WORLD HEALTH ORGANIZATION
INTERNATIONAL AGENCY FOR RESEARCH ON CANCER

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

VOLUME 70

EPSTEIN-BARR VIRUS AND KAPOSI'S SARCOMA HERPESVIRUS/HUMAN HERPESVIRUS 8

1997
IARC
LYON
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IARC MONOGRAPHS

ON THE

EVALUATION OF CARCINOGENIC RISKS TO HUMANS

Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8

VOLUME 70

This publication represents the views and expert opinions
of an IARC Working Group on the
Evaluation of Carcinogenic Risks to Humans,
which met in Lyon,

17–24 June 1997

1997

IARC MONOGRAPHS

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme on the evaluation of the carcinogenic risk of chemicals to humans involving the production of critically evaluated monographs on individual chemicals. The programme was subsequently expanded to include evaluations of carcinogenic risks associated with exposures to complex mixtures, life-style factors and biological agents, as well as those in specific occupations.

The objective of the programme is to elaborate and publish in the form of monographs critical reviews of data on carcinogenicity for agents to which humans are known to be exposed and on specific exposure situations; to evaluate these data in terms of human risk with the help of international working groups of experts in chemical carcinogenesis and related fields; and to indicate where additional research efforts are needed.

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NOTE TO THE READER

The term 'carcinogenic risk' in the *IARC Monographs* series is taken to mean the probability that exposure to an agent will lead to cancer in humans.

Inclusion of an agent in the *Monographs* does not imply that it is a carcinogen, only that the published data have been examined. Equally, the fact that an agent has not yet been evaluated in a monograph does not mean that it is not carcinogenic.

The evaluations of carcinogenic risk are made by international working groups of independent scientists and are qualitative in nature. No recommendation is given for regulation or legislation.

Anyone who is aware of published data that may alter the evaluation of the carcinogenic risk of an agent to humans is encouraged to make this information available to the Unit of Carcinogen Identification and Evaluation, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France, in order that the agent may be considered for re-evaluation by a future Working Group.

Although every effort is made to prepare the monographs as accurately as possible, mistakes may occur. Readers are requested to communicate any errors to the Unit of Carcinogen Identification and Evaluation, so that corrections can be reported in future volumes.

IARC WORKING GROUP ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS: EPSTEIN-BARR VIRUS AND KAPOSI'S SARCOMA HERPESVIRUS/HUMAN HERPESVIRUS 8

Lyon, 17–24 June 1997

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PREAMBLE

IARC MONOGRAPHS PROGRAMME ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS[1]

PREAMBLE

1. BACKGROUND

In 1969, the International Agency for Research on Cancer (IARC) initiated a programme to evaluate the carcinogenic risk of chemicals to humans and to produce monographs on individual chemicals. The *Monographs* programme has since been expanded to include consideration of exposures to complex mixtures of chemicals (which occur, for example, in some occupations and as a result of human habits) and of exposures to other agents, such as radiation and viruses. With Supplement 6 (IARC, 1987a), the title of the series was modified from *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans* to *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, in order to reflect the widened scope of the programme.

The criteria established in 1971 to evaluate carcinogenic risk to humans were adopted by the working groups whose deliberations resulted in the first 16 volumes of the *IARC Monographs series*. Those criteria were subsequently updated by further ad-hoc working groups (IARC, 1977, 1978, 1979, 1982, 1983, 1987b, 1988, 1991a; Vainio *et al.*, 1992).

2. OBJECTIVE AND SCOPE

The objective of the programme is to prepare, with the help of international working groups of experts, and to publish in the form of monographs, critical reviews and evaluations of evidence on the carcinogenicity of a wide range of human exposures. The *Monographs* may also indicate where additional research efforts are needed.

The *Monographs* represent the first step in carcinogenic risk assessment, which involves examination of all relevant information in order to assess the strength of the available evidence that certain exposures could alter the incidence of cancer in humans. The second step is quantitative risk estimation. Detailed, quantitative evaluations of epidemiological data may be made in the *Monographs*, but without extrapolation beyond the range of the data available. Quantitative extrapolation from experimental data to the human situation is not undertaken.

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[1]This project is supported by PHS Grant No. 5-UO1 CA33193-16 awarded by the United States National Cancer Institute, Department of Health and Human Services. Since 1986, the programme has also been supported by the European Commission.

The term 'carcinogen' is used in these monographs to denote an exposure that is capable of increasing the incidence of malignant neoplasms; the induction of benign neoplasms may in some circumstances (see p. 17) contribute to the judgement that the exposure is carcinogenic. The terms 'neoplasm' and 'tumour' are used interchangeably.

Some epidemiological and experimental studies indicate that different agents may act at different stages in the carcinogenic process, and several mechanisms may be involved. The aim of the *Monographs* has been, from their inception, to evaluate evidence of carcinogenicity at any stage in the carcinogenesis process, independently of the underlying mechanisms. Information on mechanisms may, however, be used in making the overall evaluation (IARC, 1991a; Vainio *et al.*, 1992; see also pp. 23–25).

The *Monographs* may assist national and international authorities in making risk assessments and in formulating decisions concerning any necessary preventive measures. The evaluations of IARC working groups are scientific, qualitative judgements about the evidence for or against carcinogenicity provided by the available data. These evaluations represent only one part of the body of information on which regulatory measures may be based. Other components of regulatory decisions vary from one situation to another and from country to country, responding to different socioeconomic and national priorities. **Therefore, no recommendation is given with regard to regulation or legislation, which are the responsibility of individual governments and/or other international organizations.**

The *IARC Monographs* are recognized as an authoritative source of information on the carcinogenicity of a wide range of human exposures. A survey of users in 1988 indicated that the *Monographs* are consulted by various agencies in 57 countries. About 4000 copies of each volume are printed, for distribution to governments, regulatory bodies and interested scientists. The Monographs are also available from the International Agency for Research on Cancer in Lyon and via the Distribution and Sales Service of the World Health Organization.

3. SELECTION OF TOPICS FOR MONOGRAPHS

Topics are selected on the basis of two main criteria: (a) there is evidence of human exposure, and (b) there is some evidence or suspicion of carcinogenicity. The term 'agent' is used to include individual chemical compounds, groups of related chemical compounds, physical agents (such as radiation) and biological factors (such as viruses). Exposures to mixtures of agents may occur in occupational exposures and as a result of personal and cultural habits (like smoking and dietary practices). Chemical analogues and compounds with biological or physical characteristics similar to those of suspected carcinogens may also be considered, even in the absence of data on a possible carcinogenic effect in humans or experimental animals.

The scientific literature is surveyed for published data relevant to an assessment of carcinogenicity. The IARC information bulletins on agents being tested for carcinogenicity (IARC, 1973–1996) and directories of on-going research in cancer epidemiology (IARC, 1976–1996) often indicate exposures that may be scheduled for future meetings. Ad-hoc working groups convened by IARC in 1984, 1989, 1991 and 1993 gave recom-

mendations as to which agents should be evaluated in the IARC Monographs series (IARC, 1984, 1989, 1991b, 1993).

As significant new data on subjects on which monographs have already been prepared become available, re-evaluations are made at subsequent meetings, and revised monographs are published.

4. DATA FOR MONOGRAPHS

The *Monographs* do not necessarily cite all the literature concerning the subject of an evaluation. Only those data considered by the Working Group to be relevant to making the evaluation are included.

With regard to biological and epidemiological data, only reports that have been published or accepted for publication in the openly available scientific literature are reviewed by the working groups. In certain instances, government agency reports that have undergone peer review and are widely available are considered. Exceptions may be made on an ad-hoc basis to include unpublished reports that are in their final form and publicly available, if their inclusion is considered pertinent to making a final evaluation (see pp. 23–25). In the sections on chemical and physical properties, on analysis, on production and use and on occurrence, unpublished sources of information may be used.

5. THE WORKING GROUP

Reviews and evaluations are formulated by a working group of experts. The tasks of the group are: (i) to ascertain that all appropriate data have been collected; (ii) to select the data relevant for the evaluation on the basis of scientific merit; (iii) to prepare accurate summaries of the data to enable the reader to follow the reasoning of the Working Group; (iv) to evaluate the results of epidemiological and experimental studies on cancer; (v) to evaluate data relevant to the understanding of mechanism of action; and (vi) to make an overall evaluation of the carcinogenicity of the exposure to humans.

Working Group participants who contributed to the considerations and evaluations within a particular volume are listed, with their addresses, at the beginning of each publication. Each participant who is a member of a working group serves as an individual scientist and not as a representative of any organization, government or industry. In addition, nominees of national and international agencies and industrial associations may be invited as observers.

6. WORKING PROCEDURES

Approximately one year in advance of a meeting of a working group, the topics of the monographs are announced and participants are selected by IARC staff in consultation with other experts. Subsequently, relevant biological and epidemiological data are collected by the Carcinogen Identification and Evaluation Unit of IARC from recognized sources of information on carcinogenesis, including data storage and retrieval systems such as MEDLINE and TOXLINE.

For chemicals and some complex mixtures, the major collection of data and the preparation of first drafts of the sections on chemical and physical properties, on analysis, on production and use and on occurrence are carried out under a separate contract funded by the United States National Cancer Institute. Representatives from industrial associations may assist in the preparation of sections on production and use. Information on production and trade is obtained from governmental and trade publications and, in some cases, by direct contact with industries. Separate production data on some agents may not be available because their publication could disclose confidential information. Information on uses may be obtained from published sources but is often complemented by direct contact with manufacturers. Efforts are made to supplement this information with data from other national and international sources.

Six months before the meeting, the material obtained is sent to meeting participants, or is used by IARC staff, to prepare sections for the first drafts of monographs. The first drafts are compiled by IARC staff and sent before the meeting to all participants of the Working Group for review.

The Working Group meets in Lyon for seven to eight days to discuss and finalize the texts of the monographs and to formulate the evaluations. After the meeting, the master copy of each monograph is verified by consulting the original literature, edited and prepared for publication. The aim is to publish monographs within six months of the Working Group meeting.

The available studies are summarized by the Working Group, with particular regard to the qualitative aspects discussed below. In general, numerical findings are indicated as they appear in the original report; units are converted when necessary for easier comparison. The Working Group may conduct additional analyses of the published data and use them in their assessment of the evidence; the results of such supplementary analyses are given in square brackets. When an important aspect of a study, directly impinging on its interpretation, should be brought to the attention of the reader, a comment is given in square brackets.

7. EXPOSURE DATA

Sections that indicate the extent of past and present human exposure, the sources of exposure, the people most likely to be exposed and the factors that contribute to the exposure are included at the beginning of each monograph.

Most monographs on individual chemicals, groups of chemicals or complex mixtures include sections on chemical and physical data, on analysis, on production and use and on occurrence. In monographs on, for example, physical agents, occupational exposures and cultural habits, other sections may be included, such as: historical perspectives, description of an industry or habit, chemistry of the complex mixture or taxonomy. Monographs on biological agents have sections on structure and biology, methods of detection, epidemiology of infection and clinical disease other than cancer.

For chemical exposures, the Chemical Abstracts Services Registry Number, the latest Chemical Abstracts Primary Name and the IUPAC Systematic Name are recorded; other

synonyms are given, but the list is not necessarily comprehensive. For biological agents, taxonomy and structure are described, and the degree of variability is given, when applicable.

Information on chemical and physical properties and, in particular, data relevant to identification, occurrence and biological activity are included. For biological agents, mode of replication, life cycle, target cells, persistence and latency and host response are given. A description of technical products of chemicals includes trade names, relevant specifications and available information on composition and impurities. Some of the trade names given may be those of mixtures in which the agent being evaluated is only one of the ingredients.

The purpose of the section on analysis or detection is to give the reader an overview of current methods, with emphasis on those widely used for regulatory purposes. Methods for monitoring human exposure are also given, when available. No critical evaluation or recommendation of any of the methods is meant or implied. The IARC published a series of volumes, *Environmental Carcinogens: Methods of Analysis and Exposure Measurement* (IARC, 1978–93), that describe validated methods for analysing a wide variety of chemicals and mixtures. For biological agents, methods of detection and exposure assessment are described, including their sensitivity, specificity and reproducibility.

The dates of first synthesis and of first commercial production of a chemical or mixture are provided; for agents which do not occur naturally, this information may allow a reasonable estimate to be made of the date before which no human exposure to the agent could have occurred. The dates of first reported occurrence of an exposure are also provided. In addition, methods of synthesis used in past and present commercial production and different methods of production which may give rise to different impurities are described.

Data on production, international trade and uses are obtained for representative regions, which usually include Europe, Japan and the United States of America. It should not, however, be inferred that those areas or nations are necessarily the sole or major sources or users of the agent. Some identified uses may not be current or major applications, and the coverage is not necessarily comprehensive. In the case of drugs, mention of their therapeutic uses does not necessarily represent current practice, nor does it imply judgement as to their therapeutic efficacy.

Information on the occurrence of an agent or mixture in the environment is obtained from data derived from the monitoring and surveillance of levels in occupational environments, air, water, soil, foods and animal and human tissues. When available, data on the generation, persistence and bioaccumulation of the agent are also included. In the case of mixtures, industries, occupations or processes, information is given about all agents present. For processes, industries and occupations, a historical description is also given, noting variations in chemical composition, physical properties and levels of occupational exposure with time and place. For biological agents, the epidemiology of infection is described.

Statements concerning regulations and guidelines (e.g., pesticide registrations, maximal levels permitted in foods, occupational exposure limits) are included for some countries as indications of potential exposures, but they may not reflect the most recent situation, since such limits are continuously reviewed and modified. The absence of information on regulatory status for a country should not be taken to imply that that country does not have regulations with regard to the exposure. For biological agents, legislation and control, including vaccines and therapy, are described.

8. STUDIES OF CANCER IN HUMANS

(*a*) *Types of studies considered*

Three types of epidemiological studies of cancer contribute to the assessment of carcinogenicity in humans — cohort studies, case–control studies and correlation (or ecological) studies. Rarely, results from randomized trials may be available. Case series and case reports of cancer in humans may also be reviewed.

Cohort and case–control studies relate the exposures under study to the occurrence of cancer in individuals and provide an estimate of relative risk (ratio of incidence or mortality in those exposed to incidence or mortality in those not exposed) as the main measure of association.

In correlation studies, the units of investigation are usually whole populations (e.g. in particular geographical areas or at particular times), and cancer frequency is related to a summary measure of the exposure of the population to the agent, mixture or exposure circumstance under study. Because individual exposure is not documented, however, a causal relationship is less easy to infer from correlation studies than from cohort and case–control studies. Case reports generally arise from a suspicion, based on clinical experience, that the concurrence of two events — that is, a particular exposure and occurrence of a cancer — has happened rather more frequently than would be expected by chance. Case reports usually lack complete ascertainment of cases in any population, definition or enumeration of the population at risk and estimation of the expected number of cases in the absence of exposure. The uncertainties surrounding interpretation of case reports and correlation studies make them inadequate, except in rare instances, to form the sole basis for inferring a causal relationship. When taken together with case–control and cohort studies, however, relevant case reports or correlation studies may add materially to the judgement that a causal relationship is present.

Epidemiological studies of benign neoplasms, presumed preneoplastic lesions and other end-points thought to be relevant to cancer are also reviewed by working groups. They may, in some instances, strengthen inferences drawn from studies of cancer itself.

(*b*) *Quality of studies considered*

The Monographs are not intended to summarize all published studies. Those that are judged to be inadequate or irrelevant to the evaluation are generally omitted. They may be mentioned briefly, particularly when the information is considered to be a useful supplement to that in other reports or when they provide the only data available. Their

inclusion does not imply acceptance of the adequacy of the study design or of the analysis and interpretation of the results, and limitations are clearly outlined in square brackets at the end of the study description.

It is necessary to take into account the possible roles of bias, confounding and chance in the interpretation of epidemiological studies. By 'bias' is meant the operation of factors in study design or execution that lead erroneously to a stronger or weaker association than in fact exists between disease and an agent, mixture or exposure circumstance. By 'confounding' is meant a situation in which the relationship with disease is made to appear stronger or weaker than it truly is as a result of an association between the apparent causal factor and another factor that is associated with either an increase or decrease in the incidence of the disease. In evaluating the extent to which these factors have been minimized in an individual study, working groups consider a number of aspects of design and analysis as described in the report of the study. Most of these considerations apply equally to case–control, cohort and correlation studies. Lack of clarity of any of these aspects in the reporting of a study can decrease its credibility and the weight given to it in the final evaluation of the exposure.

Firstly, the study population, disease (or diseases) and exposure should have been well defined by the authors. Cases of disease in the study population should have been identified in a way that was independent of the exposure of interest, and exposure should have been assessed in a way that was not related to disease status.

Secondly, the authors should have taken account in the study design and analysis of other variables that can influence the risk of disease and may have been related to the exposure of interest. Potential confounding by such variables should have been dealt with either in the design of the study, such as by matching, or in the analysis, by statistical adjustment. In cohort studies, comparisons with local rates of disease may be more appropriate than those with national rates. Internal comparisons of disease frequency among individuals at different levels of exposure should also have been made in the study.

Thirdly, the authors should have reported the basic data on which the conclusions are founded, even if sophisticated statistical analyses were employed. At the very least, they should have given the numbers of exposed and unexposed cases and controls in a case–control study and the numbers of cases observed and expected in a cohort study. Further tabulations by time since exposure began and other temporal factors are also important. In a cohort study, data on all cancer sites and all causes of death should have been given, to reveal the possibility of reporting bias. In a case–control study, the effects of investigated factors other than the exposure of interest should have been reported.

Finally, the statistical methods used to obtain estimates of relative risk, absolute rates of cancer, confidence intervals and significance tests, and to adjust for confounding should have been clearly stated by the authors. The methods used should preferably have been the generally accepted techniques that have been refined since the mid-1970s. These methods have been reviewed for case–control studies (Breslow & Day, 1980) and for cohort studies (Breslow & Day, 1987).

(*c*) *Inferences about mechanism of action*

Detailed analyses of both relative and absolute risks in relation to temporal variables, such as age at first exposure, time since first exposure, duration of exposure, cumulative exposure and time since exposure ceased, are reviewed and summarized when available. The analysis of temporal relationships can be useful in formulating models of carcinogenesis. In particular, such analyses may suggest whether a carcinogen acts early or late in the process of carcinogenesis, although at best they allow only indirect inferences about the mechanism of action. Special attention is given to measurements of biological markers of carcinogen exposure or action, such as DNA or protein adducts, as well as markers of early steps in the carcinogenic process, such as próto-oncogene mutation, when these are incorporated into epidemiological studies focused on cancer incidence or mortality. Such measurements may allow inferences to be made about putative mechanisms of action (IARC, 1991a; Vainio *et al.*, 1992).

(*d*) *Criteria for causality*

After the quality of individual epidemiological studies of cancer has been summarized and assessed, a judgement is made concerning the strength of evidence that the agent, mixture or exposure circumstance in question is carcinogenic for humans. In making its judgement, the Working Group considers several criteria for causality. A strong association (a large relative risk) is more likely to indicate causality than a weak association, although it is recognized that relative risks of small magnitude do not imply lack of causality and may be important if the disease is common. Associations that are replicated in several studies of the same design or using different epidemiological approaches or under different circumstances of exposure are more likely to represent a causal relationship than isolated observations from single studies. If there are inconsistent results among investigations, possible reasons are sought (such as differences in amount of exposure), and results of studies judged to be of high quality are given more weight than those of studies judged to be methodologically less sound. When suspicion of carcinogenicity arises largely from a single study, these data are not combined with those from later studies in any subsequent reassessment of the strength of the evidence.

If the risk of the disease in question increases with the amount of exposure, this is considered to be a strong indication of causality, although absence of a graded response is not necessarily evidence against a causal relationship. Demonstration of a decline in risk after cessation of or reduction in exposure in individuals or in whole populations also supports a causal interpretation of the findings.

Although a carcinogen may act upon more than one target, the specificity of an association (an increased occurrence of cancer at one anatomical site or of one morphological type) adds plausibility to a causal relationship, particularly when excess cancer occurrence is limited to one morphological type within the same organ.

Although rarely available, results from randomized trials showing different rates among exposed and unexposed individuals provide particularly strong evidence for causality.

When several epidemiological studies show little or no indication of an association between an exposure and cancer, the judgement may be made that, in the aggregate, they show evidence of lack of carcinogenicity. Such a judgement requires first of all that the studies giving rise to it meet, to a sufficient degree, the standards of design and analysis described above. Specifically, the possibility that bias, confounding or misclassification of exposure or outcome could explain the observed results should be considered and excluded with reasonable certainty. In addition, all studies that are judged to be methodologically sound should be consistent with a relative risk of unity for any observed level of exposure and, when considered together, should provide a pooled estimate of relative risk which is at or near unity and has a narrow confidence interval, due to sufficient population size. Moreover, no individual study nor the pooled results of all the studies should show any consistent tendency for the relative risk of cancer to increase with increasing level of exposure. It is important to note that evidence of lack of carcinogenicity obtained in this way from several epidemiological studies can apply only to the type(s) of cancer studied and to dose levels and intervals between first exposure and observation of disease that are the same as or less than those observed in all the studies. Experience with human cancer indicates that, in some cases, the period from first exposure to the development of clinical cancer is seldom less than 20 years; latent periods substantially shorter than 30 years cannot provide evidence for lack of carcinogenicity.

9. STUDIES OF CANCER IN EXPERIMENTAL ANIMALS

All known human carcinogens that have been studied adequately in experimental animals have produced positive results in one or more animal species (Wilbourn *et al.*, 1986; Tomatis *et al.*, 1989). For several agents (aflatoxins, 4-aminobiphenyl, azathioprine, betel quid with tobacco, bischloromethyl ether and chloromethyl methyl ether (technical grade), chlorambucil, chlornaphazine, ciclosporin, coal-tar pitches, coal-tars, combined oral contraceptives, cyclophosphamide, diethylstilboestrol, melphalan, 8-methoxypsoralen plus ultraviolet A radiation, mustard gas, myleran, 2-naphthylamine, nonsteroidal oestrogens, oestrogen replacement therapy/steroidal oestrogens, solar radiation, thiotepa and vinyl chloride), carcinogenicity in experimental animals was established or highly suspected before epidemiological studies confirmed their carcinogenicity in humans (Vainio *et al.*, 1995). Although this association cannot establish that all agents and mixtures that cause cancer in experimental animals also cause cancer in humans, nevertheless, **in the absence of adequate data on humans, it is biologically plausible and prudent to regard agents and mixtures for which there is *sufficient evidence* (see p. 24) of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans**. The possibility that a given agent may cause cancer through a species-specific mechanism which does not operate in humans (see p. 25) should also be taken into consideration.

The nature and extent of impurities or contaminants present in the chemical or mixture being evaluated are given when available. Animal strain, sex, numbers per group, age at start of treatment and survival are reported.

Other types of studies summarized include: experiments in which the agent or mixture was administered in conjunction with known carcinogens or factors that modify carcinogenic effects; studies in which the end-point was not cancer but a defined precancerous lesion; and experiments on the carcinogenicity of known metabolites and derivatives.

For experimental studies of mixtures, consideration is given to the possibility of changes in the physicochemical properties of the test substance during collection, storage, extraction, concentration and delivery. Chemical and toxicological interactions of the components of mixtures may result in nonlinear dose–response relationships.

An assessment is made as to the relevance to human exposure of samples tested in experimental animals, which may involve consideration of: (i) physical and chemical characteristics, (ii) constituent substances that indicate the presence of a class of substances, (iii) the results of tests for genetic and related effects, including genetic activity profiles, DNA adduct profiles, proto-oncogene mutation and expression and suppressor gene inactivation. The relevance of results obtained, for example, with animal viruses analogous to the virus being evaluated in the monograph must also be considered. They may provide biological and mechanistic information relevant to the understanding of the process of carcinogenesis in humans and may strengthen the plausibility of a conclusion that the biological agent under evaluation is carcinogenic in humans.

(*a*) *Qualitative aspects*

An assessment of carcinogenicity involves several considerations of qualitative importance, including (i) the experimental conditions under which the test was performed, including route and schedule of exposure, species, strain, sex, age, duration of follow-up; (ii) the consistency of the results, for example, across species and target organ(s); (iii) the spectrum of neoplastic response, from preneoplastic lesions and benign tumours to malignant neoplasms; and (iv) the possible role of modifying factors.

As mentioned earlier (p. 11), the *Monographs* are not intended to summarize all published studies. Those studies in experimental animals that are inadequate (e.g., too short a duration, too few animals, poor survival; see below) or are judged irrelevant to the evaluation are generally omitted. Guidelines for conducting adequate long-term carcinogenicity experiments have been outlined (e.g. Montesano *et al.*, 1986).

Considerations of importance to the Working Group in the interpretation and evaluation of a particular study include: (i) how clearly the agent was defined and, in the case of mixtures, how adequately the sample characterization was reported; (ii) whether the dose was adequately monitored, particularly in inhalation experiments; (iii) whether the doses and duration of treatment were appropriate and whether the survival of treated animals was similar to that of controls; (iv) whether there were adequate numbers of animals per group; (v) whether animals of each sex were used; (vi) whether animals were allocated randomly to groups; (vii) whether the duration of observation was adequate; and (viii) whether the data were adequately reported. If available, recent data on the incidence of specific tumours in historical controls, as well as in concurrent controls, should be taken into account in the evaluation of tumour response.

When benign tumours occur together with and originate from the same cell type in an organ or tissue as malignant tumours in a particular study and appear to represent a stage in the progression to malignancy, it may be valid to combine them in assessing tumour incidence (Huff *et al.*, 1989). The occurrence of lesions presumed to be preneoplastic may in certain instances aid in assessing the biological plausibility of any neoplastic response observed. If an agent or mixture induces only benign neoplasms that appear to be end-points that do not readily undergo transition to malignancy, it should nevertheless be suspected of being a carcinogen and requires further investigation.

(*b*) *Quantitative aspects*

The probability that tumours will occur may depend on the species, sex, strain and age of the animal, the dose of the carcinogen and the route and length of exposure. Evidence of an increased incidence of neoplasms with increased level of exposure strengthens the inference of a causal association between the exposure and the development of neoplasms.

The form of the dose–response relationship can vary widely, depending on the particular agent under study and the target organ. Both DNA damage and increased cell division are important aspects of carcinogenesis, and cell proliferation is a strong determinant of dose–response relationships for some carcinogens (Cohen & Ellwein, 1990). Since many chemicals require metabolic activation before being converted into their reactive intermediates, both metabolic and pharmacokinetic aspects are important in determining the dose–response pattern. Saturation of steps such as absorption, activation, inactivation and elimination may produce nonlinearity in the dose–response relationship, as could saturation of processes such as DNA repair (Hoel *et al.*, 1983; Gart *et al.*, 1986).

(*c*) *Statistical analysis of long-term experiments in animals*

Factors considered by the Working Group include the adequacy of the information given for each treatment group: (i) the number of animals studied and the number examined histologically, (ii) the number of animals with a given tumour type and (iii) length of survival. The statistical methods used should be clearly stated and should be the generally accepted techniques refined for this purpose (Peto *et al.*, 1980; Gart *et al.*, 1986). When there is no difference in survival between control and treatment groups, the Working Group usually compares the proportions of animals developing each tumour type in each of the groups. Otherwise, consideration is given as to whether or not appropriate adjustments have been made for differences in survival. These adjustments can include: comparisons of the proportions of tumour-bearing animals among the effective number of animals (alive at the time the first tumour is discovered), in the case where most differences in survival occur before tumours appear; life-table methods, when tumours are visible or when they may be considered 'fatal' because mortality rapidly follows tumour development; and the Mantel-Haenszel test or logistic regression, when occult tumours do not affect the animals' risk of dying but are 'incidental' findings at autopsy.

In practice, classifying tumours as fatal or incidental may be difficult. Several survival-adjusted methods have been developed that do not require this distinction (Gart *et al.*, 1986), although they have not been fully evaluated.

10. OTHER DATA RELEVANT TO AN EVALUATION OF CARCINOGENICITY AND ITS MECHANISMS

In coming to an overall evaluation of carcinogenicity in humans (see pp. 23–25), the Working Group also considers related data. The nature of the information selected for the summary depends on the agent being considered.

For chemicals and complex mixtures of chemicals such as those in some occupational situations and involving cultural habits (e.g. tobacco smoking), the other data considered to be relevant are divided into those on absorption, distribution, metabolism and excretion; toxic effects; reproductive and developmental effects; and genetic and related effects.

Concise information is given on absorption, distribution (including placental transfer) and excretion in both humans and experimental animals. Kinetic factors that may affect the dose–response relationship, such as saturation of uptake, protein binding, metabolic activation, detoxification and DNA repair processes, are mentioned. Studies that indicate the metabolic fate of the agent in humans and in experimental animals are summarized briefly, and comparisons of data on humans and on animals are made when possible. Comparative information on the relationship between exposure and the dose that reaches the target site may be of particular importance for extrapolation between species. Data are given on acute and chronic toxic effects (other than cancer), such as organ toxicity, increased cell proliferation, immunotoxicity and endocrine effects. The presence and toxicological significance of cellular receptors is described. Effects on reproduction, teratogenicity, fetotoxicity and embryotoxicity are also summarized briefly.

Tests of genetic and related effects are described in view of the relevance of gene mutation and chromosomal damage to carcinogenesis (Vainio *et al.*, 1992). The adequacy of the reporting of sample characterization is considered and, where necessary, commented upon; with regard to complex mixtures, such comments are similar to those described for animal carcinogenicity tests on p. 18. The available data are interpreted critically by phylogenetic group according to the end-points detected, which may include DNA damage, gene mutation, sister chromatid exchange, micronucleus formation, chromosomal aberrations, aneuploidy and cell transformation. The concentrations employed are given, and mention is made of whether use of an exogenous metabolic system *in vitro* affected the test result. These data are given as listings of test systems, data and references; bar graphs (activity profiles) and corresponding summary tables with detailed information on the preparation of the profiles (Waters *et al.*, 1987) are given in appendices.

Positive results in tests using prokaryotes, lower eukaryotes, plants, insects and cultured mammalian cells suggest that genetic and related effects could occur in mammals. Results from such tests may also give information about the types of genetic effect produced and about the involvement of metabolic activation. Some end-points

described are clearly genetic in nature (e.g., gene mutations and chromosomal aberrations), while others are to a greater or lesser degree associated with genetic effects (e.g. unscheduled DNA synthesis). In-vitro tests for tumour-promoting activity and for cell transformation may be sensitive to changes that are not necessarily the result of genetic alterations but that may have specific relevance to the process of carcinogenesis. A critical appraisal of these tests has been published (Montesano *et al.*, 1986).

Genetic or other activity manifest in experimental mammals and humans is regarded as being of greater relevance than that in other organisms. The demonstration that an agent or mixture can induce gene and chromosomal mutations in whole mammals indicates that it may have carcinogenic activity, although this activity may not be detectably expressed in any or all species. Relative potency in tests for mutagenicity and related effects is not a reliable indicator of carcinogenic potency. Negative results in tests for mutagenicity in selected tissues from animals treated *in vivo* provide less weight, partly because they do not exclude the possibility of an effect in tissues other than those examined. Moreover, negative results in short-term tests with genetic end-points cannot be considered to provide evidence to rule out carcinogenicity of agents or mixtures that act through other mechanisms (e.g. receptor-mediated effects, cellular toxicity with regenerative proliferation, peroxisome proliferation) (Vainio *et al.*, 1992). Factors that may lead to misleading results in short-term tests have been discussed in detail elsewhere (Montesano *et al.*, 1986).

When available, data relevant to mechanisms of carcinogenesis that do not involve structural changes at the level of the gene are also described.

The adequacy of epidemiological studies of reproductive outcome and genetic and related effects in humans is evaluated by the same criteria as are applied to epidemiological studies of cancer.

Structure–activity relationships that may be relevant to an evaluation of the carcinogenicity of an agent are also described.

For biological agents — viruses, bacteria and parasites — other data relevant to carcinogenicity include descriptions of the pathology of infection, molecular biology (integration and expression of viruses, and any genetic alterations seen in human tumours) and other observations, which might include cellular and tissue responses to infection, immune response and the presence of tumour markers.

11. SUMMARY OF DATA REPORTED

In this section, the relevant epidemiological and experimental data are summarized. Only reports, other than in abstract form, that meet the criteria outlined on p. 11 are considered for evaluating carcinogenicity. Inadequate studies are generally not summarized: such studies are usually identified by a square-bracketed comment in the preceding text.

(*a*) *Exposure*

Human exposure to chemicals and complex mixtures is summarized on the basis of elements such as production, use, occurrence in the environment and determinations in human tissues and body fluids. Quantitative data are given when available. Exposure to biological agents is described in terms of transmission and prevalence of infection.

(*b*) *Carcinogenicity in humans*

Results of epidemiological studies that are considered to be pertinent to an assessment of human carcinogenicity are summarized. When relevant, case reports and correlation studies are also summarized.

(*c*) *Carcinogenicity in experimental animals*

Data relevant to an evaluation of carcinogenicity in animals are summarized. For each animal species and route of administration, it is stated whether an increased incidence of neoplasms or preneoplastic lesions was observed, and the tumour sites are indicated. If the agent or mixture produced tumours after prenatal exposure or in single-dose experiments, this is also indicated. Negative findings are also summarized. Dose–response and other quantitative data may be given when available.

(*d*) *Other data relevant to an evaluation of carcinogenicity and its mechanisms*

Data on biological effects in humans that are of particular relevance are summarized. These may include toxicological, kinetic and metabolic considerations and evidence of DNA binding, persistence of DNA lesions or genetic damage in exposed humans. Toxicological information, such as that on cytotoxicity and regeneration, receptor binding and hormonal and immunological effects, and data on kinetics and metabolism in experimental animals are given when considered relevant to the possible mechanism of the carcinogenic action of the agent. The results of tests for genetic and related effects are summarized for whole mammals, cultured mammalian cells and nonmammalian systems.

When available, comparisons of such data for humans and for animals, and particularly animals that have developed cancer, are described.

Structure–activity relationships are mentioned when relevant.

For the agent, mixture or exposure circumstance being evaluated, the available data on end-points or other phenomena relevant to mechanisms of carcinogenesis from studies in humans, experimental animals and tissue and cell test systems are summarized within one or more of the following descriptive dimensions:

(i) Evidence of genotoxicity (structural changes at the level of the gene): for example, structure–activity considerations, adduct formation, mutagenicity (effect on specific genes), chromosomal mutation/aneuploidy

(ii) Evidence of effects on the expression of relevant genes (functional changes at the intracellular level): for example, alterations to the structure or quantity of the product of a proto-oncogene or tumour-suppressor gene, alterations to metabolic activation/-inactivation/DNA repair

(iii) Evidence of relevant effects on cell behaviour (morphological or behavioural changes at the cellular or tissue level): for example, induction of mitogenesis, compensatory cell proliferation, preneoplasia and hyperplasia, survival of premalignant or malignant cells (immortalization, immunosuppression), effects on metastatic potential

(iv) Evidence from dose and time relationships of carcinogenic effects and interactions between agents: for example, early/late stage, as inferred from epidemiological studies; initiation/promotion/progression/malignant conversion, as defined in animal carcinogenicity experiments; toxicokinetics

These dimensions are not mutually exclusive, and an agent may fall within more than one of them. Thus, for example, the action of an agent on the expression of relevant genes could be summarized under both the first and second dimensions, even if it were known with reasonable certainty that those effects resulted from genotoxicity.

12. EVALUATION

Evaluations of the strength of the evidence for carcinogenicity arising from human and experimental animal data are made, using standard terms.

It is recognized that the criteria for these evaluations, described below, cannot encompass all of the factors that may be relevant to an evaluation of carcinogenicity. In considering all of the relevant scientific data, the Working Group may assign the agent, mixture or exposure circumstance to a higher or lower category than a strict interpretation of these criteria would indicate.

(*a*) *Degrees of evidence for carcinogenicity in humans and in experimental animals and supporting evidence*

These categories refer only to the strength of the evidence that an exposure is carcinogenic and not to the extent of its carcinogenic activity (potency) nor to the mechanisms involved. A classification may change as new information becomes available.

An evaluation of degree of evidence, whether for a single agent or a mixture, is limited to the materials tested, as defined physically, chemically or biologically. When the agents evaluated are considered by the Working Group to be sufficiently closely related, they may be grouped together for the purpose of a single evaluation of degree of evidence.

(i) *Carcinogenicity in humans*

The applicability of an evaluation of the carcinogenicity of a mixture, process, occupation or industry on the basis of evidence from epidemiological studies depends on the variability over time and place of the mixtures, processes, occupations and industries. The Working Group seeks to identify the specific exposure, process or activity which is considered most likely to be responsible for any excess risk. The evaluation is focused as narrowly as the available data on exposure and other aspects permit.

The evidence relevant to carcinogenicity from studies in humans is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between exposure to the agent, mixture or exposure circumstance and human cancer. That is, a positive relationship has been observed between the exposure and cancer in studies in which chance, bias and confounding could be ruled out with reasonable confidence.

Limited evidence of carcinogenicity: A positive association has been observed between exposure to the agent, mixture or exposure circumstance and cancer for which a causal interpretation is considered by the Working Group to be credible, but chance, bias or confounding could not be ruled out with reasonable confidence.

Inadequate evidence of carcinogenicity: The available studies are of insufficient quality, consistency or statistical power to permit a conclusion regarding the presence or absence of a causal association, or no data on cancer in humans are available.

Evidence suggesting lack of carcinogenicity: There are several adequate studies covering the full range of levels of exposure that human beings are known to encounter, which are mutually consistent in not showing a positive association between exposure to the agent, mixture or exposure circumstance and any studied cancer at any observed level of exposure. A conclusion of 'evidence suggesting lack of carcinogenicity' is inevitably limited to the cancer sites, conditions and levels of exposure and length of observation covered by the available studies. In addition, the possibility of a very small risk at the levels of exposure studied can never be excluded.

In some instances, the above categories may be used to classify the degree of evidence related to carcinogenicity in specific organs or tissues.

(ii) *Carcinogenicity in experimental animals*

The evidence relevant to carcinogenicity in experimental animals is classified into one of the following categories:

Sufficient evidence of carcinogenicity: The Working Group considers that a causal relationship has been established between the agent or mixture and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols.

Exceptionally, a single study in one species might be considered to provide sufficient evidence of carcinogenicity when malignant neoplasms occur to an unusual degree with regard to incidence, site, type of tumour or age at onset.

Limited evidence of carcinogenicity: The data suggest a carcinogenic effect but are limited for making a definitive evaluation because, e.g. (a) the evidence of carcinogenicity is restricted to a single experiment; or (b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the study; or (c) the agent or mixture increases the incidence only of benign neoplasms or lesions of uncertain neoplastic potential, or of certain neoplasms which may occur spontaneously in high incidences in certain strains.

Inadequate evidence of carcinogenicity: The studies cannot be interpreted as showing either the presence or absence of a carcinogenic effect because of major qualitative or quantitative limitations, or no data on cancer in experimental animals are available.

Evidence suggesting lack of carcinogenicity: Adequate studies involving at least two species are available which show that, within the limits of the tests used, the agent or mixture is not carcinogenic. A conclusion of evidence suggesting lack of carcinogenicity is inevitably limited to the species, tumour sites and levels of exposure studied.

(*b*) *Other data relevant to the evaluation of carcinogenicity and its mechanisms*

Other evidence judged to be relevant to an evaluation of carcinogenicity and of sufficient importance to affect the overall evaluation is then described. This may include data on preneoplastic lesions, tumour pathology, genetic and related effects, structure–activity relationships, metabolism and pharmacokinetics, physicochemical parameters and analogous biological agents.

Data relevant to mechanisms of the carcinogenic action are also evaluated. The strength of the evidence that any carcinogenic effect observed is due to a particular mechanism is assessed, using terms such as weak, moderate or strong. Then, the Working Group assesses if that particular mechanism is likely to be operative in humans. The strongest indications that a particular mechanism operates in humans come from data on humans or biological specimens obtained from exposed humans. The data may be considered to be especially relevant if they show that the agent in question has caused changes in exposed humans that are on the causal pathway to carcinogenesis. Such data may, however, never become available, because it is at least conceivable that certain compounds may be kept from human use solely on the basis of evidence of their toxicity and/or carcinogenicity in experimental systems.

For complex exposures, including occupational and industrial exposures, the chemical composition and the potential contribution of carcinogens known to be present are considered by the Working Group in its overall evaluation of human carcinogenicity. The Working Group also determines the extent to which the materials tested in experimental systems are related to those to which humans are exposed.

(*c*) *Overall evaluation*

Finally, the body of evidence is considered as a whole, in order to reach an overall evaluation of the carcinogenicity to humans of an agent, mixture or circumstance of exposure.

An evaluation may be made for a group of chemical compounds that have been evaluated by the Working Group. In addition, when supporting data indicate that other, related compounds for which there is no direct evidence of capacity to induce cancer in humans or in animals may also be carcinogenic, a statement describing the rationale for this conclusion is added to the evaluation narrative; an additional evaluation may be made for this broader group of compounds if the strength of the evidence warrants it.

The agent, mixture or exposure circumstance is described according to the wording of one of the following categories, and the designated group is given. The categorization of

an agent, mixture or exposure circumstance is a matter of scientific judgement, reflecting the strength of the evidence derived from studies in humans and in experimental animals and from other relevant data.

Group 1 — The agent (mixture) is carcinogenic to humans.
The exposure circumstance entails exposures that are carcinogenic to humans.

This category is used when there is *sufficient evidence* of carcinogenicity in humans. Exceptionally, an agent (mixture) may be placed in this category when evidence in humans is less than sufficient but there is *sufficient evidence* of carcinogenicity in experimental animals and strong evidence in exposed humans that the agent (mixture) acts through a relevant mechanism of carcinogenicity.

Group 2

This category includes agents, mixtures and exposure circumstances for which, at one extreme, the degree of evidence of carcinogenicity in humans is almost sufficient, as well as those for which, at the other extreme, there are no human data but for which there is evidence of carcinogenicity in experimental animals. Agents, mixtures and exposure circumstances are assigned to either group 2A (probably carcinogenic to humans) or group 2B (possibly carcinogenic to humans) on the basis of epidemiological and experimental evidence of carcinogenicity and other relevant data.

Group 2A — The agent (mixture) is probably carcinogenic to humans.
The exposure circumstance entails exposures that are probably carcinogenic to humans.

This category is used when there is *limited evidence* of carcinogenicity in humans and sufficient evidence of carcinogenicity in experimental animals. In some cases, an agent (mixture) may be classified in this category when there is inadequate evidence of carcinogenicity in humans, *sufficient evidence* of carcinogenicity in experimental animals and strong evidence that the carcinogenesis is mediated by a mechanism that also operates in humans. Exceptionally, an agent, mixture or exposure circumstance may be classified in this category solely on the basis of limited evidence of carcinogenicity in humans.

Group 2B — The agent (mixture) is possibly carcinogenic to humans.
The exposure circumstance entails exposures that are possibly carcinogenic to humans.

This category is used for agents, mixtures and exposure circumstances for which there is *limited evidence* of carcinogenicity in humans and less than *sufficient evidence* of carcinogenicity in experimental animals. It may also be used when there is *inadequate evidence* of carcinogenicity in humans but there is *sufficient evidence* of carcinogenicity in experimental animals. In some instances, an agent, mixture or exposure circumstance for which there is *inadequate evidence* of carcinogenicity in humans but *limited evidence* of carcinogenicity in experimental animals together with supporting evidence from other relevant data may be placed in this group.

Group 3 — The agent (mixture or exposure circumstance) is not classifiable as to its carcinogenicity to humans.

This category is used most commonly for agents, mixtures and exposure circumstances for which the evidence of carcinogenicity is inadequate in humans and inadequate or limited in experimental animals.

Exceptionally, agents (mixtures) for which the evidence of carcinogenicity is inadequate in humans but sufficient in experimental animals may be placed in this category when there is strong evidence that the mechanism of carcinogenicity in experimental animals does not operate in humans.

Agents, mixtures and exposure circumstances that do not fall into any other group are also placed in this category.

Group 4 — The agent (mixture) is probably not carcinogenic to humans.

This category is used for agents or mixtures for which there is *evidence suggesting lack of carcinogenicity* in humans and in experimental animals. In some instances, agents or mixtures for which there is *inadequate evidence* of carcinogenicity in humans but *evidence suggesting lack of carcinogenicity* in experimental animals, consistently and strongly supported by a broad range of other relevant data, may be classified in this group.

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GENERAL REMARKS

This seventieth volume of the *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* is the fourth volume devoted to viruses and the fifth volume devoted to biological agents. Volume 59 (IARC, 1994a) covered three human hepatotropic viruses — hepatitis B virus, hepatitis C virus and hepatitis D virus (also known as the delta agent); Volume 64 (IARC, 1995) addressed human papillomaviruses and Volume 67 (IARC, 1996), human immunodeficiency viruses and human T-cell lymphotropic viruses. Volume 61 (IARC, 1994b) covered schistosomes, liver flukes and *Helicobacter pylori*.

Epstein-Barr virus is a gamma-1 herpesvirus found in all human populations, with a prevalence of over 90% in adults. It is found in Burkitt's lymphoma, sinonasal angiocentric T-cell lymphoma, immunosuppression-related lymphoma, Hodgkin's disease and nasopharyngeal carcinoma.

Kaposi's sarcoma herpesvirus/human herpesvirus 8 is a gamma-2 herpesvirus that was detected in 1994 in Kaposi's sarcomas in a patient with acquired immune deficiency syndrome (AIDS).

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INTRODUCTION

Herpesviruses are a family of DNA viruses found commonly in humans and animals. Nearly 100 herpesviruses have been at least partially characterized, and most animal species have been shown to be infected by at least one member of the family. The name, derived from the Greek *herpein*, to creep, refers to the characteristic lesions caused by two common human herpesviruses: fever blisters caused by herpes simplex and varicella and shingles induced by herpes zoster. The known herpesviruses have a common virion architecture and four significant biological properties:

(i) They encode a large variety of enzymes involved in nucleic acid metabolism, DNA synthesis and protein processing.

(ii) Synthesis of viral DNA and assembly of the capsid occur in the nucleus of infected cells.

(iii) The production of infectious viral progeny is usually accompanied by destruction of the infected cell.

(iv) Herpesviruses can remain latent and persist for life in their natural hosts. Latent infection occurs in specific sets of cells that differ from one virus to another. The latent viral genomes usually take the form of circular episomes, and only a small subset of viral genes is expressed.

According to the official nomenclature endorsed by the International Committee on the Taxonomy of Viruses, herpesviruses are designated by serial Arabic numbers and the family or subfamily of the natural host; e.g. human herpesviruses (HHV) 1, 2, 3 and equine herpesviruses 1, 2, 3. This nomenclature is used in the following text with the common names of the most relevant viruses.

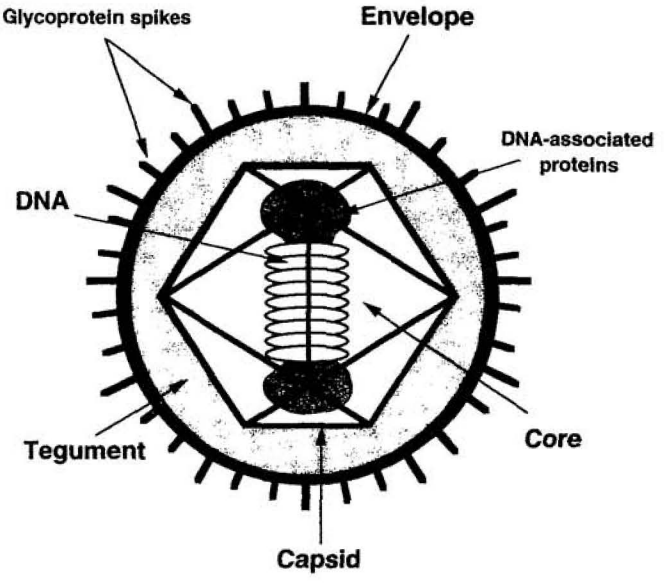
1. Structure of herpesviruses

The structure of herpesviruses has been reviewed by Roizman (1996).

1.1 *The virion*

A typical herpesvirion consists of a core containing linear double-stranded DNA, an icosahedral capsid, an amorphous tegument and a lipid envelope with viral glycoprotein spikes on its surface (Figure 1). Herpesvirus particles have a diameter of about 120 to more than 200 nm and contain, in addition to viral DNA, 25–35 virus-encoded proteins and host-specific phospholipids derived from the nuclear membrane. Many of the particles within a population of virions do not possess envelopes, and some are empty capsids.

Figure 1. Schematic structure of a herpesvirus

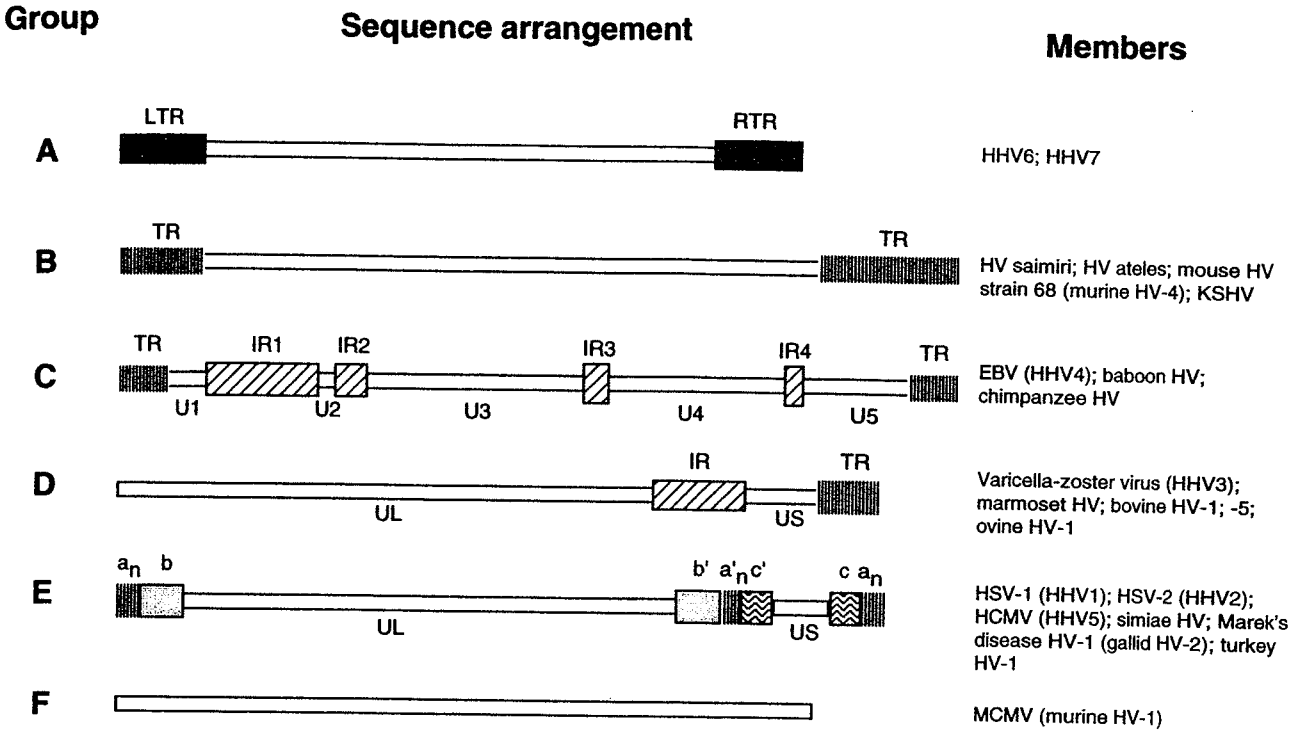


The core of the mature virion contains the double-stranded viral DNA arranged in a torus of about 75 nm in diameter. In some virions, the DNA appears to be wrapped around a DNA-associated spindle-shaped protein consisting of fibrils attached to the inside of the capsid. The icosahedral capsid is approximately 100–110 nm in diameter and contains 162 capsomers. The hexameric capsomers are 9.5 × 12.5 nm in longitudinal sections with a channel of 4 nm in diameter running along the long axis. The pentameric capsomers located at the vertices of the capsid have not been characterized. The capsid is surrounded by an amorphous material, the tegument, composed of globular proteins. The tegument is frequently distributed asymmetrically, and its thickness may vary depending on the location of the virion within the infected cells. The envelope of herpesviruses has a typical trilamellar structure and is derived from patches of altered cellular membranes. Its high lipid content results in relative instability of the virions at room temperature and their rapid inactivation by lipid solvents such as diethyl ether and chloroform and by detergents. The envelope bears numerous projections or spikes, approximately 8 nm long and consisting of glycoproteins, the composition of which varies widely among the different members of the family.

1.2 *Genomic organization*

Herpesvirus DNA can be distinguished on the basis of size, base composition and structural arrangement in unique and repeated sequences. The length of herpesvirus DNA varies from approximately 120 to more than 230 kilobases (kb) and is characteristic for each genus (e.g. simplex virus and cytomegalovirus). Minor variations in the size of individual isolates of the same virus are due mainly to variations in the number of internal and terminal repeat regions. Herpesviruses can be divided into six structurally distinct groups on the basis of the presence and location of repeated sequences greater than 100 base pairs (Figure 2). In group A viruses, exemplified by the human herpes-

Figure 2. Architecture of herpesvirus genomes



LTR, left terminal repeat; RTR, right terminal repeat; TR, terminal repeat; IR, internal repeat; U, unique sequence; UL, long unique sequence; US, short unique sequence; HV, herpesvirus

For symbols used in Group E, see text.

viruses 6 and 7 (HHV6 and HHV7), a large sequence from one terminus is directly repeated at the other terminus (left terminal repeat and right terminal repeat). In group B viruses, exemplified by Kaposi's sarcoma herpesvirus (HHV8; see the monograph in this volume), the primate herpesviruses saimiri (HVS or SHV-2) and ateles (HVA-2) and the mouse herpesvirus strain 68 (MHV-4), the terminal sequence is directly repeated numerous times at both termini. In group C viruses, exemplified by Epstein-Barr virus (EBV, HHV4; see the monograph in this volume), both terminal and internal repeat sequences are present throughout the viral genome, which subdivide it into well-defined unique sequences. In group D viruses, exemplified by varicella–zoster virus (VZV, HHV3) and by numerous viruses isolated from mammals and birds, the terminal region is repeated in an inverted orientation internally. The short unique region contained between the terminal repeat and this inverted form can assume two possible orientations relative to the long unique sequence, such that virions isolated from infected cells consist of two equimolar populations. A more complex architecture is observed in group E viruses, exemplified by herpes simplex viruses types 1 (HSV-1, HHV1) and 2 (HSV-2, HHV2), human cytomegalovirus (CMV, HHV5) and Marek's disease virus (MDV or GHV-2). Sequences from both termini (a_nb and a_nc) are repeated in inverse orientation and juxtaposed internally (a'_nb' and a'_nc'), dividing the genome into two components consisting of long and short unique sequences separated by inverted repeats. Both

components can occur in two orientations relative to each other, resulting in four equimolar populations of virions. There are no repeat regions in the genome of the group F viruses, exemplified by murine herpesvirus 1.

The base composition varies significantly among the herpesviruses, the guanine-cytosine (GC) content ranging from 46 mol % in VZV to 69 mol % in HSV (Roizman, 1996). An under-representation of the CpG dinucleotide pair and a relative excess of CpA and TpG dinucleotides has been observed in the lymphotropic herpesviruses, possibly due to methylation-dependent CpG suppression in rapidly dividing cells (Honess *et al.*, 1989). Despite these major differences, comparison of herpesvirus DNA sequences shows large regions of distant collinear homology at the predicted protein level. Relatively well-conserved genes code for *trans*-activating factors, enzymes involved in viral replication, such as DNA polymerase, ribonucleotide reductase and thymidine kinase, and some structural glycoproteins, including the major spike components (Stewart *et al.*, 1996). The similarities are largely restricted to the early and late genes expressed during the productive cycle, while genes expressed in latently infected cells are usually unique, suggesting a possible origin from cellular DNA.

2. Taxonomy of herpesviruses

Herpesviruses are classified into three subfamilies — alpha, beta and gamma — with different biological properties and tissue tropism. Further subdivision into genera is based on DNA sequence homology, similarities in genomic sequence arrangement and relatedness of viral proteins demonstrable by immunological methods (Roizman *et al.*, 1981; Roizman, 1982) (Table 1). A compilation of the major herpesviruses in animals is given in Table 2, which also includes a comparable list of human herpesviruses.

Table 1. Biological characteristics of herpesvirus subfamilies

Characteristic	Alpha	Beta	Gamma
Genus	Simplexvirus Varicellovirus	Cytomegalovirus Muromegalovirus	Lymphocryptovirus Rhadinovirus
Host range	Broad	Restricted	Restricted
Prevalent genomic organization	D, E	Variable	B, C
Productive cycle	Short	Long	Long
Spread in culture	Efficient	Moderate	Poor
Site of latency	Sensory ganglia	Lymphoreticular tissues	Lymphocytes
Proliferation of latently infected cells	No	No	Yes

Adapted from Roizman (1996)

2.1 *Alphaherpesviruses*

The subfamily Alphaherpesvirinae includes the genera *Simplexvirus* and *Varicellovirus*, of which the human HSV-1, HSV-2 and VZV (HHV1, 2 and 3, respectively) are the best-known examples. Members of the alpha subfamily have a relatively broad host range and may infect other, related species, in addition to their natural host species. These viruses have a relatively short productive cycle in epithelial cells and spread very efficiently in tissue cultures. A hallmark of the family is the capacity to establish latent infection primarily, but not exclusively, in sensory ganglia.

Table 2. International nomenclature of herpesviruses (shortened list)

Designation	Common name and synonyms	Sub-family	G+C (mol %)	Group	Size (kb)
Human viruses					
Human herpesvirus 1	Herpes simplex virus 1	α	68.3	E	152
Human herpesvirus 2	Herpes simplex virus 2	α	69	E	152
Human herpesvirus 3	Varicella-zoster virus	α	46	D	125
Human herpesvirus 4	Epstein-Barr virus	γ_1	60	C	172
Human herpesvirus 5	Cytomegalovirus	β	57	E	229
Human herpesvirus 6		β	42	A	162
Human herpesvirus 7		β		A	
Human herpesvirus 8		γ_2			230
Viruses of non-human primates					
Aotine herpesvirus 1	*Herpesvirus aotus* type 1	β	55	E	220
Aotine herpesvirus 3	*Herpesvirus aotus* type 3	β	56	D	219
Cercopithecine herpesvirus 1	B virus, *Herpesvirus simiae*	α	75	E	160
Cercopithecine herpesvirus 2	Herpesvirus simian agent 8 (SA-8)	α	67	E	150
Cercopithecine herpesvirus 3	Herpesvirus simian agent 6 (SA-6)	β	51		
Cercopithecine herpesvirus 4	Herpesvirus simian agent 15 (SA-15)	β			
Cercopithecine herpesvirus 5	African green monkey cytomegalovirus	β			
Cercopithecine herpesvirus 6	Liverpool vervet monkey virus	α	52		
Cercopithecine herpesvirus 7	Patas monkey herpesvirus; MMV or PHV delta herpesvirus	α			
Cercopithecine herpesvirus 8	Rhesus monkey cytomegalovirus	β	52		
Cercopithecine herpesvirus 9	Medical Lake macaque herpesvirus; simian varicella herpesvirus	α			
Cercopithecine herpesvirus 10	Rhesus leukocyte-associated herpesvirus strain I				
Cercopithecine herpesvirus 12	*Herpesvirus papio*, baboon herpesvirus	γ_1		C	170

Table 2 (contd)

Designation	Common name and synonyms	Sub-family	G+C (mol %)	Group	Size (kb)
Viruses of non-human primates (contd)					
Cercopithecine herpesvirus 13	*Herpesvirus cyclopis*				
Cercopithecine herpesvirus 14	African green monkey EBV-like virus	γ_1			
Cercopithecine herpesvirus 15	Rhesus EBV-like herpesvirus	γ_1			
Ateline herpesvirus 1	Spider monkey herpesvirus	α	72		
Ateline herpesvirus 2	*Herpesvirus ateles*	γ_2	48	B	135
Callitrichine herpesvirus 1	*Herpesvirus saguinus*				
Callitrichine herpesvirus 2	SSG, marmoset cytomegalo-virus	β			
Cebine herpesvirus 1	Capuchin herpesvirus (AL-5)	β			
Cebine herpesvirus 2	Capuchin herpesvirus (AP-18)	β			
Pongine herpesvirus 1	Chimpanzee herpesvirus; pan herpesvirus	γ_1		C	170
Pongine herpesvirus 2	Orangutan herpesvirus	γ_1			
Pongine herpesvirus 3	Gorilla herpesvirus	γ_1			
Saimiriine herpesvirus 1	Marmoset herpesvirus; herpes T, *Herpesvirus tamarinus*, *Herpesvirus platyrrhinae*	α	67	D	152
Saimiriine herpesvirus 2	Squirrel monkey herpesvirus, *Herpesvirus saimiri*	γ_2	46	B	155
Bovine viruses					
Bovine herpesvirus 1	Infectious bovine rhinotracheitis herpesvirus	α	72	D	140
Bovine herpesvirus 2	Bovine mammillitis virus, Allerton virus, pseudolumpy skin disease herpesvirus	α	64	E	133
Bovine herpesvirus 4	Movar herpesvirus	γ_2	50	B	145
Bovine herpesvirus 5	Bovine encephalitis herpesvirus	α	72	D	140
Ovine herpesvirus 1	Sheep pulmonary adenomatosis-associated herpesvirus			D	137
Ovine herpesvirus 2	Sheep-associated malignant catarrhal fever	γ		B	
Caprine herpesvirus 1	Goat herpesvirus	α			
Alcelaphine herpesvirus 1	Wildebeest herpesvirus, malignant catarrhal fever herpesvirus	γ	61	B	160
Alcelaphine herpesvirus 2	Hartebeest herpesvirus	γ		B	
Cervid herpesvirus 1	Red deer herpesvirus	α		D	
Cervid herpesvirus 2	Reindeer (*Rangifer tarandus*) herpesvirus	α		D	
Murid viruses					
Murid herpesvirus 1	Mouse cytomegalovirus	β	59	F	235
Murid herpesvirus 2	Rat cytomegalovirus		47		

Table 2 (contd)

Designation	Common name and synonyms	Sub-family	G+C (mol %)	Group	Size (kb)
Murid viruses (contd)					
Murid herpesvirus 3	Mouse thymic herpesvirus				
Murid herpesvirus 4	Mouse herpesvirus strain 68	γ_2		B	135
Murid herpesvirus 5	Field mouse herpesvirus; *Microtus pennsylvanicus* herpesvirus				
Murid herpesvirus 6	Sand rat nuclear inclusion agents				
Murid herpesvirus 7	Murine herpesvirus				
Gallid viruses					
Gallid herpesvirus 1	Infectious laryngotracheitis virus	α	46	D	165
Gallid herpesvirus 2	Marek's disease herpesvirus 1	α	47	E	180
Gallid herpesvirus 3	Marek's disease herpesvirus 2	α			
Gruid viruses					
Gruid herpesvirus 1	Crane herpesvirus				
Meleagrid herpesvirus 1	Turkey herpesvirus	α	48	E	150
Ranid viruses					
Ranid herpesvirus 1	Lucke frog herpesvirus		46		
Ranid herpesvirus 2	Frog herpesvirus		56		

Modified from Roizman (1996) and the International Committe on the Taxonomy of Viruses (Murphy *et al.*, 1995)

Lymphoma-associated herpesvirus, a lymphocryptovirus isolated from cynomolgus monkeys (*Macaca fascicularis*), is not yet included on this list since it has not yet been given an official name by the International Committee on the Taxonomy of Viruses.

2.2 *Betaherpesviruses*

The subfamily Betaherpesvirinae includes the genera *Cytomegalovirus* and *Muromegalovirus*, of which human CMV (HHV5) and murine CMV (MHV-1) are the prototypes, respectively. The recently discovered human lymphotropic herpesviruses HHV6 and HHV7 have been classified in this subfamily on the basis of their genetic homology with human CMV, although these viruses share several biological properties with the gammaherpesviruses. The betaherpesviruses have a restricted host range, and many animal species are infected with their own CMV. These viruses appear to replicate in a variety of cell types *in vivo*, including epithelial cells, while the host cell range is more restricted *in vitro*. The infection progresses slowly and is accompanied by cell enlargement (cytomegaly) and by the appearance of characteristic nuclear eosinophilic inclusion bodies, formed by the accumulation of defective particles containing enveloped viral proteins without DNA or assembled capsids. The viruses can be maintained in a latent

form in lymphoreticular cells, secretory glands, kidneys and other tissues. Human CMV is often isolated from explants of apparently normal human adenoids and salivary glands, as have CMVs from mice, rats, hamsters and guinea-pigs.

2.3 *Gammaherpesviruses*

The subfamily Gammaherpesvirinae includes the genera *Lymphocryptovirus* and *Rhadinovirus*. Viruses of this subfamily are characterized by their tropism for lymphoid cells and their capacity to induce cell proliferation *in vivo*, resulting in transient or chronic lymphoproliferative disorders, and *in vitro*, where many can immortalize the infected cells. Gammaherpesviruses have a narrow natural host range which is restricted to the family or order to which the natural host belongs. Most gammaherpesviruses replicate inefficiently in haematopoietic cells, but some have efficient productive cycles in epithelial cells and fibroblasts. Latent virus is usually detected in lymphoid organs. The lymphocryptoviruses (or gamma-1 herpesviruses) include EBV (HHV4) and related viruses of Old World primates such as chimpanzees (*Herpesvirus pan*), orangutans (*Herpesvirus orangutan*) and gorillas (*Herpesvirus gorilla*). These viruses share tropism for B lymphocytes, a genomic architecture of group B or C and similar gene organization. Furthermore, several of their structural and nonstructural proteins are antigenically related, especially among the primate viruses, resulting in the presence of cross-reactive antibodies (Gerber & Birch, 1967; Chu *et al.*, 1971; Landon & Malan, 1971). In contrast, there is little nucleotide sequence homology or antigenic cross-reactivity between the lymphocryptoviruses and the rhadinoviruses (or gamma-2 herpesviruses). The genus is exemplified by the herpesviruses of primates, such as the ateles virus of spider monkeys and the saimiri virus of squirrel monkeys and some viruses of horses (equid herpesvirus 2; Telford *et al.*, 1993) and mice (mouse herpesvirus strain 68; Sunil-Chandra *et al.*, 1992, 1994). The recently described human Kaposi's sarcoma-associated herpesvirus (KSHV or HHV8) has been classified in this genus owing to its close similarity to the saimiri virus. The rhadinoviruses have a group B genome.

2.4 *Current classification*

The current classification of herpesviruses, which is based mainly on biological properties, does not help in defining evolutionary relatedness. The distinction between the alpha, beta and gamma subfamilies has been somewhat blurred by more detailed molecular studies and by the discovery of new viruses that co-express the structural features of one subfamily and at least some biological properties of another. Good examples are HHV6 and HHV7, which are classified as betaherpesviruses on the basis of their genetic homology to human CMV although their primary T-cell tropism is a typical feature of gammaherpesviruses (Berneman *et al.*, 1992; Lusso, 1996), and Marek's disease virus, which is included in the alpha subfamily in spite of its lymphotropism and its capacity to induce proliferation of latently infected cells (Buckmaster *et al.*, 1988).

The rapid accumulation of DNA sequences provides increased opportunities to study molecular evolution and phylogenetic relationships. Several methods have been used for constructing phylogenetic trees for groups of organisms. These are usually based on the

alignment of homologous DNA or protein sequences, followed by tree construction based on various statistical criteria such as parsimony, distance matrices, maximum likelihood, invariants and paralinear distances. Alternatively, evolutionary distances have been assessed on the basis of the relative abundance of di-, tri- and tetranucleotides in representative DNA sequences. These studies have shown a relatively good consistency with the alpha, beta and gamma classification but have generally failed to clarify finer details of branching between subfamilies (Karlin *et al.*, 1994; McGeoch *et al.*, 1995). It was estimated that the three subfamilies arose between 180 and 200 million years ago, i.e. 100–160 million years before the emergence of mammals. The speciation within sublineages took place within the last 80 million years, probably including a major component of co-speciation with the host lineages (McGeoch *et al.*, 1995). Distance assessment based on dinucleotide-relative abundance placed the HHV6 genome in the most central position, i.e. nearest to the consensus herpesvirus genome, suggesting that it may be closest to the progenitor virus. According to this criterion, herpesvirus sequences are closer to the chicken than the human DNA sequence collection, indicating that the ancient host of the viruses was avian species (Karlin *et al.*, 1994).

3. Herpesviruses and human disease

An overview of the pathogenic properties of the known human herpesviruses is shown in Table 3.

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Table 3. Pathogenic properties of human herpesviruses

Herpesvirus	Prevalence in adults[a]	Transmission	Diseases associated with primary infection	Diseases associated with reactivation	Suspected tumour association
Herpes simplex type 1 (HHV1)	High	Direct contact	Oral and ocular herpes	Oral and ocular herpes	None
Herpes simplex type 2 (HHV2)	Low–intermediate	Sexual	Genital herpes	Genital herpes	None
Varicella-zoster virus (HHV3)	Intermediate–high	Inhalation, direct contact	Varicella (chickenpox)	Zoster (shingles)	None
Epstein-Barr virus (HHV4)	High	Saliva, blood	Infectious mononucleosis	Oral hairy leukoplakia (associated with severe immunodepression)	Multiple types, including Burkitt's lymphoma, nasopharyngeal carcinoma, Hodgkin's disease
Cytomegalovirus (HHV5)	Intermediate	Transplacental, saliva, blood?, urine?, semen?	Congenital infection, mononucleosis	e.g. Pneumonia, hepatitis (associated with severe immunodepression)	None
HHV6	High	Saliva	Exanthem subitum (mainly variant B), heterophil myeloma, infectious mononucleosis	Pneumonia, encephalitis, retinitis	None
HHV7	High	Saliva	Exanthem subitum, iosidu excitem	Unknown	None
Kaposi's sarcoma-associated herpesvirus (HHV8)	?	Unknown (semen?)	Unknown	Unknown	Multiple types, including Kaposi's sarcoma, primary effusion lymphoma, Castleman's disease

[a]May vary significantly among different populations

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THE MONOGRAPHS

EPSTEIN-BARR VIRUS

1. Virus–Host Interactions

1.1 Structure, taxonomy and viral gene products

1.1.1 *Structure*

Like other herpesviruses (see Figure 1 of Introduction), Epstein-Barr virus (EBV) has a toroid-shaped protein core that is wrapped with DNA, a nucleocapsid with 162 capsomers, a protein tegument between the nucleocapsid and the envelope and an outer envelope with external glycoprotein spikes. The major EBV capsid proteins are 160, 47 and 28 kDa, similar in size to the major capsid proteins of herpes simplex virus. The most abundant EBV envelope and tegument proteins are 350/220 and 152 kDa, respectively (Kieff, 1996).

1.1.2 *Taxonomy*

EBV is the most extensively studied gammaherpesvirus and the prototype of the lymphocryptovirus subfamily. The gammaherpesvirus classification was initially established on the basis of similarity in biological properties rather than structural characteristics. Like other gammaherpesviruses, EBV establishes latent infection in lymphocytes and can induce proliferation of the latently infected cells. Taxonomists have renamed EBV human herpesvirus 4 (HHV4).

Two major EBV types have been detected in humans (Gerber *et al.*, 1976a; Adldinger *et al.*, 1985; Zimber *et al.*, 1986; Young *et al.*, 1987; Rowe *et al.*, 1989; Sixbey *et al.*, 1989; Gratama *et al.*, 1992; Walling *et al.*, 1992). Type 1 and type 2 EBV genomes (formerly referred to as types A and B) are nearly identical, except for the genes that encode some of the nuclear proteins (EBV nuclear antigen, EBNA-2, EBNA-3A, -3B, -3C; see section 1.5.2) in latently infected cells (Nonoyama & Pagano, 1973; Bornkamm *et al.*, 1980). These differences are reflected in type-specific and type-common epitopes for antibody (Young *et al.*, 1987) and T-cell responses (Moss *et al.*, 1988). Antibodies to type-2 viruses are somewhat more prevalent in African sera than in sera from people in developed countries, but the recovery of type-2 virus from blood is unusual (Young *et al.*, 1987; Rowe *et al.*, 1989), perhaps because EBV type-2 infected lymphocytes grow less efficiently *in vitro* (Rickinson *et al.*, 1987). A naturally occurring EBV recombinant that encodes both type-1 and type-2 nuclear antigen sequences was isolated from the blood of a healthy adult in Papua–New Guinea (Burrows *et al.*, 1996a).

In addition to type-specific polymorphism, significant DNA sequence heterogeneity has been found by comparison of selected regions of the genome in EBV isolated in certain geographical areas or even from the same area. These polymorphisms define

different viral strains within types 1 and 2 (Aitken *et al.*, 1994). Some of the changes cause amino-acid substitutions in viral proteins and may even affect peptides that are important for the immune control of viral infection (de Campos-Lima *et al.*, 1993a; Lee *et al.*, 1993a; de Campos-Lima *et al.*, 1994; Lee *et al.*, 1995a; Burrows *et al.*, 1996b).

1.1.3 *Host range*

Under normal circumstances, EBV infection is restricted to humans. Antibodies to EBV have been detected in several primate species, probably due to the presence of cross-reactive antibodies against their own species-specific EBV homologues (reviewed by Kieff *et al.*, 1979). Such cross-reactive immunity may explain why these animals are usually refractory to experimental infection, although their B lymphocytes can be efficiently transformed *in vitro* (Miller *et al.*, 1972; Levine *et al.*, 1980a). New World monkeys carry no EBV-like virus, and at least three species appear to be infectable experimentally. The cotton-topped tamarin (*Saguinus oedipus oedipus*) and owl monkey (*Aotus trivirgatus*) are susceptible to EBV-induced B-cell lymphomas (Epstein *et al.*, 1973; Shope & Miller, 1975), while in more limited studies with the common marmoset (*Callithrix jacchus*) induction of a mononucleosis-like syndrome was seen in some animals (Wedderburn *et al.*, 1984). Infection of newborn marmosets with EBV resulted in the establishment of a long-term permissive infection, indicating similarities in the responses of marmosets and humans to EBV (Cox *et al.*, 1996).

1.1.4 *Target cells*

EBV is a B-lymphotropic herpesvirus, and infection of B cells is mediated through interaction of the viral envelope glycoprotein gp350 (gp340)/220 with the receptor for the C3d complement component CR2 (CD21; Fingeroth *et al.*, 1984, 1988). After binding of the viral particle to the surface of the host cell and endocytosis, the viral envelope fuses with the host cell membrane by a mechanism involving three other viral glycoproteins, gp85, gp25 and gp42 (Li *et al.*, 1995a). Interestingly, gp42 can bind to major histocompatibility complex (MHC) class II, and EBV uses this as a cofactor in the infection of B lymphocytes (Li *et al.*, 1997). The notion that EBV is a predominantly if not exclusively B-lymphotropic virus has been challenged, initially on the basis of the detection of EBV in non-B-cell tumours, notably certain T-cell lymphomas and some carcinomas. In addition, it has become apparent that expression of the C3d/EBV receptor is not restricted to B cells. Thus, identical or related molecules have been detected in T cells, follicular dendritic reticulum cells and, possibly, some epithelial cells (Reynes *et al.*, 1985; Fingeroth *et al.*, 1988; Timens *et al.*, 1991; Birkenbach *et al.*, 1992; Hedrick *et al.*, 1992; Sinha *et al.*, 1993). The cellular tropism of EBV is clearly not restricted through CD21, however; thus, the virus has been detected in CD21-negative cells. Moreover, some CD21-positive cells, such as follicular dendritic reticulum cells, have proved difficult to infect *in vitro* and have been shown consistently to contain no EBV *in vivo*.

Current evidence suggests that EBV infection in healthy chronic virus carriers is largely restricted to B cells, although in certain situations the virus can be detected in non-neoplastic epithelial cells and T lymphocytes as well (see section 1.2.1).

1.1.5 *Genome, episomal and integrated viral forms and gene products*

The EBV genome is a linear, double-stranded, 172-kb DNA molecule (Kieff, 1996). It has been speculated that the observed relative underrepresentation of CpG dinucleotides may correlate with the latent status of the viral genome (Honess *et al.*, 1989). The genome of the prototype laboratory strain B95-8 has been sequenced as a set of *Bam*HI-restriction fragments (Baer *et al.*, 1984). The nomenclature for EBV open reading frames (ORFs) is based on the *Bam*HI-restriction fragment in which they are found. For example, the *BARF1* ORF is found in the *Bam*HI A fragment and extends rightwards. The *BLLF2* ORF is the second leftwards ORF in the *Bam*HI L fragment (Table 1, Figure 1). The genome has the structural organization of group C herpesviruses with reiterated 0.5-kb terminal direct repeats and a reiterated 3-kb internal direct repeat, which divide the genome into short and long, largely unique sequences. The unique sequences contain several perfect or imperfect tandem repeat elements, many of which encode for repeat domains in proteins. EBV isolates differ in their tandem repeat reiteration frequency. Thus, differences in protein size are often useful markers of specific isolates and strains (Gratama *et al.*, 1990). Furthermore, latent EBV infection is usually characterized by persistence of the genome as an episome with a characteristic number of terminal repeats. The fact that each progeny cell tends to have the same number of terminal repeats can be used to determine whether latently infected cells have arisen from a common progenitor (Raab-Traub & Flynn, 1986).

Circular episomal EBV DNA is detected in the nuclei of blood lymphocytes infected *in vitro* within 12–16 h of infection (Hurley & Thorley-Lawson, 1988). Probably only DNA repair synthesis is required for circularization, because significant nucleotide incorporation does not occur until 48 h after infection (Sixbey & Pagano, 1985). Stable, latently infected, proliferating so-called 'lymphoblasts' contain multiple EBV episomes (Adams & Lindahl, 1975; Kaschka-Dierich *et al.*, 1976; Lindahl *et al.*, 1976). EBV episomes are replicated early in the S phase by cell DNA polymerase (Hampar *et al.*, 1974; Gussander & Adams, 1984; Shaw, 1985); the initial amplification requires DNA synthesis early in the S phase (Hampar *et al.*, 1974), and the number of episomes increases thereafter over several days (Sugden *et al.*, 1979), probably due to effects of EBNA-1 on S-phase *oriP*-dependent DNA replication, although the abundance of EBNA-1 does not appear to correlate with the episome copy number (Ernberg *et al.*, 1977; Yates *et al.*, 1984; Sternås *et al.*, 1986). Episomal DNA is likely to be necessary for lytic cycle EBV DNA replication, since viral production has not been observed in cells that contain only integrated EBV DNA, and circular DNA copy numbers increase during lytic infection (Shaw, 1985).

Although episomal forms are commonest, the EBV genome can also persist by integrating into chromosomal DNA (Henderson *et al.*, 1983; Matsuo *et al.*, 1984; Lawrence *et al.*, 1988; Hurley *et al.*, 1991a,b; Delecluse *et al.*, 1993a). Few cell lines with integrated EBV DNA have been studied in detail. In the Burkitt's lymphoma line Namalwa, EBV DNA has persisted by integrating into 1p35, and the entire block of cell DNA with the integrated EBV genome is duplicated as an inverted DNA domain

Table 1. Identified EBV gene products and their open reading frames

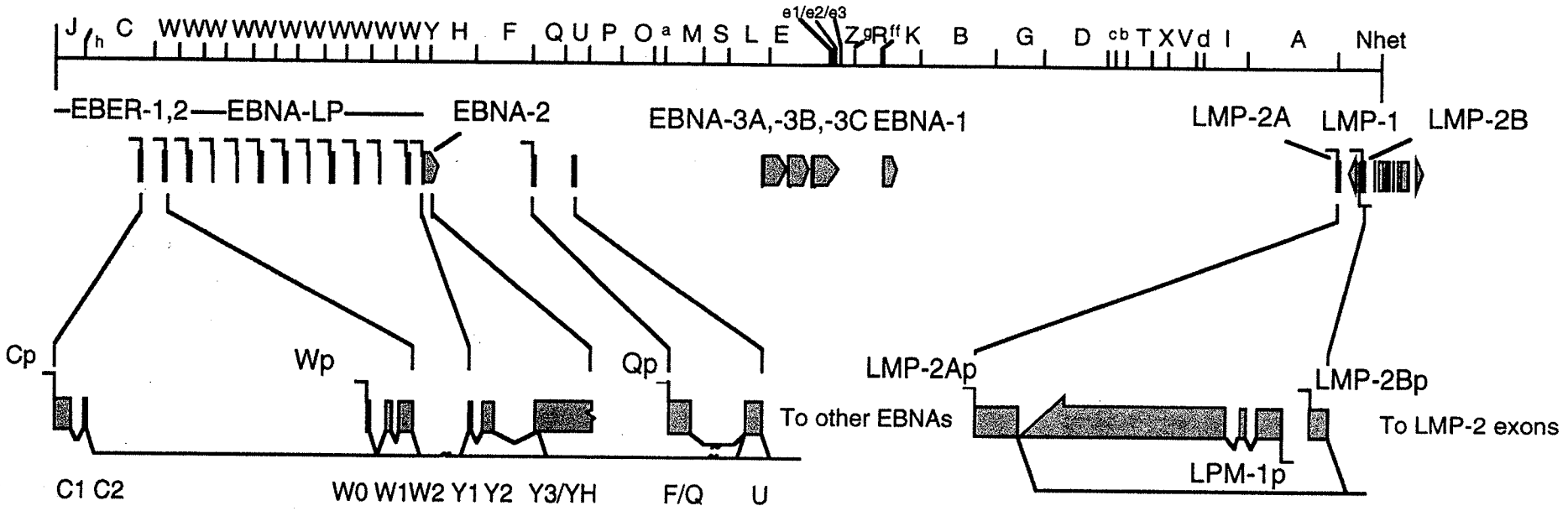
Open reading frame	Common name	Proposed function
Latent genes		
BKRF1	*EBNA-1*	Plasmid maintenance
BYRF1	*EBNA-2*	*trans*-Activation, transformation
BERF1	*EBNA-3A*	*trans*-Activation, transformation
BERF2	*EBNA-3B*	Unknown
BERF3/4	*EBNA-3C*	*trans*-Activation, transformation
BWRF1	*EBNA-LP*	*trans*-Activation, transformation
BNLF1	*LMP-1*	Transformation
BNRF1	*LMP-2A/2B*	Maintenance of latency
BARF0[a]		Unknown
Immediate early genes		
BZLF1	*ZEBRA*	*trans*-Activation, initiation of lytic cycle
BRLF1		*trans*-Activation, initiation of lytic cycle
BI'LF4		*trans*-Activation, initiation of lytic cycle
Early genes		
BMRF1		*trans*-Activation
BALF2		DNA binding
BALF5		DNA polymerase
BORF2		Ribonucleotide reductase subunit
BARF1		Ribonucleotide reductase subunit
BXLF1		Thymidine kinase
BGLF5		Alkaline exonuclease
BSLF1		Primase
BBLF4		Helicase
BKRF3		Uracil DNA glycosylase
Late genes		
BLLF1	*gp350*	Major envelope glycoprotein
BXLF2	*gp85 (gH)*	Virus–host envelope fusion
BKRF2	*gp25 (gL)*	Virus–host envelope fusion
BZLF2	*gp42*	Virus–host envelope fusion, binds MHC class II
BALF4	*gp110 (gB)*	Unknown
BDLF3	*gp100–150*	Unknown
BILF2	*gp55–80*	Unknown
BCRF1		Viral interleukin-10
BHRF1[a]		Viral *bcl-2* analogue

Adapted from Li *et al.* (1995a); Nolan & Morgan (1995)

[a] Expressed in latently infected cells as well

EBNA, EBV nuclear antigen; LP, leader protein; LMP, latent membrane protein; ZEBRA, Z EBV replication activation; gp, glycoprotein; MHC, major histocompatibility complex

Figure 1. *Bam*HI-restriction map of the EBV genome and latent transcripts



Adapted from Masucci and Ernberg (1996) and Rickinson and Kieff (1996)
EBER, EBV-encoded RNA; EBNA, Epstein-Barr nuclear antigen; LMP, latent membrane protein
Other abbreviations are explained in the text.

(Henderson *et al.*, 1983; Lawrence *et al.*, 1988). In the human fetal lymphoblastoid cell line IB4, multiple EBV genome copies are integrated in tandem into 4q25 (Matsuo *et al.*, 1984; Hurley *et al.*, 1991b). Integration occurs frequently when Burkitt's lymphoma cells are infected with EBV *in vitro* (Hurley *et al.*, 1991a). Clearly, integration is not chromosome site-specific or a regular feature of EBV infection.

In the establishment of latent infection, most of the EBV genome undergoes progressive methylation (Kintner & Sugden, 1981; Larocca & Clough, 1982; Perlmann *et al.*, 1982), whereas regulatory domains involved in maintaining latent infection, such as *oriP*, tend to remain under-methylated (Minarovits *et al.*, 1992). Extensive methylation of parts of the genome not expressed in latent infection may help to maintain latency by inhibiting lytic gene expression (Nonkwelo & Long, 1993). Treatment of latently infected cells with drugs that reduce DNA methylation increases the frequency of cells entering the productive cycle (Ben-Sasson & Klein, 1981).

EBV infection of primate B lymphocytes *in vitro* is usually nonproductive and results in the expression of a restricted number of viral genes and continuous proliferation. EBV-infected proliferating B lymphocytes are similar to lymphocytes that are proliferating in response to antigens, mitogens or interleukin (IL)-4 and anti-CD40 in that they express a similar repertoire of activation-associated markers, secrete similar immunoglobulins and have similar intercellular adherence (Åman *et al.*, 1986; Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991; Banchereau *et al.*, 1991). At least 11 EBV genes are expressed in latently infected B lymphocytes (Table 2, Figure 1). Two of these encode small, non-polyadenylated RNAs (the EBV-encoded RNAs EBER-1 and EBER-2), six encode nuclear proteins (the EBV nuclear antigens EBNA-1, EBNA-2, EBNA-3, -4 and -6, also called EBNA-3A, -3B and -3C, and EBNA-5, also called leader protein, LP), and three encode integral membrane proteins (the latent membrane proteins LMP-1 and LMP-2A and -2B, also called terminal proteins 1 and 2). Other viral mRNAs encoded for example by the *BHRF1* and *BARF0* ORFs have been detected in latently infected cells, but their products are not well characterized (reviewed by Kieff, 1996).

Different patterns of viral gene expression have been identified in latently infected, EBV-carrying cells *in vivo* (Table 3). Expression of all the latent viral genes detected in lymphoblastoid cell lines *in vitro* is a feature of the immunoblastic lymphomas of immunosuppressed patients. This form of latency is commonly defined as latency III. Latency I was first detected in EBV-positive Burkitt's lymphoma cells and is characterized by restricted expression of the EBERs, EBNA-1 and probably the product of the *BARF0* ORF. Latency II, characterized by the expression of EBERs, EBNA-1 and the three LMPs, was first recognized in a proportion of nasopharyngeal carcinomas but also seems to be common in most EBV-carrying non-B-cell tumours and in Hodgkin's disease (reviewed by Rickinson & Kieff, 1996). The latency II type has been reproduced *in vitro* by stable infection of a human T-cell line with a recombinant EBV carrying a selectable marker (Yoshiyama *et al.*, 1995). An additional form of latency characterized by restricted expression of EBERs, EBNA-1 and EBNA-2 has been described in smooth-muscle tumours arising in immunosuppressed children and AIDS patients (Lee *et al.*, 1995b).

Table 2. Nomenclature of latent viral gene products

Adopted terminology	Alternative nomenclature	
EBNA-1	EBNA-1	EBNA-1
EBNA-2	EBNA-2	EBNA-2
EBNA-3A	EBNA-3	EBNA-3A
EBNA-3B	EBNA-4	EBNA-3B
EBNA-3C	EBNA-6	EBNA-3C
EBNA-LP	EBNA-5	EBNA-4
LMP-1		
LMP-2A	TP-1	
LMP-2B	TP-2	
EBER-1		
EBER-2		

EBNA, EBV nuclear antigen; LP, leader protein; LMP, latent membrane protein; EBER, EBV-encoded RNA; TP, terminal protein

Table 3. Patterns of EBV latent gene expression

Type of latency	Gene product	Examples	Reference
I	EBERs, EBNA-1	Burkitt's lymphoma	Rowe *et al.* (1987a)
		Gastric carcinoma	Imai *et al.* (1994a)
II	EBERs, EBNA-1, LMP-1, -2A, -2B, BARF0	Hodgkin's disease	Deacon *et al.* (1993)
		Nasopharyngeal carcinoma	Hitt *et al.* (1989); Brooks *et al.* (1992)
III	EBERs, EBNA-1, -2, -3A, -3B, -3C, -LP LMP-1, -2A, -2B	Post-transplant lympho-proliferative disorder	Young *et al.* (1989a)
		Infectious mononucleosis	Tierney *et al.* (1994)
Other	EBERs, EBNA-1, -2	Smooth-muscle tumours	Lee *et al.* (1995b)

EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; LMP, latent membrane protein; LP, leader protein

With a frequency characteristic of each EBV-infected B-lymphocyte line, some progeny spontaneously become permissive for viral replication. In these cells, the viral DNA is amplified several hundred-fold by a lytic origin of DNA replication, *oriLyt* (Hammerschmidt & Sugden, 1988). Multimeric forms produced from presumably circular templates are generated from *oriLyt* following a pattern that could result from rolling-circle DNA replication (Pfüller & Hammerschmidt, 1996). The expression of viral genes associated with productive infection follows a temporal and sequential order (reviewed by Kieff, 1996). Some viral genes are expressed early after induction, indepen-

dently of new protein synthesis, and are therefore classified as immediate early genes. Early lytic genes are expressed slightly later, and their expression is not affected by inhibition of viral DNA synthesis. Genes the expression of which is blocked by inhibitors of viral DNA synthesis are categorized as late genes. Because epithelial cells are fully permissive for lytic EBV infection *in vivo* (Lau *et al.*, 1993; Ryon *et al.*, 1993), considerable efforts have been made to adapt EBV to grow in organ cultures of transformed epithelial cell lines. These cultures can be infected, but the infection is often abortive or inefficient and very little virus is produced (Li *et al.*, 1992). Therefore, most of our knowledge of latent or lytic EBV products is derived from the study of EBV-infected B lymphocytes.

1.1.5.1 *EBV-encoded RNAs*

The two EBV-encoded, small, non-polyadenylated RNAs (EBER-1 and -2) are by far the commonest viral RNAs in latently infected cells, with an estimated abundance of 10^7 copies per cell (Howe & Steitz, 1986; Howe & Shu, 1989). They are usually transcribed by RNA polymerase III, although polymerase II may also be involved (Howe & Shu, 1989). Most EBERs are located in the nucleus and are associated at the 3′ terminus with the cellular La antigen (Howe & Steitz, 1986) and other cellular proteins, including the EBER-associated protein, which shows strong resemblance to an endodermal, developmentally regulated sea-urchin protein called 217 (Toczyski & Steitz, 1991). The primary sequences of EBER-1 and -2 show extensive similarity to those of adenovirus VAI and VAII and cell U6 small RNAs (Glickman *et al.*, 1988). These RNAs form stable secondary structures that allow extensive intermolecular base-pairing and may have, therefore, similar functions. On the basis of the known function of the RNAs of VA and U6, two alternative roles have been proposed for the EBERs. In adenovirus infection, VAI RNA inhibits the activation of an interferon-induced protein kinase which blocks transcription by phosphorylating the protein-synthesis initiator factor eIF-2 α. EBER-1 and -2 can partially complement the replication of an adenovirus with null mutations in VAI and VAII, but their effect on eIF-2 α kinase activity is significantly smaller and they are not found in the cytoplasm (Kieff, 1996). A possible role of the EBERs in the splicing of the primary EBNA and LMP mRNA transcripts is suggested by the partial complementarity to RNA splice sites. Complexes between U6 and U4 RNAs are required for RNA splicing *in vitro*, and the EBER-2 RNA contains a single-stranded loop with six of seven nucleotides identical to those required for U6–U4 interaction (Glickman *et al.*, 1988). Both of the proposed functions of EBERs are somewhat incompatible with the observation that their expression is delayed until after EBNA and LMP gene expression and initiation of DNA synthesis (Alfieri *et al.*, 1991). Nevertheless, the earlier events in primary B-cell infection are sensitive to interferon (IF) (Thorley-Lawson, 1980, 1981), and EBERs may play a role in blocking eIF-2 kinase. EBV recombinants in which the EBERs have been deleted can initiate primary B-cell infection and growth transformation in the same way as wild-type virus, and no differences were seen in the growth of lymphoblastoid cell lines infected with EBER-deleted and control virus or in the permissiveness of these cells for lytic infection (Swaminthatan *et al.*, 1991). The EBER

transcription unit was found to be unmethylated in all types of EBV-carrying cells (Minarovits *et al.*, 1992).

1.1.5.2 *Nuclear proteins*

Transcription of nuclear proteins is initiated at polymerase II-dependent promoters in the *Bam*HI C (Cp) and *Bam*HI W (Wp) regions (reviewed by Rogers *et al.*, 1992). Approximately at the time of genome circularization, the Wp promoter initiates rightwards transcription that proceeds through almost the entire strand of the genome (Bodescot *et al.*, 1986). The mRNAs contain a 40-base first exon, W0 (Rogers *et al.*, 1992), which is spliced to alternating W1 and W2 exons derived from successive reiteration of the internal *Bam*HI W repeats (Bodescot & Perricaudet, 1986; Sample *et al.*, 1986). The use of an alternative splice acceptor site in W1 results in generation of an ATG codon in about half of the transcript. This initiates translation of an ORF encoded by the repeating W1 and W2 exons and by two short exons in the *Bam*HI Y region (Y1 and Y2). The resulting protein, called EBNA-LP, is encoded in the leader of the EBNA mRNAs and is therefore also called leader protein (LP) (Kieff, 1996).

The Y2 exon is spliced to a long exon, YH, that spans the right end of *Bam*HI W and the left end of *Bam*HI H (Kieff, 1996). The ORF of this exon encodes the nuclear protein EBNA-2. EBNA-LP and EBNA-2 are the first viral proteins expressed in infected B lymphocytes. They are already detected 12 h after infection and reach the levels maintained in transformed lymphoblastoid cell lines within 24–32 h after infection (Alfieri *et al.*, 1991). Probably as a consequence of the action of EBNA-2 on response elements located in the upstream *Bam*HI C fragment, a switch from Wp to Cp usage occurs in most of the infected cells (Woisetschlaeger *et al.*, 1990). Two exons downstream of Cp, C1 and C2, replace the W0 exon in those transcripts. As with the W0 exon, alternative splicing of C2 to W1 or W0 results in mRNAs incapable or capable of translating EBNA-LP, respectively. A potential splice donor site near the beginning of the YH exon, but preceding the EBNA-2 initiation codon, is activated in some of the transcripts and is spliced to a downstream acceptor in *Bam*HI U. The U exon is further spliced to any of four alternate acceptor sites that begin the ORFs encoding EBNA-3A, EBNA-3B, EBNA-3C or EBNA-1. Thus, as a consequence of alternate 5′ splicing of Cp- or Wp-initiated RNAs, some RNAs will encode EBNA-LP or EBNA-LP and EBNA-2, while other RNAs will exclusively code for EBNA-3A, -3B, -3C or EBNA-1. The turning on of EBNA-3A, -3B, -3C and EBNA-1 expression is probably explained by the strength of the Cp, which leads to significantly more transcription. Polyadenylation may determine splicing and thereby regulate the expression of the six EBNA mRNAs from the same promoter. *cis*- or *trans*-Acting factors, such as the expression of certain viral products or the formation of secondary structures in the primary EBNA transcripts, may also play a role in splice choice.

In cells that express a latency I or II phenotype, the Cp and Wp are silenced and extensively methylated (Ernberg *et al.*, 1989; Schaefer *et al.*, 1997). Transcription of the EBNA-1 message is initiated at an alternative promoter located in the FQ transition region and, like the Cp- and Wp-initiated messages, splices to the U and K exons.

Characterization of the transcripts originating from this region has been complicated by the presence of several transcription–initiation start sites and by the overlapping of promoters that are active during the latent and lytic phases of the cycle. It seems now clear that the originally described Fp is a lytic promoter (Nonkwelo *et al.*, 1995, 1996), while transcription of the latency I EBNA-1 mRNAs begins at the adjacent Qp (Nonkwelo *et al.*, 1997).

(*a*) *EBNA-LP*

The size of EBNA-LP varies among isolates, owing to varying numbers of internal repeats (IR1) that contain the W1 and W2 exons of LP (Dillner *et al.*, 1986; Finke *et al.*, 1987; Wang *et al.*, 1987a). The W2 exon encodes for a 44-amino acid repeat containing basic Arg-Arg-His-Arg and Arg-Arg-Val-Arg-Arg-Arg domains that could be nuclear localization signals. EBNA-LP is phosphorylated, probably on serine residues located in the W2 exon near a casein kinase II phosphorylation site (Petti *et al.*, 1990; Kieff, 1996). The protein is strongly associated with the nuclear matrix and has an unusual location, as seen by immunofluorescence microscopy (Petti *et al.*, 1990; Jiang *et al.*, 1991). Some EBNA-LP is diffusely spread through the nucleus, while the rest is concentrated in a few granules, frequently distributed in curved linear arrays.

Molecular analysis of EBNA-LP has focused on the last two exons that are deleted in the nontransforming P3HR-1 EBV strain. Deletion of the exons resulted in EBV recombinants with significantly reduced growth transforming ability under standard in-vitro culture conditions (Allan *et al.*, 1992); however, only a modest reduction of transformation efficiency was observed by plating infected B lymphocytes in soft agarose over fibroblast feeder layers (Hammerschmidt & Sugden, 1989; Mannick *et al.*, 1991). The mutant virus-infected lymphoblastoid cell lines also tended to be more differentiated towards immunoglobulin secretion. The unusual fibroblast dependency and enhanced differentiation suggest that EBNA-LP may indirectly or directly up-regulate the expression of autocrine factors required for B-cell growth. Transient transfection of EBNA-LP and EBNA-2 into primary B lymphocytes co-stimulated with gp350 indicated that the two proteins cooperate in the induction of G0 to G1 transition, as marked by induction of cyclin D2 (Sinclair *et al.*, 1994), but the mechanism of this effect remains unknown.

(*b*) *EBNA-2*

EBNA-2 differs extensively between the EBV type-1 and type-2 isolates (Aitken *et al.*, 1994) and is the primary determinant of the biological differences that enable the type-1 strains to transform B lymphocytes with greater efficiency (Rickinson *et al.*, 1987). The two proteins consist of 484 and 443 amino acids, respectively; they are overall acidic, phosphorylated on threonine and serine residues and contain at least seven discrete domains including a polyproline repeat that varies by 10–40 amino acids in different EBV isolates. The carboxy-terminal domain contains two Arg-Gly and Lys-Arg-Pro-Arg repeat sequences that are required for nuclear localization (Cohen *et al.*, 1991). EBNA-2 localizes in large nuclear granules and is associated with nucleoplasmic, chromatin and nuclear matrix fractions (Petti *et al.*, 1990).

EBNA-2 is a specific *trans*-activator of cellular genes, such as the B-cell activation markers CD23 (Wang *et al.*, 1987b, 1990a, 1991) and CD21 (Wang *et al.*, 1990a) and the *c-fge* oncogene (Knutson, 1990), and viral genes including *LMP-1* (Abbot *et al.*, 1990; Ghosh & Kieff, 1990; Wang *et al.*, 1990b; Tsang *et al.*, 1991; Fåhraeus *et al.*, 1993) and *LMP-2* (Tsang *et al.*, 1991) and the *cis*-acting element upstream of the Cp (Sung *et al.*, 1991; Walls & Perricaudet, 1991; Sjöblom *et al.*, 1995a). The EBNA-2-responsive elements have been partially defined by deletion analysis of EBV promoter constructs or by positioning near a heterologous promoter (Laux *et al.*, 1994a). Molecular analysis of EBNA-2 by rescue of transforming virus from P3HR1-infected cells or the *trans*-activation of LMP-1 in transiently transfected Burkitt's lymphoma cells as phenotypic markers reveals a correlation between the sequences of EBNA-2 that are essential for transformation and those important for promoter *trans*-activating activity (Cohen *et al.*, 1991). Two large deletions, between amino acids 112–230 and 463–483, were shown to reduce transformation and *trans*-activating activity but still result in virus capable of transforming cells, albeit with low efficiency. Deletion of the Arg-Gly oligomer domain in residues 337–357 resulted in a 90% reduction of transforming efficiency but increased *trans*-activation of the LMP-1 promoter by at least 10-fold (Tong *et al.*, 1994; Kieff, 1996). This domain can interact with histones, potentially facilitating the interaction of EBNA-2 with DNA. Only three regions, located between residues 95–110, 280–337 and 425–462, appear to be stringently required for transformation and *trans*-activating activity. The 425–462 region is essential for its acidic *trans*-activating characteristics, while the 280–337 region mediates the interactions with DNA sequence-specific binding proteins that bring EBNA-2 to its responsive elements. The function of the 95–110 region is unclear (Kieff, 1996). Detailed analysis of the 425–462 region indicates that this domain is similar in many respects to the prototype VP16 acidic domain (Cohen & Kieff, 1991; Cohen, 1992), part of which can substitute for part of the EBNA-2 domain in the context of reconstituted EBV recombinants (Cohen, 1992). The two domains share affinity for the transcription factors TFIIB, TAF40, TFIIH and RPA70, suggesting a critical role of the 425–462 region in recruiting these factors to EBNA-2-responsive promoters (Tong *et al.*, 1995).

EBNA-2 does not interact directly with its responsive elements. The response elements in the LMP-1, LMP-2 and Cp promoters have been intensively investigated by electrophoretic mobility shift assays with nuclear extracts from EBNA-2-positive cells. Each responsive element contains at least two gel shift activities: the major activity corresponds to an oligonucleotide that includes the GTGGGAA motif (Zimber-Strobl *et al.*, 1991; Jin & Speck, 1992; Ling *et al.*, 1993a). A 28-amino acid polypeptide corresponding to residues 310–336 of EBNA-2 was used to affinity purify a 63-kDa nuclear protein that reproduced the gel shift pattern (Yalamanchili *et al.*, 1994). Sequencing of p63 showed it to be the previously characterized recombination signal sequence binding protein Jκ, a widely expressed and highly conserved protein that probably acts as a key adapter for transcription regulatory factors of cellular genes (Grossman *et al.*, 1994; Henkel *et al.*, 1994). Additional protein binding sites are present in the EBNA-2-responsive elements. Mutation of the PU.1 binding site has a profound effect on the EBNA-2 responsiveness of the LMP-1 promoter (Johannsen *et al.*, 1995; Sjöblom *et al.*,

1995a,b). PU.1 is also likely to be an important factor for many of the cellular genes that are activated by EBNA-2, as it is frequently involved in B lymphocyte-specific gene transcription.

(*c*) *EBNA-3A, -3B, -3C*

EBNA-3A, -3B and -3C are encoded by three genes placed tandemly in the EBV genome. Each protein is encoded by a short and a long exon 3′ to the respective mRNA (Hennessy *et al.*, 1985, 1986; Kallin *et al.*, 1986; Joab *et al.*, 1987; Petti & Kieff, 1988; Petti *et al.*, 1988; Ricksten *et al.*, 1988). The mRNAs that encode these proteins are the least abundant EBNA mRNAs, few molecules occurring in each latently infected cell. The proteins encoded by type-1 and type-2 EBV strains are only 84, 80 and 72% identical in the predicted primary amino acid sequence (Sample *et al.*, 1990). Each protein contains different repeating polypeptide domains near their carboxy termini that are responsible for size variation in different viral isolates. These EBNAs are remarkably hydrophobic and contain repeats of hydrophobic leucines, isoleucines or valines that could facilitate homo- and heterodimerization. They are located in large nuclear clumps in the nuclear matrix, chromatin and nucleoplasmic fractions but not in the nucleolus (Petti *et al.*, 1990).

Because of their structure and sequence similarity, these proteins are likely to have similar functions in latent EBV infection and transformation. EBNA-3C was shown to up-regulate the expression of CD21 mRNA in transfected Burkitt's lymphoma cells (Wang *et al.*, 1990a) and the expression of LMP-1 in Raji cells (Allday *et al.*, 1993; Allday & Farrell, 1994), while expression of EBNA-3B was shown to correlate with up-regulation of vimentin and CD40 and down-regulation of CD77 (Silins & Sculley, 1994). EBNA-3A, -3B and -3C can inhibit EBNA-2-activated transcription, probably by their capacity to bind to Jκ (Robertson *et al.*, 1995a; Krauer *et al.*, 1996; Robertson *et al.*, 1996). The complexes do not bind to DNA *in vivo*, suggesting a possible mechanism by which these proteins could modulate B-cell activation.

(*d*) *EBNA-1*

EBNA-1 is the only EBNA that is associated with chromosomes during mitosis (Reedman *et al.*, 1974; Ohno *et al.*, 1977; Grogan *et al.*, 1983). The predicted sequence of the prototype EBV type 1 strain consists of 641 amino acids that form four obvious domains: an amino terminus of 89 amino acids rich in basic residues, a 239-amino acid Gly-Ala copolymer that could form β sheets and may participate in intermolecular interactions, a short basic domain and a long hydrophobic domain from residues 459–607 which have sequence-specific DNA binding and dimerization activities (Ambinder *et al.*, 1991; Shah *et al.*, 1992; Kieff, 1996). *EBNA-1* is phosphorylated on serine residues in the carboxy terminus of the molecule (Hearing & Levine, 1985; Polvino-Bodnar *et al.*, 1988).

EBNA-1 binds to DNA by recognizing the partial palindrome TAGGATAGCATA-TGCTACCCAGATCCAG that is found at three sites in the EBV genome (Rawlins *et al.*, 1985; Ambinder *et al.*, 1990; Kieff, 1996). The site with the greatest affinity consists of 20 tandem direct repeats of the cognate sequence about 7 kb from the left end

of the genome. The second highest affinity site is 1 kb to the right and consists of two cognate sequences in dyad symmetry and two in tandem. The third site is in the *Bam*HI Q fragment about 10 kb downstream of the *EBNA-2* coding exon (Rawlins *et al.*, 1985). This site is composed of two divergent tandem repeats and appears to be important for negative regulation of the alternative promoter for latent *EBNA-1* transcription in *Bam*HI FQ (Lear *et al.*, 1992; Sample *et al.*, 1992). Binding of *EBNA-1* to the tandem repeat and dyad symmetry sites enables covalently closed, circular DNA molecules to replicate and persist as episomes (Reisman *et al.*, 1985; Yates *et al.*, 1985; Yates & Guan, 1991). The EBV DNA segment containing both sites is therefore designated *oriP*, for origin of plasmid DNA replication. The tandem repeat component acts as an *EBNA-1*-dependent enhancer on heterologous or neighbouring EBV promoters in transient transfection assays (Reisman & Sugden, 1986). The dyad symmetry component is stringently required for episome replication. The structure is partially denatured as a consequence of *EBNA-1* binding, forming bubble and cruciform structures that are characteristic of other efficient origins (Williams & Kowalski, 1993). The interaction of *EBNA-1* with the tandem repeat and dyad symmetry sites is cooperative and results in higher-order structures that lead to bending of the DNA, distortion of the duplex and looping out of the intervening sequences (Frappier & O'Donnell, 1991; Orlowski & Miller, 1991; Frappier & O'Donnell, 1992). The amino terminal of the dimerization domain is important for the formation of macromolecular complexes of *EBNA-1* homopolymers after association with the DNA templates (Goldsmith *et al.*, 1993). The carboxy terminus of *EBNA-1* determines its nuclear location by interacting with a specific protein that is homogeneously distributed on chromosomes (Ohno *et al.*, 1977; Harris *et al.*, 1985; Petti *et al.*, 1990). This property is likely to be important for segregation of episomes into progeny nuclei during mitosis. Part of *EBNA-1* is also associated with the nuclear matrix. EBNA-1 is the only EBNA that continues to be made during lytic infection. The lytic mRNA begins at a *Bam*HI F promoter near 62.2 kb (Lear *et al.*, 1992; Kieff, 1996). The two *EBNA-1* binding sites that are not part of *ori-P* are downstream of the adjacent latency I promoter in *Bam*HI Q (kb 62.3). Thus, *EBNA-1* is likely to play a role in regulation of these promoters (Sample *et al.*, 1992; Kieff, 1996).

1.1.5.3 *Latent membrane proteins*

(*a*) *LMP-1*

LMP-1 mRNA is the second most abundant viral transcript in latently infected cells (Fennewald *et al.*, 1984; Sample & Kieff, 1990). The product, encoded by three exons, is an integral membrane protein with at least three domains: a 20-amino acid hydrophilic amino terminus; six hydrophobic, 20-amino acid, alpha helical transmembrane segments separated by five reverse turns, each five to 10 amino acids in length and a 200-amino acid carboxy terminus, rich in acidic residues. The results of studies of protein cleavage in live cells are consistent with a model of plasma membrane insertion in which both the N and C termini are positioned on the cytoplasmic side and there are only three short reverse turns on the extracellular side of the membrane (Liebowitz *et al.*, 1986). At least half of LMP-1 is associated with the cytoskeleton, as defined by resistance to extraction

with nonionic detergents and co-localization with the intermediate filament protein vimentin (Mann *et al.*, 1985; Liebowitz *et al.*, 1986; Moorthy & Thorley-Lawson, 1990, 1993a). Nascent, nonionic detergent-soluble LMP-1 has a half-life of less than 2 h and is converted to an insoluble, closely cytoskeleton-associated form that is phosphorylated on serine and threonine residues in the carboxy-terminal domain (Moorthy & Thorley-Lawson, 1990, 1993a). The cytoskeletal form has a half-life in the order of 3–15 h. After phosphorylation, *LMP-1* is cleaved near the beginning of the carboxy-terminal domain, resulting in a soluble product of about 25 kDa (Moorthy & Thorley-Lawson, 1990, 1993b). LMP-1 forms patches in the cell membrane that are often organized into a single cap-like structure (Hennessy *et al.*, 1984; Liebowitz *et al.*, 1986). Although vimentin co-localizes to the patches, patch formation does not require vimentin or other EBV proteins (Wang *et al.*, 1988a; Liebowitz *et al.*, 1992) and is likely to be due to the capacity of LMP-1 to interact with itself through the transmembrane domains. Like *EBNA-1*, *LMP-1* is transcribed during lytic infection and can be induced by treatment with activators of protein kinase C (Rowe *et al.*, 1987a; Laux *et al.*, 1988a). In late lytic infection, a promoter in the third *LMP-1* exon transcribes the part of the ORF that encodes for the last two transmembrane domains and the cytoplasmic domain (Hudson *et al.*, 1985). This truncated *LMP-1* does not associate with vimentin or other cytoskeletal elements and does not have transforming or cell-activating properties (Mann *et al.*, 1985; Wang *et al.*, 1985, 1988a,b; Liebowitz *et al.*, 1992). The full-size LMP-1 is incorporated into virions (Mann *et al.*, 1985), indicating that the virion-associated products may affect the growth of newly infected cells.

Transfection of *LMP-1* with heterologous promoters has transforming effects in rodent fibroblast cell lines (Wang *et al.*, 1985; Baichwal & Sugden, 1988; Wang *et al.*, 1988a; Moorthy & Thorley-Lawson, 1993a). LMP-1 expression in Rat-1 or NIH 3T3 correlates with altered cell morphology and growth in low concentrations of serum. The cells lose anchorage dependence, acquire growth capacity in soft agar and become tumorigenic in nude mice. Expression at levels above those detected in ordinary lymphoblastoid cell lines results in toxicity (Wang *et al.*, 1988a). LMP-1 also dramatically alters the growth of EBV-negative B lymphoblasts and induces many of the changes that are usually associated with EBV infection of primary B cells, including cell clumping, increased numbers of villous projections and vimentin expression, increased surface expression of CD23, CD39, CD40, CD44, class II MHC and the cell adhesion molecules LFA-1, ICAM-1 and LFA-3 and decreased expression of CD10 (Wang *et al.*, 1988b; Birkenbach *et al.*, 1989; Wang *et al.*, 1990a; Liebowitz *et al.*, 1992; Peng & Lundgren, 1992; Zhang *et al.*, 1994a; Kieff, 1996). LMP-1 has been shown to protect B lymphocytes from apoptosis by inducing *bcl-2* (Rowe *et al.*, 1994) and probably also *A20* (Henderson *et al.*, 1991; Martin *et al.*, 1993; Fries *et al.*, 1996). In addition, LMP-1 was shown to up-regulate certain cytokines with B-cell promoting activity, such as IL-10 (Nakagomi *et al.*, 1994). *LMP-1* expression also alters the growth of multipotent haematopoietic stem cells and epithelial cells (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a; Hu *et al.*, 1993). In monolayer cultures of immortalized human keratinocytes, LMP-1 induces morphological changes, alters cytokeratin expression and inhibits cell differen-

tiation (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a). Similar effects were observed in the hyperplastic skin of *LMP-1* transgenic mice.

Studies with *LMP-1*-deletion mutants in rodent and human cells and the isolation of EBV recombinants specifically mutated in *LMP-1* have yielded information on the role of different protein domains in cell growth and transformation. Transfection studies with *LMP-1*-deletion mutants indicate that the cytoplasmic amino terminus is not responsible for the activating effects, the transmembrane domains are critical, probably due to their importance for LMP-1 aggregation in the plasma membrane, and the carboxy-terminal domain is essential (Wang *et al.*, 1988a,b; Baichwal & Sugden, 1989; Martin & Sugden, 1991; Liebowitz *et al.*, 1992; Moorthy & Thorley-Lawson, 1993a,b). As expected, EBV recombinants lacking *LMP*-1 were unable to induce growth transformation of primary B cells (Kaye *et al.*, 1993). Growth transformation was also abolished by deletion of progressively large parts of the amino terminal and transmembrane domains, and the same was true for deletion of all of the carboxy-terminal cytoplasmic domain. Primary B lymphocytes infected with EBV recombinants that express the entire amino terminus and transmembrane domain and the first 44 amino acids of the carboxy-terminal domain grow well on diploid fibroblast feeder layers, suggesting that at least two functional domains exist in the carboxy terminus (Kaye *et al.*, 1995). A cellular protein interaction with the first 44 amino acids of the C-terminal domain was identified in a two-hybrid screen. The LMP-1-associated protein has a ring finger and extended coiled-coil structure and is homologous to factors associated with the murine tumour necrosis factor receptor. A second LMP-1-associated protein, EBI6, is the human homologue of the murine factors. The LMP-1-associated protein binds directly not only to the LMP-1 C-terminal domain but also to the p80 tumour necrosis factor receptor, CD40 and lymphotoxin-β receptor cytoplasmic domains (Mosialos *et al.*, 1995) and to lesser extent to the cytoplasmic domains of Fas/apo-1 protein and p60 tumour necrosis factor receptor. Thus, LMP-1 seems to interact with cellular proteins that are mediators of cytoplasmic signalling from the family of tumour necrosis factor receptors and thereby induce constitutive activation of the growth, death and necrosis factor-κB signalling pathways of those receptors (Hammarskjöld & Simurda, 1992). An additional domain in the distal C-terminal end of LMP-1 seems to be involved in necrosis factor-κB activation (Huen *et al.*, 1995).

Although the standard type-1 and type-2 EBV strains are closely similar (Sample *et al.*, 1994), numerous sequence variations were identified in *LMP-1* genes from different EBV isolates. A major (*Xho*)I restriction enzyme polymorphism was detected in type-1 strains from southeast China (Hu *et al.*, 1991a,b) and in type-2 strains from Alaska. In addition, consistent nucleotide variations in the amino terminus of *LMP-1* were identified in strains marked by the *Xho*I polymorphism. Amino acids 343–352 are deleted in the Chinese strains but not in those from Alaska, and numerous other changes were detected in the amino terminus that did not co-segregate with either disease phenotype, EBV type or specific geographic region (Miller *et al.*, 1994a; see also section 1.3.2).

(*b*) *LMP-2A and -2B*

LMP-2A and -2B are encoded by spliced mRNAs transcribed from the circularized EBV genome across the terminal repeats (Laux *et al.*, 1988a). The *LMP-2A* transcription starts 3 kb downstream of the *LMP-1* polyadenylation site, whereas that of *LMP-2B* starts 0.2 kb upstream of the *LMP-1* transcription start site (Laux *et al.*, 1988a; Sample *et al.*, 1989). The *LMP-2B* and *LMP-1* promoters form a bi-directional transcription unit containing a common *EBNA-2* response element, while a separate *EBNA-2* response element regulates *LMP-2A* transcription (Zimber-Strobl *et al.*, 1993). The two proteins have unique first exons but share the remaining exons derived from the terminal repeats. These encode for 12 hydrophobic transmembrane sequences separated by short reverse turns and a 27-amino acid hydrophilic C-terminal domain. LMP-2 co-localizes with LMP-1 in the plasma membrane of latently EBV-infected B lymphocytes (Longnecker & Kieff, 1990; Longnecker *et al.*, 1991).

1.1.5.4 *Other latent viral genes*

BHRF1 (Austin *et al.*, 1988) and *Bam*HI A (Brooks *et al.*, 1993a) mRNAs have been identified in strictly latently infected cells. *BHRF1* RNA and protein are abundantly expressed early in lytic infection, but the protein is not found in latently infected cells or newly infected primary B lymphocytes (Alfieri *et al.*, 1991). An ORF, *BARF0*, is contained in a highly spliced RNA originally identified from cDNA cloning of nasopharyngeal carcinoma RNA (Sadler & Raab-Traub, 1995a). Antibodies specific for the in-vitro translated polypeptide can be detected in sera from healthy people and from nasopharyngeal carcinoma patients (Gilligan *et al.*, 1991). The antisera produced to a synthetic peptide were used to identify a doublet of 30–35 kDa in immunoblots of EBV-carrying but not EBV-negative lines and in biopsy samples of nasopharyngeal carcinoma. Cellular fractionation indicates that the protein is associated with the membrane. Although its function in EBV infection is unknown, a recombinant virus carrying a deletion encompassing the *BARF0* coding region can transform lymphocytes *in vitro* (Robertson *et al.*, 1994).

1.1.5.5 *Genes of the productive viral cycle*

Only a small fraction of latently infected B lymphocytes spontaneously enters the productive cycle; thus, lytic infection is usually induced by chemicals (Luka *et al.*, 1979; Saemundsen *et al.*, 1980; Laux *et al.*, 1988b). Of the latently infected cell lines, marmoset cells tend to be more inducible than adult human lymphoblasts, and neonatal human lymphoblasts are the least inducible (Miller & Coope, 1974). A few lymphoblastoid cell lines can be induced to permit viral replication in approximately 10% of the cells. The Akata cell line, which carries an *LMP-2A*-deleted virus, can be induced by cross-linking of surface immunoglobulin (Ig) to the extent that more than 50% of the cells enter the lytic cycle (Takada, 1984; Takada & Ono, 1989). A second approach to studying viral replication is to induce the lytic cycle by superinfection of Raji cells with defective EBV from the P3HR-1 cell line (Mueller-Lantzsch *et al.*, 1980). Raji is an EBV-positive Burkitt's lymphoma line with an unusually high EBV episome copy

number. The Raji EBV genome has at least two deletions that make it defective for DNA replication and late gene expression; the cell line is, therefore, tightly latent (Polack *et al.*, 1984a). Defective virions from P3HR-1 contain rearranged DNA molecules in which the immediate early *trans*-activators of the lytic cycle are expressed after superinfection (Cho *et al.*, 1984; Miller *et al.*, 1984).

Cells that have become permissive for viral replication undergo cytoplasmic changes that are typical of herpesviruses, including margination of nuclear chromatin, synthesis of viral DNA, assembly of nucleocapsids, envelopment of the virus by budding through the inner nuclear membrane and inhibition of host macromolecular synthesis (Gergely *et al.*, 1971). Expression of the CD21 EBV receptor is down-regulated in 12-*O*-tetradecanoyl phorbol-13-acetate (TPA)-treated EBV-positive but not EBV-negative cells, probably as a consequence of binding of viral particles (Yamamoto *et al.*, 1982).

(*a*) *Immediate early genes*

Three leftward mRNAs are transcribed after P3HR-1 superinfection of Raji or surface Ig cross-linking of Akata cells in the presence of protein synthesis inhibitors. The *BZLF1*, *BRLF1* and *BI'LF4*-encoded proteins are potent *trans*-activators of early EBV gene expression (Takada & Ono, 1989; Marschall *et al.*, 1991; Kieff, 1996). Two key early promoter regulatory elements with left and right duplications that include the origins of lytic viral DNA replication are co-ordinately up-regulated by *BZLF1* and *BRLF1* (Hudewentz *et al.*, 1982; Hummel & Kieff, 1982a; Freese *et al.*, 1983; Nuebling & Mueller-Lantzsch, 1991). The two proteins acted synergistically on the bi-directional BHRF1 and BHLF1 promoter of left duplication in assays for transient transfection (Cox *et al.*, 1990). The *BSMLF1* and *BMRF1* promoters are also coordinately regulated, probably by binding to specific response elements (Holley-Guthrie *et al.*, 1990).

The R protein is a DNA sequence-specific acidic *trans*-activator that has distant homology to *c-myb* (Gruffat & Sergeant, 1994; Kieff, 1996). The Z protein varies slightly in size among EBV strains, but the sequence differences have not been investigated (Packham *et al.*, 1993). The *BZLF1* mRNA is spliced and consists of three exons (Lieberman & Berk, 1990): the first exon encodes amino acids 1–167 which include the *trans*-activating domain (Taylor *et al.*, 1991; Chi & Carey, 1993); the second encodes amino acids 168–202 which include a strong basic domain that has homology to a conserved region of the *c-jun/c-fos* family of transcription modulators (Chang *et al.*, 1990; Lieberman & Berk, 1990) and confers the ability to interact with AP1-related sites in DNA and also targets Z to the nucleus (Mikaélian *et al.*, 1993); the third exon encodes amino acids 203–245 which include a perfect leucine or isoleucine heptad repeat capable of coiled-coil dimer formation (Chang *et al.*, 1990; Kouzarides *et al.*, 1991). This domain is required for interaction of *Z* with *p53* (Zhang *et al.*, 1994b). Over-expression of wild-type *p53* inhibits the ability of *Z* to disrupt viral latency. Thus, direct interaction between *Z* and *p53* may play a role in regulating the switch from latency to lytic infection. Dimerization may facilitate the interaction with templates that have multiple Z responsive elements. The similarity between TPA response elements, AP1 sites and *Z* recognition sites may partially explain the capacity of TPA to activate the lytic cycle (Borras *et al.*, 1996).

(*b*) *Early genes*

The early genes are expressed when the lytic cycle is induced in the presence of inhibitors of DNA synthesis. By this criterion, at least 30 EBV mRNAs are early gene products (Hummel & Kieff, 1982a,b; Baer *et al.*, 1984). Because of the difficulty in studying EBV replicative functions, proteins encoded by early genes have been identified or assigned functions by analysis of their predicted sequences or by conducting functional assays of proteins translated *in vitro* or expressed *in vivo* (Wong & Levine, 1986; Nuebling & Mueller-Lantzsch, 1991). In many cases, functions have been suggested from comparison with the primary amino-acid sequence of the herpesvirus proteins of known function. Two very abundant early proteins have been mapped to specific DNA sequences. The BALF2 protein is homologous to the HSV DNA binding protein ICP8 and is important in DNA replication (Hummel & Kieff, 1982a; Kieff, 1996). The BHRF1 protein, which is expressed in moderate abundance, has extensive collinear homology with bcl-2 (Pearson *et al.*, 1983a; Austin *et al.*, 1988). BHRF1 can protect EBV-negative Burkitt's lymphoma cells from apoptosis (McCarthy *et al.*, 1996); however, EBV recombinants lacking the *BHRF1* ORF are fully able to initiate and maintain cell growth transformation and they can also enter the lytic cycle and produce virus (Lee & Yates, 1992; Marchini *et al.*, 1991).

Several of the early genes are linked to DNA replication. These include the genes for DNA polymerase (*BALF5*), the major DNA binding protein (*BALF2*), ribonucleotide reductase (*BORF2* and *BARF1*), thymidine kinase (*BXLF1*) and alkaline exonuclease (*BGLF5*), which are distributed through the unique long domain of EBV DNA (Kieff, 1996). The ribonucleotide reductase is confined to multiple discrete regions in the cytoplasm of productively infected cells (Pearson *et al.*, 1983b, 1987). Acetone fixation destroys its immunological reactivity, suggesting that it may be a major component of the restricted early antigen complex originally identified in EBV-immune sera from African Burkitt's lymphoma patients. Transfection experiments demonstrate that some of these genes are activated in the process of cell differentiation in the absence of other viral gene products, suggesting a possible role of cellular factors in regulating the productive cycle, at least in certain cell types (Marschall *et al.*, 1991).

(*c*) *Late genes*

The late genes with partially known products or which can be identified by homology with other herpesviruses code for structural glycoproteins or proteins that modify the infected cells in order to permit viral envelopment or egress. Among the non-glycoproteins, the major nucleocapsid protein is probably encoded by *cLF1*, *NRF1* encodes the major external non-glycoprotein of the virion, and *BXRF1* is likely to encode a basic core protein (Kieff, 1996). The *BFRF3* ORF encodes a highly basic viral structural capsid protein or tegument protein, VCA p18, which is strongly immunogenic in humans (van Grunsven *et al.*, 1994). The known EBV glycoprotein genes are *BLLF1* (gp350/220), *BALF4* (gp110), *BXLF2* (gp85), *BILF2* (gp55/80), *BDLF3* (gp100–150) and *BZLF2* (gp42) (see Table 1) (Hummel *et al.*, 1984; Beisel *et al.*, 1985; Gong *et al.*, 1987; Heineman *et al.*, 1988; Oba & Hutt-Fletcher, 1988; Li *et al.*, 1995a; Nolan & Morgan, 1995; Kieff, 1996). Gp110 is one of the most abundant late EBV proteins (Emini *et al.*,

1987; Gong *et al.*, 1987; Gong & Kieff, 1990). Immune light microscopy and electron microscopy have been used to localize gp110 to the inner and outer nuclear membrane and to the cytoplasmic membranes frequently surrounding enveloped virus but not to the Golgi or plasma membrane, suggesting that the protein is not processed in the Golgi apparatus. In contrast, gp85 and gp350/220 are processed efficiently through the Golgi apparatus and are found on the virus and in the plasma membrane of lytically infected cells (Gong & Kieff, 1990; Kieff, 1996). Gp85 is a relatively minor viral component that appears to be important in fusion between the virus and cell membranes (Miller & Hutt-Fletcher, 1988). Viral penetration involves two other EBV glycoproteins, gp25 and gp42, which form a tripartite complex with gp85. Gp42 also binds to HLA DR; thus, MHC class II molecules may serve as cofactors in human B-cell infection (Li *et al.*, 1997). Gp350/220 is the dominant external viral protein involved in mediating viral binding to the B-lymphocyte receptor CD21 and is the major target of the human EBV neutralizing antibody response (Thorley-Lawson & Geilinger, 1980; Thorley-Lawson & Poodry, 1982; Tanner *et al.*, 1988; Zhang *et al.*, 1991). Injection of purified gp350/220 or infection with vaccinia recombinants that express this protein protects cotton-topped tamarins against a lethal, lymphomagenic EBV challenge (Morgan *et al.*, 1988a,b).

The late *BCRF1* gene, which is located in the middle of the EBNA regulatory domain between *ori-P* and the Cp, is a close homologue of the human *IL-10* gene, with nearly 90% collinear identity in amino-acid sequence (Moore *et al.*, 1990; Vieira *et al.*, 1991; Touitou *et al.*, 1996). *BCRF1* has most of the activities of human *IL-10*, including negative regulation of macrophage and NK cell functions and inhibition of IF γ production. Thus, virally expressed *IL-10* may have a local effect on these responses to reactivated infection. Nonsense or deletion mutations involving *BCRF1* have no effect on the ability of EBV recombinants to initiate growth transformation or to maintain latent infection or on the ability of B lymphocytes to enter the lytic cycle *in vitro* (Swaminathan *et al.*, 1993).

1.2 Methods of detection

1.2.1 *Assays to detect antibodies to EBV*

The measurement of antibodies to EBV in biological fluids remains the major means of diagnosis of EBV infection. As discussed in section 1.2.3, distinct patterns of antibody response have been identified during primary infection, latent infection of immunocompetent carriers and viral reactivation and in various EBV-associated diseases (Evans, 1972).

1.2.1.1 *Immunofluorescence*

Immunofluorescence assays are, in general, labour-intensive and time-consuming, and experience is required to interpret the resulting patterns. Preparation of the antigen slides is also subject to batch variation. Although some commercial kits are available, their use has not been accepted universally.

Antibodies to viral capsid antigen, early antigen and membrane antigen: Detection of anti-viral capsid antigen (VCA) and anti-early antigen (EA) in serum by immunofluorescence was one of the earliest tests developed and is currently the 'gold standard' with which other EBV antibody assays are compared (Henle & Henle, 1967, 1985). Burkitt's lymphoma cell lines (e.g. Raji) superinfected with P3HR1 virus or induced with TPA and/or sodium *n*-butyrate are used as the source of VCA and EA antigens. After reaction with the test serum and its dilutions, the antibody is revealed by fluorescein isothiocyanate-conjugated, class-specific anti-human Ig. Detection of specific antibodies of different isotypes is used for the diagnosis and investigation of various EBV-associated diseases such as infectious mononucleosis (IgM), nasopharyngeal carcinoma (IgA) and Burkitt's lymphoma and Hodgkin's disease (IgG). EAs can be divided into restricted (R) and diffuse (D) forms on the basis of their pattern of distribution and sensitivity to methanol (Henle *et al.*, 1971a,b). As discussed in section 1.3.3.1, antibodies against EA(D) and EA(R) may show different prevalence patterns in different EBV-associated diseases.

In order to detect anti-membrane antigen by immunofluorescence, live cells must be used.

EBNA antibodies: Anti-EBNA antibodies are detected by the anti-human complement immunofluorescence assay first developed by Reedman and Klein (1973; Reedman *et al.*, 1974). While this test is generally used for the detection of EBNA complex antibodies, more detailed analysis is now possible using cell lines transfected with individual EBNA genes.

1.2.1.2 *Enzyme-linked immunosorbent assay*

Some of the technical difficulties associated with immunofluorescence tests have been overcome by the development of specific enzyme-linked immunosorbent assays (ELISAs). The specificity of most ELISAs used in EBV serology suffers, however, in comparison with that of immunofluorescence assays. A variety of antibodies to EBV antigens can be detected in ELISA with affinity-purified native or recombinant proteins. Serological reactions to antibody-specific epitopes have been mapped with the use of synthetic peptides. ELISAs have been developed for EBNAs, EA(D), EA(R), VCA, membrane antigen and ribonucleotide reductase (Luka *et al.*, 1984; Halprin *et al.*, 1986; Uen *et al.*, 1988; Weber *et al.*, 1996). Recombinant gp350 is used for the detection of antibodies to membrane antigen in ELISAs.

1.2.1.3 *Immunoblotting*

Lysates of EBV-positive cell lines fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis are used to detect anti-EBV antibodies in western blot methods. Individual antibody polypeptides can be distinguished by their characteristic size and migration patterns.

1.2.1.4 *Complement fixation*

Soluble complement-fixing antibodies were characterized when tested against soluble, non-sedimentable, nonstructural antigens extracted from non-virus-producing cells such as Raji, and were shown to appear only months after primary EBV infection. The results of this test parallel the presence of EBNA in cases of asymptomatic infectious mononucleosis, Burkitt's lymphoma and nasopharyngeal carcinoma (Gerber & Birch, 1967; Sohier & de Thé, 1972; Vonka *et al.*, 1972).

1.2.1.5 *Functional assays*

Antibodies to EBV-encoded proteins have been detected by their capacity to neutralize viral function or to mediate lysis of virus-infected target cells. For the detection of EBV DNase neutralizing antibodies, the EBV-encoded enzyme is purified from productively infected cells or expressed as a recombinant protein (Cheng *et al.*, 1980; Stolzenberg *et al.*, 1996). The last serum dilution capable of neutralizing a given amount of the enzyme (e.g. six units) gives the anti-DNase titre. In the antibody-dependent cellular cytotoxicity assay, antibodies to EBV membrane antigen are detected by their capacity to mediate killing of ^{51}Cr-labelled, productively EBV-infected cells by Fc-receptor-positive killer cells (Chan *et al.*, 1979a; Mathew *et al.*, 1981). The significance of the functional activity of these antibodies for the control of EBV infection *in vivo* is uncertain.

1.2.2 *Detection of EBV in tissues*

While serological studies can provide indirect evidence of an association of EBV with human tumours, definite identification of EBV-associated tumours rests ultimately on the detection of viral genomes and/or viral gene products in tumour tissues. Over the years, a number of methods have become available, which can be divided broadly into those based on the extraction of nucleic acids from tissues (Southern blot hybridization and the polymerase chain reaction (PCR)) and those which detect viral genomes or gene products *in situ* in single cells (in-situ hybridization and immunohistology). Some methods are used to detect and characterize viral genomes (Southern blot hybridization, PCR, DNA in-situ hybridization), and others to detect viral gene products (reverse transcriptase (RT)-PCR, RNA in-situ hybridization, immunohistology). The latter require that the viral genome be actively expressed; it is important to realize that this is also the case for EBER in-situ hybridization (see below), which has largely replaced EBV DNA in-situ hybridization for the identification of EBV. Thus, ultimate proof that the virus is absent from a given tissue can come only through the demonstration that the viral genome is absent. The suitability of these methods for the detection of EBV in tissues will be discussed using Hodgkin's disease as an example. Hodgkin's disease is particularly suited to illustrate the advantages and disadvantages of the various techniques because of its unique pathological features (see section 2.3.1), including the scarcity of neoplastic cells, the Hodgkin and Reed-Sternberg (HRS) cells, which rarely amount to more than 2% of the total cell population in an affected lymph node and which are embedded in a

background of abundant reactive cells (Herbst & Niedobitek, 1993). This scarcity made the association between EBV and Hodgkin's disease particularly difficult to establish.

1.2.2.1 *Southern blot hybridization and the polymerase chain reaction*

Prompted by seroepidemiological studies, several groups have attempted to detect viral genomes in extracts of DNA from Hodgkin's disease tissues. Studies with nucleic acid reassociation kinetics failed to do so, probably because of the limited sensitivity of this method (zur Hausen, 1976). With the development of Southern blot hybridization, a more sensitive and specific technique became available and, when probes directed, e.g. against the *Bam*HI W internal repetitive fragment of the viral genome, were used, EBV DNA was indeed detected in approximately 20% of cases of Hodgkin's disease (Weiss *et al.*, 1987; Herbst *et al.*, 1989). Southern blot hybridization involving probes for characterization of the terminal repeat region of the viral genome was used to demonstrate that in most cases the viral genomes are present as monoclonal episomes (Raab-Traub & Flynn, 1986; Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989; Masih *et al.*, 1991). This suggested that the virus-carrying cell population is monoclonal and implicated the HRS cells as the likely cellular source. Several groups have subsequently used PCR to detect viral genomes in Hodgkin's disease tissues. These experiments were often designed to amplify sequences from the *Bam*HI W internal repetitive fragment of the viral genome, since this promised maximal sensitivity. Using this approach, viral genomes were detected in 50–80% of biopsy samples from cases of Hodgkin's disease in western countries (Herbst *et al.*, 1990; Knecht *et al.*, 1991; Shibata *et al.*, 1991a).

Interpretation of the results obtained with Southern blot hybridization and PCR has, however, been difficult. The sensitivity of Southern blot hybridization is limited, and target DNA must be present in at least 1% of the total cell population. Furthermore, because DNA extracts from heterogeneous cell populations are analysed, the cellular source of any viral DNA detected cannot be determined unequivocally. Examination of the configuration of the terminal repeat region of the EBV genome by Southern blot hybridization can provide evidence for the clonal composition of the viral genomes. Detection of monoclonal viral genomes in DNA from clonal neoplasms indicates that the tumour cells are the cellular source of the viral DNA and that EBV infection took place before clonal expansion of the neoplastic cell population. The nature and clonal origin of HRS cells are, however, uncertain; the results of a few recent studies suggest the possibility that HRS cells may be polyclonal at least in some cases (Hummel *et al.*, 1995a; Delabie *et al.*, 1996).

Analysis of DNA extracts by PCR faces the same difficulties, which are compounded by the high sensitivity of this method. It is now clear that circulating, EBV-carrying, non-neoplastic lymphocytes are found in a variety of tissues, including Hodgkin's disease lymph nodes (Herbst *et al.*, 1992; Khan *et al.*, 1992; Niedobitek *et al.*, 1992a). Thus, the demonstration of EBV DNA in any tumour by PCR cannot be taken as evidence of viral infection in the tumour-cell population. A number of RT-PCR strategies have been devised for the analysis of EBV gene expression in peripheral blood or tissues (Raab-Traub *et al*,. 1991; Brooks *et al.*, 1992; Busson *et al.*, 1992a; Qu & Rowe, 1992; Tierney

et al., 1994). With the possible exception of the demonstration of EBER expression, these methods are not suitable for diagnostic purposes but rather represent a tool for the characterization of EBV infection under conditions in which the association with EBV is known.

1.2.2.2 *In-situ hybridization and immunohistochemistry*

The study of the relationship between EBV infection and Hodgkin's disease illustrates the necessity of identifying the cellular location of the virus. The first unequivocal demonstration of EBV within the HRS cells of Hodgkin's disease came from two independent studies in which DNA in-situ hybridization was used with ^{35}S-labelled probes. Again in these studies, the *Bam*HI W internal repetitive fragment of the viral genome was used as a target, because it promises greater sensitivity than other unique genome fragments. In both studies, EBV DNA was detected exclusively in the HRS cells of a few selected cases (Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989a). These results were subsequently confirmed, with the use of both radioactive and non-radioactive DNA probes (Herbst *et al.*, 1990; Uccini *et al.*, 1990; Coates *et al.*, 1991a,b; Delsol *et al.*, 1992). The detection rate of DNA in-situ hybridization was always lower than that of PCR, indicating comparatively low sensitivity. Moreover, when biotin-labelled probes are used, the presence of endogenous biotin may give rise to artefactual cytoplasmic labelling (Niedobitek *et al.*, 1989a; Hawkins *et al.*, 1990; Santucci *et al.*, 1993).

A major breakthrough in the analysis of EBV infection by in-situ hybridization came with the introduction of probes specific for the small nuclear EBV-encoded RNAs, *EBER-1* and *-2* (Wu *et al.*, 1990). The EBERs are abundantly expressed in all forms of EBV latency and are therefore uniquely suitable as targets for in-situ hybridization. Moreover, because of their high copy number, EBERs can be detected with non-radioactive RNA and oligonucleotide probes, making this method suitable for many laboratories (Wu *et al.*, 1991). Because of its high sensitivity, this method allows precise determination of the incidence of EBV infection in HRS cells. Thus, EBER in-situ hybridization has revealed that EBV infection in Hodgkin's disease is not restricted to HRS cells but may occur in non-neoplastic, so-called 'bystander' lymphocytes (Herbst *et al.*, 1992; Khan *et al.*, 1992; Jiwa *et al.*, 1993a). This method has been applied in many laboratories to the study of not only Hodgkin's disease but also other malignancies, leading to the identification of a variety of new EBV-associated tumours.

In parallel with these developments, several monoclonal antibodies have become available which are suitable for the detection of EBV-encoded latent and replicative proteins in sections of routinely formalin-fixed, paraffin-embedded tissues (Rowe *et al.*, 1987b; Young *et al.*, 1989b, 1991; Grässer *et al.*, 1994). This technique has been vastly improved by the development of antigen retrieval techniques with microwave irradiation or pressure cooking (Shi *et al.*, 1997), allowing the detection of epitopes in paraffin sections masked by formalin fixation and which have been undetectable hitherto. Thus, monoclonal antibodies specific for EBNA-1, EBNA-2, LMP-1 and LMP-2A can now be used for the immunohistochemical analysis of latent EBV gene expression (Grässer *et al.*, 1994; Delecluse *et al.*, 1995a; Murray *et al.*, 1996; Niedobitek *et al.*, 1997a).

Similarly, monoclonal antibodies and probes that recognize several lytic cycle gene products, e.g. BZLF1 and EA(D), are available (Young *et al.*, 1991; Brousset *et al.*, 1992, 1993; Rowlands *et al.*, 1993; Ryon *et al.*, 1993; Niedobitek *et al.*, 1997a). Unlike transcriptional studies involving PCR or western blot analysis, immunohistochemistry allows the analysis of EBV gene expression in single cells. This is particularly relevant for the study of Hodgkin's disease because of the frequent presence of EBV-carrying, non-neoplastic lymphocytes in such tissues. With the exception of EBNA-1, however, these viral proteins are not invariably expressed in virus-associated tumours. *EBNA-1* is expressed only weakly in many cases of Hodgkin's disease and is therefore often not detectable by immunohistochemistry (Grässer *et al.*, 1994). For these reasons, immunohistology cannot substitute for in-situ hybridization for the detection of latent EBV infection. Immunohistology for the detection of LMP-1 in Hodgkin's disease represents a possible exception to this rule, since this protein has been shown to be expressed in virtually all cases (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a).

1.3 Biology of EBV infection

1.3.1 *Target tissues*

1.3.1.1 *Infection* in vitro

A characteristic feature of EBV is its ability to infect human B cells *in vitro* and to convert them into permanently growing lymphoblastoid cell lines. Several studies have shown that the virus not only immortalizes mature B cells but can also infect precursor B cells, including those with immunoglobulin heavy and light chain genes in germ-line configuration (Hansson *et al.*, 1983; Ernberg *et al.*, 1987; Gregory *et al.*, 1987a). Moreover, EBV infection of immature B cells induces the expression of 'late' lymphocyte activation antigens (CD30, CD70), as also seen in Ig-expressing lymphoblastoid cell lines (Gregory *et al.*, 1987a). This finding is consistent with the detection of EBV in $CD30^+$ HRS cells with pro-B- and pre-B-cell phenotypes and genotypes (Herbst *et al.*, 1993).

The efforts to generate in-vitro models for EBV-associated non-B-cell neoplasia have been particularly successful for T lymphocytes, in which EBV infection through CD21 or a related molecule has been achieved. Several groups have recently reported the infection of immature thymocytes with EBV. Interestingly, the viral genome in these cells appears to be maintained in linear form, without episome formation. Qp-derived EBNA-1 mRNA expression has been demonstrated in such cells, and there is a high degree of lytic cycle antigen expression (Watry *et al.*, 1991; Kaufman Paterson *et al.*,. 1995; Kelleher *et al.*, 1995). This suggests that the virus–cell interaction in these cells is fundamentally different from that in B-cell lines. A more conventional result was reported by Yoshiyama *et al.* (1995), who infected a human T-cell lymphotropic virus (HTLV)-I-carrying T-cell line with EBV. This resulted in a stable latent infection with viral episomes and expression of EBNA-1 and LMP-1, reminiscent of the type-II latency seen in many EBV-associated T-cell lymphomas. In contrast to EBV-infected B cells, up-regulation of CD23, LFA-1, LFA-3 and ICAM-1 was not observed in these cells; and in

contrast to the results obtained with immature thymocytes, lytic cycle antigens were not inducible.

In view of the frequent association of EBV with carcinomas, efforts have been made to infect epithelial cells with the virus *in vitro*; however, this has proved difficult. Some reports suggest that direct infection of nasopharyngeal and cervical epithelial cells with EBV may be possible (Sixbey *et al.*, 1986; Furukawa *et al.*, 1990; Yoshizaki *et al.*, 1994); however, in most recent studies mechanisms to facilitate this process had to be devised. One such mechanism is transfection of CR2-negative keratinocytes with the gene that encodes the B-cell EBV receptor: thus, Li *et al.* (1992) achieved transient infection of CR2-transfected human keratinocytes. Subsequently, Knox *et al.* (1996) generated stable EBV-infected subclones from such cells. In these cells, EBNA-1 and low levels of LMP-1 were expressed, reminiscent of the situation in nasopharyngeal carcinomas. Moreover, the ability of these cells to undergo terminal squamous differentiation was impaired, suggesting that stable EBV infection of epithelial cells requires an undifferentiated cellular environment.

A different approach was chosen by Sixbey and co-workers, who demonstrated that coating EBV with gp350-specific polymeric IgA could facilitate binding to and infection of a human colon carcinoma cell line which was otherwise refractory to EBV infection. The EBNA complex, EBNA-2, and lytic cycle antigens were expressed in a fraction of these cells (Sixbey & Yao, 1992). The relevance of this model for EBV infection *in vivo* is as yet uncertain.

Infection of a hepatoma cell line with EBV has been reported (Lisi *et al.*, 1995), but this model has yet to be fully characterized. Of uncertain significance also is the reported generation of precursor monocyte cell lines (Revoltella *et al.*, 1989) which are EBV DNA-positive, as demonstrated by Southern blot hybridization and expressed EBNA complex.

Lindhout *et al.* (1994) achieved infection of follicular dendritic cells with EBV, presumably mediated through CR2, generating permanently growing cell lines that express LMP-1 and EBNA-2. A report of EBV infection of mouse fibroblasts after CR2 transfection demonstrates that, at least in certain cell types, the absence of CR2 is the main obstacle for EBV infection (Ahearn *et al.*, 1988).

Yet another mechanism that may be important for EBV infection of CR2-negative cells of various lineages was suggested by the results of Bayliss and Wolf (1980, 1981), who demonstrated that EBV induces cell fusion between virus-carrying B cells and other cell types, including T cells and fibroblasts. The importance of this mechanism is suggested by the reported EBV infection of endothelial cells by co-cultivation with irradiated EBV-carrying B-cell lines (Jones *et al.*, 1995). While the mechanism of entry of the virus into endothelial cells was not elucidated in this study, it suggests that it is due to cell–cell contact. This result, together with the detection of the virus in CD21-negative tumour cells, raises the possibility that cell fusion may facilitate the entry of EBV in the absence of the C3d/EBV receptor (Bayliss & Wolf, 1980; Ahearn *et al.*, 1988).

1.3.1.2 *Infection of non-neoplastic cells* in vivo

As anticipated, it has been shown in several studies that EBV-carrying B cells are readily identifiable in lymphoid tissues during infectious mononucleosis and in persistent infection. When EBER in-situ hybridization is used, numerous EBV-infected cells are found in lymphoid tissues from patients with infectious mononucleosis, mainly in the expanded paracortical areas (Niedobitek *et al.*, 1992a; Hamilton-Dutoit & Pallesen, 1994; Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). These cells are mostly of B-cell phenotype, and a proportion has been shown to undergo plasma-cell differentiation (Robinson *et al.*, 1981; Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). Significantly, the expression of lytic-cycle gene products has been reported in plasma cells during infectious mononucleosis (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b). Small numbers of EBV-positive lymphocytes are detectable in lymphoid and other tissues as well as in peripheral blood from chronic virus carriers, again, usually in the paracortex of lymphoreticular tissues; many display the phenotype of small, resting B cells (Niedobitek *et al.*, 1992a; Deamant *et al.*, 1993; Miyashita *et al.*, 1995). The number of EBV-positive cells can increase dramatically under conditions of impaired T-cell immunity, e.g. in individuals infected with the human immunodeficiency virus (HIV). On rare occasions, expansion of EBV-infected cells in germinal-centre reactions has been observed, suggesting that at least some EBV-positive circulating B cells show physiological response to antigenic stimulation (Niedobitek *et al.*, 1992a).

The possibility of infection of other cell types by EBV is more controversial. The ability of EBV to infect T cells is demonstrated by the frequent detection of the virus in T-cell non-Hodgkin lymphomas (Pallesen *et al.*, 1991b). Moreover, EBV infection has been demonstrated in certain atypical non-neoplastic T-cell proliferations, often in association with primary infection. Kikuta *et al.* (1988) demonstrated EBV infection in $CD4^+$ T cells from a boy with chronic active EBV infection and Kawasaki disease. Yoneda *et al.* (1990) reported the detection of EBV in a transient polyclonal T-cell proliferation in a young man. Moreover, several groups have demonstrated the presence of EBV in non-neoplastic T cells and natural killer (NK) cells in patients with virus-associated haemophagocytic syndrome (Kawaguchi *et al.*, 1993; Su *et al.*, 1994; Dolezal *et al.*, 1995). In agreement with these observations, EBV infection of T cells has been reported in patients with infectious mononucleosis as well as in persistent infection (Deamant *et al.*, 1993; Anagnostopoulos *et al.*, 1995, 1996); however, Niedobitek *et al.* (1992a, 1997b) could not confirm this finding, and EBV infection of T cells in healthy individuals appears to be a rare event.

Similarly, the question of the infection of normal epithelial cells with EBV is controversial. There is unequivocal evidence that in oral hairy leukoplakia, an AIDS-associated lesion of the tongue, viral replication occurs in the differentiated upper epithelial cell layers (Greenspan *et al.*, 1985; Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Young *et al.*, 1991; see also section 1.2.2); however, several authors have indicated that this is not accompanied by latent EBV infection in the basal epithelial cells of the lesion, suggesting that hairy leukoplakia represents a focus of isolated EBV replication without

a detectable latent phase (Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Sandvej *et al.*, 1992; Murray *et al.*, 1996). Lemon *et al.* (1977) and Sixbey *et al.* (1984) reported the detection by in-situ hybridization of EBV DNA in oropharyngeal epithelial cells from patients with infectious mononucleosis and in one chronic virus carrier. More recent studies of tonsils from patients with infectious mononucleosis, however, have shown no evidence of EBV infection in the tonsillar epithelium (Niedobitek *et al.*, 1989b; Weiss & Movahed, 1989; Niedobitek *et al.*, 1997b), and Karajannis *et al.* (1997) demonstrated the presence of isolated EBV-positive B cells in throat washings from infectious mononucleosis patients in the absence of detectable virus in desquamated epithelial cells. Moreover, there is no evidence of EBV infection in epithelial cells of normal nasopharyngeal mucosa, in tissues from either healthy individuals or from the vicinity of EBV-positive nasopharyngeal carcinomas (Niedobitek *et al.*, 1991b; Sam *et al.*, 1993; Tao *et al.*, 1995). The recent description of EBV infection in nasopharyngeal carcinoma *in situ* indicates that viral infection of the nasopharyngeal mucosa takes place before the infiltrative growth of the carcinoma commences (Pathmanathan *et al.*, 1995a); however, when exactly in the pathogenic process of nasopharyngeal carcinoma this occurs is as yet unclear, and present evidence would suggest that EBV infection of normal oro- and nasopharyngeal epithelial cells is at best a rare event. Similarly, EBV infection of normal gastric mucosa has not yet been demonstrated convincingly (Rowlands *et al.*, 1993; Fukayama *et al.*, 1994; Osato & Imai, 1996).

A few studies have suggested that EBV may persist in the epithelial cells of the parotid and other salivary glands, first on the basis of reassociation kinetics and DNA in-situ hybridization studies of parotid glands from healthy individuals (Wolf *et al.*, 1984). Subsequent studies using EBV DNA in-situ hybridization and immunohistochemistry showed the presence of EBV in ductal epithelial cells of salivary and lachrymal glands from patients with Sjögren's syndrome (Pflugfelder *et al.*, 1993; Wen *et al.*, 1996). Interestingly, a difference between the results of EBV DNA and EBER in-situ hybridization was noted in one of these studies. Thus, EBV DNA was localized to lymphocytes and epithelial cells, whereas latent EBV infection was detected by EBER in-situ hybridization only in lymphocytes and not in epithelial cells. This result is difficult to explain, particularly in view of the reported absence of antigens associated with lytic EBV infection (Wen *et al.*, 1996). Other studies of salivary glands from patients with Sjögren's disease and of normal tissue adjacent to salivary gland carcinomas by EBER and EBV DNA in-situ hybridization also showed no detectable EBV infection in salivary gland epithelial cells. This issue therefore remains controversial (Hamilton-Dutoit *et al.*, 1991a; DiGuiseppe *et al.*, 1994; Hamilton-Dutoit & Pallesen, 1994; Leung *et al.*, 1995a). Interestingly, EBV-infected hepatocytes and adrenocortical epithelial cells have been demonstrated occasionally in transplant recipients and in one patient with virus-associated haemophagocytic syndrome (Randhawa *et al.*, 1992; Kikuta *et al.*, 1993). These reports suggest that the cellular tropism of EBV may be broader in patients with impaired EBV-specific immunity.

Convincing evidence of infection of normal follicular dentritic cells, endothelial cells, smooth muscle cells and other cell types has not yet been reported.

1.3.1.3 *Infection in neoplasms*

The detection of EBV in certain B-cell non-Hodgkin's lymphomas is in keeping with the known ability of the virus to infect and immortalize human B cells *in vitro*. The association of EBV with endemic Burkitt's lymphoma is well documented (Epstein *et al.*, 1964; Lenoir & Bornkamm, 1987; Magrath *et al.*, 1992). Moreover, the virus is present in the majority of B-cell lymphoproliferations that develop in immunosuppressed transplant patients and in a large proportion of AIDS-related B-cell lymphomas (Hamilton-Dutoit *et al.*, 1991b; Craig *et al.*, 1993). The presence and role of EBV in tumour cells is discussed in Section 4. EBV has been detected in T-cell non-Hodgkin's lymphomas of the $CD4^+$, $CD8^+$ and NK cell phenotypes (Harabuchi *et al.*, 1990; Su *et al.*, 1990; Pallesen *et al.*, 1993). The ability of EBV to infect a broad range of target cells within the lymphoid system has also been demonstrated by the frequent detection of the virus in HRS (Herbst *et al.*, 1991a, 1993). While the precise nature and clonal origin of HRS cells are uncertain, these cells may express B- or T-cell antigens (Herbst *et al.*, 1993; Haluska *et al.*, 1994).

The ability of EBV to infect cells of non-lymphoid lineages was first illustrated by its detection in undifferentiated nasopharyngeal carcinoma (Klein, 1979). The presence of EBV in squamous-cell nasopharyngeal carcinomas and in gastric and other adenocarcinomas further demonstrates the ability of the virus to infect variously differentiated epithelial cells (Raab-Traub *et al.*, 1987; Shibata & Weiss, 1992; Pathmanathan *et al.*, 1995b; Osato & Imai, 1996).

The virus has also been detected in tumours derived from smooth-muscle cells, i.e. in leiomyomas and leiomyosarcomas, in immunocompromised patients (Prévot *et al.*, 1994; Lee *et al.*, 1995b; McClain *et al.*, 1995). Intriguingly, in some of these cases, EBNA-2 appears to be expressed in the absence of detectable LMP-1 expression (Lee *et al.*, 1995b). Moreover, the presence of clonal EBV genomes has been reported in so-called inflammatory pseudotumours. In some of these cases, the virus-carrying cells were of smooth-muscle phenotype, while in other cases the immunophenotype was consistent with derivation from follicular dendritic cells (Arber *et al.*, 1995; Selves *et al.*, 1996a). In one case with a follicular dendritic cell phenotype, LMP-1 expression was detected by immunostaining (Selves *et al.*, 1996a).

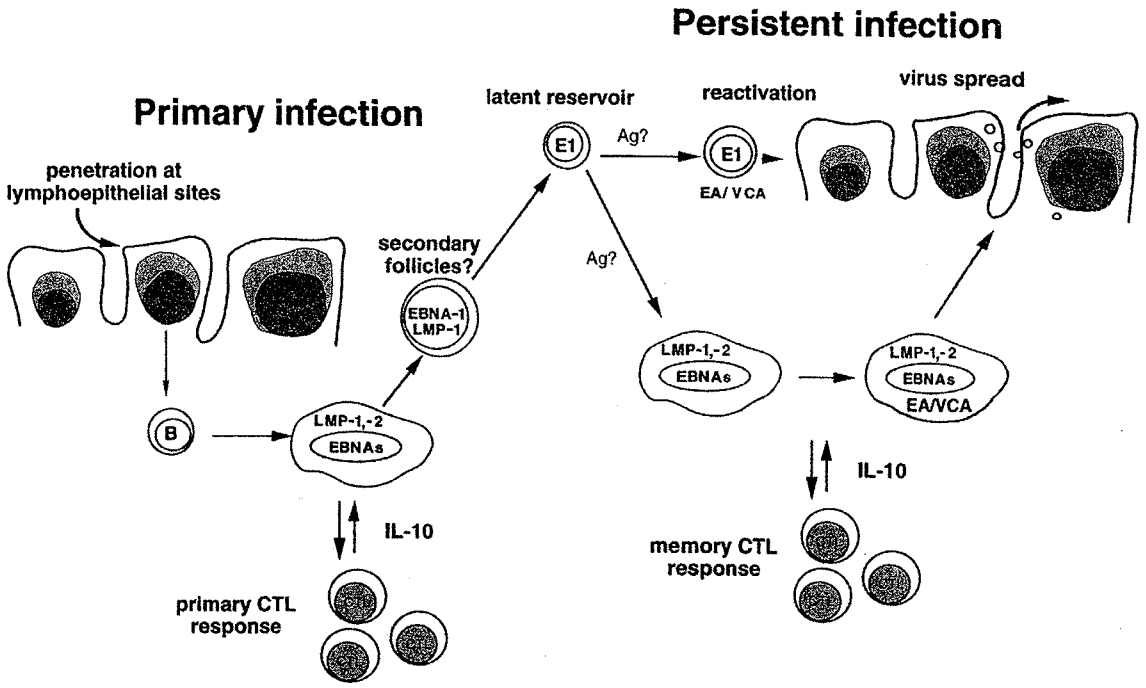
The expanding list of virus-associated tumours serves to illustrate that the target cell tropism of EBV *in vivo* is much broader than was originally anticipated. The conditions and mechanisms that allow EBV infection of these diverse cell types are, however, in most cases unknown.

1.3.2 *Persistence and latency*

Figure 2 illustrates the cellular events thought to be involved in EBV infection and persistence. Primary infection, occurring via transmission of cell-free virus and/or of productively infected cells in saliva, is associated with entry of the virus into the circulating B-cell pool. The detection of productively infected epithelial cells in the throat washings of patients in the acute phase of infectious mononucleosis has been reported (Sixbey *et al.*, 1984), and EBV can also replicate in the stratified squamous epi-

thelium lining the oral cavity, as clearly demonstrated in hairy leukoplakia lesions in immunosuppressed individuals (Greenspan *et al.*, 1985; Niedobitek *et al.*, 1991a; Lau *et al.*, 1993). It is not clear, however, whether epithelial cells are the primary targets of orally transmitted virus or whether the virus must be amplified in locally infiltrating B lymphocytes, e.g. in the tonsillary crypts. Uncertainty about the role of epithelial cells is further increased by the failure to demonstrate a putative EBV receptor on these cells. In a recent study of throat washings from infectious mononucleosis patients, EBV was not detected in desquamated epithelial cells (Karajannis *et al.*, 1997). A 200-kDa glycoprotein expressed on differentiating squamous epithelium has only some antigenic similarity to the CD21 molecule on B cells, and its presence does not correlate with EBV-binding activity (Young *et al.*, 1986; Birkenbach *et al.*, 1992). Very low levels of CD21 have been detected in some epithelial tumour lines *in vitro* (Birkenbach *et al.*, 1992) and in transplantable nasopharyngeal carcinomas, suggesting that low levels of the receptor could be expressed *in vivo*. Other routes of viral entry, such as fusion with EBV-infected, infiltrating B lymphocytes (Bayliss & Wolf, 1981) or transport as an immune complex (Sixbey & Yao, 1992), may be more relevant to epithelial-cell infection.

Figure 2. Model of EBV–host interaction during primary infection and persistence



Ag, antigen; EA, early antigen; VCA, viral capsid antigen; EBNA, EBV nuclear antigen; E1, EBV nuclear antigen 1; LMP, latent membrane protein; IL, interleukin; CTL, cytotoxic T lymphocyte

The initial events of B-cell infection *in vivo* appear to be similar to those seen on experimental infection of resting B cells *in vitro*, i.e. transcription from Wp, Cp and LMP promoters and expression of the full spectrum of latent proteins (Falk *et al.*, 1990; Tierney *et al.*, 1994). This pattern of latent viral gene expression has two important effects, both serving to increase the chances that viral persistence will be established in the lymphoid system. First, it can drive rapid polyclonal expansion of the infected B-cell

pool in the very early phases of the infection, before an effective cellular immune response has developed. Second, LMP-1 can up-regulate at least two cellular proteins, bcl-2 and A20, that are capable of extending cell survival *in vivo*. Virus-induced entry of infected cells into the long-lived B-cell pool could be important for viral persistence, since the majority of B lymphocytes are short-lived, and only a very small fraction of the newly generated cells is selected in the long-lived compartment by physiological means.

The early phase of virus-induced B-cell proliferation is curtailed by the developing T-cell response, and large numbers of infected cells appear to be eliminated by virus-specific cytotoxic T lymphocytes (CTLs) at this stage (Klein *et al.*, 1981; Svedmyr *et al.*, 1984; Callan *et al.*, 1996; Steven *et al.*, 1996), which correlates with the decrease in virus shedding in the oropharynx. Despite the strength of this primary immune response, viral latency within the B-cell pool and viral replication in the oropharynx are never completely eliminated. The B-cell compartment appears to be the true reservoir of latent virus, since pharmacological elimination of the autologous lymphoid system in patients receiving allogeneic bone-marrow transplants may result in eradication of the virus (Gratama *et al.*, 1988). Furthermore, treatment with the nucleoside analogue, acyclovir, abolished virus production in the oropharynx but had no effect on the number of latently infected cells in the blood (Yao *et al.*, 1989a). B Lymphocytes cultured from peripheral blood or lymphoid tissues show spontaneous outgrowth of lymphoblastoid cell lines, due predominantly to reactivation of viral replication and infection of co-resident B cells (Rickinson & Epstein, 1978; Rickinson *et al.*, 1978). In a very few cases, direct outgrowth of B lymphocytes latently infected *in vivo* has been suggested by the establishment of spontaneous lymphoblastoid cell lines in cultures supplemented with inhibitors of viral replication and virus-neutralizing antibodies (Lewin *et al.*, 1987, 1988). When assays for spontaneous outgrowth are performed in the presence of cyclosporin A, to prevent T-cell activation, the minimum number of cells required to be seeded for spontaneous outgrowth varies between 10^4 and 10^6 B cells per culture (von Knebel Doeberitz *et al.*, 1983; Rickinson *et al.*, 1984). Similar figures were recently obtained by semi-quantitative PCR analysis of purified B lymphocytes (Chen *et al.*, 1995a; Miyashita *et al.*, 1995). In healthy virus carriers, the same EBV strain appears to predominate in the blood and in throat washings (Gratama *et al.*, 1990, 1992). In the few cases in which detailed studies have been conducted, it was noted that individuals with large numbers of EBV-infected cells in the blood also tended to shed relatively high levels of virus in the throat (Yao *et al.*, 1985a,b). Altogether, these findings are consistent with the idea that latently infected B cells are necessary and sufficient for persistence and that epithelial infection may depend upon seeding from the lymphoid reservoir .

The persistence of EBV-infected B cells in healthy, immunocompetent virus carriers appears to involve a form of latency that is different from that seen in B cells during primary infection. PCR analysis of viral transcripts in circulating B cells from healthy donors showed expression of *EBNA-1* and *LMP-2A* mRNAs only (Qu & Rowe, 1992; Tierney *et al.*, 1994; Chen *et al.*, 1995a). Such a restricted pattern of latent viral gene expression is consistent with the conclusion that virus-carrying cells have a resting phenotype (Miyashita *et al.*, 1995) and could explain how these cells can persist in the face of efficient CTL surveillance (reviewed by Masucci & Ernberg, 1994). A switch

from the activated to the resting cell pattern of viral gene expression has never been seen to occur spontaneously in lymphoblastoid cell lines *in vitro* but can be induced experimentally by enforcing a change in cellular phenotype in cell hybrids (Contreras-Salazar *et al.*, 1989; Contreras-Brodin *et al.*, 1991; Kerr *et al.*, 1992). A similar switch might occur naturally *in vivo* if some of the infected cells could progress down a normal B-cell differentiation pathway.

Cells that express the full repertoire of growth transformation-associated antigens are likely be generated continually in asymptomatic virus carriers, since memory CTLs that are reactive against most EBNAs are maintained at high levels for life. Furthermore, some B cells must be continually triggered into the lytic cycle in order to maintain oropharyngeal shedding of infectious virus. Both events are likely to occur by reactivation from a latently infected memory B-cell pool. An interesting possibility is that both events may be governed by physiological signals that control normal B-cell behaviour. The switching of EBV-infected B cells to a blast-like phenotype and pattern of viral gene expression may therefore be triggered by conventional antigen-driven activation of the latently infected cells; similarly, physiological stimuli may be delivered to circulating B cells at mucosal surfaces which can trigger initiation of the lytic cycle.

1.3.3 *Immune responses*

1.3.3.1 *Antibody responses*

As outlined in Section 1.2, the seroepidemiology of EBV infection still relies on a set of immunofluorescence assays developed within the first decade of EBV research. These are used to measure antibody responses to the nuclear antigens (EBNA; Reedman & Klein, 1973); EA(D) and EA(R); the VCA, expressed in the cells late during the productive virus cycle (de Schryver *et al.*, 1972) and the membrane antigen, expressed on the surface of lytically infected cells (Klein *et al.*, 1969). Each of these antigens is a composite of several viral products, and detection of individual reactivity requires complex assays. Several partially successful attempts have been made to replace the fluorescence tests with ELISA-based and other assays in which recombinant proteins or synthetic peptides are used as substrates (Sternås *et al.*, 1986; Zhang *et al.*, 1991).

Studies of infectious mononucleosis patients have provided the bulk of the information available on primary antibody responses. By the time of onset of clinical symptoms, most such patients have substantial titres of IgM antibodies to VCA and rising IgG titres to both EA and VCA (Henle & Henle, 1973a; Horwitz *et al.*, 1975; Svedmyr *et al.*, 1984; Horwitz *et al.*, 1985; Marklund *et al.*, 1986; van Grunsven *et al.*, 1994). IgA antibodies to these antigens may also appear at this stage. Anti-VCA IgM disappears over the next few months, whereas anti-VCA IgG titres rise to a peak that may fall slightly and anti-EA IgG becomes either undetectable or stabilizes at a very low level. Neutralizing antibodies are detectable during the acute phase of infectious mononucleosis but only at very low titres; these increase to stable levels thereafter (Horwitz *et al.*, 1975; Lennette *et al.*, 1982). An interesting feature of the serology of infectious mononucleosis is the pattern of anti-EBNA responses. Patients in the acute phase of the disease show an IgG response to EBNA-2 (and probably also to EBNA-3A, -3B and

-3C), whereas an IgG response to EBNA-1 is not usually detectable until convalescence (Henle *et al.*, 1987). IgM antibodies to EBNA-1 are usually detected during the acute phase, but these are thought to be part of an autoantibody response with cross-reactivity to the Gly-Ala repeat domain, which EBNA-1 probably shares with several cellular proteins (Rhodes *et al.*, 1987). Anti-EBNA persists for life, whereas the persistence of other EBV antibodies, including anti-EBNA-2, varies (Henle *et al.*, 1987). The delayed antibody response to EBNA-1 is difficult to understand. The original hypothesis that it is due to delayed destruction of latently infected cells *in vivo* is difficult to reconcile with the detection of good responses to EBNA-2. One possible explanation is that EBNA-1 is somewhat protected from certain pathways of antigen processing. Alternatively, the changes in antibody reactivity between primary and persistent infection could reflect the predominance of different types of latently infected cells. In addition to EBV-specific antibodies, the early phase of infectious mononucleosis is characterized by a general increase in total IgM, IgG and IgA, which is consistent with virus-driven polyclonal activation of the B-cell system. Heterophile antibodies with the capacity to agglutinate sheep and horse erythrocytes are the basis of the Paul-Bunnel-Davidson test for the diagnosis of classic EBV-associated infectious mononucleosis (Fleisher *et al.*, 1979; Halbert *et al.*, 1982).

Healthy virus carriers consistently have antibodies to VCA, neutralizing anti-gp350 antibodies and antibodies to EBNA-1 (Henle & Henle, 1976a; Henle *et al.*, 1987). The titres can differ markedly among individuals and are usually stable over time. Only a proportion of healthy carriers have antibodies to EA or to one or more of the other EBNA proteins. Usually, there is no direct correlation between the titres of anti-VCA or anti-EA antibodies and the levels of virus replication, at least as measured by the titres of transforming virus in throat washings (Yao *et al.*, 1985a,b,c), although some individuals with extremely high titres of anti-VCA or anti-EA and low anti-EBNA-1 antibodies show signs of chronically active EBV infection (Johansson *et al.*, 1971; DuBois *et al.*, 1984; Straus *et al.*, 1985; Henle *et al.*, 1987; Miller *et al.*, 1987). The role of antibodies in maintaining persistent infection is not clear. Anti-gp350 antibodies may sensitize lytically infected cells to lysis by $CD16^+$ effectors (Pearson *et al.*, 1978a, 1979), but cannot recognize latently infected cells. Gp350 antibodies can neutralize a broad spectrum of EBV strains (Thorley-Lawson & Geilinger, 1980), however, and this may be one of the mechanisms that helps to prevent superinfection of the immune host with orally transmitted virus.

1.3.3.2 *Cell-mediated responses*

Clinically manifested primary EBV infection is associated with an unusually strong cell-mediated immune response. 'Atypical' mononuclear cells, the large numbers of which are a key feature of infectious mononucleosis, are predominantly T lymphocytes of the $CD8^+$ subset, with a small contribution of $CD4^+$ cells (Sheldon *et al.*, 1973; Svedmyr *et al.*, 1984). These activated T cells express the CD45RO marker and, when expanded *in vitro*, are programmed to die by apoptosis unless rescued by exogenously added cytokines (Moss *et al.*, 1985). The main stimulus for this response appears to be

latently infected B lymphocytes. In this context, it is possible that viral IL-10 produced at foci of lytic replication in the oropharynx serves to dampen local cellular immune responses. IL-10 also acts as a B-cell growth factor (Rousset *et al.*, 1992). Both these effects could promote spread of the virus from the initial focus of infection into the circulating B-cell pool which later elicits the generalized T-cell response.

Functional characterization of infectious mononucleosis T cells has been the subject of numerous investigations. Early observations demonstrated that freshly isolated infectious mononucleosis cells can kill a wide range of EBV-positive cells of different HLA type while not recognizing EBV-negative targets (Svedmyr & Jondal, 1975; Seeley *et al.*, 1981). This apparent lack of MHC restriction has now been resolved by the demonstration that, although $CD8^+$ CTLs with a classical pattern of MHC class I-restricted virus-specific reactivity are activated during primary infection (Strang & Rickinson, 1987a,b), these cells often exhibit cross-reactive recognition of certain allo-antigens (Schendel *et al.*, 1992; Burrows *et al.*, 1994). This appears unlikely to be a complete explanation, however, and other $CD8^+$ T-cell clones coincidentally expanded *in vivo* alongside the EBV-specific response may well contribute to the alloreactivity (Klein *et al.*, 1981). The activated $CD4^+$ population may also contain an EBV-specific component (Misko *et al.*, 1991). The magnitude of the response in acute infectious mononucleosis has led to the speculation that EBV may encode, or induce the expression of, a superantigen with the capacity to activate certain T-cell subsets in a polyclonal manner. This possibility has recently received some experimental support (Sutkowski *et al.*, 1996a,b). It has become clear that an important component of the primary EBV-specific response in infectious mononucleosis is indeed the expansion of specific CTL clones that show the same pattern of EBV antigen recognition and epitope choice as the memory response (Steven *et al.*, 1996).

A range of cell-mediated immune mechanisms is probably involved in the control of persistent EBV infection. Early work on experimentally infected cultures allowed identification of a number of activities that can delay the initial B-cell proliferation. Many of these are independent of immune status and have been ascribed to $CD16^+$ NK cells (Masucci *et al.*, 1983), to IF α release (Thorley-Lawson, 1981) or to IF γ release by $CD4^+$ T cells (Farrell *et al.*, 1997); however, these reactivities can only rarely prevent the transformation process. Complete regression of lymphoblastoid cell growth occurs specifically in cultures from virus-immune donors and is mediated *in vitro* by CTL reactivated from the pool of circulating memory T cells (Moss *et al.*, 1978, 1979; Rickinson *et al.*, 1979). The frequency of CTL precursors, estimated from regression assays, is between 1 in 10^3 and 1 in 10^4 circulating T cells (Rickinson *et al.*, 1981a). A prospective study of infectious mononucleosis patients showed that CTL memory increases during convalescence and soon reaches a stable steady state (Rickinson *et al.*, 1980a).

Techniques for expansion of reactivated CTLs, as either bulk T-cell lines or clones in IL-2-conditioned medium, have greatly facilitated analysis of their function (Wallace *et al.*, 1981, 1982a,b,c). Most of the CTL precursors are $CD8^+$ cells and recognize autologous and MHC class I-matched lymphoblastoid cells but not autologous mitogen-activated B blasts, while a much smaller fraction of the EBV-specific CTLs are $CD4^+$- and MHC class II-restricted (Rickinson *et al.*, 1980b, 1981b; Misko *et al.*, 1984). The

importance of this T-cell subpopulation in the control of latent EBV infection is supported by the finding that $CD4^+$ CTLs are associated with protective immunity in the cotton-topped tamarin model (Wilson *et al.*, 1996a). $CD4^+$ cells could also express their function by triggering Fas/apo-1 protein/Fas–ligand interaction and inducing apoptosis (Durandy *et al.*, 1997). The $CD8^+$ effectors recognize small peptide fragments of endogenously synthesized viral proteins presented on the cell surface in the peptide binding groove of HLA class I molecules. This implies that all EBV antigens constitutively expressed on lymphoblastoid cells are potential sources of immunogenic peptides. Further, since antigen presentation selects for peptides with high affinity for a particular binding groove, the identity of the dominant peptide epitopes, and hence the proteins from which they are derived, differs between individuals, depending on their HLA class I type.

Studies on a large panel of virus-immune donors, encompassing a wide range of HLA types, have shown that reactivity against epitopes from EBNA-3A, -3B and -3C forms a substantial part of the EBV-specific CTL response in a high proportion of individuals (Gavioli *et al.*, 1992; Khanna *et al.*, 1992; Murray *et al.*, 1992a; Gavioli *et al.*, 1993). To some extent, this reflects the fact that class I alleles that are common in Caucasian populations, such as A3, B7, B8 and B44, all tend to select epitopes from these proteins. In addition, certain alleles such as A11 and B44, when present, tend to be the dominant restricting determinant for EBV-specific responses and focus the reactivity to epitopes in EBNA-3B and EBNA-3C, respectively. CTL responses to EBNA-2, EBNA-LP, LMP-1 and LMP-2 have also been identified in some individuals, while class I-restricted CTLs that recognize EBNA-1-expressing cells have not been demonstrated to date. The failure to trigger EBNA-1-specific class I-restricted CTLs seems to be due to a *cis*-acting inhibitory effect of the internal Gly-Ala repeat on MHC class I-restricted presentation (Levitskaya *et al.*, 1995). While this may not necessarily prevent the triggering of CTL responses, EBNA-1-specific MHC class II-restricted CTLs were in fact demonstrated (Khanna *et al.*, 1995). The inability of the immune system to recognize EBNA-1 expressed in latently infected targets would be a major advantage to the virus in its strategy for persistence in immunocompetent hosts.

An interesting feature of the EBV system is the extent to which an individual HLA allele focuses the CTL response, not only against a single viral protein but against a single immunodominant peptide epitope within that protein. Well-documented examples of immunodominant EBV peptides and their restricting class I alleles are listed in Table 4. Rare components of the memory response can be selectively activated *in vitro* by stimulation with autologous cells that express individual EBV antigens carried by recombinant adenovirus vectors (Morgan *et al.*, 1996). The precise constraint on both peptide–HLA interaction and recognition of the complex by the T-cell receptor imply that quite small changes in peptide sequence may abrogate CTL recognition. Some CTL responses are type-specific, in that they recognize proteins encoded by type-1 but not type-2 EBV strains, while other responses recognize epitopes that are shared between EBV types (Burrows *et al.*, 1990a,b; Brooks *et al.*, 1993b; Lee *et al.*, 1993a; Hill *et al.*, 1995; Lee *et al.*, 1995a). Sequence polymorphism affecting CTL epitope regions can also occur between strains of the same type. For example, the major A11-restricted epitope in

EBNA-3B residues 416–424 is conserved in most EBV type-1 strains worldwide but is specifically mutated in all the type-1 strains from Southeast Asian populations, in whom the A11 allele itself is unusually prevalent (de Campos-Lima *et al.*, 1993a; Burrows *et al.*, 1996b). The mutated peptide sequences either do not bind to HLA A11 or, in some cases, do bind but are no longer immunogenic for T-cell responses (de Campos-Lima *et al.*, 1994). This raises the possibility that viral strains with mutations in an otherwise immunodominant epitope may have enjoyed selective advantage in a population in which the presenting allele is expressed in a high proportion of the individuals.

Table 4. Cytotoxic T lymphocyte target epitopes in latent EBV antigens

Antigen	EBV type	Amino acid residues	Cognate peptide	HLA class I restriction
EBNA-2	1	67–76	DTPLIPLTIF	B51/A2
EBNA-3A	1	158–166	QAKWRLQTL	B8
	1	325–333	FLRGRAYGL	B8
	1 and 2	596–604	SVRDRLARL	A2
	1	609–617	RLRAEAQVK	A3
	1	379–387	RPPIFIRRL	B7
	1	458–466	YPLHEQHGM	B35
EBNA-3B	1	101–115	NPTQAPVIQLVHAVY[a]	A11
	1	399–408	AVFDRKSDAK	A11
	1	416–424	IVTDFSVIK	A11
	1	481–495	LPGPQVTAVLLHEES[a]	A11
	1	551–564	DEPASTEPVHDQLL[a]	A11
	1	243–253	RRARSLSAERY	B27
EBNA-3C	1 and 2	881–889	QPRAPIRPI	B7
	1	258–266	RRIYDLIEL	B27
	1 and 2	290–299	EENLLDFVRF	B44
	1	335–343	KEHVIQNAF	B44
LMP-2A/2B	1 and 2	426–434	CLGGLLTMV	A0201
	1 and 2	329–337	LLWTLVVLL	A0201
	1 and 2	453–461	LTAGFLIFL	A0206
	1 and 2	340–349	SSCSSCPLSK	A1101
	1 and 2	419–427	TYGPVFMCL	A2404
	1 and 2	200–208	IEDPPFNSL	B40011
	1 and 2	236–244	RRRWRRLTV	B27

Adapted from de Campos-Lima *et al.* (1996)
EBNA, EBV nuclear antigen; LMP, latent membrane protein
[a]The minimal epitopes have not been identified.

Given the unique features of the in-vitro model available for the EBV system, it is not surprising that much more is known about cell-mediated immune responses to latent than to lytic infection; however, recent evidence suggests that several of the immediate and early antigens of the EBV lytic cycle can serve as targets for specific $CD8^+$ CTLs

(Bogedain *et al.*, 1995; Steven *et al.*, 1997). Cells moving through the lytic cycle may therefore be subject to a number of lytic antigen-specific responses operating either through direct cytotoxicity and/or lymphokine release. Studies with in-vitro models also show that lytically infected cells become more susceptible to lysis by NK cells (Blazar *et al.*, 1980; Patarroyo *et al.*, 1980) and, in the presence of IgG antibodies to gp350, are sensitized to antibody-dependent cellular cytotoxicity effectors (Pearson *et al.*, 1978a, 1979).

1.4 Epidemiology of EBV infection

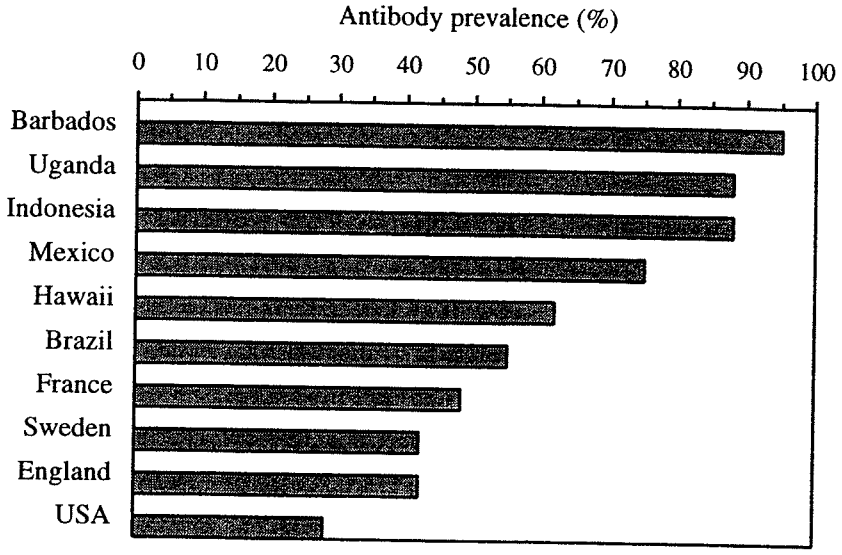
Extensive seroepidemiology studies have shown that EBV is ubiquitous throughout the world (Evans & Niederman, 1989), including the most remote populations, such as the Aleutian Islanders in Alaska (Tischendorf *et al.*, 1970) and Amazonian tribes, in whom other viral infections, such as measles, are absent (Black *et al.*, 1970).

By the third decade of life, 80–100% of individuals have become carriers of the infection. The age-specific seroprevalence of younger persons generally varies inversely with level of socioeconomic development, family size and hygienic standards. The oral route is the primary route of transmission of the virus; however, transmission by transfusion has been documented. In developing countries, infection is acquired in the first few years of life (Figure 3). Crowding and/or the practice of pre-chewing food for infants may be contributing factors. In the developed world, infection is often delayed to adolescence, when transmission is more likely because of intimate oral exposure (Evans & Niederman, 1989) (Figure 4). About 50% of primary infections during young adulthood result in clinical infectious mononucleosis (see section 1.4.1; Evans *et al.*, 1968; University Health Physicians & PHLS Laboratories, 1971; Hallee *et al.*, 1974; Gratama & Ernberg, 1995). Psychological and behavioural factors may influence the severity of clinical manifestations of EBV infections occurring after childhood (Kasl *et al.*, 1979).

The antibody response in healthy carriers is to a symptomless primary infection, as described in section 1.3.3 and reviewed recently by Evans and Kaslow (1997).

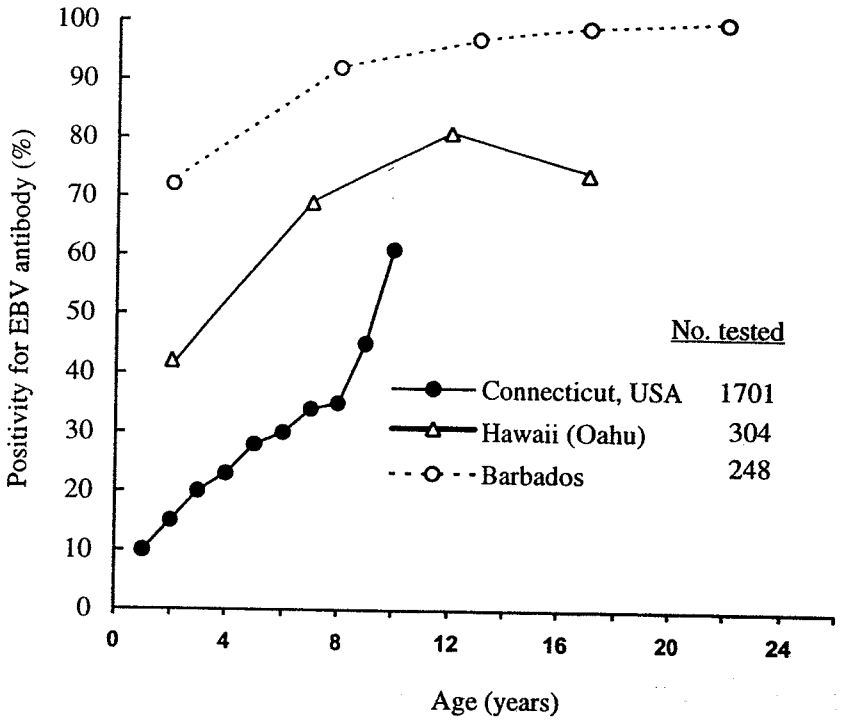
Use of molecular methods to characterize EBV genomes in human tumours led to the identification of several non-random polymorphisms, which are variably distributed between the two major EBV types and among several strains. As a result, there has been some controversy about the relationships between these polymorphisms. The major polymorphic markers identified to date and their geographic distribution are listed in Table 5. Little information is available about the incidence of these polymorphisms in the general population. On the basis of the results of a comparative study, Khanim *et al.* (1996) suggested that detection of EBV gene polymorphisms in human tumours simply reflects their overall prevalence in the respective normal population. Thus, although there is evidence from studies *in vitro* to suggest that some of these polymorphisms may be of functional relevance (see sections 4.3.1.2 and 4.5.1.6), their implication in the pathogenesis of EBV-associated tumours is not firmly established.

Figure 3. EBV antibody prevalence at age 4–6 years in various populations



From Evans & Niederman (1989)

Figure 4. Acquisition of EBV antibody by age in three areas



From Evans & Niederman (1989)

Table 5. EBV strain polymorphisms and their geographical distribution

Gene	Polymorphic marker	Goegraphical distribution	Reference
EBNA-2	Type 1 and type 2	Type 2 prevalent in central Africa and Alaska	de Campos-Lima *et al.* (1993a); Gratama & Ernberg (1995)
EBNA-3A, -3B, -3C	Type 1 and type 2	Type 2 prevalent in central Africa and Alaska	Gratama & Ernberg (1995)
EBNA-3B	Amino acid 424 K to R, T, N	South-east Asia, Papua–New Guinea	de Campos-Lima *et al.* (1993b, 1994)
EBNA-1	Amino acid 487: A, T, P, L, V	Worldwide (generated in individual virus carriers)	Bhatia *et al.* (1996); Gutiérrez *et al.* (1997)
LMP-1	C variant (loss of *Bam*HI site between W1 and I1 regions)	Southern China, Japan	Lung *et al.* (1990); Hu *et al.* (1991a); Chen *et al.* (1992a); Miller *et al.* (1994a); Gratama & Ernberg (1995)
	f variant (additional *Bam*HI site in *Bam*HI F)	Southern China	
	*Xho*I polymorphism	Southern China, Alaska	
	30-bp deletion (site loss)	China, Brazil, Africa, Europe (20–100% of the isolates)	
	33-bp repeat variation	Worldwide (generated in individual virus carriers)	
ZEBRA	Akata type (102 416 T to C mutation)	Far East	Packham *et al.* (1993)
	B95-8 type (102 743 G to A mutation)	United States, Europe, North Africa	
	Jijoye type (102 591 C to T mutation)	Central Africa	

EBNA, EBV nuclear antigen; LMP, latent membrane protein; ZEBRA, Z EBV replication activator; bp, base pair

1.5 Clinical conditions other than malignancy

1.5.1 *Infectious mononucleosis*

Infectious mononucleosis (glandular fever) is characterized clinically by fever, sore throat, tonsillitis, lymphadenopathy and splenomegaly. Tonsillitis may give rise to ulcers, leading to an erroneous diagnosis of bacterial infection. The diagnosis of infectious mononucleosis relies on the detection of atypical lymphoid cells in the peripheral blood, the occurrence of so-called heterophile antibodies and EBV seroconversion. In most cases, the disease runs its course within a few weeks, but more protracted cases are occasionally observed (Evans, 1972).

As primary EBV infection is usually asymptomatic, most of what is known about it has come from the study of patients with infectious mononucleosis and is based on the implicit assumption that asymptomatic primary EBV infection is milder than but not fundamentally different from infectious mononucleosis. This disease represents a benign lymphoproliferative disorder with prominent expansion of the paracortex of lymphoid tissues by numerous activated B blasts. Morphological, molecular and tissue culture studies have demonstrated the proliferation of EBV-infected polyclonal B blasts, accompanied by the growth of activated T cells (Svedmyr & Jondal, 1975; Tosato *et al.*, 1979; Svedmyr *et al.*, 1984; Brown *et al.*, 1986; Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Callan *et al.*, 1996; Niedobitek *et al.*, 1997b).

In-situ hybridization for the detection of EBERs has shown numerous latently EBV-infected cells in the paracortical areas of the tonsils of patients with infectious mononucleosis (Niedobitek *et al.*, 1992a; Anagnostopoulos *et al.*, 1995; Reynolds *et al.*, 1995; Niedobitek *et al.*, 1997b). Morphologically, these cells are a mixture, ranging from large immunoblasts, including HRS-like cells, to small lymphoid cells. Phenotypic studies in conjunction with in-situ hybridization have shown that the cells are largely B cells which often undergo plasma-cell differentiation (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b), in keeping with the results of Robinson *et al.* (1981), who showed that during acute infectious mononucleosis up to 80% of EBNA-positive cells in peripheral blood may express cytoplasmic immunoglobulins. The detection of EBV infection in cells with evidence of plasmacytic differentiation could be due to infection of cells already committed to plasma-cell differentiation; alternatively, and more probably, this process may be secondary to EBV infection, as indicated by the demonstration that EBV infection can induce polyclonal immunoglobulin synthesis in B cells *in vitro* (Rosén *et al.*, 1977; Kirchner *et al.*, 1979). It has been suggested that plasma-cell differentiation of EBV-infected cells may in part explain the occurrence of heterophile antibodies in infectious mononucleosis patients (Robinson *et al.*, 1981).

In addition to latently EBV-infected cells, a small number of cells expressing lytic cycle antigens, such as BZLF1 and EA, are detectable in tissues from patients with infectious mononucleosis. These tend to be small lymphoid cells that are often found adjacent to crypt epithelium. Phenotypic characterization by double-labelling immunohistochemistry has shown that BZLF1-positive cells with features of plasmacellular differentiation are frequent (Niedobitek *et al.*, 1997b), confirming the results of a study showing expression of VCA in B cells with plasmacytoid differentiation *in vitro* (Crawford & Ando, 1986). Although the expression of structural viral antigens has not been analysed, it would appear that plasma cells may sustain full replication of EBV and may represent a cellular source of infectious virus in the saliva of patients with infectious mononucleosis.

Transcriptional analysis suggests that a type-III EBV latency prevails in infectious mononucleosis, with expression of the full set of EBV latent genes, including Cp/Wp-driven *EBNA-1* (Falk *et al.*, 1990; Tierney *et al.*, 1994). A more detailed analysis of EBV gene expression at the level of the single cell, reveals, however, a more heterogeneous picture. Only a subset of cells co-expresses *EBNA-2* and *LMP-1*, characteristic of type-III latency. Most cells appear to be EBER-positive but negative for EBNA-2 and LMP-1,

suggesting a type-I latency, and some large immunoblasts are seen which appear to express LMP-1 in the absence of EBNA-2 (type-II latency). Moreover, there are many small lymphocytes that express EBNA-2 but no detectable LMP-1 (Niedobitek *et al.*, 1997b). It is uncertain if this represents a new type of EBV latency or merely a transitory phenomenon. The latter possibility is supported by the observation that *EBNA-2* expression precedes that of *LMP-1* in EBV-induced B-cell immortalization *in vitro* (Alfieri *et al.*, 1991).

While infectious mononucleosis is usually a benign, self-limiting disease, complications may ensue, including rupture of the spleen when there is pronounced splenomegaly. Neurological complications, interstitial nephritis with renal failure, hepatitis, interstitial pneumonia and anaemia have also been reported (Lloyd-Still *et al.*, 1986; Imoto *et al.*, 1995; Mayer *et al.*, 1996; López-Navidad *et al.*, 1996; Morgenlander, 1996; Schuster *et al.*, 1996; Sriskandan *et al.*, 1996). The underlying mechanisms of these diseases remain uncertain. It has been suggested that such complications of acute infectious mononucleosis are due mainly to tissue infiltration by EBV-carrying B cells and reactive activated T cells or are mediated by autoantibodies (Purtilo & Sakamoto, 1981). Indeed, Sriskandan *et al.* (1996) reported recently the detection of EBV-positive lymphoid cells in the interstitium of the lungs of a patient with interstitial pneumonia after acute infectious mononucleosis. Similarly, EBV-carrying lymphocytes have been demonstrated in the portal tracts and sinusoids of the liver in patients with EBV-associated hepatitis (Markin *et al.*, 1990; Randhawa *et al.*, 1990; Hubscher *et al.*, 1994; Imoto *et al.*, 1995); however, EBV infection of occasional hepatocytes has also been reported in acute cases (Imoto *et al.*, 1995). In a renal biopsy sample from a patient with renal failure in conjunction with acute infectious mononucleosis, no EBER-positive cells were found. It has been suggested that at least some of the cases with renal impairment can be explained by a concurrent streptococcal infection causing glomerulonephritis (Mayer *et al.*, 1996).

Control of primary EBV infection and transition into a state of asymptomatic viral persistence is mediated by T-cell immunity (Rickinson & Kieff, 1996). A clonal antigen-driven expansion of $CD8^+$ T cells was recently demonstrated in acute infectious mononucleosis (Callan *et al.*, 1996). The clonal response has been shown to include virus-specific components against both latent and lytic-cycle viral antigens and to disappear during convalescence (Callan *et al.*, 1996; White *et al.*, 1996). Cases in which T-cell control of primary EBV infection fails and fatal infectious mononucleosis ensues may be due to defined genetic defects such as in X-linked lymphoproliferative syndrome. Patients with this disorder suffer from an immune defect which makes them sensitive to EBV-induced diseases; most affected men die by the age of 40 (Purtilo, 1991). Sporadic cases of fatal infectious mononucleosis without defined defects in T-cell immunity also occur. In the majority of such cases, the host appears to be overrun by excessive, uninhibited proliferation of polyclonal B cells (Falk *et al.*, 1990; Purtilo, 1991), considered to be EBV-driven on the basis of the observation of a type-III latency. In some cases, however, the proliferating B cells appear to be monoclonal, and development of morphologically malignant lymphoma has also been reported (Falk *et al.*, 1990; Purtilo, 1991; Schuster *et al.*, 1996). Thus, patients with X-linked lymphoproliferative syndrome

may develop Burkitt's lymphoma carrying *c-myc* translocations (Purtilo, 1991). Moreover, cases with transition of an infectious mononucleosis-like illness into malignant lymphoma have been reported. These include high-grade B-cell non-Hodgkin's lymphomas, acute lymphoblastic leukaemia and even cases of Hodgkin's disease (Abo *et al.*, 1982; Veltri *et al.*, 1983; Finlay *et al.*, 1986). In some of these cases, detection of EBV in the tumour cell populations was not attempted or was unsuccessful; thus, the relationship with the virus remains tenuous.

These findings suggest a scenario in which patients with impaired T-cell immunity develop massive polyclonal EBV-driven B-cell lymphoproliferation, which may show transition into classical malignant lymphoma. The observation that Burkitt's lymphomas in patients with X-linked lymphoproliferative syndrome harbour *c-myc* translocations (Purtilo, 1991) indicates that the transition from polyclonal lymphoproliferative disorder to frankly malignant lymphoma requires additional genetic changes.

1.5.2 *Oral hairy leukoplakia*

Oral hairy leukoplakia is an epithelial lesion of the tongue which was originally described in HIV-infected individuals but was subsequently also found in immunosuppressed transplant recipients. Oral hairy leukoplakia manifests itself as a raised white lesion, typically located at the lateral border of the tongue, but which may extend to other parts of the oral mucosa. Histologically, this lesion is characterized by intracellular oedema of the more superficial squamous cells, some nuclear alterations and pronounced hyperparakeratosis. While some of these changes suggested that oral hairy leukoplakia represents a human papillomavirus-induced lesion, this has not been substantiated. In 1985, EBV DNA was detected in this lesion, and the virus has been localized to the superficial epithelial cells. Linear virion DNA and the expression of viral lytic cycle antigens, e.g., BZLF1 and VCA, have been shown, indicating that epithelial cells may support EBV replication (Greenspan *et al.*, 1985; Gilligan *et al.*, 1990a; Young *et al.*, 1991). Walling *et al.* (1992) reported the presence of multiple viral strains in patients with oral hairy leukoplakia and suggested that the intense viral reproduction in this lesion contributes to the generation of EBV variants.

Morphological studies have demonstrated that the expression of lytic cycle antigens such as BZLF1 and VCA is restricted to the more differentiated upper epithelial cell layers (Greenspan *et al.*, 1985; Young *et al.*, 1991). Similarly, the expression of some latent proteins, such as EBNA-1 and LMP-1, has been reported in these cells in the context of lytic EBV infection (Thomas *et al.*, 1991a; Niedobitek *et al.*, 1991a; Sandvej *et al.*, 1992; Murray *et al.*, 1996). Current evidence suggests that EBERs are expressed at low levels, if at all (Gilligan *et al.*, 1990a; Niedobitek *et al.*, 1991a; Lau *et al.*, 1993).

In contrast to the abundance of the virus in upper epithelial cells, viral genomes and EBV gene products associated with latent infection are consistently absent from the basal or parabasal epithelial cells of oral hairy leukoplakia (Niedobitek *et al.*, 1991a; Thomas *et al.*, 1991a; Sandvej *et al.*, 1992). Together with the absence of a detectable episomal population of EBV genomes (Gilligan *et al.*, 1990a), this indicates that oral hairy leukoplakia is an isolated focus of lytic EBV infection, with no detectable latent phase.

Furthermore, oral hairy leukoplakia can be induced to regress by treatment with acyclovir, indicating that this lesion is indeed caused by EBV infection (Resnick *et al.*, 1988).

1.5.3 *The X-linked lymphoproliferative syndrome*

The X-linked lymphoproliferative syndrome is a hereditary immunodeficiency disorder characterized by a self-destructive immune response to primary EBV infection (Provisor *et al.*, 1975; Purtilo, 1976; Turner *et al.*, 1992). Patients are usually asymptomatic until they encounter EBV, but may present symptoms of immunodeficiency prior to EBV infection. After primary EBV infection, the majority of patients develop a fulminant infectious mononucleosis with a virus-associated haemophagocytic syndrome, which results in liver and bone-marrow destruction, with a fatal outcome. Patients who survive the primary infection are at high risk for developing malignant lymphoma, hypogammaglobulinaemia or aplastic anaemia. Because the immune response to EBV is ineffective, a serological response to the EBV infection may be weak or missing (Seemayer *et al.*, 1995). The *XLP* gene has been localized to Xq25 (Skare *et al.*, 1987; Sylla *et al.*, 1989), and the region spanning the smallest deletion in patients has been completely cloned (Lamartine *et al.*, 1996; Lanyi *et al.*, 1997) and sequenced; nevertheless, a good candidate gene has still to be identified. Identification of the function of the *XLP* gene is of prime importance for better understanding the complex interaction between EBV and its host.

Antiviral agents such as IF γ and α and acyclovir are ineffective, and the only curative treatment for X-linked proliferative disorder is allogeneic bone-marrow transplantation (Williams *et al.*, 1993).

1.6 Control and prevention

1.6.1 *Drugs*

Few drugs prevent replication of EBV, and even fewer are clinically useful; in fact, there are no clinically effective anti-EBV drugs with acceptable side-effects. Most of the drugs that prevent EBV replication were originally tested in studies with herpes simplex virus (HSV). The principal reagent effective against EBV is 9-(2-hydroxyethoxymethyl)-guanine, known as acyclovir when phosphorylated (Colby *et al.*, 1980; King, 1988), which is a substrate for EBV deoxynucleoside kinase and inhibits EBV DNA polymerase (King, 1988). Related compounds that are being evaluated include gancyclovir [9-(1,3-dihydroxy-2-propoxymethyl)guanine] (Cheng *et al.*, 1983), famcyclovir, phosphonoformic acid (Foscarnet) and valacyclovir (L-valyl ester of acyclovir); these are being used particularly in the management of viral infections in patients who have received bone-marrow transplants, with varying degrees of success (Dix & Wingard, 1996). Other drugs with activity against EBV include acyclic nucleoside phosphonate analogues (Lin *et al.*, 1991), a thymidine L-nucleoside (Yao *et al.*, 1996), (*E*)-5-(2-bromovinyl)- and 5-vinyl-1-β-D-arabinofuranosyluracil (Lin *et al.*, 1992), phosphorothioate oligodeoxynucleotides without sequence specification (Yao *et al.*, 1993), cucurbitane triterpenoids

(Konoshima *et al.*, 1994, 1995) and triterpenoids from ferns (Konoshima *et al.*, 1996) and quinones (Konoshima *et al.*, 1989).

A distinction should be made between those compounds that directly inhibit viral functions and those that indirectly inhibit EBV replication by interfering with interactions between host-cell components and viral components. For example, retinoic acid down-regulates *BZLF1* expression, thus indirectly reducing lytic cycle induction (Sista *et al.*, 1995), and ciclosporin A (see IARC, 1990) affects lytic cycle EBV gene expression induced by anti-immunoglobulin (Goldfeld *et al.*, 1995; Liu *et al.*, 1997). Retinoids irreversibly inhibit the growth of EBV lymphoblastoid cell lines *in vitro* by unknown mechanisms (Pomponi *et al.*, 1996). Other forms of second-messenger regulation of *BZLF1* expression undoubtedly exist (Daibata *et al.*, 1994). Induction of the EBV lytic cycle by *n*-butyrate and phorbol esters (Ito *et al.*, 1981) may also be blocked by a number of compounds; however, the effect may occur not by direct interference with EBV DNA replication but by blocking of the induction pathway activated by phorbol esters and *n*-butyrate (Daniel *et al.*, 1984; Lin & Smith, 1984). Cucurbitane and fern triterpenoids and quinones fall into this category (Konoshima *et al.*, 1989, 1994, 1995, 1996). The anti-HIV agent zidovudine (AZT) also appears to reduce EBV lytic cycle replication, but again probably by an indirect action on the intracellular environment and not directly on EBV replicative functions (Lin & Machida, 1988; Mar *et al.*, 1995).

Antiviral drugs are ineffective in the treatment of infectious mononucleosis. The clinical symptoms were not reduced by acyclovir in a number of double-blind trials. Viral excretion in throat washings was reduced in acyclovir-treated patients but resumed on cessation of treatment (Andersson & Ernberg, 1988; van der Horst *et al.*, 1991). Because EBV infection is primarily latent and only very few infected cells actually enter the lytic replicative cycle, drugs that interfere with the replication cycle are clearly of limited use in clearing EBV infection. Thus, administration of acyclovir to EBV-seropositive patients, while reducing the shedding of infectious virus in the saliva, has no effect on the level of EBV-infected circulating B lymphocytes (Yao *et al.*, 1989a,b). Treatments with acyclovir in combination with prednisolone had beneficial effects in patients with fulminant mononucleosis (Andersson & Ernberg, 1988) but had no effect on the course of less severe disease in a multicentre, placebo-controlled, double-blind trial, although oropharyngeal shedding of EBV was reduced (Tynell *et al.*, 1996). Oral hairy leukoplakia regresses on treatment with acyclovir (Resnick *et al.*, 1988), but treatment of X-linked proliferative syndrome with acyclovir has been unsuccessful (Sullivan & Woda, 1989).

The search continues for drugs that block EBV replication efficiently but have low toxicity. Their toxicity appears to be due to their incorporation into chromosomal or mitochondrial DNA. Nucleoside analogues continue to be a rich source of potentially useful compounds, the most recent being a thymidine L-nucleoside, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil, which appears to act by a different mechanism, as it is not incorporated into DNA and is of comparatively low toxicity *in vitro* (Yao *et al.*, 1996).

The EBV latency C promoter is the origin of transcripts for six EBV latent viral proteins; it is active in lymphoblastoid cell lines but silent in many EBV-associated tumours and tumour cell lines. As the C promoter is hypermethylated in these tumour lines, only *EBNA-1* is expressed. 5-Azacytidine inhibits DNA methyltransferase, resulting in demethylation of EBV genomes, activates Cp transcription and induces the expression of *EBNA-2* (Masucci *et al.*, 1989; Robertson *et al.*, 1995b). 5-Azacytidine and similar reagents may therefore render EBV tumour cells susceptible to T-cell control by inducing expression of EBV genes other than *EBNA-1* (Robertson *et al.*, 1995c). Numerous other pharmaceutical agents have been investigated that are cytotoxic to EBV tumour cells. These reagents and the effects of interferons, interleukins and RNA antisense oligomers are not considered here. Clearly, much progress will be required before drugs that are effective against EBV replication and/or transcription and are of low toxicity become available in the clinical setting.

1.6.2 *Prospects for vaccines*

The rational design of an EBV vaccine depends on an understanding of the EBV life cycle and the natural immune responses generated by the virus in humans *in vivo*. How the virus sustains a persistent latent infection in the face of a healthy immune response is not fully understood. Consequently, the development of EBV vaccines has so far been speculative. Since immunosuppressed patients are predisposed to EBV B-cell lymphomas and increased shedding of virus in the saliva (Yao *et al.*, 1985a; Thomas *et al.*, 1991b), immune control must normally exist *in vivo*. Immune responses to EBV include the generation of virus-neutralizing antibodies against envelope glycoproteins (Pearson *et al.*, 1970; de Schryver *et al.*, 1974), recognition of latent antigens by MHC class I-restricted CTLs (Rickinson *et al.*, 1980b), antibody-dependent cellular cytotoxicity against cells carrying surface gp350 (Khyatti *et al.*, 1991) and MHC class II-restricted T-cell responses (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991; Lee *et al.*, 1993b; White *et al.*, 1996). Primary MHC class II-restricted CTLs have been found in seronegative people (Misko *et al.*, 1991). More recently, an EBV superantigen activity was discovered that stimulates Vβ 13 $CD4^+$ T cells from cord blood (Sutkowski *et al.*, 1996a). The various strategies adopted for EBV vaccination have changed as the understanding of the biology of the virus has evolved.

The development of a vaccine to control diseases associated with EBV must be based on the premise that most primary infections occur during the first few years of life and that sooner or later almost the entire population is exposed. The simplest view is that prevention of EBV infection, if attainable, will prevent the diseases associated with EBV, but prophylactic, post-infection and therapeutic EBV vaccination strategies should be distinguished:

- The aim of prophylactic vaccination is to prevent or modify primary infection.
- The aim of post-infection vaccination is to modify the existing immune status of an infected individual.
- Therapeutic vaccination is selectively targeted against viral antigens expressed in tumour cells.

One rationale for developing post-infection vaccines is that the rapid rise in serum IgA antibodies against lytic cycle antigens could serve as a diagnostic indicator of the onset of nasopharyngeal carcinoma and could be used to identify high-risk populations (Zeng, 1985). This marker presumably represents an increase in viral replication at some site that is linked to the emergence of the tumour. Intervention with a gp350 vaccine before this event takes place might well alter the immune balance at a crucial time and prevent development of nasopharyngeal carcinoma. Similarly, high anti-VCA titres have been used as indicators of the development of endemic Burkitt's lymphoma in Ugandan children (de Thé *et al.*, 1978a).

Therapeutic vaccination is intended to enhance the immune response against EBV-associated tumours that have already developed. In this approach, strategies involving the few EBV latent genes that are expressed in the tumours themselves, such as *EBNA-1*, *LMP-1* and *LMP-2*, would be most appropriate (Rickinson, 1995; Ambinder *et al.*, 1996; Moss *et al.*, 1996). The use of EBV latent antigen peptides representing MHC class I-restricted CTL epitopes in EBNA-3A to induce CTL memory by vaccination has recently been reviewed (Moss *et al.*, 1996). No studies on therapeutic or post-infection vaccines have yet been reported.

Current approaches to the development of prophylactic vaccines may also have applications for post-infection and therapeutic vaccines. The latter strategies have obvious target populations; the main purpose of prophylactic EBV vaccination in western countries would be to prevent infectious mononucleosis, which occurs in 50% of persons in whom EBV infection is delayed until adolescence. The incidence of infectious mononucleosis in the United States is approximately 65/100 000 (Evans, 1993), which is greater than the incidence of all other reportable diseases except gonorrhoea. Prophylactic vaccination to prevent or modify EBV infection and disease in the less developed world would require vaccination of children during the first year of life.

1.6.2.1 *Selection of an EBV vaccine antigen molecule*

Immunization can prevent tumour induction in non-human primates infected with *Herpesvirus saimiri* (Laufs & Steinke, 1975). Attenuated and killed EBV variants cannot be used as vaccines because of their oncogenic potential. The first prototype EBV vaccine was based on a purified viral envelope glycoprotein, since a correlation had been observed in serum samples between their viral neutralization activity and the reactivity of antibodies against viral membrane antigens on productively infected lymphocytes *in vitro* (Pearson *et al.*, 1970; de Schryver *et al.*, 1974). When these virus-neutralizing antibodies were used to immunoprecipitate viral glycoproteins from lysates of infected and radiolabelled cells, the viral membrane antigens were found to consist of three principal glycoprotein components (North *et al.*, 1980; Qualtière & Pearson, 1980; Thorley-Lawson & Geilinger, 1980). The larger and most abundant of the three are gp350 (gp340) and gp220, the latter being a spliced variant of the former which is encoded by the same ORF, *BLLF1* (Beisel *et al.*, 1985). Gp350 contains up to 50% carbohydrate, much of which is *O*-linked (Morgan *et al.*, 1984; Serafini-Cessi *et al.*, 1989). At least 20 ORFs in the EBV genome could potentially code for glycoproteins as

they have potential sites for *N*-linked sugars (Baer *et al.*, 1984). With the exceptions of the 110-kDa EBV counterpart of HSV gB coded for by *BALF4* (Gong & Kieff, 1990; Gong *et al.*, 1987), the product of *BILF2* (Mackett *et al.*, 1990), gp85, which is the HSV gH analogue (Heineman *et al.*, 1988; Miller & Hutt-Fletcher, 1988), and the product of *BDLF3* (Nolan & Morgan, 1995), none have been identified or characterized and certainly not evaluated for incorporation into a vaccine. Several EBV glycoprotein products will perhaps have to be incorporated into any vaccine formulation.

A number of interesting properties of gp350 have emerged since the first studies showed it to be a protective immunogen in an animal model of EBV-induced lymphoma (Epstein *et al.*, 1985). Gp350 is the virus ligand which binds to the complement receptor CD21 (Tanner *et al.*, 1987, 1988; Moore *et al.*, 1989; Birkenbach *et al.*, 1992). Cross-linking of CD21 by gp350 induces synthesis of IL-6 (Tanner *et al.*, 1996) and can modulate the synthesis of IL-1 and tumour necrosis factor (Gosselin *et al.*, 1992). CD21 is part of a membrane-signalling complex involved in the activation of B-cell immune responses, and cross-linking by gp350 may be an important early event in driving infected B cells into the cell cycle before immortalization (Sinclair & Farrell, 1995). Indeed, it might be expected that gp350 could affect other cells that express CD21, including follicular dendritic cells, T cells, monocytes and epithelial cells. These possible effects of gp350 could be both advantageous and disadvantageous in vaccination; however, the potential effects of gp350 vaccines in these respects have not been evaluated.

As serious problems have arisen in designing vaccines against HIV and influenza virus because of their capacity to generate antigenic variants, the question has arisen as to whether wild-type variation in the gp350 antigen is significant and would render a conventional vaccine based on a single laboratory strain ineffective. The sequences of *gp350* genes from several type-1 and type-2 virus isolates have been compared and no significant differences found. Furthermore, a panel of monoclonal antibodies recognizes the gp350s from all sources equally well (Lees *et al.*, 1993).

As mentioned above, the selection of a viral molecule for use as a subunit vaccine was based originally on the assumption that protective immunity would be provided by virus-neutralizing antibodies directed at glycoproteins on the surface of the virus. This is clearly not the case either in the tamarin model or the common marmoset model (see below) and is unlikely to be the only immunological criterion for protection against EBV-associated diseases in humans.

1.6.2.2 *Animal models of EBV infection, disease and vaccination*

Two primate model systems have been used in the development of a vaccine against EBV: the cotton-topped tamarin (*Saguinus oedipus oedipus*) (Miller *et al.*, 1977) and the common marmoset (*Callithrix jacchus*) (Wedderburn *et al.*, 1984; Emini *et al.*, 1986). Both have shortcomings which must be taken into account in interpreting results. Early studies showed that inoculation of EBV into the common marmoset gives rise to a poorly defined, mononucleosis-like syndrome. These observations have been extended to show that infection of the common marmoset with the M81 strain of EBV gives rise to the

long-term maintenance of antibodies to viral antigens. Furthermore, the presence of EBV DNA has now been reliably demonstrated in tissues and oral fluids of infected animals by PCR analysis (Farrell *et al.*, 1997). A crucial finding was that when infected common marmosets were paired in the same cages with uninfected animals, the uninfected animals seroconverted within four to six weeks, indicating that this animal model of EBV infection will be much more useful than previously thought (Cox *et al.*, 1996).

Injection of large doses of EBV into cotton-topped tamarins gives rise to multicentric oligoclonal B-cell lymphomas, which closely resemble those found in immunosuppressed human patients, all of which express a type-III EBV latency pattern (Cleary *et al.*, 1985; Young *et al.*, 1989b). There appears to be a threshold dose below which lymphomas do not develop. Cotton-topped tamarins have never been shown to be infected by the oral route and do not support persistent latent infection at the same level as humans, although small numbers of EBV-positive B cells have been detected in animals that have been immunized and protected from a lymphomagenic dose of EBV (Niedobitek *et al.*, 1994). This finding indicates that sterilizing immunity is not induced. The model is useful for testing the efficacy of an EBV vaccine, since protection can be achieved against a massive tumorigenic dose of virus injected intraperitoneally. This severe challenge contrasts with the normal mode of infection of humans, who receive a very small quantity of virus orally.

Other animal models have been developed for EBV-related herpesviruses. Malignant lymphomas can be induced in rabbits by an EBV-related herpesvirus from *Macaca arctoides* (Wutzler *et al.*, 1995); and a murine herpesvirus, MHV-68, although a gamma-2 herpesvirus (see Section 5 and Table 2 in the introduction) may be useful in vaccine development since it can cause lymphomas in mice and maintains a persistent infection in B cells, appearing to be a reservoir for infection *in vivo* (Nash *et al.*, 1996). Inoculation of mice with severe combined immunodeficiency with human EBV-infected lymphocytes gives rise to human B-cell lymphomas (Mosier, 1996), but this model has not yet been exploited in the development of a vaccine. [In the absence of a satisfactory animal system, it seems reasonable to progress directly to human trials, after immunogenicity and toxicity have been evaluated in animals, because of the difficulty of relating data obtained in the available animal models to human EBV infection and disease.]

1.6.2.3 *Natural gp350 subunit vaccines*

The first demonstration that gp350 is an effective subunit vaccine in the tamarin lymphoma model was obtained using material isolated from large bulk cultures of cells infected with the B95-8 laboratory isolate of EBV and induced to productive infection with sodium butyrate and phorbol esters (Morgan *et al.*, 1983). Only very small quantities of protein were isolated, which were purified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, eluted from the gel and renatured after removal of sodium dodecyl sulfate. These small quantities of protein were incorporated into artificial liposomes made from phosphatidylcholine. Despite the small quantities of protein and the crude adjuvant system, complete protective immunity was induced in tamarins against a tumorigenic dose of EBV (Epstein *et al.*, 1985).

Gp350 prepared by mono-Q anion exchange (David & Morgan, 1988) and incorporated into immunostimulating complexes (iscoms) (Morein *et al.*, 1984; Morgan *et al.*, 1988a; Morein *et al.*, 1995) or into Syntex adjuvant formulation (Allison & Byars, 1986; Morgan *et al.*, 1989) induces protective immunity in tamarins against the standard lymphomagenic dose of virus and results in high titres of virus-neutralizing antibodies. Protective immune responses were obtained with a dose of 5 μg or less of antigen when Syntex adjuvant formulation or iscoms were used.

1.6.2.4 *Recombinant gp350 subunit vaccines*

In order to overcome the problems of yield, purity and the presence of EBV DNA, efforts have been made to express the *gp350* gene in bacteria, yeast and eukaryotic cells. Parts of the gene have been expressed in bacteria (Beisel *et al.*, 1985; Zhang *et al.*, 1991; Pither *et al.*, 1992a,b), and the complete gene has been expressed in yeast (Schultz *et al.*, 1987); however, glycosylation does not occur in bacteria, and carbohydrates different from those found on mammalian cells are added when the gene is expressed in yeast (Emini *et al.*, 1988). Emphasis was also placed on producing gp350 that could induce virus-neutralizing antibodies, since this was believed to be the key immune response required to obtain protective immunity against the virus. Although the bacterial product was recognized by antibodies from normal seropositive individuals, none of the antibodies neutralized the virus (Pither *et al.*, 1992a,b). The yeast gp350 product was not sufficiently antigenically similar, presumably because of glycosylation, and this approach was discontinued (Emini *et al.*, 1988). Cell-mediated immune responses to gp350 are known to be protective against EBV-induced lymphoma in the tamarin, however, and are likely to be important in protective immunity in humans. Since tertiary structure and glycosylation are unlikely to impede generation of the appropriate T-cell responses, both the bacterial and yeast products should be re-examined as candidate vaccines. A truncated version of *gp350* has been expressed in a baculovirus system, but the product has not been characterized in terms of its ability to induce virus-neutralizing antibodies or to induce protective immunity in the tamarin lymphoma model (Nuebling *et al.*, 1992).

The *gp350* gene has now been expressed in a number of mammalian cell systems in which glycosylation and post-translational modifications occur, which are closely similar to those found on the natural product (Motz *et al.*, 1986; Whang *et al.*, 1987; Conway *et al.*, 1989; Hessing *et al.*, 1992; Madej *et al.*, 1992). No difference has been found between these products and the natural product gp350 in terms of their ability to induce virus-neutralizing antibodies, bind a range of monoclonal antibodies and, in some studies, to stimulate the proliferation of gp350-specific T cells (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991). In some studies, the membrane anchor sequence has been removed from the gene, allowing secretion of the expressed eukaryotic product into the culture medium (Whang *et al.*, 1987; Hessing *et al.*, 1992; Madej *et al.*, 1992). This approach offers major advantages in the large-scale preparation of a defined product that is relatively easy to purify. A bovine papillomavirus expression system and a Chinese hamster ovary cell system have been adapted to secrete a gp350 product in large quantities

(Hessing *et al.*, 1992; Madej *et al.*, 1992). This product is also indistinguishable from authentic gp350 in immunological terms. Another major benefit of use of this system is the guaranteed absence of potentially oncogenic EBV DNA.

1.6.2.5 *T- and B-cell epitopes on the gp350 molecule*

The generation of a panel of monoclonal antibodies (Qualtière *et al.*, 1987) allowed categorization of gp350 B-cell epitopes into at least seven groups on the basis of antibody competition. The mapping of particular immune functions to specific regions of the molecule could ultimately allow the design of a synthetic peptide or a recombinant-derived vaccine of predetermined immunological specificity. The immunological profile of gp350 is certainly complex. Most, if not all, of those epitopes associated with virus-neutralizing antibodies are discontinuous and are dependent on the conformation of the molecule, including the contribution of the large mass of carbohydrate. A possible exception is a linear epitope in the amino-terminal region between residues 317 and 327, which is recognized by antibodies from rabbits immunized either with gp350 bacterial fusion proteins containing the sequence, with intact virus or with gp350 iscoms. One approach for the identification of B- and T-cell epitopes is to express overlapping fragments of the gene in bacteria as β-galactosidase fusion proteins (Pither *et al.*, 1992a,b). The ability of antibodies made against gp350 in vaccinated animals and present in normal human sera to bind to the various fragments of gp350 expressed as bacterial fusion proteins was determined by western blotting. This procedure allows the detection only of linear epitopes, and a number of immunodominant epitopes were recognized with antisera from normal seropositive individuals. None of these epitopes was able to bind virus-neutralizing antibodies. Clear differences in the recognition patterns of sera from normal seropositive people and from immunized animals were seen, which may simply reflect species differences in immune responses but which may also reflect different immune responses after natural infection and after vaccination with a purified molecule. This difference might explain why neutralizing antibodies against linear epitopes were found in sera from vaccinated rabbits but not in sera from naturally infected humans. No method is yet available for identifying or reconstructing discontinuous B-cell epitopes, although the use of random-sequence synthetic peptides or random peptide sequences in phage libraries should be pursued (Geysen *et al.*, 1986; Cwirla *et al.*, 1990; Scott & Smith, 1990).

Gp350 bacterial fusion proteins have also been used to detect T-cell epitopes and, in some cases, to map them. It would be expected that, although the bacterial products do not retain the native conformation of gp350 and do not carry their carbohydrate complement, they should still have the capacity to be recognized by T cells after MHC class I or class II presentation. Bacterial fusion proteins were screened for their ability to induce proliferation in previously isolated gp350-specific T-cell clones from normal seropositive individuals. A large number of potential T-cell epitopes have been tentatively identified using an algorithm for this purpose, but only a few epitopes have been located in the amino-terminal region (Wallace *et al.*, 1991; Pither *et al.*, 1992a,b). Given the difficulty of identifying appropriate epitopes and the inevitable variation in

epitopes among individuals, the development of synthetic gp350 peptide vaccines seems to be remote.

1.6.2.6 *Choice of adjuvant*

Most proteins and glycoproteins are weakly immunogenic when inoculated alone into animals, and gp350 is no different in this respect. An adjuvant is invariably required to stimulate the immune response to the antigen, except when it is presented as part of a live virus or other vehicle, in which case a range of natural immune responses is triggered. A variety of effective immunological adjuvants is now available, and new ones appear from time to time (Gupta & Siber, 1995). They probably do not all work in the same way and are certainly not equally effective. Antigen presentation, targeting and delayed decomposition may all be important functions of the delivery vehicle. The mode of action of the small immuno-activator molecule may include lymphokine production, antigen processing, mitogenicity and upregulation of HLA expression. A key element in the action of adjuvants is probably their effect on the differentiation of $CD4^+$ T cells into T-helper 1 or 2 subsets after vaccination, which can influence the ratio of the two types (Dotsika *et al.*, 1997). T-helper 1 cells produce IL-2 and IF γ and enhance cell-mediated immune responses, while T-helper 2 cells produce IL-4, -5 and -10 and augment humoral immune responses (Mosmann & Coffman, 1989). It has been shown that IL-12 can substitute for certain bacterial adjuvants in enhancing cell-mediated immune responses, at least against the protozoan parasite *Leishmania major* (Afonso *et al.*, 1994). Studies of protection in the tamarin lymphoma model with natural product gp350 subunit vaccines have so far been confined to the use of artificial liposomes (North *et al.*, 1982), a threonyl derivative of muramyl dipeptide in pluronic triblock copolymers (Syntex adjuvant formulation) (Allison & Byars, 1986, 1987), iscoms (Morgan *et al.*, 1988a; Dotsika *et al.*, 1997) and aluminium salts (Finerty *et al.*, 1994).

Because some of the more powerful adjuvants have not yet been licensed for human use, studies have focused on evaluating aluminium salts, the only adjuvants licensed for use in humans, in the tamarin lymphoma model. In experiments with recombinant gp350 (Finerty *et al.*, 1994), the formulations raised protective immunity against EBV-induced lymphoma. It is worth remembering that challenge with a lymphomagenic dose of virus represents an extreme, completely unphysiological event and, in some respects, a very stringent test of the efficacy of any vaccine. Given that three out of five animals immunized with gp350 in alum were protected against lymphoma, this formulation might be sufficiently effective in humans. It also induced protective immunity in the *Callithrix jacchus* model of an EBV-induced mononucleosis-like syndrome (Emini *et al.*, 1986, 1989).

1.6.2.7 *Live virus–vector recombinants*

The disadvantage of subunit vaccines most often cited is that they generate poor and sometimes inappropriate responses, although the new generation of adjuvants mentioned above should overcome this objection. Any failure to induce a broad, powerful immune response should be set against the advantages of using biologically dead material of

absolutely defined composition (Murphy, 1989; Moss, 1996). The choice will depend on a variety of factors, including the immune responses that are required, assuming that the induction of both T- and B-cell responses is necessary together with the establishment of immunological memory. Both iscoms (Takahashi *et al.*, 1990) and Syntex adjuvant formulation induce proliferative, cytotoxic T-cell responses and memory (Byars *et al.*, 1991). Aluminium salts also allow the induction of limited cell-mediated immune responses (Dillon *et al.*, 1992).

Many recombinant viruses have been made that express one or more important vaccine molecules; so far, these have been used only rarely in humans. Gp350 has been expressed in vaccinia virus (Mackett & Arrand, 1985), adenovirus (Ragot *et el.*, 1993) and varicella virus (Lowe *et al.*, 1987). Recombinant vaccinia viruses expressing gp350 have been derived from both the relatively virulent WR laboratory strain and the attenuated Wyeth vaccine strain and both have been tested in the tamarin lymphoma model (Morgan *et al.*, 1988b). Protective immunity was induced in three of four animals by the recombinant WR strain but in none of the animals immunized with the Wyeth strain derivative. Both groups of animals responded to vaccination, with high levels of antibody against vaccinia proteins, but no antibodies against gp350 could be detected in any animal. The levels of antibodies to vaccinia proteins were substantially lower when the Wyeth derivative was used, and this is presumably a reflection of the degree to which the strain has been attenuated. The key observation in these experiments is that the WR strain derivative gives protective immunity in the absence of antibodies to gp350, so that the immunity in this case is provided by some form of cell-mediated immune response. When protective immunity was induced in the tamarin lymphoma model by injection of a replication-defective adenovirus expressing gp350, antibodies against gp350 were induced but had no capacity to neutralize EBV *in vitro* (Ragot *et al.*, 1993).

More work is needed to develop effective vaccinia recombinants which strike the correct balance between attenuation and immunogenicity. The *gp350* gene may be expressed in a canarypox vector (Taylor *et al.*, 1992) or in a vaccinia recombinant derived from the Copenhagen strain which has been specifically attenuated by the removal or inactivation of individual genes (Tartaglia *et al.*, 1992). The vaccinia gp350 recombinant previously tested in the tamarin model (Morgan *et al.*, 1988b) was also tested in the common marmoset model but with the M81 EBV strain, which resembles more closely the wild-type strains of EBV in the general population than the standard B95-8 strain. Replication of the challenge virus in the vaccinated group was reduced in comparison with control groups (Mackett *et al.*, 1996). The Oka varicella–zoster viral strain has been used to make recombinants expressing *gp350* (Lowe *et al.*, 1987), but protection was not induced in animals.

A number of recombinant adenoviruses that express foreign antigens have been constructed, and recombinant adenovirus-expressing gp350 has been tested in the tamarin model (Ragot *et al.*, 1993). Replication-defective recombinants were made in which the E1 region is deleted and the virus can be propagated only in a helper cell line which provides the deleted E1 function (Graham *et al.*, 1977). Adenoviruses have the capacity to down-regulate HLA expression in the cells they infect, but the E3 region responsible for this function has also been deleted in these replication-defective recom-

binants. Several features of adenoviruses have made them attractive for vaccine delivery. Firstly, adenoviruses type-4 and type-7 vaccines have already been used on a large scale in the United States Armed Forces to prevent respiratory disease, with a good safety record (Top *et al.*, 1971a,b). Secondly, the adenovirus can be encapsulated and given orally; although primary immune contact is in the gut lymphoid tissue, mucosal immunity is induced in the respiratory tract. Mucosal immune responses may be advantageous, but they could allow IgA-enhanced entry into epithelial cells (Sixbey & Yao, 1992).

The most significant results obtained with a recombinant virus expressing gp350 are those of a study in which the Chinese vaccinia strain (*Tien Tan*) was used to vaccinate a small group of seronegative and seropositive children in southern China (Gu *et al.*, 1995). Antibody levels to gp350 were reported to have been raised in subjects who were already seropositive and to have been induced in those who were seronegative at the beginning of the trial. Six of nine vaccinated children who were seronegative for EBV at the time of vaccination showed no evidence of natural EBV infection 16 months after vaccination.

1.6.2.8 *Cell-mediated immune responses to gp350*

Tamarins mount cell-mediated immune responses after inoculation with a lymphomagenic dose of EBV, as measured in a regression assay in which the outgrowth of autologous EBV-infected B cells was inhibited in the presence of blood lymphocytes from tamarins with spontaneously regressed EBV-induced tumours. This capacity to inhibit growth could be boosted by reinoculation of the tamarins with a sub-lymphomagenic dose of virus 18 months after the first challenge (Finerty *et al.*, 1988). More recently, EBV-specific CTLs which are $CD4^+$ MHC class II-restricted have been identified in the re-stimulated animals (Wilson *et al.*, 1996a).

Demonstration of the presence of gp350-specific T cells in vaccinated animals which can proliferate in response to gp350 *in vitro* is an immediate goal. Efforts are also being made to detect gp350-specific CTLs, but the creation of an effective target cell is a problem. Recombinant vaccinia virus expressing gp350 could be used to infect autologous fibroblasts or lymphocytes, as was done for the latent antigens (Khanna *et al.*, 1992; Murray *et al.*, 1992a). T-Cell clones specific for gp350 have been detected in normal seropositive individuals (Ulaeto *et al.*, 1988; Wallace *et al.*, 1991; Lee *et al.*, 1993b), and gp350 can stimulate the production of T cells that prevent EBV-induced transformation *in vitro* (Bejarano *et al.*, 1990). In certain circumstances, cells that express gp350 can be good targets for antibody-dependent cellular cytotoxicity (Khyatti et al., 1991). This activity is readily detected in sera from rabbits immunized with whole EBV, but its detection in sera from tamarins or rabbits vaccinated with purified gp350 has not been reported. The mechanism of cell-mediated immune protection in the tamarin lymphoma model is difficult to explain if a protective function for antibody is excluded, since protection is gp350-specific. The tumour cells themselves seem unlikely targets for gp350-specific responses as they do not express gp350 in readily detectable amounts. Cell-mediated responses induced by vaccinia–gp350 vaccination may in some way reduce the effective viral challenge dose to below the threshold required for tumour

induction. Immune responses in tamarins that had spontaneously recovered from a lymphomagenic viral challenge were possibly directed through MHC class II-restricted T cells positive for both CD4 and CD8 (Wilson *et al.*, 1996a). These cells may inhibit proliferation of lymphoblastoid cell lines by other mechanisms than perforin-mediated lysis.

1.6.2.9 *Vaccines against EBV latent antigens*

A completely different approach to EBV vaccination has emerged during the past few years, based on the knowledge that $CD8^+$ T cells are responsible, at least in part, for limiting the number of EBV-infected B lymphocytes that express most or all of the viral latency genes in the circulation of normal seropositive individuals. Could induction of these immune cells before primary infection be effective in preventing or modifying primary infection? The central problem with this approach is the same as that with gp350. How does the virus persist despite the presence of presumably effective cellular and humoral immune responses? Normal seropositive individuals have CTLs that are specific for EBNA-3A and EBNA-3C and some with activity against LMP-2 and EBNA-2. An important observation is that a large proportion of the total CTL activity in normal seropositive individuals cannot be accounted for by the EBV latent genes (Khanna *et al.*, 1992; Murray *et al.*, 1992a). The target antigens for this proportion of the CTL population have yet to be identified.

A number of epitopes that are recognized by CTLs have been located in EBNA-3A. Moss *et al.* (1996) consider that these epitopes could be used in a vaccine to elicit T-cell memory, which could then be activated to produce EBNA-3A-specific CTLs. Since the target epitope varies between HLA types, several synthetic peptides corresponding to different epitopes would have to be incorporated in such a vaccine. A relatively small number of CTL epitopes would be able to elicit an immune response in the vast majority of the population. Phase 1 trials are in progress of an EBNA-3A peptide, FLRGRAYGL, which is restricted through the HLA-B8 allele. Some circulating, latently infected B cells in bone marrow and/or the circulation, however, express only EBNA-1, which is refractory to MHC class I-restricted antigen processing (Levitskaya *et al.*, 1995; Frisan *et al.*, 1996); these B cells thus cannot be targets for MHC class I-restricted CTLs induced by either vaccination or natural infection.

1.6.2.10 *Conclusions*

The potential usefulness of gp350-based EBV vaccines depends on the roles of gp350 and lytic replication in infection and disease. Primary infection in the oropharynx, an essential stage in the infectious cycle, is mediated by gp350 in the viral envelope. Vaccine-induced mucosal IgA against gp350 could well act at this level, controlling to some extent but probably not preventing infection (Yao *et al.*, 1989a), but there is also the theoretical possibility that mucosal IgA could enhance EBV infection (Sixbey & Yao, 1992). It is impossible to know at this stage what effects gp350 vaccine-induced immune responses will have on primary EBV infection. Will it be possible to induce sterilizing immunity, or is latent infection inevitable, and, once latency has been established, will

the immune responses of the vaccinee be more effective in preventing EBV disease than those of a normal, unvaccinated, seropositive person?

Patients with infectious mononucleosis have some circulating B cells that express lytic cycle antigens (Anagnostopoulos *et al.*, 1995; Niedobitek *et al.*, 1997b), but acyclovir treatment has no effect on the course or symptoms of the disease, although virus shedding is reduced (Tynell *et al.*, 1996). It is possible that $CD4^+$ T cells primed by gp350 vaccination would be reactivated on viral challenge. Such cells could influence the course of infectious mononucleosis by inducing apoptosis of EBV-infected B cells and by down-regulating the large monoclonal or oligoclonal populations of $CD8^+$ T cells which account for much of the lymphocytosis symptomatic of infectious mononucleosis (Callan *et al.*, 1996).

The observations that the onset of nasopharyngeal carcinoma is accompanied by a rise in the titre of serum IgA antibodies against lytic cycle antigens (Zeng, 1985) and that high anti-VCA titres in Ugandan children are prognostic for the development of endemic Burkitt's lymphoma (de Thé *et al.*, 1978a) also indicate a potential role for intervention with a gp350 vaccine. Answers to the above questions will not be obtained until candidate vaccines have been evaluated in human trials. The major EBV envelope glycoprotein gp350, when expressed as a genetically engineered product in mammalian cells, could now be evaluated in trials in which the target population is first-year university students — a population at high risk of contracting infectious mononucleosis. The endpoints of a trial in this population would be EBV seroconversion, possible development of infectious mononucleosis and changes in a range of immunological parameters, such as virus-neutralizing antibodies. Any further progress in EBV vaccine development will depend heavily on the outcome of such trials.

1.6.3 *Passive immunotherapy*

As discussed in section 1.3.3, although CTLs cannot eliminate EBV from the body, they appear to be essential for maintaining control of latently infected cells. Thus, infusion of such cells should be a safe, effective form of treatment for immunodeficient patients with EBV-related lymphoproliferation. This therapeutic approach has been explored in a number of studies and clinical trials of bone-marrow transplant recipients. Papadopoulos *et al.* (1994) and Heslop *et al.* (1994) used unseparated leukocytes from EBV-seropositive donors to treat patients with EBV-associated immunoblastic lymphomas that arose after transplantation of T cell-depleted bone-marrow allografts. All of the patients responded, as judged by clinical and laboratory criteria, but some developed fatal pulmonary complications. Severe graft-versus-host disease was observed in the survivors. Thus, although adoptive transfer of unseparated leukocytes seems to be effective, this therapeutic strategy may not be generally applicable owing to the high risk of secondary complications. The problem of graft-versus-host disease has been overcome in more recent studies in which EBV-specific CTLs of donor origin reactivated *in vitro* were infused into allograft recipients (Rooney *et al.*, 1995; Heslop *et al.*, 1996). The patients did not develop complications that could be attributed to the infusion, and in three patients with signs of EBV reactivation (> 100-fold increase in EBV DNA concen-

tration measured by semiquantitative PCR), infusion of CTLs reduced the EBV load to control levels within three to four weeks. In one case, clinical resolution of the immunoblastic lymphoma was observed. Genetic marking of the infused CTLs was used to demonstrate that specific T cells that responded to challenge with virus-infected cells *in vivo* or *ex vivo* persisted for as long as 18 months. These findings support more widespread use of CTLs in the treatment of infections and cancer. Their applications may extend to other EBV-associated malignancies, such as Hodgkin's disease and nasopharyngeal carcinoma, which express a relatively restricted range of EBV-encoded antigens that could serve as target cells for CTL therapy (see Tables 3 and 6).

2. Studies of Cancer in Humans

2.1 Burkitt's lymphoma

2.1.1 *Clinical features and pathology*

2.1.1.1 *Clinical features*

The jaw is the most frequently involved site and the commonest presenting feature in patients with Burkitt's lymphoma in equatorial Africa (Burkitt, 1958, 1970a) and Papua–New Guinea (ten Seldam *et al.*, 1966; Burkitt, 1967; Magrath *et al.*, 1992). The tumour frequently affects multiple jaw quadrants (Figure 5). Jaw involvement is age-dependent, occurring much more frequently in young children, since it arises in close proximity to the developing molar tooth buds. In series of cases of Burkitt's lymphoma in Uganda, 70% of children under five years of age and 25% of patients over 14 had jaw involvement (Burkitt, 1970a). Very young children who do not have overt jaw tumours often have orbital involvement (Olurin & Williams, 1972; Figure 6); some of these orbital tumours may arise in the maxilla. In general, jaw involvement appears to be more frequent in regions of higher incidence, even within equatorial Africa; however, patients from, for example, highland regions in Africa, in which the annual incidence rate of Burkitt's lymphoma is much lower, are also of higher median age, and this probably accounts for the lower frequency of jaw tumours (Burkitt & Wright, 1966; Kitinya & Lauren, 1982). It has been suggested that the frequency of jaw tumours is decreasing in some regions of equatorial Africa, with a corresponding increase in the fraction of abdominal tumours but with no clear change in the age-related incidence (Nkrumah, 1984).

Abdominal involvement is found in a little more than half of equatorial African patients at presentation (Burkitt & Wright, 1963; Burkitt, 1970b; Williams, 1975) and as many as 80% of patients in other countries (Magrath, 1991, 1997). There appear to be differences in the intra-abdominal sites of involvement in endemic countries (e.g. equatorial Africa and Papua–New Guinea) and in those where the disease is sporadic (Europe, Australia and North America). Presentation with a resectable mass in the right iliac fossa or with intussusception (arising from intraluminal tumours), for example, both of which are common in sporadic tumours, is uncommon in African patients. Thus, Wright (1970)

Table 6. Cellular origin and patterns of viral gene expression in EBV-associated malignancies

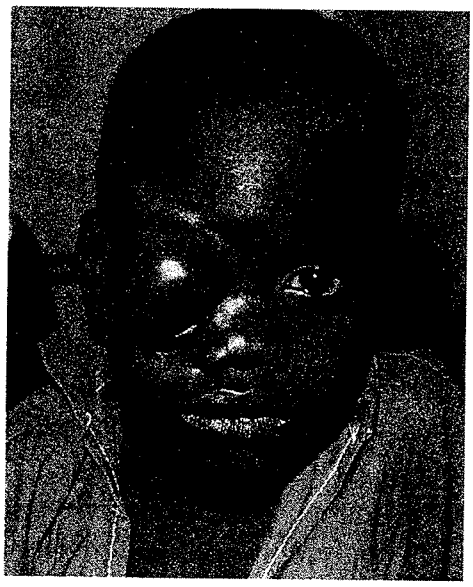
Cell type	Tumour	EBV association (%)	EBV gene expression			Latency type	Reference
			EBNA-1	*EBNA-2, -3A, -3B, -3C, -LP*	*LMP-1/2*		
B Lymphocyte	Immunoblastic B lymphoma	100	+	+	+	III	Thomas *et al.* (1990)
B Lymphocyte	Endemic Burkitt's lymphoma	> 95	+	–	–	I	Rowe *et al.* (1987a)
T Lymphocyte	Immunoblastic T lymphoma	50–90	+	–	+	II	Jones *et al.* (1988); Kikuta *et al.* (1988)
T lymphocyte	Midline granuloma	100	+	–	+	II	Minarovits *et al.* (1994a)
HRS cells	Hodgkin's disease	40–50	+	–	+	II	Herbst *et al.* (1991a); Pallesen *et al.* (1991c); Deacon *et al.* (1993); Grasser *et al.* (1994)
Nasal epithelium	Nasopharyngeal carcinoma	100	+	–	+ (60%)	II	Fåhraeus *et al.* (1988); Brooks *et al.* (1992); Busson *et al.* (1992a); Smith & Griffin (1992)
Thymic epithelium	Thymic carcinoma	Case report	+	–	+	II	Leyvraz *et al.* (1985)
Gastric epithelium	Gastric carcinoma	90	+	–	–	I	Imai *et al.* (1994a)
Smooth muscle	Leiomyosarcoma	100	+	+	ñ	IV	Lee *et al.* (1995b)

Viral antigen expression was demonstrated by immunohistochemistry and/or reverse transcriptase–polymerase chain reaction.
HRS, Hodgkin and Reed-Sternberg

Figure 5. Ugandan patients with Burkitt's lymphoma involving the jaw

From WHO (1969); left, mandibular tumour; right, maxillary tumour

Figure 6. Orbital Burkitt's lymphoma in a Ugandan child



From WHO (1969)

reported no case of intussusception in Uganda among over 500 cases. In contrast, involvement of the mesentery and omentum (i.e. extraluminal) is common in African cases of Burkitt's lymphoma, hence the rarity of Burkitt's lymphoma-associated intussusception in Africa. Ascites may be a manifestation of abdominal disease regardless of geographic location. Precise figures for the involvement of various intra-abdominal and

retroperitoneal structures at presentation are not available from Africa, as relatively few centres have adequate radiological facilities. The available data are based largely on the results of clinical examinations, sometimes supplemented by laparotomy, or by autopsy studies dating from the 1960s (O'Conor, 1961; Wright, 1964, 1970; Williams, 1975; see below).

Bone-marrow involvement is seen in some 7–8% of Ugandan patients at presentation and relapse but in about 20% of patients in Europe and the United States. An additional fraction of patients in Europe and the United States presents with a leukaemic syndrome, referred to as the French–American–British subtype 'L3' or acute B-cell Burkitt's lymphoma (Magrath & Ziegler, 1980).

Central nervous system involvement — including cerebral spinal fluid pleocytosis, cranial nerve palsy and paraplegia due to paraspinal disease — is relatively common in Africa, being found in about one-third of patients at presentation (Ziegler *et al.*, 1970), but is much less common in regions of sporadic incidence (Magrath, 1997). Interestingly, cranial nerve involvement (usually due to direct infiltration by tumour cells) is frequently not associated with cerebral spinal fluid pleocytosis at presentation, but malignant cells are nearly always detectable in cerebral spinal fluid of patients with cranial nerve palsy at relapse (Magrath, 1991). The optic nerve is frequently compressed and the surrounding subarachnoid space involved, however, when an intraorbital tumour is present (Ifekwunigwe *et al.*, 1966). Cranial nerves and meninges have been described as the only sites of disease at presentation (Osuntokun *et al.*, 1973). Paraplegia is the presenting feature in 15% of Ugandan patients but in less than 1% of patients in the United States (Magrath, 1991, 1997). In Ugandan patients, the spine is quite frequently the only evident site of disease, such that lamininectomy is required to make a diagnosis (and to relieve spinal cord pressure). Intracerebral disease is very uncommon and usually occurs in patients who have had persistent or multiple relapsed cranial nerve palsy or cerebral spinal fluid pleocytosis (Wright, 1970; Magrath *et al.*, 1974); however, presentation with raised intracranial pressure from parenchymal brain infiltration is observed, albeit very uncommonly (Odeku *et al.*, 1973; Osuntokun *et al.*, 1973). Other sites of disease that are occasionally observed include the salivary glands, thyroid, breast (in pubertal girls or lactating women), testis, bone, pleura and heart, with involvement of the pericardium or, infrequently, cardiac muscle (Burkitt, 1970a; Aderele *et al.*, 1975; Durodola, 1976; Magrath, 1991, 1997). Interestingly, pharyngeal involvement, peripheral lymphadenopathy and splenic involvement are rare in the African patient (although there is frequently splenomegaly from malaria) and rather more common in patients in regions of sporadic disease, although both splenic and lymph-node involvement are significantly more frequently observed in cases of Burkitt-like lymphoma.

2.1.1.2 *Gross pathology*

Burkitt's lymphoma, unlike follicular lymphomas and diffuse large B-cell lymphomas, arises predominantly at extranodal sites. The kidneys are a frequent site of disease in sub-Saharan Africa, being the organ involved in 80% of cases at autopsy; the ovaries are frequently bilaterally involved in young females, representing the site seen in

81% of autopsies in females. The adrenals are the third most commonly involved intra-abdominal organ, are more often involved at autopsy than the liver or spleen and are about as frequently involved in children as the jaw (58%). Bowel involvement is common, but other organs are involved less frequently. The lung parenchyma is very rarely involved, but serosal infiltration of the pleura and peritoneum, resulting in effusion, is frequent. Wright (1964, 1970) never observed involvement of the thymus. Fatal Burkitt's lymphoma is usually accompanied by widespread dissemination.

Grossly, the tumour is fleshy, creamy and soft. Areas of necrosis and haemorrhage are seen only in very large tumours. The tumours can locally infiltrate surrounding tissues and may spread via the lymphatics or blood vessels. In the central nervous system, choroid plexus involvement is not uncommon and is usually associated with cerebrospinal fluid pleocytosis. Haematogenous dissemination may result in parenchymal involvement of the brain and spinal cord, in which case cerebrospinal fluid pleocytosis may be difficult to detect.

2.1.1.3 *Histological characteristics*

Burkitt's lymphoma (small non-cleaved-cell lymphoma, Burkitt's type) is classified as a non-Hodgkin's lymphoma and is characterized by a monomorphic cytoarchitecture composed of medium-sized cells (between those of large B-cell lymphoma and small-cell lymphocytic lymphoma). These cells do not have the characteristics of plasmacytoid cells or mature lymphocytes; they have a high nucleus to cytoplasm ratio, a round or oval nucleus with a coarse or 'open' chromatin pattern and usually two to five readily discernible nucleoli. A few cells may have a single large nucleolus, but when more than a few such cells are present the tumour is probably a Burkitt-like lymphoma. The cytoplasm is intensely basophilic, staining strongly with methyl-green pyronine (pyroninophilic) because of the abundant free ribosomes, which are readily visible on transmission electron microscopy. Intracytoplasmic lipid vacuoles which stain with oil-red O and Sudan black are usually apparent on imprint or smear cytology (Berard *et al.*, 1969). Histological sections often reveal the presence of tingible body macrophages scattered among the tumour cells, giving rise to a 'starry sky appearance' in which nuclear debris from apoptotic tumour cells is discernible. This appearance is not, however, pathognomonic of Burkitt's lymphoma and may be seen in other lymphomas (O'Conor, 1961).

Burkitt-like (small non-cleaved, non-Burkitt's) lymphomas are similar in appearance to Burkitt's lymphoma, but are distinguishable by a greater degree of pleomorphism, a fraction of the cells being similar in size to those of large-cell or centroblastic lymphomas, and a higher frequency of cells with a single large nucleolus in the neoplastic population. This entity may simply represent the borderline between Burkitt's lymphoma and large-cell lymphoma.

Burkitt's lymphoma is invariably of B-cell origin, the presence of surface immunoglobulin being first shown in 1967 (Klein *et al.*, 1967), and has the immunophenotypic characteristics of a subset of germinal-centre cells; hence, the cells do not or very uncommonly express terminal deoxyribonucleotide transferase. B-Cell lineage markers such as CD19, CD20, CD22 and CD79a and surface immunoglobulin are always

demonstrable. The surface immunoglobulin is usually IgM, but IgG and IgA are occasionally present and kappa or lambda immunoglobulin light chains are nearly always detected. Other surface antigens that are expressed in most Burkitt's lymphomas include CD10 and CD77, but CD23 and CD5 are absent (Harris *et al.*, 1994). Burkitt's lymphoma cells express low levels of HLA class I adhesion and activation molecules such as CD54, CD11a/18 and CD58 (Masucci *et al.*, 1987; Billaud *et al.*, 1989; Andersson *et al.*, 1991). Some EBV-negative Burkitt's lymphomas of American origin synthesize and secrete immunoglobulin (Benjamin *et al.*, 1982).

2.1.2 *Descriptive epidemiology*

2.1.2.1 *Historical aspects*

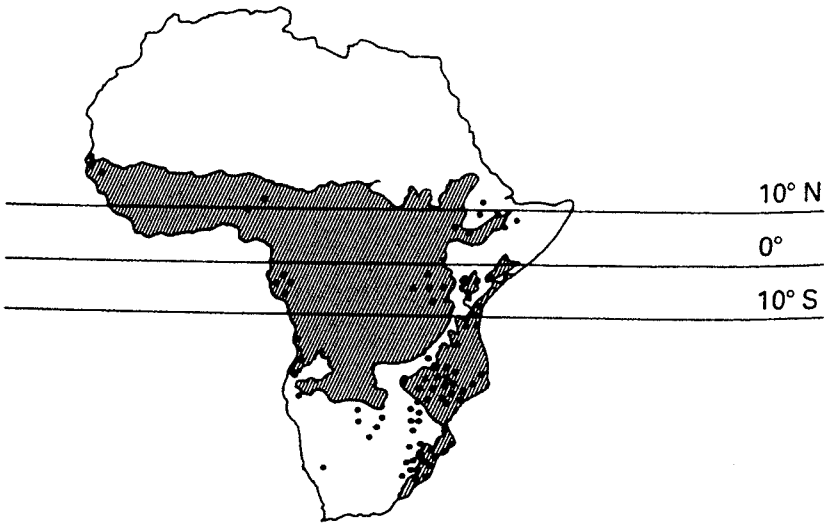
Burkitt's lymphoma was first identified in Africa, where there is every reason to believe that it has existed for millennia. Its presence prior to its description by Europeans is attested to by wooden masks depicting jaw and orbital tumours (Pulvertaft, 1965). It seems probable that the environmental factors relevant to its pathogenesis, with the possible exception of HIV, were relatively constant prior to the lifestyle changes brought about by the technological revolution of this century. The first known medical description is that of Sir Albert Cook who, with his brother, established the first mission hospital in Uganda in 1897. Cook's meticulous records were analysed many years later by Davies *et al.* (1964a,b), who reproduced in their report a drawing made by Cook in 1910 of a malignant jaw tumour in a child. Subsequently, a number of expatriate pathologists working in Africa noted that facial 'sarcomas' and lymphomas occurred at high frequency in African children. Most of these tumours were probably Burkitt's lymphomas.

Smith and Elmes (1934) described a series of 500 malignant tumours collected in Lagos, Nigeria, which included 16 jaw tumours recorded as sarcomas, three of which were in children, and 10 'round-cell sarcomas of the orbit', all in children under 10 years of age. More than a decade later, Davies (1948) observed that neoplasms of the reticuloendothelial system occurred at high frequency in Uganda, and Edington (1956; Edington & Giles, 1968) working in Ghana, then known as the Gold Coast, commented on the relatively high frequency of maxillary lymphosarcoma in children. Thijs (1957) reported from the Belgian Congo that 74 of 145 children with malignant tumours had lymphosarcoma. Interestingly, jaw tumours accounted for only 11 of the latter cases. De Smet (1956), also working in the Belgian Congo, mentioned in his report on children with lymphosarcoma that multiple organ sites, including the maxilla, orbit, abdomen and thyroid, were frequently involved.

Denis Burkitt and his pathologist colleagues, Davies and O'Conor noted, like De Smet, that children with jaw tumours often had histologically similar tumours at multiple organ sites, particularly in the abdomen (Burkitt, 1958; O'Conor & Davies, 1960). They subsequently demonstrated that the tumour occurred at high frequency in a broad belt across Africa, extending approximately 15° N and S of the equator, with a southern prolongation on the eastern side of the continent (Burkitt, 1962a,b,c; Figure 7). While Burkitt was initially under the impression that the tumour he was studying was a

sarcoma, O'Conor and Davies, in a review of the malignant tumours of children in the Kampala Cancer Registry in Uganda, recognized, as had Thijs, that malignant lymphoma accounted for some 50% of all childhood malignant tumours in the registry (O'Conor & Davies, 1960; O'Conor, 1961).

Figure 7. Distribution of Burkitt's lymphoma in Africa



From Haddow (1963)
The shaded area represents the area in which, on climatological grounds, Burkitt's lymphoma might be expected to occur. The black points show the distribution of the series of cases compiled by D. Burkitt. The method used was to fill in any degree-square in which the condition had been recorded, irrespective of the number of cases.

Soon after these observations in Africa, O'Conor, Wright (who had also worked in Uganda) and others reported histologically indistinguishable tumours in children in Europe and the United States (Dorfman, 1965; O'Conor *et al.*, 1965; Wright, 1966). At about the same time, strong similarities between the distribution of Burkitt's lymphoma and that of yellow fever were seen (Burkitt & Davies, 1961; Haddow, 1963), and it was therefore suggested that Burkitt's lymphoma might be caused by a vectored virus.

2.1.2.2 *Incidence*

Estimates made some 30 years ago suggested a relatively high incidence rate of Burkitt's lymphoma in Africa, with about 5–10 cases per 100 000 children below the age of 16 years and a peak incidence between five and 10 years of age. In Nigeria, the incidence in 1960–63 was reported to be 22 per 100 000 in 5–9-year-old boys and 10 per 100 000 in girls (Edington & MacLean, 1964). Williams (1975) found that Burkitt's lymphoma accounted for 682 of 1325 tumours in a hospital-based tumour registry in Ibadan, Nigeria, in 1960–72.

Burkitt's lymphoma now accounts for 30–70% of childhood cancers in equatorial Africa. In the United States, the incidence of Burkitt's lymphoma is 2–3 per million children per year. Recent estimates of incidence rates worldwide range from zero, which

may be due to differences in nomenclature or diagnostic practices, to 3.6 per 100 000 per year.

2.1.2.3 *Climatic determinants*

The very high frequency of Burkitt's lymphoma in equatorial Africa and its climatically determined distribution, first described in the early 1960s, focused attention on the possibility of a causal association with an environmental agent. The evidence that malaria is such an agent is based largely on the similarity of the distribution of holoendemic malaria and African Burkitt's lymphoma and on associations between rates of parasitaemia and the likelihood of developing Burkitt's lymphoma (see section 2.1.4.1).

The peri-equatorial distribution of patients in Africa with a clinical syndrome consistent with Burkitt's lymphoma was established by the initial investigations of Burkitt and several colleagues throughout Africa and on responses on questionnaires administered in a large number of hospitals on the continent (Burkitt, 1962a,b,c; Haddow, 1963; Burkitt, 1985). These investigators observed that the tumour occurred in a belt across equatorial Africa, with a prolongation to the south-east (Mozambique and Natal) (see Figure 7). Within this region, however, the tumour was rare or absent in a number of densely populated areas (Burkitt, 1963, 1969, 1970a). These included south-west Uganda, Rwanda and Burundi, the highlands of Kenya and the United Republic of Tanzania, the islands of Pemba and Zanzibar and some parts of Zaire. Several sharply delineated transitions from high to low incidence were observed, the majority between river valleys or lake shores and highland regions. In Nigeria, the relative rarity of the tumour in the arid (30 in [76 cm] of rainfall per year between the months of June and September) but densely populated northern region, Kano, contrasted with its much greater prevalence in wetter (up to 400 in [10 m] of rainfall per year), more sparsely populated regions less than 400 miles [640 km] to the south. Interestingly, this pattern applied to West Africa in general, the tumour being common throughout the southern parts of these countries (Accra in Ghana being an exception) but rare in the north. With some exceptions (e.g. the islands of Pemba and Zanzibar, Kinshasa, Brazzaville and Lambarene), the distribution was clearly determined by altitude in East Africa and by rainfall in West Africa. Thus, in East Africa, the tumour did not occur at any notable frequency above 1500 feet (450 m), or in regions where the mean temperature fell below 15.5 °C in any month (Haddow, 1963), and in West Africa the tumour did not in occur in regions in which the annual rainfall was less than 50 cm.

Burkitt and Wright (1966) showed in their series of histologically proven cases that the incidence of the tumour was much higher in the northern, eastern and central regions of Uganda. In Kigezi, in the south-west, for example, the incidence of 0.6 per 100 000 was estimated to be one-twentieth of that in the north-west. The low-incidence areas are highlands over 5000 feet (1500 m) above sea level, e.g. Kigezi, which continues westwards into the high central plateau that forms much of Rwanda and Burundi and extends into north-western Tanzania. Less than 4% of the cases in the Kampala registry occurred in children living in the south-western districts, which accounted for 25% of the population of Uganda; however, some 20% of the population of the central region of Uganda,

Buganda, is comprised of peoples from the low-incidence south-western region (Rwanda, Burundi and Kigezi), and about 19% of the cases of Burkitt's lymphoma recorded at Mulago Hospital up to the time of the report occurred in these immigrant peoples. Moreover, the average age of patients in the immigrant groups was 16.5 years, while that of patients from high-incidence areas was 8.1 years. Some of the immigrants with Burkitt's lymphoma had been living in the area for fewer than three years. The average age of patients from low-incidence areas was also higher than that of patients from high-incidence areas. These data strongly suggested that differences in the geographical distribution of the tumour were not a consequence of genetic differences among the tribes of Uganda but were related to environmental differences, being entirely consistent with the previously described barriers of temperature (altitude) and rainfall.

Haddow (1963) subsequently refined the map of the distribution of Burkitt's lymphoma in relation to climatic parameters and pointed out that the high-frequency regions were those in which the rainfall was more than 20 in (50 cm) per year (Figure 7). This finding is consistent with the hypothesis that Burkitt's lymphoma is caused by a vectored virus, since investigators at the East African Virus Research Institute in Entebbe, which Haddow directed, had shown that the yellow fever virus cannot replicate in mosquito vectors when temperatures fall below 60 °F (15 °C), accounting for the virtual absence of yellow fever in people living above 5000 feet (1500 m). In addition, adult mosquitoes do not survive in dry weather, but rely upon drought-resistant eggs in regions prone to dry spells. In West Africa, such regions were defined as those in which the annual rainfall was less than 20 in (50 cm). By using maps in which the distribution of Burkitt's lymphoma was superimposed on isotherms and isohets, it was possible to determine that only 5% of cases of Burkitt's lymphoma fell outside these limits of temperature and rainfall.

The distribution of Burkitt's lymphoma was also shown (Burkitt, 1963) to be very similar to that of a mosquito-vectored arbovirus disease, *o'nyong nyong* fever, in Uganda (Shore, 1961). Thus, it seemed highly probable that insect vectors were in some way involved in transmission of the disease. Tumour-free areas were readily explained on the basis of the absence of the vector, the vectored microorganism or both.

Wright and Roberts (1966), who studied 324 histologically proven cases of Burkitt's lymphoma seen in Uganda between 1959 and 1964 and 425 cases of other types of lymphoma seen during the same period, showed that the remarkably precise climatic determinants of the distribution of Burkitt's lymphoma did not apply to the distribution of other types of lymphoma in Uganda, which varied, instead, solely with the density of the population. Similar data were reported from northern Nigeria by Edington (1978) in respect of other malignant lymphomas.

Dalldorf *et al.* (1964) reported on the distribution of Burkitt's lymphoma in Kenya. The lowest incidence was found in the Kalenjin tribe, living in the highland belt (above 5000 feet [1500 m]), and the highest incidence in coastal or lake-shore dwelling tribes (below 5000 feet). There was no significant difference in the incidence of squamous-cell carcinoma among these tribal groups. These authors also reported on exposure to natural radiation, to arboviruses (by serology) and to malaria. Children of the Kalenjin tribe had

a variable rate of splenomegaly, this region being considered to be free of malaria in the recent past, in contrast to coastal tribes and the Luo, in regions where malaria was considered to be holoendemic. The prevalence of malaria in these tribes was confirmed by the rates of sickle-cell trait (negatively correlated) and glucose-6-phosphate dehydrogenase deficiency (positively correlated) (Bienzle *et al.*, 1981).

Eshleman (1966) reported on 31 cases of Burkitt's tumour seen at the Shirati Hospital in the United Republic of Tanzania. All of the patients came from lowland areas, less than 4500 feet (1350 m) above sea level, although the other patients seen at the hospital came from the Mara region with a height above sea level varying from 3700 to 5500 feet (1110–1650 m).

Goma (1965) carried out a survey of the environment in Uganda within a two-mile zone of 21 huts in which cases of Burkitt's lymphoma had occurred. He found permanent or semi-permanent water nearby and usually dense vegetation — ideal breeding conditions for mosquitoes. Goma easily trapped mosquitoes from the vicinity of the huts and was able to identify 42 different species. Williams (1967) pointed out that, among the large variety of arthropods known to transmit human diseases, only *Mansonia* and *Anopheles* species had similar distributions in Uganda to Burkitt's lymphoma.

More recent data (Kitinya & Lauren, 1982) confirm the relationship between the incidence of Burkitt's lymphoma and altitude. On Mount Kilimanjaro in northern Tanzania, the relative frequency of Burkitt's lymphoma (2.2% of all malignancies) was less than that in the coastal and low-lying regions of Uganda and Tanzania. Moreover, on the slopes of Mount Kilimanjaro, cases of Burkitt's lymphoma occurred predominantly in the sparsely populated areas below 1000 m, although some cases were found in densely populated areas up to 1500 m. As in earlier studies in East Africa, the average age of patients from this low-incidence area was higher (16.4% were over 20 years of age) than that of patients from high-incidence areas (in Uganda, for example, only 5% of patients were over 20), while the frequency of jaw tumours was lower (22%, compared with 55% or more in Uganda).

2.1.2.4 *Time–space clustering*

Pike *et al.* (1967) first reported on time–space clustering of Burkitt's lymphoma in the West Nile District of Uganda between 1961 and 1965. They concluded that patients whose dates of onset were close tended to live close together. This finding was highly statistically significant and suggested an 'epidemic' character.

Williams *et al.* (1969) provided further evidence of time–space clustering in the West Nile District, refining previously collected information and extending the study period to 1967. While the new data confirmed the results of the earlier analysis, the authors reported that an unpublished analysis conducted in the East and West Mengo districts in central Uganda did not show a similar phenomenon.

Baikie *et al.* (1972) subsequently reviewed the evidence for clustering of Burkitt's lymphoma. Of particular interest is a cluster observed in Bwamba County, in a low-incidence area in the south-west of Uganda, close to the border with the Congo. The only seven cases known to have occurred in the county were diagnosed between October 1966

and December 1968. Two of these cases were in a brother and sister, and five occurred during a six-month period from July to December 1968. No other clusters, apart from those in the West Nile, were observed in either Uganda or Tanzania (Brubaker *et al.*, 1973).

2.1.2.5 *Familial cases*

X-Linked lymphoproliferative syndrome, characterized by an abnormal response to EBV, predisposes males to the development of lymphomas, some of which may resemble Burkitt's lymphoma. Familial Burkitt's lymphomas, apparently unrelated to this syndrome and in some cases associated with a familial predisposition to other tumours, such as chronic myeloid leukaemia and nasopharyngeal carcinoma, have also been described.

Brubaker *et al.* (1980) reported the occurrence of Burkitt's lymphoma in North Mara District, Tanzania, in five families in which several members were afflicted, sometimes with another malignancy. The families in which multiple cases of Burkitt's lymphoma had occurred included two brothers and a half-brother and two brothers. In two other families, there were cases of chronic myeloid leukaemia and of Burkitt's lymphoma, in young siblings in one family and in a father and a son in the other; in another family, one woman who had nasopharyngeal carcinoma had a son with Burkitt's lymphoma. Nasopharyngeal carcinoma and chronic myeloid leukaemia are both rare tumours in the North Mara region.

Stevens *et al.* (1972) described two siblings with Burkitt's lymphoma, one of whom had Burkitt's leukaemia, in the United States. Joncas *et al.* (1976) described Burkitt's lymphoma in first-degree cousins, a young woman aged 16 and a young man of 17, in a large French-Canadian family. Two of the siblings of one of the patients with Burkitt's lymphoma had nasopharyngeal carcinomas, and an additional first-degree cousin had a plasmacytoma. Several siblings and cousins had low IgA values. Poulsen *et al.* (1991) described a case of EBV-negative Burkitt's lymphoma of the breast in two sisters aged 18 and 21 years in Denmark.

Familial Burkitt's lymphoma has also been described in three families in Papua–New Guinea (Winnett *et al.*, 1997). The first family had three cases of Burkitt's lymphoma between 1964 and 1965 in full brothers. In the second, two cases of Burkitt's lymphoma occurred between 1983 and 1984 in a full brother and sister; and in the third family, three cases arose between 1980 and 1983 in two sons and one daughter of one man and his two wives.

2.1.3 *Epidemiology of Burkitt's lymphoma in association with EBV*

The EBV was identified in an attempt to confirm the hypothesis that African Burkitt's lymphoma is caused by a vectored virus. Epstein (1985) examined tumour samples sent by Burkitt by electron microscopy, as the viral culture systems available at the time gave negative results. No virus was found, however, despite the most exhaustive searches, until he and his colleagues, Achong and Barr, succeeded in growing cell lines from the tumours. They believed that the virus could grow in cells that did not have host defences,

as is the case for avian tumour virus. Viral particles with a morphology consistent with that of the herpes family were identified in the first grid square (Epstein *et al.*, 1964). Subsequently, antigenic relationships with other herpesviruses, including the Lucké virus, Marek's disease virus and infectious bovine rhinotracheitis, were demonstrated by immunological methods (Ono *et al.*, 1970; Stevens *et al.*, 1971; Evans *et al.*, 1972). Evidence that this virus — EBV, as it came to be known — is frequently associated with Burkitt's lymphoma was based initially on serological evidence, made possible by the development of immunofluorescence tests for viral antibodies by Werner Henle and Gertrude Henle (1966). This test was later shown to reflect the level of VCA in the cells; cells that gave positive results in immunofluorescence assays were shown to contain viral particles by electron microscopy (Henle & Henle, 1967; Epstein & Achong, 1968). It was found, however, that control sera also generally showed anti-EBV antibodies, and the late viral antigens detected by the fluorescence antibody tests were rarely, if ever, detected in fresh tumour cells, although they were detected in a small percentage of cells in cell lines derived from tumours. Confirmation that viral genomes are present in every tumour cell therefore had to await the development of fluorescence tests for latent antigens and in-situ hybridization techniques.

zur Hausen and Schulte-Holthausen (1970) were the first to detect EBV DNA, using a nucleic acid hybridization technique, in a cell line in which viral particles could not be demonstrated. This result was then confirmed by Nonoyama and Pagano (1971). These results strongly suggested that viral DNA is present in cells in which late viral antigens or viral particles cannot be detected, indicating that the virus must be able to latently infect cells, i.e. remain within the cell without replicating viral particles.

2.1.3.1 *Case series*

Differences in the frequency of the association of Burkitt's lymphoma with EBV, as determined by the presence of EBV DNA (or RNA) or EBV antigens, are found in different regions of the world, as reflected in differences in serology. Table 7 gives the results found in case series in endemic and non-endemic areas of the world with respect to the association between Burkitt's lymphoma and markers of infection with EBV.

(*a*) *African patients*

Some 95% of African Burkitt's lymphomas are associated with EBV, as demonstrated by the presence of either EBNA or EBV DNA in the tumour cells, and such patients have higher geometric mean titres of antibodies against EBV-associated antigens. For some time, investigators had recognized the presence of complement-fixing antibodies in the sera of patients with Burkitt's lymphoma (Armstrong *et al.*, 1966). Pope *et al.* (1969) subsequently identified an EBV-associated complement-fixing antigen in soluble extracts of EBV-transformed lymphoblastoid cell lines and the Raji cell line, then thought to be 'virus free'. Reedman and Klein (1973) identified these antigens at the cellular level with an anticomplement immunofluorescence test in the nuclei of over 90% of the cells of EBV-transformed lymphoblastoid cell lines, regardless of whether they produced virus or not, and in a similar fraction of cells from two Burkitt's lymphoma biopsy samples. Comparison with other antibodies against EBV in 52 sera used to demonstrate the

antigen showed that the 32 with anti-VCA and complement-fixing antibodies also showed anticomplement immunofluorescence. Five of six sera which had no anti-VCA but had complement-fixing antibodies gave positive or equivocal results for anticomplement immunofluorescence, and 14 sera that had no anti-VCA and no complement-fixing antibodies gave negative results. A variety of control cells also gave negative results. This complement-fixing antigen became known as the EBV nuclear antigen (EBNA).

Table 7. Association between Burkitt's lymphoma and EBV in endemic and non-endemic regions

Country	No. of cases	Association with EBV	Reference
Endemic areas of Africa			
	23	EBV DNA in 22 (low level in 1 case)	Nonoyama *et al.* (1973)
	13	11 EBNA-positive	Reedman *et al.* (1974)
	27	EBV DNA in 26; 25 EBNA-positive	Lindahl *et al.* (1974)
Cameroon and Gabon	10	EBV DNA in 10	Prévot *et al.* (1992)
Ghana	4	EBV RNA identified	Dambaugh *et al.* (1979)
	23	EBV DNA in 23	Shiramizu *et al.* (1991)
Uganda	27	EBNA-positive; satisfactory biopsies	Olweny *et al.* (1977)
	15	EBV DNA in 14; all EBNA-positive	
	5	All EBNA-positive; IF on extracted DNA	Luka *et al.* (1978)
Malawi	44	42 EBER-1- or *Bam*HI W-positive by ISH	Labrecque *et al.* (1994)
Non-endemic areas			
Americas			
Argentina	16	EBER-1 in 4	Drut *et al.* (1994)
Argentina, Chile	27	EBV DNA in 13 (48%)	Gutiérrez *et al.* (1992)
Brazil	12	EBV DNA in 7 (58%)	
Brazil	24	EBER-1 in 17 (71%)	Bacchi *et al.* (1996a)
Brazil	54	47 EBER-1 positive (87%)	Araujo *et al.* (1996)
Cuba	7	6 VCA and EA(R)-positive	Riverend *et al.* (1984)
USA	4	No EBV DNA	Pagano *et al.* (1973)
USA	1	EBV DNA present	Gravell *et al.* (1976)
USA	12	EBV DNA in 5	Andersson *et al.* (1976);
	20	EBV DNA in same 5	Ziegler *et al.* (1976)
USA	3	EBV DNA in 3	Judson *et al.* (1977)
USA	32	EBV DNA in 12 (38%)	Shiramizu *et al.* (1991)
Asia			
China	18	EBER-1 in 5 (28%)	Chan *et al.* (1995a)

Table 7 (contd)

Country	No. of cases	Association with EBV	Reference
Non-endemic areas (contd)			
Egypt	41	30 EBER-1-positive (73%)	Anwar *et al.* (1995)
India	2	EBNA-positive	Venkitaraman *et al.* (1983)
Japan	14	2 EBNA-positive	Miyoshi (1983)
Japan	7	EBV DNA in 4	Okano *et al.* (1992)
Europe			
France	67	EBV DNA in 24%	Philip (1985)
Germany	1	EBV DNA present	Bornkamm *et al.* (1976)
Germany	1	EBNA-positive	Kachel *et al.* (1980)
Italy	15	EBV DNA in 1/12	Tirelli *et al.* (1984)
Sweden	1	EBV DNA present	Biberfeld *et al.* (1981)
Turkey	15	EBV DNA in 14	Çavdar *et al.* (1994)
Oceania			
Australia	1	EBNA-negative	Roeser *et al.* (1977)

EBNA, EBV nuclear antigen; EBER, EBV-encoded RNA; IF, immunofluorescence; ISH, in-situ hybridization

Nonoyama *et al.* (1973) reported that 22 of 23 biopsy samples from African patients with Burkitt's lymphoma contained EBV DNA as ascertained by nucleic acid hybridization, most containing 15–113 genome copies per cell (average, 38). Reedman *et al.* (1974) subsequently examined biopsies from 19 African Burkitt's lymphomas by complement-fixation tests and anticomplement immunofluorescence for EBV-associated antigens. Extracts from 12 of these tumours reacted in complement fixation tests with EBV-seropositive sera but not with EBV-seronegative sera. Eleven of 13 biopsy samples showed anticomplement fluorescence, and the reactive antibodies could be absorbed out with lymphoblastoid cell lines. In all cases, the fluorescence was coarsely granular and present in almost all the tumour cells. The remaining biopsy samples were considered not to contain EBV. None of 13 samples from tumours other than Burkitt's lymphoma showed anticomplement immunofluorescence.

Lindahl *et al.* (1974) reported on the correlation between the presence of EBNA and the detection of EBV DNA by nucleic acid hybridization with complementary RNA probes. Twenty-six of 27 histologically confirmed cases of African Burkitt's lymphoma were shown to contain 10–101 viral genomes per cell (average, 39) by hybridization, and 25 of these 26 cases also contained EBNA.

Magrath *et al.* (1975) reported on a patient who presented with Hodgkin's disease and then Burkitt's lymphoma. The patient had a high titre of anti-VCA (> 1:320) but not anti-EA at the time of presentation with Hodgkin's disease. The anti-VCA titres continued to rise, albeit at a slow pace, over the ensuing months, to > 1:1250, and those of anti-EA and anti-EBNA increased rapidly to 1:1250 shortly before presentation of widespread

Burkitt's lymphoma nine months later. Anti-membrane antigen antibodies, although continuously present, did not change in titre in association with the onset of Burkitt's lymphoma. This case demonstrates that antibody titres to EBV are raised many months before the onset of Burkitt's lymphoma, consistent with the results of the prospective study in the West Nile District, and also that rapid changes in EA titres may occur at the time of tumour growth.

Olweny *et al.* (1977) examined the expression of EBNA in 34 patients with Burkitt's lymphoma in Uganda and 25 patients with other malignancies (predominantly other lymphomas). All 27 biopsy samples from cases of Burkitt's lymphoma considered to be in satisfactory condition contained EBNA. Fourteen of 15 of these samples examined by nucleic acid hybridization were shown to contain EBV DNA, with a mean genome copy number of 39. The one case in which fewer than two genome copies (the level of detection of the test) were found was serologically negative for EBV antibodies and was in a patient who had lived in a malaria-free highland area before migrating two months before the diagnosis of Burkitt's lymphoma to a malaria-endemic region. None of the 25 non-Burkitt's lymphoma biopsy specimens contained EBNA, and all 15 subjected to nucleic acid hybridization showed no EBV DNA. The results of EBV serology were consistent with those reported previously, the anti-VCA titre being fourfold higher and anti-EA being detectable in 59% of the group with Burkitt's lymphoma.

Luka *et al.* (1978) examined five biopsy samples from Burkitt's lymphoma patients in Africa previously shown to contain EBNA by standard anticomplementary immunofluorescence, DNA extraction, separation on DNA–cellulose columns and fixation to chicken erythrocytes before immunofluorescence testing. All five samples contained EBV DNA.

Dambaugh *et al.* (1979) studied four Burkitt's lymphoma biopsy samples from Accra, Ghana, and demonstrated RNA homologous to at least 3–6% of the DNA of EBV; they also identified the general regions of the EBV genome from which the RNA was transcribed.

Prévot *et al.* (1992) studied tissues from 12 cases of Burkitt's lymphoma and 12 cases of non-Burkitt's lymphoma from Cameroon and Gabon by in-situ hybridization with a DNA probe derived from the *Bam*HI W repeat region of EBV. All 10 samples from Burkitt's lymphoma considered technically satisfactory were shown to contain EBV DNA, and all 12 of the non-Burkitt's lymphoma samples gave negative results.

Shiramizu *et al.* (1991) studied tissues from 23 cases of Burkitt's lymphoma from Ghana by hybridization with a probe derived from the *Bam*HI K fragment of the EBV genome. All 23 samples showed the presence of EBV DNA, which was confirmed with probes from the terminal repeat region of EBV.

Labrecque *et al.* (1994) in Malawi used in-situ hybridization on material obtained from Burkitt's lymphomas by aspiration biopsy, with two probes: EBER-1 and the *Bam*HI W fragment of EBV. Of 66 cases of suspected Burkitt's lymphoma, 44 were confirmed cytopathologically, and 42 showed the presence of EBV DNA with EBER-1 and/or *Bam*HI W. Of the 22 cases considered not be Burkitt's lymphoma, only one contained EBV.

(*b*) *Non-African patients*

Some 10–30% of European and American tumours appear to be associated with EBV, while 50–90% of tumours in developing countries are EBV-associated. The fact that only a proportion of cases are associated with EBV should be borne in mind when interpreting the results of serological studies, as the available data indicate that only patients with EBV present in the tumour cells have raised antibody titres to EBV. Thus, serological results reflect the fact that these series include both EBV-associated and EBV-negative cases. Furthermore, age may be an important factor in the frequency of association with EBV.

Goldberg and Drut (1986) reviewed their 16-year experience of Burkitt's lymphoma in Argentina; 73% of patients had elevated antibody titres to EBV. Drut *et al.* (1994) examined 16 Argentine patients with Burkitt's lymphoma aged 2–8 years for EBV by PCR and in-situ hybridization (using a NotI/PstI fragment). Four cases gave positive results in both tests, 70–90% of the cells giving a signal in in-situ hybridization. Interestingly, all of these cases were seen in the same year, 1984. Gutiérrez *et al.* (1992) reported 39 cases of Burkitt's lymphoma from Argentina, Chile and Brazil, mostly in children, the age range being 1–28, with only two patients over 15 years of age. Overall, 51% of these cases (48% from Argentina and Chile and 58% from Brazil) were EBV-associated, as determined by Southern blotting. The EBV in these tumours was shown with the EBV terminal-repeat region probe to be monoclonal.

Bacchi *et al.* (1996a) reported the results of in-situ hybridization for EBER-1 in 24 cases (mostly in children, but some ages unknown) of Burkitt's lymphoma from Campinas and São Paulo in Brazil. Seventeen (71%) gave positive results, the signal being present in virtually all of the neoplastic cells of each case. Only one of these patients had jaw involvement. Araujo *et al.* (1996) described 54 children (median age, six years) with Burkitt's lymphoma, four of whom had mandibular tumours, in Bahia, in tropical Brazil and reported the results of in-situ hybridization with an EBER-1 probe. Forty seven (87%) of the cases showed the presence of EBV DNA. Interestingly, the fraction of positive cases appeared to be related to age: 39 of 41 patients under nine years of age and six of 11 aged nine years or more had EBER-1-positive tumours. Essentially 100% of the cells showed positive results. LMP-1 was not expressed in the tumours, except in a few cells in proximity to ova of *Schistosoma mansoni* in two cases. Of 27 EBV-positive tumours tested for EBV subtype, 20 (74%) were type 1 and the remaining seven (26%) were type 2. This result is intermediate between that of African Burkitt's lymphoma (50% type 1) and North American Burkitt's lymphoma (10% type 2) (Goldschmidts *et al.*, 1992).

Riverend *et al.* (1984) described seven children with Burkitt's lymphoma in Cuba. Five of the patients had high antibody titres to VCA (1:640–1:5120) and to EA(R) (1:10–1:2560), one had low titres to these antigens (1:20 and 1:5, respectively) and one had negative titres. Interestingly, three of the patients with high antibody titres had jaw tumours.

In spite of the serological evidence that Burkitt's lymphoma in the United States is also EBV-associated, Pagano *et al.* (1973) failed to detect hybridizable EBV DNA in

tumour-infiltrated tissue from four cases of Burkitt's lymphoma; however, Gravell *et al.* (1976) detected EBV DNA in an American patient with Burkitt's lymphoma.

Andersson *et al.* (1976) and Ziegler *et al.* (1976) studied an additional 12 American patients with Burkitt's lymphoma. Nine of the patients had low anti-VCA titres, but two had titres of 1:320 and 1:160 and one gave a negative result. Five of the tumours had EBV DNA that was readily detectable by liquid hybridization studies; the remainder had less than one genome copy per cell. These data suggested that the majority of American Burkitt's lymphomas lack EBV DNA. This suggestion was confirmed by Shiramizu *et al.* (1991), who studied tissues from 32 cases of Burkitt's lymphoma in the United States by hybridization with a probe derived from the *Bam*HI K fragment of the EBV genome. Twelve samples (38%) showed the presence of EBV DNA, which was confirmed with probes from the terminal repeat region of EBV. Judson *et al.* (1977), however, described four patients with Burkitt's lymphoma living within 50 km of each other in the United States. All showed anti-EBV antibodies similar to those observed in African patients (e.g. anti-VCA titre > 1:640 in all cases). Similarly, viral DNA and EBNA were found in tumour samples from three of the patients; the fourth was not tested.

Chan *et al.* (1995a) reported on the association between EBV and Burkitt's lymphoma in a Chinese population in Hong Kong. Among 18 patients who ranged in age from 4 to 85 years (median, 35.5), five aged 4, 6, 18, 57 and 58 years showed EBER-1 by in-situ hybridization in essentially all tumour cells. There were no cases of jaw tumour. Two cases (a T cell-rich large B-cell lymphoma and an anaplastic large-cell lymphoma) out of 54 of non-Hodgkin's lymphoma also showed positive results, the Reed-Sternberg-like cells found in one case being positive.

Anwar *et al.* (1995) found positive results by in-situ hybridization in 30 of 41 patients with Burkitt's lymphoma in Egypt.

Venkitaraman *et al.* (1983) reported on two Indian cases of Burkitt's lymphoma in children aged 5 and 11 years. Their anti-VCA titres were 1: 640 and 1:80; those of anti-EBNA were 1:1280 and 1:320, and those of anti-EA(R) were 1:80 and < 1:5, respectively. The presence of EBV in the tumour cells was not assessed.

Miyoshi *et al.* (1978) reported a case of EBNA-positive Burkitt's lymphoma in a person aged 29 years in Okayama, Japan, and referred to an earlier EBV-negative case (Tanaka *et al.*, 1976; Miyoshi *et al.*, 1977). Miyoshi (1983) subsequently described 14 patients (age range, 4–52 years), including these two cases, with Burkitt's lymphoma, five of which were jaw tumours. Two were EBV-seronegative, nine had anti-VCA titres of 1:40–1:1280, and three were not tested. The anti-EBNA titres in the six tested ranged from 1:20 to 1:80. Four of these patients had bone-marrow involvement at the time of presentation. Two of 14 tumours tested for EBNA by anticomplement immunofluorescence showed positive results. Okano *et al.* (1992) reported on seven patients in Hokkaido, Japan, aged 4–39 years. Five had anti-VCA titres of ≥ 1:320, including three cases with anti-VCA titres of 1:2560 and one with a titre of 1:320, with correspondingly high titres of antibodies to EA; two had titres of 1:40 and 1:80 and no anti-EA antibodies.

Four of these seven cases were EBNA-positive and three of the six tested showed EBV DNA by nucleic acid hybridization.

Philip (1985) mentioned the unpublished results of G. Lenoir showing that 16 of 67 Burkitt's lymphomas in Caucasian patients were EBNA-positive; 42 of the patients were from Lyon, France, of whom 6% had EBNA. Bornkamm *et al.* (1976) studied a single patient among a series of lymphoma patients in Germany and detected EBV DNA by reassociation kinetics. A single case of EBV-associated Burkitt's lymphoma that was EBNA-positive, with high titres to anti-VCA and anti-EA (1:2056), was reported in a 34-year-old woman in Germany (Kachel *et al.*, 1980). Among 15 patients, five of whom were 15 years of age or less, with Burkitt's lymphoma reported in north-east Italy, only one was serologically positive for EBV among 12 patients tested (Tirelli *et al.*, 1984). Biberfeld *et al.* (1981) reported an EBV-associated case in a 25-year-old man in Sweden. Çavdar *et al.* (1994) found EBV DNA by Southern blotting in 14 of 15 tumours examined in Turkey.

Roeser *et al.* (1977) studied a single Australian case of Burkitt's lymphoma and found the patient to be EBV-seronegative; no EBNA was found in the tumour cells.

In summary, the frequency of EBV association in Burkitt's lymphoma varies with geographical location. In most developed countries, some 20% or less of tumours are positive in most series, while in equatorial Africa about 95% are positive. In temperate regions in South America, such as Argentina and Chile, the rate of EBV association is lower than in tropical regions in the north of the continent; however, differences in the climate and in socioeconomic circumstances may account for the apparent differences in EBV association. If age at EBV infection is a primary determinant of association, socioeconomic or lifestyle factors are likely to be of paramount importance. Too few data are available to assess the importance of age as a determinant of EBV status in countries in which the frequency of EBV-negative cases is sufficiently high to make such an investigation worthwhile.

2.1.3.2 *Case–control studies*

Because the prevalence of antibodies to EBV changes with age (see section 1.4), cases and controls should be closely matched for age; however, in most of the studies described below, the closeness of age-matching is not stated.

(*a*) *African patients*

Henle *et al.* (1969) compared antibody titres to EBV (using an indirect immunofluorescent test for IgG antibodies to Burkitt's lymphoma tumour cells) in sera from Burkitt's lymphoma patients and various comparison groups, including 94 'recent onset' cases from East Africa (58 from Nairobi, Kenya and 36 from Kampala, Uganda). The comparison groups [selection criteria unspecified] comprised 62 children who were matched to cases for age, sex and tribe, 72 siblings and neighbours aged 1–15 years, 62 children from 'regions of high Burkitt's lymphoma incidence', 50 children from 'regions of low Burkitt's lymphoma incidence', 113 children aged six months to five years from 'well-baby' clinics in Nairobi and 130 patients without cancer from the paediatric wards

of Kenya Hospital, Nairobi. All of the Burkitt's lymphoma patients had EBV antibody titres ≥ 1:10, 87.2% having titres of ≥ 1/160. The geometric mean titre (GMT) was 1:326 (1:382 in Kenya, 1:243 in Uganda). In the 'control' groups, 18% had no antibody (13% in age-, sex- and tribe-matched controls), and only 14% had titres ≥ 1:160 (11% in age-, sex- and tribe-matched control [odds ratio, 54]). The GMT in the control children was 1:37 (1:36 in age-, sex- and tribe-matched controls).

Klein *et al.* (1970) investigated the presence of anti-EBV antibody in sera from 19 confirmed cases of Burkitt's lymphoma and 27 controls [selection criteria unspecified, but probably not matched in any way to the cases], using intracellular immunofluorescence in fixed smears of EBV-carrying lymphoblastoid (P3J) cell lines (anti-EBV), blocking of membrane fluorescence or immunoprecipitation against a soluble antigen from an EBV-carrying cell line. Of the 19 patients with confirmed Burkitt's lymphoma, 15 were highly reactive in all three tests, while only three of the 27 controls were; 19 of the controls had low titres in all three tests.

Henle *et al.* (1971b) compared the anti-EA antibody titres in sera from 156 Burkitt's lymphoma patients recruited from hospitals in Kampala, Uganda, and Nairobi, Kenya, with those in sera from 200 control children from the West Nile District of Uganda. The controls were selected to include children with low or absent titres of anti-VCA titre [but presumably were not matched to the cases]. At each level of anti-VCA titre, more of the Burkitt's lymphoma cases were anti-EA-positive. [The Mantel-Haenszel odds ratio (stratified for anti-VCA titre) associated with positive anti-EA is 19 (95% confidence interval [CI], 9.6–39)].

Hirshaut *et al.* (1973) studied cases of Burkitt's lymphoma from Africa (as well as from the United States, see below) with respect to anti-EBV antibody (measured by immunofluorescence and immunodiffusion) and compared them with age- and sex-matched controls from the same district and parents and siblings. When tested by immunofluorescence, EBV antibody was present in all 21 cases tested and in 21/27 of the age- and sex-matched controls, 76% of cases and 12% of controls having titres > 1:640 (odds ratio, 26; [95% CI, 5.3–120]). When tested by immunodiffusion, 67 of 73 cases and 19/30 controls gave positive results (odds ratio, 6.5; [95% CI, 2.1–20]).

Nkrumah *et al.* (1976) examined sera from 141 patients with Burkitt's lymphoma in Ghana. For a subset of 75 patients, they compared the anti-VCA antibody titres with those of 54 siblings and 50 age- and sex-matched neighbourhood controls. The GMT of antibody in the cases was significantly higher (1:424; $p < 0.001$) than that in either siblings (1:56) or neighbours (1:62).

(*b*) *Non-African patients*

Levine *et al.* (1972) examined patients with histologically identified Burkitt's lymphoma in the United States for anti-VCA antibodies against EBV. Twenty-four of 29 cases gave positive results, in comparison with 31/57 age- and sex-matched controls, and the antibody GMTs were higher (1:94) than in controls (1:11) or in 26 patients with lymphoblastic leukaemia (1:16), but were not as high as in African Burkitt's lymphoma patients (1:3338). A subset of patients under eight years of age all had anti-VCA anti-

bodies, however with a GMT of 1:425 (in comparison with 1:2848 in 11 African children), which was markedly higher than that in the 34 controls (1:4), 62% of whom had no anti-VCA.

Hirshaut *et al.* (1973) compared the EBV antibody titres of 15 patients with Burkitt's lymphoma in the United States with those in 15 age- and sex-matched controls and 25 parents and siblings of the cases. As ascertained by immunofluorescence, 10 cases and seven controls had antibodies. Although two patients and one control had antibody levels ≥ 1:640, the GMT was not significantly different between the two groups (5.2 versus 4.4). When 16 cases and 16 controls were ascertained by immunodiffusion, seven cases and six controls were found to have anti-EBV antibodies.

The data of Levine *et al.* (1972) were extended by Ablashi *et al.* (1974), who studied a wider range of antibodies in 21 patients in the United States with Burkitt's lymphoma, and compared them with 10 control subjects. [It is not clear how the controls were selected; one was a relative of a case.] All 15 sera tested for anti-VCA, anti-EA, anti-EBNA and complement-fixing antibodies had titres of at least one of the antibodies, whereas only one of seven control sera did. Five of the case sera did not show anti-VCA antibodies, but three of these sera had antibodies against EBNA. The GMTs for the patients were 1:111 for VCA, 1:17 for EA, 1:4.2 for complement fixation and 1:59 for EBNA; those of controls were 1:24, 1:2.7, 1:2.6 and 1:1.5, respectively.

Gotlieb-Stematsky *et al.* (1976) reported on 16 children with Burkitt's lymphoma in Israel. Although some of the 10 Arab children did not have elevated antibody titres against EBV (only one had negative serology), elevated titres against VCA were observed in the Burkitt's lymphoma patients, four having titres ≥ 1:160 (GMT, 1:26), whereas controls matched for age, sex and ethnicity showed no such increase, except for one subject with a titre of 1:160 (GMT, 1: 5.6).

Çavdar *et al.* (1994) reported on 81 children, of a median age of five years, with Burkitt's lymphoma in Turkey. In 32 of these patients in whom antibody titres against EBV were examined, they were found to be high, with 100% positive for anti-EBNA and a GMT of 1:320 for VCA. In 311 healthy children [unmatched for age or sex], the GMT for VCA was 1:93. [The proportion that was anti-EBNA positive was not reported.]

2.1.3.3 *Cohort study*

Between February 1972 and September 1974, serum samples were collected from about 42 000 children aged four to eight years in four counties in the West Nile District of Uganda. As of November 1977, 13 incident cases of histologically confirmed Burkitt's lymphoma and one case of 'unclassified lymphoma' had developed among the cohort members. The interval between initial serum collection and diagnosis of Burkitt's lymphoma ranged from seven to 54 months. EBV was found in seven of nine tumours tested by nucleic acid hybridization and in eight of these tumours by testing for EBNA. For each case of Burkitt's lymphoma, five control subjects from the cohort matched for age, sex and locality to the index case were chosen for a nested case–control analysis (de Thé *et al.*, 1978a). The 14 Burkitt's lymphoma patients had significantly higher pre-diagnostic anti-VCA titres than control subjects (GMT, 425.5 versus 125.8), but no

difference was observed between cases and controls in the titres of anti-EA and anti-EBNA. No difference in anti-VCA antibody titres was seen before and after diagnosis in the Burkitt's lymphoma patients, but seven of them developed anti-EA(R) antibodies; only one patient had anti-EA(D) antibodies before developing Burkitt's lymphoma. Similar temporal changes were not observed in the controls. Antibody titres to herpes simplex virus, cytomegalovirus and measles were unchanged, and the malaria parasitaemia rates before development of the tumour did not differ in patients and controls. This study clearly demonstrated that antibodies to EBV are present months to years before the development of Burkitt's lymphoma.

In 1982, Geser *et al.* reported the final results of this study. Two additional EBV-associated, histologically confirmed cases were detected up to 1979, both of which had high anti-VCA titres before the onset of Burkitt's lymphoma. One had anti-EA antibodies and the other did not, both before and after onset of tumour; anti-EBNA antibodies were also found before and after development of the tumour in both patients. This study showed that anti-VCA titres can be elevated as long as six years before the onset of Burkitt's lymphoma and as early as three months after birth. The relative risk for developing Burkitt's lymphoma increased multiplicatively by a factor of 5.1 for each two-fold dilution in anti-VCA titre for all cases of Burkitt's lymphoma and by a factor of 9.2 when the analysis was confined to cases in which EBV DNA was present in the tumour.

2.1.4 *Cofactors*

2.1.4.1 *Malaria*

Morrow (1985) summarized the data that suggest that malaria is a cofactor in the development of Burkitt's lymphoma:

- The incidence of Burkitt's lymphoma correlates within countries and internationally with the incidence of malaria and with parasitaemia rates.
- The age at which peak levels of antimalarial antibodies are acquired (5–8 years) corresponds to the peak age incidence of Burkitt's lymphoma.
- Individuals who live in urban areas where malarial transmission rates are lower also have a lower incidence of Burkitt's lymphoma.
- In regions in which death rates due to malaria have declined, there is a corresponding decline in the incidence of Burkitt's lymphoma.
- The age at onset of Burkitt's lymphoma in immigrants from malaria-free areas to malarious areas is higher than that of the original inhabitants.
- There is an inverse relationship between the age at onset of Burkitt's lymphoma and the intensity of infection with *Plasmodium falciparum.*
- There is an apparently reduced incidence (though not statistically significant) of Burkitt's lymphoma in individuals with the sickle-cell trait, which also protects against malaria.
- There is some evidence for a seasonal variation in the onset of Burkitt's lymphoma and for time–space clustering (see section 2.1.2.4).

(*a*) *Ecological studies*

Dalldorf (1962) first suggested that malaria is relevant to the development of Burkitt's lymphoma. Subsequently, he examined the distribution of Burkitt's lymphoma in Kenya (Dalldorf *et al.*, 1964). Because of marked variability in the endemicity of malaria in Kenya, primarily related to the suitability of various environments for breeding of the mosquito vector, he was able to show that the highest incidence of Burkitt's lymphoma occurred in areas where malaria was holoendemic, namely in the coastal and lakeside regions (see Figure 7). The major vectors in these areas are *Anopheles gambiae* and *A. funestus*. Dalldorf pointed out that malaria affects the reticuloendothelial system and as such could well influence the development of Burkitt's lymphoma. Infestation rates by malarial parasites rise rapidly during the first year of life and often persist up to the age of three. Similarly high rates of infestation occurred in Papua–New Guinea (ten Seldam *et al.*, 1966), the only other region in the world where malaria was known to be holoendemic and where the incidence of Burkitt's lymphoma was similarly high.

Burkitt (1969) also noted that high incidences of Burkitt's lymphoma occurred only in regions where malaria was holo- or hyperendemic (equatorial Africa, Papua–New Guinea and parts of Malaysia) and that the disease was rare in regions in which malaria eradication campaigns had been successful (the islands of Zanzibar and Pemba, Singapore, Sri Lanka, the West Indies and India) or showed a marked decrease in incidence in regions where malaria eradication had been undertaken only recently.

Not all areas believed in the 1960s to be lymphoma-free, however, were known to be malaria-free. Kinshasa, for example, and Lambarene appeared to have a markedly lower incidence of Burkitt's lymphoma than surrounding areas (Kafuko & Burkitt, 1970); however, similar anomalies of distribution apply to known vectored viral diseases, such as yellow fever, and could be related to the paucity of suitable mosquito breeding grounds (e.g. in many urban regions) or even differences in exposure to mosquitoes related to differences in life style.

Interestingly, ten Seldam *et al.* (1966) were not convinced that similar climatic determinants of the distribution of Burkitt's lymphoma pertained in the territories of Papua–New Guinea, largely because of their observation that four of their 35 cases occurred in highlands above 5000 feet (1500 m). Booth *et al.* (1967) found, however, that the distribution of the 37 cases they reported (many of which had been reported by ten Seldam *et al.*) was consistent with that observed in Africa. Thus, 34 of their cases came from the coastal regions or plains immediately adjacent to the coast, and only three came from the highlands. They also remarked that the capital (Port Moresby) appeared to be tumour-free. They equated this with the six dry months of the year in Port Moresby and drew a parallel with the situation in Accra, Ghana. They estimated that the incidence of Burkitt's lymphoma in the highlands of New Guinea, where 40% of the people live, was 1 per 442 000 children, as compared with 1 in 29 000 in children living in the coastal region — a difference of some 14- or 15-fold. These figures, are, however, based on rather small numbers of observed cases. Tefuarani *et al.* (1988) later estimated the incidence of Burkitt's lymphoma in Papua–New Guinea on the basis of 109 cases. The incidence of all childhood tumours was 36.5 per 100 000 (based on a total of 680 cases) per annum,

and that of Burkitt's lymphoma, representing 16% of all tumours, was about 6 per 100 000 — closely similar to that in Africa.

Kafuko and Burkitt (1970) summarized the available information on the influence of malaria on the risk for developing Burkitt's lymphoma. They stated that the disease is not common in any area where malaria transmission occurs for less than six months in the year. They quoted an unpublished report by P.J. Cook and D.P. Burkitt on the frequency of Burkitt's lymphoma in relationship to gastric cancer, liver cancer, Kaposi's sarcoma and epithelioma at the site of a chronic tropical ulcer in a large number of hospitals in Uganda, Kenya and Tanzania, who found that the hospitals with the highest relative fraction of Burkitt's lymphoma were all in highly malarious areas. A survey of spleen size and malarial parasite rates was carried out in Uganda between 1963 and 1966 by testing children in over 100 schools and conducting 86 mass surveys; the degree of malarial endemicity was calculated according to accepted criteria based on parasitaemia rates in children of various ages. A close correlation was found between malarial endemicity and the incidence of Burkitt's lymphoma, although no statistical analysis was performed. In addition, it was shown that the parasite density index was highest in the 0–4-year-old age group and higher in the 0–10-year-old age group than in older individuals. Thus, the peak incidence of Burkitt's lymphoma corresponds to the age range in which malarial infestation rates are highest. It was also noted that adults who emigrate to malarious regions from malaria-free regions develop an intense parasitaemia not seen in the resident adults, who have acquired immunity. This is consistent with the greater incidence of Burkitt's lymphoma in immigrants than in the regions from which they come.

Morrow *et al.* (1976) and Morrow (1985) studied the incidence of Burkitt's lymphoma in various counties in the Mengo districts of central Uganda. On the basis of 130 cases from East and West Mengo, 100 with a confirmed histological diagnosis and 11 with a typical clinical syndrome of Burkitt's lymphoma, seen at Mulago Hospital between 1959 and 1968, they recorded a marked variation in the incidence of Burkitt's lymphoma which, they stated, corresponded to the recorded incidence rates of malaria in those regions. They observed a gradual decline in the incidence rate of Burkitt's lymphoma throughout this period, in spite of improved case ascertainment, as evidenced by a tripling of the number of other cancers reported from the Mengo districts to the Kampala Cancer Registry during the same period. The decline was particularly noticeable among the Ganda tribe, native to this region, which they suggested was due to the greater availability and use of chloroquine from Government and private dispensaries, with a consequent decline in the incidence of severe malarial infection. They showed that immigrants into Mengo from highland regions with low malarial prevalence who developed Burkitt's lymphoma were significantly older (median, 12 years) at the onset of Burkitt's lymphoma than patients from meso-endemic Mengo (median, 8 years; $p < 0.008$), while patients from Mengo were significantly older than patients from hyper- or holoendemic regions at lower altitude (median, 6 years; $p < 0.04$). These observations were considered to be consistent with the hypothesis that Burkitt's lymphoma is most likely to occur within a few years of a first intense infection with malaria. The authors also showed a seasonal variation in the onset of Burkitt's lymphoma, more cases occurring in the first half of the year. No time–space clustering was observed.

In Ghana, Biggar *et al.* (1981) showed a significant difference in malaria parasitaemia (*P. falciparum*) rates in urban (1.4%) and rural populations (22%), accompanied by similar differences in antimalarial antibody titres. Persons who had taken chloroquine for treatment of suspected malaria had a lower antibody frequency and lower titres than those who did not use chloroquine. This difference correlated with the distribution of Burkitt's lymphoma in Ghana (Biggar & Nkrumah, 1979).

Morrow (1985) reported a significant correlation ($p < 0.001$ by Spearman rank coefficient) between malaria parasitaemia (*P. falciparum*) rates and the incidence of Burkitt's lymphoma in various districts in Uganda. The parasitaemia rates ranged from 7.9% (in Ankole) to 75.2% (in Madi), and the incidence of Burkitt's lymphoma in children aged 0–14 from 0 (0.09 in Ankole) to 6.0 (in Lango) per 100 000. He also suggested that differences in vectorial capacity, i.e. the rate of potentially infective contacts per person by a vector, and the consequent levels of parasitaemia may account for differences in the likelihood that Burkitt's lymphoma will occur. In holoendemic areas, the peak age of prevalence and of the density of falciparum parasitaemia is two to three years, whereas the maximal level of antimalarial and non-antimalarial immunoglobulin occurs two to five years later, coinciding with the peak age of incidence of Burkitt's lymphoma in these regions (Molineaux & Gramiccia, 1980). No differences have been reported in the levels of malarial antibodies between patients and controls.

(*b*) *Relationship between Burkitt's lymphoma and sickle-cell trait*

Since sickle-cell trait (AS haemoglobin) was shown to protect substantially against severe *P. falciparum* malaria (Allison, 1963), several attempts have been made to determine whether children with AS haemoglobin are less likely to develop Burkitt's lymphoma than others.

Williams (1966) compared the haemoglobin electrophoretic patterns of 100 children of the Yoruba tribe in Nigeria who had Burkitt's lymphoma (78% AA, 17% AS and 5% SC, SS, C or AC haemoglobin) with those of 331 similarly aged control patients from the same hospital (68% AA). Children with AA haemoglobin were more susceptible to Burkitt's lymphoma ($p = 0.03$). In another study of children over five years of age from a single Yoruba village near Ibadan, Nigeria, 66% had AA haemoglobin, 26% AS and 8% SC, SS, CC or AC, and there was no significant difference between patients and village controls (Gilles, 1963). Pike *et al.* (1970) conducted a case–control study in Uganda in which the controls were matched for age, sex, tribe and place of residence. Although the AS haemoglobin type appeared to be protective, the results were not statistically significant.

Studies in Uganda are difficult to conduct owing to the marked variation in the frequency of AS haemoglobin disease in different districts. Nkrumah and Perkins (1976) studied 112 patients with Burkitt's lymphoma, using the patients' nearest neighbours of the same age, sex and tribe as controls. Once again, no statistically significant difference in the frequency of patients with AS and other variations from AA haemoglobin was observed. The issue of whether individuals with variant haemoglobins are protected against the development of Burkitt's lymphoma therefore remains unanswered, largely

because of the lack of large enough studies. If there is a protective effect, it is presumably a modest one.

(*c*) *Intervention study*

Geser *et al.* (1989) undertook a study to determine whether suppression of malaria in the North Mara District of Tanzania by distributing chloroquine regularly to a cohort of children below the age of 10 years would result in a decrease in the incidence of Burkitt's lymphoma. This trial provided confirmation of the relationship between malaria prevalence rates and the incidence of Burkitt's lymphoma. Thus, before the trial (1964–76), all 85 cases of Burkitt's lymphoma in North Mara occurred in the lowlands (near Lake Victoria), with a high malarial parasitaemia rate (28–48%), and none occurred in the high plateau (over 1500 m) bordering Kenya, with a low malarial parasitaemia rate (5–24%). The prevalence of both malaria (parasitaemia rates of 11 and 13% in 1977 and 1978, with corresponding reductions in antimalarial fluorescence antibody titres) and Burkitt's lymphoma fell transiently during and after the period of chloroquine administration (1977–82), the latter to the lowest level ever recorded in the region: 0.5 per 100 000 in 1979 and 1981 in comparison with 2.6–6.9 per 100 000 before the trial. After 1979, the prevalence of malaria rapidly rose again to pre-trial levels, apparently because of problems in chloroquine distribution, although the incidence of Burkitt's lymphoma remained low until approximately two years after the distribution of chloroquine was stopped, when it reached a high of 7.1 in 1984. Interestingly, the prevalence of parasitaemia in South Mara, where chloroquine distribution was not conducted, rose throughout the trial from the pre-trial level of 23–28% to a high of 57% in 1985. Chloroquine resistance was not reported in the area until 1982 (Draper *et al.*, 1985). As anticipated, 90% of malaria detected was due to *P. falciparum*, the remainder being due to *P. malariae*. The authors reported some evidence of a trend towards a lower incidence of Burkitt's lymphoma in North Mara before the trial, although this did not reach statistical significance, whereas the reduction in the incidence of Burkitt's lymphoma between 1964 and 1982 was highly significant ($p < 0.001$).

2.1.4.2 Euphorbia tirucalli *and other medicinal plants*

Another possible cofactor in the pathogenesis of Burkitt's lymphoma in Africa is the plant *Euphorbia tirucalli*, which is used quite widely in equatorial Africa for medicinal purposes. Phorbol esters present in this plant have been reported to increase the ability of EBV to transform B lymphocytes and to increase the likelihood that a chromosomal translocation will develop in transformed cells (see section 4.2.4). Epidemiological information relevant to this issue is limited, although the distribution of *Euphorbia* in Africa has been examined.

Osato *et al.* (1987, 1990) found *Euphorbia tirucalli* around almost all houses, fields and reservoirs in villages surrounding Lake Victoria and in other high-incidence regions in Kenya and Tanzania. The plant was reported to be uncommon in areas in these countries in which Burkitt's lymphoma is uncommon.

van den Bosch *et al.* (1993) reported this plant to be frequent within the 'lymphoma belt' in equatorial Africa and to be used significantly more commonly in the homes of patients from Malawi with Burkitt's lymphoma than in control patients. A number of other plants of this family (Euphorbiaceae) and a variety of medicinal plants were also common. The tobacco plant was also significantly more commonly used in the homes of patients with Burkitt's lymphoma than those of controls, but use of other plants was equally distributed (Ito *et al.*, 1983).

van den Bosch *et al.* (1993) mentioned three patients who developed Burkitt's lymphoma after being treated for an illness with an extract of *Terminalia sericea*.

2.1.5 *Molecular epidemiology*

The identification of specific chromosomal abnormalities in patients with Burkitt's lymphoma (see section 4.2.1.1) permitted the examination of differences in the translocation-dependent structural alterations in *c-myc* in different regions of the world (Pelicci *et al.*, 1986; Shiramizu *et al.*, 1991; Gutiérrez *et al.*, 1992). In some tumours, the chromosomal breakpoint is quite distant from *c-myc* — often as much as several hundred kilobases upstream (in t(8;14)) or downstream (in variant translocations). This is the most frequent location of the breakpoint in African Burkitt's lymphoma, occurring in 75% of tumours, and is observed in about 50% of Brazilian tumours. Sometimes the breakpoint is close to the gene, i.e. within its 5′ flanking region, arbitrarily defined as extending from the upstream *Hind*III restriction enzyme site to exon 1. This is found in approximately half of the tumours occurring in Chile and Argentina (Gutiérrez *et al.*, 1992). Of tumours in North America, 60% have the breakpoints within the gene, i.e. within exon 1 or intron 1, and only 9% outside the *Hind*III fragment (Shiramizu *et al.*, 1991). Intron and exon breakpoints separate the coding region of *c-myc* from its major promoters, and transcripts are initiated from regions within the first intron. This is a marked difference from African Burkitt's lymphomas in which the *c-myc* gene is grossly intact, although point mutations in regulatory and coding regions are nearly always observed, implying different mechanisms leading to *c-myc* deregulation. Factors that are relevant to the induction of specific chromosomal breakpoint locations have not been identified but are likely to be environmental.

Interestingly, there is an apparent geographical association between the frequency of breakpoints outside the *c-myc* gene and the fraction of tumours associated with EBV (Magrath, 1997). In the United States, for example, only 15–20% of tumours are EBV-associated and only 9% have a breakpoint outside *c-myc*. In Africa, 95% of tumours are EBV-associated and 75% have breakpoints outside *c-myc*. Tumours from South American countries are intermediate in both respects. The nature of this relationship is uncertain, since, for example, non-African tumours with breakpoints outside *c-myc*, which occur at highest frequency (in countries for which data are available) in Brazil are not necessarily EBV-associated.

2.2 Non-Hodgkin's lymphomas other than Burkitt's lymphoma

2.2.1 *Pathology*

Non-Hodgkin's lymphomas are a numerous, heterogeneous group of malignancies that originate from lymphocytes. They can develop either from within organized lymphoid tissues, such as lymph nodes, or from other sites. Many lymphoid neoplasms pass through both solid tumour and circulating (leukaemic) phases.

Classification of non-Hodgkin's lymphomas is a complex and evolving process, and a thorough description of these diseases and their diagnostic criteria is beyond the scope of this volume. Several schemes of nomenclature have been proposed and used in different parts of the world, and many lymphoma entities that are recognized clinically and pathologically have been described by different names in these classifications. Lymphomas are divided into B-cell and T-cell neoplasms, according to their immunophenotypic characteristics. Within each group, there are numerous specific disease entities, many of which are associated with specific karyotypic abnormalities. Neoplasms of putative NK cell origin are provisionally grouped with the T-cell malignancies.

The International Lymphoma Study Group proposed in 1994 (Harris *et al.*, 1994) a 'Revised European–American Lymphoma (REAL) Classification' that attempted to link the major classification systems then in use in Europe and the United States. These included the Kiel classification, widely used in Europe (Stansfeld *et al.*, 1988), and the Working Formulation used in clinical trials in the United States (Anon., 1982). Table 8, adapted from Harris *et al.* (1994), is a list of non-Hodgkin's lymphoid neoplasms recognized by the International Lymphoma Study Group. The disease entities mentioned in section 2.2.2 are named in most cases according to the REAL classification; the pathology of Burkitt's lymphoma, a B-cell neoplasm, is presented in section 2.1.1. Hodgkin's disease, which is also considered within the REAL classification, is discussed separately in section 2.3.1.

2.2.2 *Epidemiology*

2.2.2.1 *Descriptive epidemiology*

Non-Hodgkin's lymphoma is estimated to account for 2.5% of all cancer cases worldwide (Pisani *et al.*, 1997). The incidence varies approximately sixfold, the highest reported rates being seen in whites in the United States and the lowest in Southeast Asia, India and sub-Saharan Africa (Parkin *et al.*, 1997). The incidence rises steeply with age. In the United States, the age-adjusted rates for 1990–94 were 9.2 cases per 100 000 annually among people under 65 years of age and 73.5 per 100 000 for those aged 65 and over (Ries *et al.*, 1997). Males are at higher risk, having incidence rates approximately 50–100% higher than females in most countries (Parkin *et al.*, 1997). The rates have been increasing steadily throughout the world, for reasons that are largely unexplained (Hartge *et al.*, 1994). Immunodeficiency of various etiologies, including HIV infection (see section 2.2.3), iatrogenic immunosuppression (see section 2.2.3) and congenital immunodeficiency (see section 2.2.4), is associated with a greatly increased risk, but the etiology

Table 8. The Revised European–American Lymphoma classification of non-Hodgkin's lymphomas

B-Cell neoplasms

I. Precursor B-cell neoplasm: Precursor B-lymphoblastic leukaemia/lymphoma
II. Peripheral B-cell neoplasms
  1. B-Cell chronic lymphocytic leukaemia/prolymphocytic leukaemia/small lymphocytic lymphoma
  2. Lymphoplasmacytoid lymphoma/immunocytoma
  3. Mantle-cell lymphoma
  4. Follicle-centre lymphoma, follicular
  5. Marginal zone B-cell lymphoma
     Extranodal (MALT-type +/– monocytoid B cells)
  6. Provisional entity: Splenic marginal zone lymphoma
     (+/– villous lymphocytes)
  7. Hairy-cell leukaemia
  8. Plasmacytoma/plasma-cell myeloma
  9. Diffuse large B-cell lymphoma[a] (centroblastic)
     Subtype: Primary mediastinal (thymic) B-cell lymphoma
  10. Burkitt's lymphoma (small noncleaved cell)
  11. Provisional entity: High-grade B-cell lymphoma, Burkitt-like[a]

T-Cell and putative NK-cell neoplasms

I. Precursor T-cell neoplasm: Precursor T-lymphoblastic lymphoma/leukaemia
II. Peripheral T-cell and NK-cell neoplasms
  1. T-Cell chronic lymphocytic leukaemia/prolymphocytic leukaemia
  2. Large granular lymphocyte leukaemia
     T-cell type
     NK-cell type
  3. Mycosis fungoides/Sezary syndrome
  4. Peripheral T-cell lymphomas, unspecified[a]
     Provisional cytological categories: Medium-sized cell, mixed medium and large cell, large cell, lymphoepithelioid cell
  5. Angioimmunoblastic T-cell lymphoma
  6. Angiocentric lymphoma
  7. Intestinal T-cell lymphoma (+/– enteropathy associated)
  8. Adult T-cell lymphoma/leukaemia
  9. Anaplastic large-cell lymphoma, $CD30^+$, T- and null-cell types
  10. Provisional entity: Anaplastic large-cell lymphoma, Hodgkin's-like

From Harris *et al.* (1994)

[a]These categories are thought likely to include more than one disease entity.

of most cases of non-Hodgkin's lymphoma remains unknown. As immunodeficiency-associated lymphomas differ in a number of respects, the remainder of this section addresses only lymphomas in individuals with no evidence of prior immunocompromise.

2.2.2.2 *Case reports and case series*

Non-Hodgkin's lymphomas are pathologically diverse, as noted above, and their association with EBV differs accordingly.

(*a*) *B-Cell non-Hodgkin's lymphoma*

Although the B cell is the usual target in latent infection, the rate of detection of EBV was relatively low in three large series of B-cell lymphomas. Hamilton-Dutoit and Pallesen (1992) found that four of 105 B-cell lymphomas expressed LMP-1 and only one expressed EBNA-2. Hummel *et al.* (1995b) found EBER transcription in 54 (26%) of 208 tumours; in half of the positive cases, this was localized to non-neoplastic bystander cells. Of 27 cases with EBER in the tumour cells, more than 80% of tumour cells in 17 cases expressed EBER. d'Amore *et al.* (1996) found EBER transcription in 25 (6.5%) of 386 tumours, but only 6 (2%) had more than 10 EBER-positive tumour cells per medium-power (× 200) field on light microscopic examination. The three studies showed no consistent association between histological subtype and the presence of EBV (Table 9).

Primary central nervous system lymphoma, which is nearly always of B-cell origin, has been of particular interest since the first description by Hochberg *et al.* (1983) of an EBV-containing tumour detected by Southern blot, as EBV is almost universally present in brain lymphomas secondary to immunodeficiency (see section 2.2.3). In the series of central nervous system lymphomas reported by Murphy *et al.* (1990) and by DeAngelis *et al.* (1992), nearly half of the tumours were shown to contain EBV DNA by in-situ hybridization or PCR. These two initial reports were not, however, confirmed by many other studies of DNA (Bashir *et al.*, 1990; Nakhleh *et al.*, 1991; Geddes *et al.*, 1992) and RNA (MacMahon *et al.*, 1991; Chang *et al.*, 1993a; Bashir *et al.*, 1994; Bergmann *et al.*, 1995) by in-situ hybridization, in which EBV was detected in no more than 12% of cases (Table 10).

B-Cell lymphoma of mucosa-associated lymphoid tissue (MALT), which is found in the stomach and elsewhere, warrants attention because of the association between gastric adenocarcinoma and EBV (see section 2.5). Liu *et al.* (1995) found one of 16 Japanese cases of gastric MALT lymphoma to contain EBER-1 by in-situ hybridization, and 20% of the tumour cells contained EBV. An additional case showed no EBV in the primary gastric tumour, but an EBER-positive tumour was found in a regional lymph node. d'Amore *et al.* (1996) found rare EBER-1/2-positive tumour cells in three of 28 MALT tumours in Denmark. Lee *et al.* (1997) found no EBER-1-positive cases among eight gastric MALT tumours in the Republic of Korea. A similarly low frequency of EBV-positivity has been found in MALT tumours outside of the stomach: using PCR, Diss *et al.* (1995) found EBV DNA in three of 36 parotid MALT tumours in the United

Kingdom; in the one case with EBV DNA detected by in-situ hybridization, less than 5% of tumour cells contained EBER.

Table 9. Presence of EBV in B-cell non-Hodgkin's lymphoma tissue

Reference	Study area	Detection method	Histology	No. of cases	No. with EBV	Tumour cells with EBV
Hamilton-Dutoit & Pallesen (1992)	Denmark	LMP-1, IHC	Diffuse large cell	37	0	
			Immunoblastic	54	3	< 50%
			Small non-cleaved cell	10	0	
			Anaplastic large cell	4	1	
Hummel *et al.* (1995b)	Germany	EBER-1/2, ISH	Low-grade	65	3	Variable
			Diffuse large cell	39	3	
			Immunoblastic	28	4	
			Small non-cleaved cell	36	7	
			Anaplastic large cell	16	3	
			Other high grade	24	7	
		LMP, IHC	Low grade	65	1	Low
			Diffuse large cell	39	2	
			Immunoblastic	28	2	
			Small non-cleaved cell	36	0	
			Anaplastic large cell	16	3	
			Other high grade	24	3	
d'Amore *et al.* (1996)	Denmark	EBER-1/2, ISH	Low grade	154	9	Few
			Diffuse large cell	74	3	
			Immunoblastic	21	1	
			Small non-cleaved cell	24	3	
			Anaplastic large cell	7	0	
			Other high grade	106	9	

All in non-immunocompromised individuals
EBER, EBV-encoded RNA; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein

(*b*) *Angiocentric T-cell lymphoma*

Sinonasal T-cell lymphoma, which encompasses entities also referred to as lethal midline granuloma and midline reticulosis, is the non-Hodgkin's lymphoma most strongly associated with EBV. Series in Asia (Harabuchi *et al.*, 1990; Ho *et al.*, 1990; Chan *et al.*, 1994a; Ko & Lee, 1994; Lee *et al.*, 1994a; Peh *et al.*, 1995; Harabuchi *et al.*, 1996), South America (Arber *et al.*, 1993), Europe (O'Leary & Kennedy, 1995; Dictor *et al.*, 1996; Kanavaros *et al.*, 1996) and the United States (Weiss *et al.*, 1992a; Davison *et al.*, 1996) consistently showed the presence of EBV in the great majority of cases, EBV being present in most tumour cells (Table 11). CD56-positive tumours, which are suggested to be of NK-cell derivation, were uniformly EBV-positive, whereas CD56-negative tumours were more frequently EBV-negative (Chan *et al.*, 1994a; Harabuchi

et al., 1996; Kanavaros *et al.*, 1996). EBV-positive tumours consistently expressed the transforming EBV protein LMP-1 (Harabuchi *et al.*, 1996; Kanavaros *et al.*, 1996), and EBV terminal repeat sequences have been shown to have monoclonal or biclonal infection (Ho *et al.*, 1990; Harabuchi *et al.*, 1996).

Table 10. Presence of EBV in primary central nervous system non-Hodgkin's lymphoma tissue in HIV-negative cases

Reference	Study area	Detection method	No. of cases	No. with EBV
Hochberg *et al.* (1983)	USA	EBV DNA, SB	1	1
Bashir *et al.* (1990)	USA	*Bam*HI V, ISH	10	0
Murphy *et al.* (1990)	UK	*Bam*HI W, ISH	24	11
Rouah *et al.* (1990)	USA	EBV DNA, ISH	5	2
		EBV DNA, PCR	7	2
Bignon *et al.* (1991)	France	EBV DNA, PCR	9	1
MacMahon *et al.* (1991)	USA	EBER-1, ISH	14	1
Nakhleh *et al.* (1991)	USA	*Bam*HI W, ISH	17	2
Geddes *et al.* (1992)	UK	*Bam*HI W, ISH	43	2
DeAngelis *et al.* (1992)	USA	EBV DNA, PCR	13	7
Chang *et al.* (1993a)	USA	EBER-1, ISH	27	1
Bashir *et al.* (1994)	USA	EBER-1, ISH	9	0
Bergmann *et al.* (1995/96)	Germany	EBER, ISH	36	0

*Bam*HI V and *Bam*HI W, DNA restriction fragments; EBER, EBV-encoded RNA; ISH, in-situ hybridization; PCR, polymerase chain reaction; SB, Southern blot

In contrast, EBV is much less frequently found in sinonasal B-cell lymphoma. Chan *et al.* (1994a) and Peh *et al.* (1995) found one EBV-positive case among ten and nine tumours respectively, and Kanavaros *et al.* (1996) found one among 10 tumours; a somewhat higher frequency was found by Weiss *et al.* (1992a) (Table 11).

B-Cell lymphomas of the nasopharynx, tonsil and tongue ('Waldeyer's ring') less frequently contain EBV, the frequency ranging from 0 to 13% in six series (Chan *et al.*, 1994a; Ko & Lee, 1994; Lee *et al.*, 1994a; O'Leary & Kennedy, 1995; Peh *et al.*, 1995; Kanavaros *et al.*, 1996), with a slightly higher frequency in the report of Weiss *et al.* (1992a) (Table 11). T-Cell tumours of Waldeyer's ring have a more variable association with EBV: 0/8 and 3/6 tumours were found to contain EBV in the two largest series (Ko & Lee, 1994; Kanavaros *et al.* 1996).

Lymphomatoid granulomatosis, which resembles sinonasal lymphoma histologically, is another condition in which EBV is highly prevalent. In eight series, EBV was detected in the majority of cases (Katzenstein & Peiper, 1990; Medeiros *et al.*, 1991; Guinee *et al.*, 1994; Tsang *et al.*, 1994; Myers *et al.*, 1995; Nicholson *et al.*, 1996; Takeshita *et al.*, 1996; Wilson *et al.*, 1996b); in two other series it was detected at lower frequency (Sabourin *et al.*, 1993; Kobashi *et al.*, 1996) (Table 12). The diagnostic classification of

Table 11. Presence of EBV in sinonasal and Waldeyer's ring non-Hodgkin's lymphomas

Reference	Study area	Detection method	Immuno-phenotype	Sinonasal		Waldeyer's ring		Tumour cells with EBV	Comments
				No. of cases	No. with EBV	No. of cases	No. with EBV		
Harabuchi *et al.* (1990)	Japan	*Bam*HI W, ISH	T Cell	5	5			80–90%	
		EBNA, IFA	T Cell	5	5				
Ho *et al.* (1990)	Hong Kong	*Bam*HI W, SB	T Cell	7	7				EBV mono- or biclonal
			B Cell	3	2				
Weiss *et al.* (1989a; 1992a)	USA	*Bam*HI W, ISH	T Cell	3	3			Uniform	
			B Cell	5	2	10	2		
Arber *et al.* (1993)	Peru	EBER-1, ISH	T Cell	11	11			Many	
			B Cell	2	1				
			Indeter-minate	1	1				
Chan *et al.* (1994a)	Hong Kong	EBER-1/2, ISH	T Cell	30	25	1	0	Most	21/21 $CD56^+$ and 4/9 $CD56^-$ contained EBV
			B Cell	10	1	20	0		
Ko & Lee (1994)	Republic of Korea	EBER, ISH	T Cell	10	7	8	0		
			B Cell			12	1		
Lee *et al.* (1994a)	Republic of Korea	EBER-1/2, ISH	T Cell	12	12	2	2	Many	
			B Cell	1	0	16	2		
O'Leary & Kennedy (1995)	Ireland	EBER-1/2, ISH	T Cell	8	6			Many	
			B Cell	3	0	10	2		
Peh *et al.* (1995)	Malaysia	EBER-1, ISH	T Cell	10	9	3	2	70–100%	
			B Cell	9	1	7	0		
Davison *et al.* (1996)	USA	EBER-1, ISH	T Cell	30	29			Majority	
Dictor *et al.* (1996)	Sweden	EBER-1, ISH	T Cell	12	12			50–100%	

Table 11 (contd)

Reference	Study area	Detection method	Immuno-phenotype	Sinonasal		Waldeyer's ring		Tumour cells with EBV	Comments
				No. of cases	No. with EBV	No. of cases	No. with EBV		
Harabuchi *et al.* (1996)	Japan	EBER-1, ISH	T Cell	16	16			> 50%	EBV monoclonal; 9/9 $CD56^+$; includes 6 cases from Harabuchi *et al.* (1990)
		LMP-1, IHC		9	9			> 50%	
Kanavaros *et al.* (1996)	France and Greece	EBER-1/2, ISH	B Cell	17	16	6	3	> 50%	11/11 $CD56^+$ and 1/3 $CD56^-$ contained EBV
				10	1	22	1		
		LMP-1, IHC	T Cell	17	16	6	3	1–50%	
			B Cell	10	0	22	0		

All in non-immunocompromised individuals
EBER, EBV-encoded RNA; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein; SB, Southern blot

Table 12. Presence of EBV in lymphomatoid granulomatosis tissue

Reference	Study area	Detection method	Immunophenotype	No. of cases	No. with EBV	Tumours with EBV	Comments
Katzenstein & Peiper (1990)	USA	EBV DNA, PCR		29	21		
Medeiros *et al.* (1991)	USA	*Bam*HI W, PCR	T Cell	5	3		
		EBV, SB	T Cell	7	2		
Sabourin *et al.* (1993)	France	EBER-1/2, ISH	T Cell	6	2	Numerous	
			B Cell	1	0		
		LMP, IHC	T Cell	6	2		
			B Cell	1	0		
Guinee *et al.* (1994)	USA	EBER-1, ISH	T and B cell	10	10	Only in B cells	B cells monoclonal, T cells polyclonal
Tsang *et al.* (1994)	Hong Kong	EBER-1/2, ISH, IHC	NK Cell	15	10	Most	
Myers *et al.* (1995)	USA	EBER-1, ISH	T Cell	6	0		
			T and B cell	11	10	Only in B cells	
Kobashi *et al.* (1996)	Japan	EBER-1/2, ISH	NK Cell	9	3	Most	EBV mono- or biclonal
		LMP-1, IHC	NK Cell	9	2		
Nicholson *et al.* (1996)	UK	EBER-1, ISH	T and B cell	7	4	Only in B cells	
Takeshita *et al.* (1996)	Japan	EBER-1, ISH	T and B cell	3	2	Some to many	1 only in B cells
			B Cell	9	4		
			NK Cell	2	2		
		LMP, IHC	T and B cell	3	2		1 only in B cells
			B Cell	9	4		
			NK Cell	2	1		
Wilson *et al.* (1996b)	USA	EBER-1, ISH	T and B cell	4	4	Only in B cells	

All in non-immunocompromised individuals

*Bam*HI W, DNA restriction fragment; EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; IFA, immunofluorescence assay; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein; PCR, polymerase chain reaction; SB, Southern blot; NK, natural killer

this disorder has recently undergone revision. Guinee *et al.* (1994) found that despite a predominance of T cells in these tumours, a minor population of monoclonal B cells is present within a polyclonal population of T cells. Furthermore, in all 10 cases, EBV was present in the B-cell population only. Myers *et al.* (1995), Nicholson *et al.* (1996) and Takeshita *et al.* (1996) reported a similar restriction of EBV to B cells in tumours of mixed immunophenotype. Kobashi *et al.* (1996) found monoclonal or biclonal EBV by terminal-repeat fragment analysis in three of three EBV-positive tumours.

(*c*) *Other peripheral T-cell lymphomas*

These entities have been reviewed by Pallesen *et al.* (1993). Other peripheral T-cell lymphomas commonly contain EBV (Table 13). In 12 series from North America, Europe and Asia, the frequency of EBV positivity ranged from 18 to 70% in all but one study (Herbst *et al.*, 1991b; Lee *et al.*, 1991; Kanavaros *et al.*, 1992; Ott *et al.*, 1992; Weiss *et al.*, 1992b; Korbjuhn *et al.*, 1993; Tsang *et al.*, 1994; Zhou *et al.*, 1994; Lopategui *et al.*, 1995; d'Amore *et al.*, 1996; Hirose *et al.*, 1996). Hamilton-Dutoit and Pallesen (1992) found only 10% positivity, but used an assay with low sensitivity. The presence of EBV in tumour cells varies, some tumours showing its presence uniformly and others in only a fraction of cells. Terminal-repeat assays have been used to confirm the mono- or oligoclonality of latent EBV episomes (Ott *et al.*, 1992; Hirose *et al.*, 1996).

There is some variation by histological subtype. Most angioimmunoblastic lymphomas contain EBV (Ott *et al.*, 1992; Weiss *et al.*, 1992b; Zhou *et al.*, 1994; d'Amore *et al.*, 1996; Hirose *et al.*, 1996), although in one study tumour cells containing EBV were rare (Tsang *et al.*, 1994). Most cases of anaplastic large-cell lymphoma do not contain EBV (Herbst *et al.*, 1991b; Hamilton-Dutoit & Pallesen, 1992; Kanavaros *et al.*, 1992; Ott *et al.*, 1992; Zhou *et al.*, 1994; Lopategui *et al.*, 1995; d'Amore *et al.*, 1996). Pleomorphic and other tumour types, such as lymphoepithelioid and T-zone tumours, show an intermediate frequency, about one-third of the reported cases containing EBV (Hamilton-Dutoit & Pallesen, 1992; Ott *et al.*, 1992; Korbjuhn *et al.*, 1993; Tsang *et al.*, 1994; Zhou *et al.*, 1994; d'Amore *et al.*, 1996; Hirose *et al.*, 1996; Table 13).

Enteropathy-associated T-cell lymphoma is a distinct entity which has been investigated for the presence of EBV by EBER in-situ hybridization in four studies. Pan *et al.* (1993) detected EBV in four of 11 cases in the United Kingdom; in the cases with EBV, over 80% of tumour cells contained EBER, and the EBV was shown to be monoclonal by terminal-repeat analysis. These findings were not confirmed in other studies. Korbjuhn *et al.* (1993) found EBV in two of 10 cases in Germany; in both cases, less than 20% of the tumour cells contained EBER. Similarly, Ilyas *et al.* (1995) found EBV in none of seven cases in the United Kingdom, and Walsh *et al.* (1995) found EBV in only one of 16 cases in Ireland.

EBV was detected infrequently in low-grade cutaneous lymphomas (mycosis fungoides and Sézary syndrome) in three studies in which EBER-1/2 was analysed by in-situ hybridization. Anagnostopoulos *et al.* (1996) found EBER in four of 42 cases in Germany, but less than 1% of the tumour cells contained EBV. Angel *et al.* (1996) in the

Table 13. Presence of EBV in peripheral T-cell lymphoma tissue

Reference	Study area	Detection method	Histological type	No. of cases	No. with EBV	Tumour cells with EBV	Comments
Herbst *et al.* (1991b)	Germany	*Bam*HI W, PCR	Anaplastic large cell	18	5		
		LMP, IHC		18	2	50–90%	
Lee *et al.*(1991)	Taiwan	EBV DNA, SB		6	4		Paediatric series
Hamilton-Dutoit & Pallesen (1992)	Denmark	LMP-1, IHC	Pleomorphic	50	7	< 50%	
			Angioimmunoblastic	4	1		
			Anaplastic large cell	19	0		
			Other	9	0		
Kanavaros *et al.* (1992)	Netherlands	EBV DNA, PCR, ISH	Anaplastic large cell	3	2	50%	
		LMP, IHC		11	1	Variable	
Ott *et al.* (1992)	Germany	*Bam*HI W, SB	Pleomorphic	14	5	Variable	EBV mono- or biclonal
			Angioimmunoblastic	14	8		
			Anaplastic large cell	8	3		
			Other	10	2		
Weiss *et al.* (1992b)	USA	EBER-1, ISH	Angioimmunoblastic	23	19	Variable	Mostly B cells
Korbjuhn *et al.* (1993)	Germany	EBER, ISH	Pleomorphic	81	38	1–100%	
Tsang *et al.* (1994)	Hong Kong	EBER-1/2, ISH	Pleomorphic	7	3	≥ 90%	
			Angioimmunoblastic	6	0		Rare EBER-positive cells in 4 cases
			Other	2	0		
Zhou *et al.* (1994)	China	EBER ISH	Pleomorphic	27	18	≥ 50%	
			Angioimmunoblastic	6	4		
			Anaplastic large cell	2	0		
			Other	4	2		
		LMP-1, IHC	Pleomorphic	26	13		
			Angioimmunoblastic	6	4		
			Anaplastic large cell	2	0		
			Other	4	2		

Table 13 (contd)

Reference	Study area	Detection method	Histological type	No. of cases	No. with EBV	Tumour cells with EBV	Comments
Lopategui *et al.* (1995)	USA and Hong Kong	EBER-1, ISH	Anaplastic large cell	15	3	25–90%	
		LMP-1, IHC		15	0		
d'Amore *et al.* (1996)	Denmark	EBER-1/2, ISH	Pleomorphic	67	24	< 10–> 50%	
			Angioimmunoblastic	13	11		B cells also positive
			Anaplastic large cell	9	0		
			Other	21	0		
		LMP-1, IHC	Pleomorphic	24	8		
			Angioimmunoblastic	11	2		
			Other	5	2		
Hirose *et al.* (1996)	Japan	EBER, ISH	Pleomorphic	10	4	Few to extensive	
			Angioimmunoblastic	9	7		B cells also positive; EBV mono- or biclonal
			Other	4	4		
		LMP-1, IHC	Pleomorphic	10	3		
			Angioimmunoblastic	9	1		
			Other	4	0		

All in non-immunocompromised individuals

*Bam*HI W, DNA restriction fragment; EBER, EBV-encoded RNA; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein; PCR, polymerase chain reaction; SB, Southern blot

United Kingdom and d'Amore *et al.* (1996) in Denmark found no EBV in 25 and 13 cases, respectively.

2.2.2.3 *Cohort studies*

The association between abnormal EBV seroreactivity and the risk for subsequent non-Hodgkin's lymphoma has been examined in two studies. Mueller *et al.* (1991) performed a nested case–control study based on sera from four banks established between 1964 and 1974 containing specimens from over 240 000 people in Norway and the United States, 104 of whom had developed non-Hodgkin's lymphoma an average of 63 months after their serum had been collected. They were compared with 259 controls matched for age, sex, ethnic group and date of serum collection. Immunofluorescence assays showed that elevated levels of IgG (titre, ≥ 1:320) and IgM (titre, ≥ 1:5) antibodies against EBV VCA were associated with relative risks for non-Hodgkin's lymphoma of 2.5 (95% CI, 1.1–5.7) for IgG and 3.2 (95% CI, 1.3–7.5) for IgM. Low titres (≤ 1:5) of antibody to EBNA were associated with a decreased risk for lymphoma, although the effect was significant only for cases that developed within five years of serum collection. No differences were reported in subgroup analyses of follicular versus diffuse, large-cell versus mixed- and small-cell, or low- versus intermediate- versus high-grade lymphomas. [The Working Group noted that no T versus B immunophenotyping or tissue analyses for EBV markers were reported, and that the relative risks may have been attenuated by the inclusion of all types of non-Hodgkin's lymphoma.]

Lehtinen *et al.* (1993) performed a nested case–control study of 39 000 Finnish adults from whom serum was collected between 1968 and 1972. Eleven subjects who developed non-Hodgkin's lymphoma 1–12 years after serum collection were compared with 22 controls matched for age, sex, municipality and date of phlebotomy. Enzyme immunoassays showed no significant differences in antibodies to VCA, EA or EBNA. [The Working Group noted that immunofluorescence is the standard method for detecting EBV antibodies and the relevance of the authors' enzyme immunoassay is uncertain.]

2.2.3 *Human immunodeficiency virus as a cofactor*

HIV and HHV8 also play a role in the pathogenesis of EBV-associated non-Hodgkin's lymphoma. The monograph on HHV8 in this volume covers its interaction with EBV; this section deals only with HIV.

Lymphomas in HIV-infected patients are nearly always of B-cell origin. Under the Working Formulation classification (Rosenberg, 1982), they include tumours of the small non-cleaved cell (diffuse large B cell in the REAL classification), large cell diffuse and immunoblastic types; anaplastic large cell and unclassified high-grade types are also observed. Like other immune-deficient conditions, HIV infection is associated in particular with primary central nervous system lymphoma. The association of EBV with HIV-related lymphomas varies.

2.2.3.1 *Primary central nervous system lymphomas*

Unlike central nervous system lymphomas in immunocompetent individuals, those found in association with HIV infection nearly always contain detectable EBV. In nine reported series, EBV was detected in all cases in five studies (MacMahon *et al.*, 1991; Chang *et al.*, 1993a; Cinque *et al.*, 1993; Bashir *et al.*, 1994; Arribas *et al.*, 1995) and in the majority of cases in the remaining four studies (Bashir *et al.*, 1990; DeAngelis *et al.*, 1992; Morgello, 1992; Bergmann *et al.*, 1995; Table 14). In-situ hybridization has been used to show that most or all of the tumour cells contained EBV RNA (MacMahon *et al.*, 1991; Chang *et al.*, 1993a; Cinque *et al.*, 1993; Bashir *et al.*, 1994). LMP expression is more variable; nine of 21 tumours reported by MacMahon *et al.* (1991) and 13 of 19 reported by Bergmann *et al.* (1995/96) were shown by immunohistochemistry to contain LMP.

Table 14. Presence of EBV in HIV-associated primary central nervous system non-Hodgkin's lymphoma tissue

Reference	Study area	Detection method	No. of cases	No. with EBV	Tumour cells with EBV
Bashir *et al.* (1990)	USA	*Bam*HI V, ISH	5	4	Variable
MacMahon *et al.* (1991)	USA	EBER-1, ISH	21	21	Uniform
		LMP, IHC	21	9	Many cells
DeAngelis *et al.* (1992)	USA	*Bam*HI W, PCR	13	11	
Morgello (1992)	USA	EBNA-1, PCR	12	6	
Chang *et al.* (1993a)	USA	EBER-1, ISH	5	5	Uniform
Cinque *et al.* (1993)	Sweden and Italy	EBER, ISH	16	16	40–90%
Bashir *et al.* (1994)	USA	EBER-1, ISH	5	5	Most
Arribas *et al.* (1995)	USA	LMP, IHC	6	6	Variable
Bergmann *et al.* (1995/96)	Germany	EBER, ISH	19	15	
		LMP, IHC	19	13	

*Bam*HI V and *Bam*HI W, DNA restriction fragments; EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein; PCR, polymerase chain reaction

2.2.3.2 *Systemic non-Hodgkin's lymphomas*

EBV is found in a large fraction of systemic tumours. In 13 series of 10 or more patients, the frequency of EBV positivity ranged from 38 to 79% (Subar *et al.*, 1988; Borisch-Chappuis *et al.*, 1990a,b; Boyle *et al.*, 1991; Guarner *et al.*, 1991; Borisch *et al.*, 1992a; Ballerini *et al.*, 1993; Carbone *et al.*, 1993a,b; Hamilton-Dutoit *et al.*, 1993a; Shibata *et al.*, 1993; Raphael *et al.*, 1994; Bacchi *et al.*, 1996b; Carbone *et al.*, 1996; Table 15). In many of the tumours, the majority of the cells contained EBV (Borisch-Chappuis *et al.*, 1990a,b; Guarner *et al.*, 1991), although in several studies the fraction of EBV positivity was somewhat variable (Borisch *et al.*, 1992a; Carbone *et al.*, 1993a;

Table 15. Presence of EBV in HIV-associated systemic non-Hodgkin's lymphoma tissue

Reference	Study area	Detection method	Histological type	No. of cases	No. with EBV	Tumour cells with EBV	Comments
Subar *et al.* (1988)	USA	EBV DNA, SB	Diffuse large cell	4	1		
			Immunoblastic	2	2		
			Small non-cleaved cell	10	3		
		EBNA, IFA	Diffuse large cell	3	1	80%	
			Immunoblastic	1	1		
			Small non-cleaved cell	4	2		
Borisch-Chappuis *et al.* (1990a,b)	Germany	*Bam*HI W, ISH	Diffuse large cell	1	1	> 50%	
			Immunoblastic	2	1		EBV-positive tumour, T immunophenotype
			Small non-cleaved cell	9	4		
			Anaplastic large cell	1	0		
			Other high grade	1	1		
Boiocchi *et al.* (1990)	Italy	*Bam*HI W, SB	Immunoblastic	2	1		EBV monoclonal
			Small non-cleaved cell	3	0		
Boyle *et al.* (1991)	Australia	EBNA-1, PCR	Immunoblastic	12	6		
			Small non-cleaved cell	7	3		
			Other high grade	1	1		
Guarner *et al.* (1991)	USA	EBV DNA, ISH	Diffuse large cell	4	2	> 80%	
			Immunoblastic	2	1		
			Small non-cleaved cell	6	6		
			Other	2	0		
MacMahon *et al.* (1991)	USA	EBER-1, ISH	NR	7	3		
Neri *et al.* (1991)	USA	*Bam*HI	Diffuse large cell	1	1		EBV monoclonal in 10/10
			Immunoblastic	1	1		
			Small non-cleaved cell	5	5		
			Other	3	3		

Table 15 (contd)

Reference	Study area	Detection method	Histological type	No. of cases	No. with EBV	Tumour cells with EBV	Comments
Borisch *et al.* (1992a)	Switzerland	EBER-1, ISH	Diffuse large cell	3	3	Variable	
			Immunoblastic	8	8		
			Small non-cleaved cell	2	0		
			Anaplastic large cell	1	0		
		LMP, IHC	Diffuse large cell	3	1		
			Immunoblastic	8	3		
			Small non-cleaved cell	2	0		
			Anaplastic large cell	1	0		
Ballerini *et al.* (1993)	USA	EBV DNA, SB and EBNA-1, IHC	Diffuse large cell	4	1		EBV monoclonal
			Immunoblastic	4	4		
			Small non-cleaved cell	16	5		
Carbone *et al.* (1993a)	Italy	EBV DNA, ISH	Immunoblastic	4	1	Variable	
			Small non-cleaved cell	9	4		
			Anaplastic large cell	9	7		
Carbone *et al.* (1993b)	Italy	EBER-1/2 or *Bam*HI W, ISH	Diffuse large cell	6	1		
			Immunoblastic	7	3		
			Small non-cleaved cell	15	6		
			Anaplastic large cell	12	10		
		LMP, IHC	Diffuse large cell	6	0		
			Immunoblastic	7	3		
			Small non-cleaved cell	15	0		
			Anaplastic large cell	12	9		
Hamilton-Dutoit *et al.* (1993a)	France and Denmark	EBER-1, ISH or *Bam*HI W, SB LMP-1, IHC	Immunoblastic	30	22	30–90%	
			Small non-cleaved cell	19	11		
			Immunoblastic	30	16		
			Small non-cleaved cell	19	3		

Table 15 (contd)

Reference	Study area	Detection method	Histological type	No. of cases	No. with EBV	Tumour cells with EBV	Comments
Shibata *et al.* (1993)	USA	EBNA-1, PCR and EBER-1, ISH	Diffuse large cell	11	6		EBV monoclonal
			Immunoblastic	20	17		
			Small non-cleaved cell	28	16		
Raphael *et al.* (1994)	France	*Bam*HI W, SB or EBER, ISH	Diffuse large cell	3	0		
			Immunoblastic	9	8		
			Small non-cleaved cell	16	8		
			Anaplastic large cell	1	0		
			Other high grade	3	1		
Bacchi *et al.* (1996b)	Brazil	EBER-1, ISH	Diffuse large cell	11	6	40–100%	1 EBV-negative tumour T immunophenotype
			Immunoblastic	4	3		
			Small non-cleaved cell	5	2		
Carbone *et al.* (1996)	Italy	EBER-1/2, ISH	Small non-cleaved cell	11	4	25–75%	EBV monoclonal
			Anaplastic large cell	5	4		
		LMP, IHC	Small non-cleaved cell	10	0		
			Anaplastic large cell	5	3		

*Bam*HI V and *Bam*HI W, DNA restriction fragments; EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; IFA, immunofluorescence assay; IHC, immunohistochemistry; ISH, in-situ hybridization; LMP, latent membrane protein; PCR, polymerase chain reaction; SB, Southern blot; NR, not reported

Hamilton-Dutoit *et al.*, 1993a; Bacchi *et al.*, 1996b; Carbone *et al.*, 1996). Immunoblastic lymphomas (classified as diffuse large-cell lymphomas in the REAL classification) tend to be associated with more advanced immunosuppression, and these tumours more commonly contain EBV than other diffuse large-cell and small non-cleaved-cell lymphomas (Ballerini *et al.*, 1993; Hamilton-Dutoit *et al.*, 1993a; Raphael *et al.*, 1994); Carbone *et al.* (1993a,b; 1996) also noted a higher frequency of EBV positivity in anaplastic large-cell tumours. EBV monoclonality has been consistently observed by terminal-repeat sequence analysis (Boiocchi *et al.*, 1990; Neri *et al.*, 1991; Ballerini *et al.*, 1993; Shibata *et al.*, 1993; Carbone *et al.*, 1996).

2.2.4 *Congenital immunodeficiency syndromes*

Patients with X-linked lymphoproliferative syndrome (see section 4.2) are at increased risk for non-Hodgkin's lymphoma, which occurs in up to 25% of affected individuals (Purtilo, 1981; Sullivan & Woda, 1989). Wiskott-Aldrich syndrome, ataxia telangiectasia and other primary immunodeficiency syndromes are also associated with greatly increased risks for lymphoma (Filipovich *et al.*, 1992; Schuster *et al.*, 1995).

Patients with X-linked lymphoproliferative syndrome have a specific defect in their control of infection with EBV. EBV was found in all of 82 lymphomas associated with this condition (Sullivan & Woda, 1989). Several case reports of lymphomas associated with Wiskott-Aldrich syndrome (Okano *et al.*, 1984; Nakhleh *et al.*, 1991; Nakanishi *et al.*, 1993), ataxia telangiectasia (Saemundson *et al.*, 1981; Mselati *et al.*, 1983) and severe combined immunodeficiency (Garcia *et al.*, 1987) have confirmed the near universal presence of EBV in lymphomas associated with congenital immunodeficiency, although one EBV-negative case has been reported (Okano *et al.*, 1984; Table 16).

Table 16. Prevalence of EBV-associated lymphomas arising in children with primary immunodeficiency

Reference	Study area	Detection method	Primary immunodeficiency	No. of cases	No. with EBV
Sullivan & Woda (1989)	USA and Europe	Immunofluorescence	X-linked lymphoproliferative	82	82
Okano *et al.* (1984)	Japan	Immunofluorescence and EBV DNA	Wiskott-Aldrich	2	1
Nakanishi *et al.* (1993)	Japan	EBV DNA	Wiskott-Aldrich	1	1
Nakhleh *et al.* (1991)	USA	EBV DNA, in-situ hybridization	Wiskott-Aldrich	1	1
Saemundsen *et al.* (1981)	Turkey	EBV DNA	Ataxia telangiectasia	1	1
Mselati *et al.* (1983)	France	Serology	Ataxia telangiectasia	2	2
Garcia *et al.* (1987)	USA	EBV DNA	Severe combined	1	1

2.3 Hodgkin's disease

2.3.1 *Pathology and clinical features*

Histologically, Hodgkin's disease is characterized by mononuclear Hodgkin cells and their multinucleated variants, the Reed-Sternberg cells, together abbreviated as HRS cells. HRS cells are embedded in a background of abundant reactive cells, including lymphocytes, plasma cells, histiocytes and eosinophils (Lukes & Butler, 1966). Typically, HRS cells account for only a small proportion of cells in an affected lymph node, rarely amounting to more than 2% of the total cell population. The Rye classification distinguishes four major types of Hodgkin's disease: nodular lymphocyte predominant, nodular sclerosis, mixed cellularity and lymphocyte-depleted (Lukes & Butler, 1966; Herbst & Niedobitek, 1993; Harris *et al.*, 1994). It is now accepted that lymphocyte-depleted Hodgkin's disease represents a separate tumour entity probably derived from germinal-centre B cells, and this is considered separately from the other three 'classical' forms of Hodgkin's disease. Lymphocyte-depleted Hodgkin's disease is encountered only rarely at primary diagnosis of Hodgkin's disease. It is usually seen as recurrent Hodgkin's disease in patients in whom nodular sclerosis or mixed cellularity Hodgkin's disease had been diagnosed previously. Moreover, many cases diagnosed as lymphocyte-depleted Hodgkin's disease are now reported as CD30-positive anaplastic large-cell lymphomas. There is thus increasing evidence to suggest that Hodgkin's disease is not a single entity but rather a heterogeneous group of diseases. This prompted inclusion of Hodgkin's disease in the Revised European–American Lymphoma (REAL) classification (Harris *et al.*, 1994).

The nature and clonal origin of HRS cells are a matter of controversy: most of the constituent cells of the lymph node have been proposed as HRS precursor cells at some stage (Herbst *et al.*, 1996a). HRS cells are characterized immunophenotypically by the expression of lymphocyte-activation antigens, such as CD25, CD30 and CD70 (Herbst *et al.*, 1993; Harris *et al.*, 1994). In a proportion of cases of Hodgkin's disease, HRS cells express B-cell antigens, and in a number of cases antigens characteristic of T cells can be demonstrated (Kadin *et al.*, 1988; Schmid *et al.*, 1991; Herbst *et al.*, 1996a); however, in most cases, no lineage-specific antigens are seen (Herbst *et al.*, 1993). Most recent studies indicate that HRS cells in many (but not all) cases are derived from B cells (Schmid *et al.*, 1991; Küppers *et al.*, 1994), and a germinal-centre origin has been proposed (Kanzler *et al.*, 1996).

The clinical presentation of Hodgkin's disease varies in different geographical locations. In this section, the clinical features seen in the western world are described briefly. Hodgkin's disease usually arises as a unifocal lesion in cervical lymph nodes. Contiguous spread of the tumour to adjacent lymph nodes gives rise to palpably enlarged nodes. With spread of the tumour through lymphatic channels, other organs are involved, the preferential sites of involvement including the spleen and distant lymph nodes. Subsequently, as the disease becomes more aggressive, other organs are involved, including the liver and the kidneys (Kaplan, 1980).

The presence of Hodgkin's disease at each of 17 sites of potential nodal involvement was determined in 719 patients in the United States. The predominant sites of lymph

node involvement were mediastinal (59%), left neck (58%) and right neck (55%), and at least one of these sites was involved in 92% of the patients (Mauch *et al.*, 1993). The spleen was involved in 27% of patients; the epitrochlear, popliteal and mesenteric lymph nodes were not commonly involved. Abdominal lymph node involvement without splenic involvement was rare, and the risk for abdominal lymph node involvement increased with increasing splenic size.

Bone-marrow involvement in Hodgkin's disease is indicative of extensive tumour infiltration and is associated with systemic symptoms including leukopenia, anaemia, thrombocytopenia and elevated levels of alkaline phosphatases. Other organs occasionally involved in Hodgkin's disease include the skin, subcutaneous tissue and breast. Involvement of the central nervous system is relatively rare, except through invasion and extension of the epidural space for enlarged para-aortic nodes. Hilar and mediastinal adenopathy may predispose to pleural involvement, with pleural effusions and pericardial involvement (DeVita *et al.*, 1993).

2.3.2 *Epidemiology*

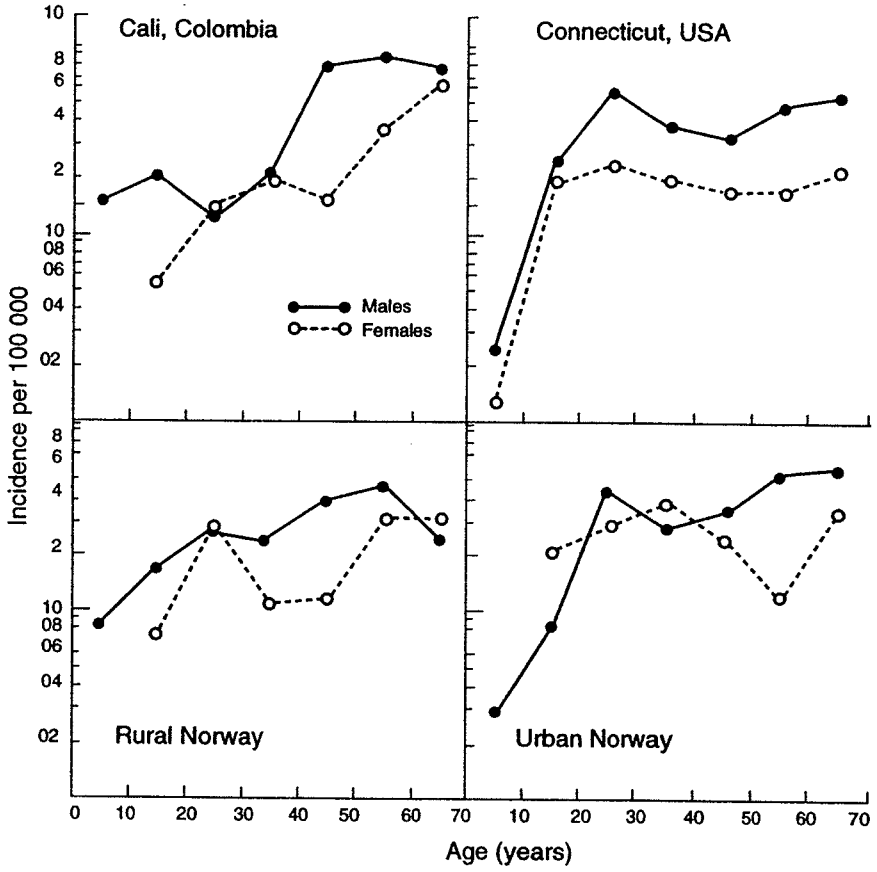
2.3.2.1 *Descriptive epidemiology*

The distinguishing epidemiological feature of Hodgkin's disease characteristically seen in most western populations such as the United States is the bimodal age–incidence curve (Figure 8). In such populations, very few cases occur among children; a rapid increase in incidence among teenagers peaks at about age 25; the incidence then decreases to a plateau through middle age, after which the rates increase with age to a second peak. There is an excess among males, which is pronounced at older ages. MacMahon (1957) proposed that the bimodality results from the overlap of two disease distributions with peaks at different ages. He further suggested that Hodgkin's disease in young adults is caused by a biological agent of low infectivity, while the cause among the elderly is probably similar to those of other lymphomas (MacMahon, 1966).

In 1971, Correa and O'Conor noted a different age pattern among poor populations, with an initial peak in childhood only among boys, relatively low rates among young adults, followed by a late peak among those of advanced age. They further described an intermediate pattern, contrasting data for rural and urban Norwegians in the 1960s (see Figure 8). The shift from a 'developing' to an 'intermediate' pattern in parallel with economic development has been noted by others (Glaser, 1990; Hartge *et al.*, 1994). An intermediate pattern of the occurrence of Hodgkin's disease was seen in African Americans in the late 1970s (Olisa *et al.*, 1976; Cozen *et al.*, 1992), which is less evident in recent data (Parkin *et al.*, 1997).

Currently, essentially all of the majority populations in Europe and North America have a well-defined 'developed' pattern of incidence of Hodgkin's disease. The peak of occurrence in young adulthood varies within this set of countries, being high in Canada, France, Switzerland and the United States and lower in southern Europe. The pattern within eastern European countries is variable. In contrast, the pattern in Asia and Africa is generally 'intermediate' or 'developing' (Parkin *et al.*, 1997).

Figure 8. Age-specific incidence rates of Hodgkin's disease per 100 000 population of each sex in (a) Cali, Colombia (1962–66), (b) Connecticut, United States (1960–62), (c) rural Norway (1964–66) and (d) urban Norway (1964–66)



From Correa and O'Conor (1971)

Alexander *et al.* (1991a,b) used a large, population-based registry of leukaemia and lymphoma covering about half of the United Kingdom during a five-year period to evaluate the characteristics of over 1800 cases of Hodgkin's disease by area-based indices of socioeconomic status and population density. They reported that significantly more of the 486 cases diagnosed in people under the age of 25 occurred in areas of higher socioeconomic status (relative risk [RR], 1.2), and there was a significant trend to increased incidence in areas closer to 'built-up areas' (mutually adjusted). For cases among people aged less than 35 years at the time of diagnosis, there was a significantly positive association with social class, while a negative association was found for cases grouped by ages 35–49 and 50–79, the trend for the latter being significant.

Descriptive studies have shown that the association between higher social class and Hodgkin's disease in young adults is specific for the nodular sclerosis subtype. Henderson *et al.* (1979) computed the incidence rates of Hodgkin's disease of specific histological types in Los Angeles County (United States) in 1972–75 by social class. They reported that the incidence of the nodular sclerosis type was directly related to social class; there was no consistent association for the other histological types. These data were confirmed and extended through 1985 by Cozen *et al.* (1992), who also found

an increase in incidence between 1972 and 1985 only among cases of the nodular sclerosis subtype. They further reported that the risk pattern for Hodgkin's disease of mixed cellularity was quite distinct and negatively associated with social class. This latter finding fits the general observation that cases of Hodgkin's disease occurring in economically developing populations (Mueller, 1987) and among groups of lower social class in developed populations (Hu *et al.*, 1988) are predominantly of the mixed cellularity and lymphocyte depletion subtypes.

These data are consistent with the rates in the United States in 1969–80 (Glaser, 1987): thus, the incidence rates for young adults were positively correlated with community-level indicators of social class and the incidence of the nodular sclerosis subtype increased in parallel with regional indices of social class.

There is a consistent body of evidence that the risk for Hodgkin's disease occurring from early childhood through middle age is associated with factors in the childhood environment that influence the age at infection with a virus such as EBV. The pattern of association with these factors varies with the age at diagnosis. The data have been reviewed (Jarrett *et al.*, 1996; Mueller, 1996).

Because Hodgkin's disease in childhood occurs primarily in developing countries, the children at risk for the disease would appear to be of lower social class and thus to be infected earlier. This has been a consistent finding in the few case–control studies that have been reported (Sobrinho-Simões & Areias, 1978; Gutensohn & Shapiro, 1982; Bogger-Goren *et al.*, 1983). A shift occurs in young adults (generally defined as between 15 and 40 years of age), who have been found quite consistently to be of higher social class than expected, as measured by occupation or educational attainment (Cohen *et al.*, 1964; Gutensohn & Cole, 1977; Abramson *et al.*, 1978; Serraino *et al.*, 1991). In addition, there is an inverse association between risk for Hodgkin's disease and number of siblings and also with birth order (Gutensohn & Cole, 1977, 1981; Bernard *et al.*, 1987; Bonelli *et al.*, 1990). The young adult patients also lived in less crowded conditions during childhood. Among the oldest patients, however, no consistent association with social class was seen (Abramson *et al.*, 1978; Gutensohn, 1982).

As is generally recognized for most haematopoietic malignancies, Jews are at somewhat higher risk for Hodgkin's disease than non-Jews (MacMahon, 1960). In a study of cases occurring in the 1950s in Brooklyn, New York, United States, MacMahon (1957) reported that older, but not younger, Jews were at increased risk. In a population-based case–control study conducted in Boston–Worcester (United States) in the 1970s, however, Jewish people of all ages in the population were at particularly high risk (Gutensohn & Cole, 1981). Bernard *et al.* (1984, 1987) also noted an excess of Jewish cases in their population-based studies in Yorkshire, United Kingdom. Similarly, an increased incidence among Jews was documented in Los Angeles County, United States (Cozen *et al.*, 1992).

In summary, there is evidence that the risk for Hodgkin's disease in young adulthood through middle age is associated with higher education, higher social class, fewer siblings, less crowded housing and early birth rank. All of these factors lead to susceptibility to late infections with the common childhood infections. As in the model of

paralytic polio, such late infections tend to be more severe than those in younger children; however, social class does not appear to predict the occurrence of Hodgkin's disease in the late decades of life, when primary infection with EBV is unlikely.

2.3.2.2 *Association with EBV*

(*a*) *Case reports and case series*

There have been numerous case reports of Hodgkin's disease developing in close association with serologically documented primary infection with EBV (Kaplan, 1980) but with no direct evidence of the presence of the virus in the tumour itself.

(i) *Infectious mononucleosis*

Poppema *et al.* (1985) described a case of mixed cellularity Hodgkin's disease that developed after heterophil-negative infectious mononucleosis. The patient had been followed clinically by multiple node biopsies and serology during this period and was described as having a clinical and serological picture consistent with chronic EBV infection. The investigators demonstrated the presence of EBNA in HRS cells from involved lymph nodes.

(ii) *Serology*

Few data are available on the relationship between EBV serology and EBV status. Ohshima *et al.* (1990) reported a single case of an eight-year-old boy with EBV-positive Hodgkin's disease, as determined by Southern blot and PCR. His titres were 1:1280 anti-VCA IgG, 1:10 anti-VCA IgA, 1:10 anti-VCA IgM, 1:160 anti-EA and 1:40 anti-EBNA, consistent with an active EBV infection. In an overlapping series of 107 cases, Brousset *et al.* (1991) and Delsol *et al.* (1992) concluded that there was no association between the presence of EBV and a serological pattern of reactivation, which they defined as > 1:640 anti-VCA, > 1:40 anti-EA and > 1:160 anti-EBNA; however, only one of 35 EBV-negative and none of 16 EBV-positive cases had this rather extreme pattern. Levine *et al.* (1994) assessed the relationship of the EBV status of 39 cases with serological results published previously. No differences in anti-VCA or anti-EA titres were seen between EBV-positive and EBV-negative cases. When the sera of 19 of these cases were subsequently tested for anti-EBNA-1 and anti-EBNA-2, none of the 5 EBV-positive cases showed elevated titres (> 1:320) of EBNA-1, whereas 5 of 14 EBV-negative cases did so. Conversely, 2 of the 5 positive cases and 2 of the 14 negative cases had elevated titres (> 1:80) of EBNA-2. Neither of these differences was statistically significant (Mueller, 1997).

In preliminary reports from two groups, specimens from 54 patients with Hodgkin's disease who had reported a history of infectious mononucleosis were tested for the presence of the EBERs. They were found in only one (Mueller, 1997).

(iii) *Viral nucleic acid and protein*

With the advent of highly sensitive molecular probes, dozens of case series have now been published on the detection of genomic segments, transcripts and viral products of EBV in a substantial proportion of biopsy samples from patients with Hodgkin's disease.

Many of these reports (restricted to HIV-1-negative cases) are summarized in Table 17. Weiss *et al.* (1987, 1988) and Staal *et al.* (1989) first reported the detection of monoclonal EBV genome in Hodgkin's disease tissue, Staal *et al.* noting that the number of viral episomes per cell was low. Weiss *et al.* (1989a), Uccini *et al.* (1989) and Anagnostopoulos *et al.* (1989) all reported detection of the viral genome within HRS cells (and their variants) themselves. In 1990, Wu *et al.* reported detection of RNA EBER transcripts in HRS cells. Pallesen *et al.* (1991a) and Herbst *et al.* (1991a) reported that the EBV in Hodgkin's disease has a restricted latent phenotype of LMP-1 expression without detectable EBNA-2, as in nasopharyngeal carcinoma. These findings have been replicated in dozens of populations by a large number of laboratories.

EBV nucleic acid has also been found in a fraction of small lymphocytes in both EBV genome-positive and -negative tissue from patients with Hodgkin's disease (Masih *et al.*, 1991; Weiss *et al.*, 1991; Herbst *et al.*, 1992; Khan *et al.*, 1992; Ambinder *et al.*, 1993; Bhagat *et al.*, 1993; Chang *et al.*, 1993b; Jiwa *et al.*, 1993b), and at a low frequency in normal lymph nodes (Niedobitek *et al.*, 1992a).

Consistency of viral marker status of individual patients: EBV status is consistent among anatomically distinct sites involved at diagnosis. Vasef *et al.* (1995) evaluated 14 cases with two to five involved sites for the concordance of EBV status among patients. All of the biopsy samples from eight patients were EBV genome-negative and all those from six patients were EBV genome-positive. In five of the latter cases, the investigators analysed the 3′ end of the *LMP-1* gene at all sites of disease by PCR. In three patients, all of the sites had a 30-base pair deletion; in the other two cases, there was discordance in the presence of this deletion, some sites having the germ-line configuration.

EBV status appears to be stable if not identical over time. Delsol *et al.* (1992) reported that EBV status was consistent in subsequent biopsy samples at relapse (range, 14–126 months) in 12 cases, of which seven were initially EBV-positive. Two EBV-positive cases showed substantial reduction or loss of staining for LMP-1 in their later biopsies. Coates *et al.* (1991a) found that sequential biopsy samples from three EBV-positive patients (age range, 2–10 years) contained EBV at about the same level as in the initial sample. Boiocchi *et al.* (1993a) reported the same clonal EBV genome in each of two patients from whom two or three biopsy samples had been taken over a period of up to 11 months. Brousset *et al.* (1994) evaluated 12 cases of relapsed Hodgkin's disease for consistency of EBV status: five showed no EBV at either initial diagnosis or relapse, while seven that were initially EBV-positive remained positive at relapse. For two of the latter, additional assays — including terminal repeat polymorphism by Southern blot and LMP-1 polymorphism sequencing by PCR — were carried out on tissue taken at both diagnosis and relapse. In one case, the EBV genome appeared to be identical in sequential biopsy samples taken nine years apart. In the other case, a monoclonal episome was detected by Southern blot in the initial sample but not in that taken at relapse eight years later. The authors noted that the latter finding may be due to a low viral load that cannot be detected by Southern blot. Both samples from this patient contained an identical mutation in EBV *LMP-1*.

Table 17. Detection of the EBV genome or gene products in tissues from HIV-1-negative cases of Hodgkin's disease

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Weiss *et al.* (1987, 1988)	21	4	SB, slot–blot	More mixed cellularity than nodular sclerosis types positive; no cytomegalovirus; overlaps with next study
Weiss *et al.* (1989a)	16	3	SB, slot–blot, ISH	
Staal *et al.* (1989)	28	8	SB	More mixed cellularity than nodular sclerosis types positive
Uccini *et al.* (1989)	32	6	SB, ISH	
Boiocchi *et al.* (1989)	17	7	SB	
Herbst *et al.* (1989)	39	5	SB	Overlaps with next study
Anagnostopoulos *et al.* (1989)	42	7	SB, ISH	
Uhara *et al.* (1990)	31	8	PCR, ISH	More mixed cellularity than nodular sclerosis types positive
Bignon *et al.* (1990)	16	8	PCR	Mostly young adults
Wu *et al.* (1990)	8	6	ISH (EBER)	EBV-positive cases from Staal *et al.* (1989)
Herbst *et al.* (1990)	198	114	PCR, ISH	No variation by age or sex
Libetta *et al.* (1990)	34	15	SB	No variation by age
Uccini *et al.* (1990)	20	3	SB, ISH	None contained VCA or EA; all positive cases were of mixed cellularity type
Ohshima *et al.* (1990)	7	2	SB, PCR	Serology available for one case: high titre of anti-VCA and EA IgG, positive for anti-VCA IgM and IgA
Pallesen *et al.* (1991a)	84	40	mAb to LMP-1 and EBNA-2	Positive only for LMP-1; more mixed cellularity than nodular sclerosis types positive
Gledhill *et al.* (1991)	35	11	PCR	8 of 8 analysed were of EBV type 1; no association with histology and little with age

Table 17 (contd)

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Brousset *et al.* (1991)	54	16	ISH ('mRNA')	More mixed cellularity than nodular sclerosis types positive; no correlation with serology (one case seronegative); results later interpreted as detection of EBV DNA (Delsol *et al.*, 1992)
Herbst *et al.* (1991a)	47	32 with DNA 18 with LMP	PCR, mAb to LMP-1, EBNA-2 and gp350/250	All LMP-1-positive positive by PCR; no variation by histology
Knecht *et al.* (1991)	48	38	PCR (semi-quantitative)	About two-thirds of cases had numerous HRS; no association with histology or proportion of HRS
Masih *et al.* (1991)	52	30	PCR, slot–blot, SB	More mixed cellularity than nodular sclerosis types positive, 43% of controls with hyperplastic lymph nodes were EBV-positive; two of six tested were clonal
Pallesen *et al.* (1991c)	96	47	mAb to LMP-1, ZEBRA, EA, VCA, MA	Includes cases from Pallesen *et al.* (1991a); no cytomegalovirus; three positive for LMP-1 also positive for ZEBRA; none positive for EA, VCA or MA
Vestlev *et al.* (1992)	66	27	Follow-up of cases from above	LMP-1-positive cases more likely to be of mixed cellularity type, male, less likely to have mediastinal involvement; LMP-1 status not associated with prognosis
Jarrett *et al.* (1991)	95	43	SB	48 cases selected for age and histological type; includes cases from Gledhill *et al.* (1991); positivity highest among children and older adults; 30 of 30 samples tested were of EBV type 1
Weiss *et al.* (1991)	36	14	PCR, ISH (EBER-1)	More mixed cellularity than nodular sclerosis types positive; some background B and T cells positive

Table 17 (contd)

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Coates *et al.* (1991a)	55	9	ISH	More cases of nodular sclerosis (7/24) than of mixed cellularity (2/16) positive; no difference in mean age of EBV-positive and EBV-negative cases; variation in amount of virus between cases; 3 patients ≤ 10 years with sequential biopsies positive at same level in all specimens; in 8 other cases, EBV found only in non-neoplastic cells
Brocksmith *et al.* (1991)	57	33	SB, PCR	More mixed cellularity than nodular sclerosis types positive; no variation with age
Delsol *et al.* (1992)	107	37	ISH, mAb to LMP-1, EBNA-2	Overlaps with Brousset *et al.* (1991a); more mixed cellularity types positive; all 12 cases tested at diagnosis and relapse remained concordant for EBV; one LMP-1-positive case became LMP-negative and one LMP-1-positive case became intermediate; no correlation with serology or short-term prognosis; all 13 positive cases EBNA-2-negative
Khan *et al.* (1992)	33	12	ISH (EBER)	In six cases, EBER localized to non-neoplastic small lymphocytes only
Herbst *et al.* (1992)	46	26	ISH (EBER-1/2); mAb to LMP-1	18 positive for LMP-1 (all EBER-positive); EBER-positive small lymphocytes found in 39 cases at low levels, 3 at high levels
Fellbaum *et al.* (1992)	187	66	PCR	More mixed cellularity types positive; no association with survival
Murray *et al.* (1992b)	46	22	mAb to LMP-1	Positivity and proportion of LMP-positive HRS cells increased with 'histological grade'
Ambinder *et al.* (1993)	36	20	ISH (EBER-1); mAb to LMP1	All cases < 15 years; 11 of 11 cases from Honduras and 9 of 25 from USA positive; no cytomegalovirus; EBV-positive small lymphocytes also seen in 9 positive cases; more mixed cellularity than nodular sclerosis types positive in US series

Table 17 (contd)

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Boyle *et al.* (1993)	12	3	PCR, ISH	Positivity associated with younger age
Brousset *et al.* (1993)	35	3	ISH (ZEBRA, EBER-1/2), mAb to LMP-1, SB	All patients had relapsed; all consistent in EBV status at sequential biopsies; in 2 cases analysed, EBV appeared to be identical in both samples
Chang *et al.* (1993b)	32	30	ISH (EBER-1), mAb to LMP-1, MA	Patients from Peru; positivity by age: 19 of 19 ≤ 15 years, 5/6 15–39, 6/7 older; some small lymphocytes positive
Carbone *et al.* (1993c)	39	15	ISH, mAb to LMP-1, vimentin	More mixed cellularity than nodular sclerosis types positive; vimentin found in 24 cases localized to HRS cells, including all 11 LMP-1-positive cases
Deacon *et al.* (1993)	23	16	ISH (EBER-1/2), mAb to LMP-1	Transcription analysis demonstrated EBV latency pattern found in nasopharyngeal carcinoma
Jiwa *et al.* (1993b)	33	19	PCR, ISH (EBER-1/2), mAb to LMP, bcl-2, c-myc	Almost all cases nodular sclerosis type; some small lymphocytes also positive; 20 of 29 cases expressed bcl-2 and 30 of 32 expressed c-myc in HRS cells independently of EBV status
Niedobitek *et al.* (1993a)	116	33	ISH (EBER), mAb to p53	More mixed cellularity than nodular sclerosis types positive; 37 positive for p53 in HRS cells independently of histology, less frequent in EBV-positive (21%) than EBV-negative (36%) [$p = 0.12$]
Lin *et al.* (1993a)	23	16	PCR (EBER) (multiple gene loci)	2 of 10 reactive hyperplasia nodes were EBV-positive
Bhagat *et al.* (1993)	11	4	ISH (EBER-1), mAb to bcl-2	Most cases nodular sclerosis: 3 of 7 with t(14;8) and 5 of 6 without t(14;18) were bcl-2-positive; bcl-2 antibody reacted with HRS cells but also in majority of small lymphocytes; EBV-positivity not correlated with presence of t(14;18) or bcl-2

Table 17 (contd)

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Khan *et al.* (1993)	77	25	ISH (EBER), mAb to LMP-1	Cases selected on basis of histology and age; high positivity associated with mixed cellularity type (68%); some increase with age; some small lymphocytes positive; no cytomegalovirus or HHV6
Kanavaros *et al.* (1994)	22	12	ISH (EBER), mAb to LMP-1	Paediatric cases from Greece; more mixed cellularity than nodular sclerosis types positive
Gulley *et al.* (1994)	125	58	SB, ISH (EBER)	79 cases from USA, 31 from Mexico and 15 from Costa Rica; more mixed cellularity than nodular sclerosis types positive; Hispanic ethnicity associated with positivity (RR = 4.3)
Poppema & Visser (1994)	72	19	ISH (EBER-1/2), mAb to LMP-1, HLA-A2	Cases from Canada; more mixed cellularity types positive; no association with HLA-A2
Zarate-Osorno *et al.* (1994)	27	18	ISH (EBER-1)	Cases from Mexico; more mixed cellularity types positive
Quintanilla-Martínez *et al.* (1995)	50	35	mAb to LMP-1	Cases from Mexico; more mixed cellularity than nodular sclerosis types positive
Preciado *et al.* (1995)	41	22	SB; ISH (EBER), mAb to EBNA-2, LMP-1	Paediatric cases from Argentina; more mixed cellularity than nodular sclerosis types and more younger children (< 7) positive
Chan *et al.* (1995b)	23	15	ISH (EBER-1), mAb to BHLF1	Cases from Hong Kong; more mixed cellularity than nodular sclerosis types positive for EBER; none positive for BHLF1; least positivity among persons aged 15–49 years
Li *et al.* (1995b)	40	17	ISH (EBER-1), mAb to LMP-1	Cases from Japan; more mixed cellularity than nodular sclerosis types positive; positive cases more likely to have some positive small lymphocytes in background

Table 17 (contd)

Reference	No. of cases	No. with EBV genome or gene products	Method of detection	Comments
Vasef *et al.* (1995)	14	6	ISH (EBER-1, LMP-1)	Patients had two to five separate involved sites; all biopsy samples consistent for EBV status; three positive cases had identical LMP-1 genes; 2 patients had discordant LMP-1 genes at different sites
Leoncini *et al.* (1996)				
Kenya	92	85	ISH (EBER)	More mixed cellularity types positive in both series; positivity among cases of nodular sclerosis in Kenya higher than that in cases in Italy. In Kenya, more children ≤ 15 years (41 of 42) positive than adults > 15 years (44 of 50)
Italy	65	31		
Weinreb *et al.* (1996a)	101	85	ISH (EBER-1/2), mAb to LMP-1	Cases from Kenya. Positivity varied by age: 100% in 53 paediatric cases, 67% in 48 adult cases
Tomita *et al.* (1996)	50	32	PCR, SB, ISH (EBER)	Cases from Japan; more mixed cellularity than other types, more older (> 40) than younger (< 40 years) and more males positive
Weinreb *et al.* (1996b)	277	196	ISH (EBER-1/2), mAb to LMP-1	Children in 10 countries; positivity 50–100%, more mixed cellularity type and more males positive [HIV status unknown]
Huh *et al.* (1996)	87	60	ISH, ISH (EBER, BHLF), mAb to LMP-1	Cases from Republic of Korea; positivity increased with stage; more mixed cellularity than nodular sclerosis positive; only 1 case positive for *Bam*HI H left-frame transcript

Adapted from Mueller (1996)

HRS, Hodgkin and Reed-Sternberg; ISH, in-situ hybridization (for EBV genome probe, unless otherwise specified); SB, Southern blot; PCR, polymerase chain reaction; LMP-1, latent membrane protein 1; EBNA, EBV nuclear antigen; EBER, EBV-encoded RNA; mAb, monoclonal antibody; EA, early antigen; ZEBRA, Z EBV replication activator; gp350/250, envelope glycoprotein; VCA, viral capsid antigen; MA, membrane antigen

Relationship to clinical features: Few clinical or other molecular features appear to be related to EBV status. O'Grady *et al.* (1994) reported that EBV positivity in stage-I disease is associated with cervical neck node presentation, irrespective of histological subtype. This could be related to primary EBV infection in the oropharynx and would appear to strengthen the association between Hodgkin's disease and previous infectious mononucleosis. Similarly, Kapadia *et al.* (1995) reported a relatively high rate of EBV positivity in 8 of 12 cases of Hodgkin's disease occurring in Waldeyer's tonsillar ring. Primary Hodgkin's disease of the tonsils is rare, however, and recent studies have suggested that HRS cells in cases of Hodgkin's disease arising in association with infectious mononucleosis are predominantly EBV-negative (Mueller, 1997). EBV genome status has not been found to be an independent predictor of prognosis (Delsol *et al.*, 1992; Fellbaum *et al.*, 1992; Vestlev *et al.*, 1992).

Relationship to histology, age and ethnicity: Several factors appear to be predictive of EBV status. Viewing the data on the two most common subtypes overall (Table 17), cases of the mixed cellularity subtype generally occur at higher rates than those of nodular sclerosis. Given the difference in the age and sex distribution of these two subtypes of Hodgkin's disease (Medeiros & Greiner, 1995), the presence of EBV should have a U-shaped relationship with age, with the highest rates at the extremes of age and the lowest in young adulthood. The general consensus is that EBV infection of HRS cells is at best rare in cases of the nodular lymphocyte-predominant type, consistent with the notion that these are an entity distinct from the 'classical' forms of Hodgkin's disease (Pallesen *et al.*, 1991c; Shibata *et al.*, 1991a; Alkan *et al.*, 1995). Few cases of lymphocyte-depletion Hodgkin's disease have been reported in the literature, and their association with EBV is highly variable, consistent with the origin of such cases from Hodgkin's disease of either nodular sclerosis or mixed cellularity type and with the proposed overlap with CD30-positive anaplastic large-cell lymphomas (Zhou *et al.*, 1993; Bai *et al.*, 1994; Quintanilla-Martínez *et al.*, 1995; Herbst *et al.*, 1996a).

In studies of cases in developing countries, where patients generally present with more advanced disease, the rate of positivity is notably high, particularly among children. Gulley *et al.* (1994) analysed 125 cases from Costa Rica, Mexico and the United States by multivariate analysis and found that Hispanic ethnicity *per se* was an independent predictor of the presence of the EBV genome, with a RR of 4.3. Murray *et al.* (1992b) noted that the proportion of HRS cells containing LMP-1 in EBV-positive specimens increased in parallel with a less favourable histological subtype: 41% in one of 12 cases of lymphocyte-predominant disease, 49–78% in 12 of 24 cases of the nodular sclerosis type, 63–81% in six of seven cases of the mixed cellularity type and 89–97% in all three cases of lymphocyte-depletion Hodgkin's disease.

Glaser *et al.* (1997) analysed the data from a number of investigators and previously unpublished data by multivariate analysis, with a total series of 1546 HIV-1-negative cases of Hodgkin's disease, in order to identify characteristics that differentiate EBV-positive and EBV-negative cases. Cases of mixed-cell disease were significantly more likely to contain EBV than those of nodular sclerosis in all age groups: < 15 years, RR, 7.3; 15–49 years, RR, 13; ≥ 50 years, RR, 4.9. Patients under 15 years of age in developing countries were significantly more likely to have EBV than those from more deve-

loped countries [RR, 6.0]; similar results were not found for older individuals. Men aged 15–49 years, but not those in other age groups, were significantly more likely to have tumours containing the EBV genome (RR, 2.5). Overall, it appears that the presence of EBV is related to a less favourable host response.

HIV infection: Essentially all HIV-1-infected patients with Hodgkin's disease have a higher rate of EBV positivity: 8 of 10 (Moran *et al.*, 1992), 11 of 11 (Hamilton-Dutoit *et al.*, 1993b) and 14 of 18 (Tirelli *et al.*, 1995). In general, these patients present with advanced Hodgkin's disease and have a poor prognosis. [HIV-1-infected patients may be at increased risk for Hodgkin's disease (IARC, 1996).]

(*b*) *Case–control studies*

(i) *Infectious mononucleosis*

The association between history of infectious mononucleosis and Hodgkin's disease has been evaluated in several case–control studies (Table 18). A weak positive association is usually seen, particularly in young adults and patients with the nodular sclerosis subtype.

(ii) *Serology*

In case–control studies of more than 2000 patients with Hodgkin's disease of all ages, the proportion who had IgG antibodies to VCA, indicative of prior infection, was similar to that of controls (Table 19); however, the cases consistently had higher mean antibody titres than controls, except in two studies involving paediatric patients (Lange *et al.*, 1978; Shope *et al.*, 1982). Similarly, in most studies, the cases had a higher prevalence of antibodies (as well as higher titres) against the EA complex of EBV, indicative of active viral replication (Table 20). Evans and Gutensohn (1984) found that subjects with a history of infectious mononucleosis had higher GMTs against VCA and against both EA(D) and EA(R) than did subjects who did not have such a history. This was true among both cases as a group and among the sibling controls as a group, although only sera in which antibody was present at the lowest dilution were included in calculating GMTs.

In eight studies, antibodies against EBNA were also evaluated (Table 21). Of these, three showed no higher levels in diagnosed cases than in controls. Lennette *et al.* (1993) and Merk *et al.* (1995) also tested for antibodies against specific components of the EBNAs, using recombinant proteins as targets. In a study of 20 Hodgkin's disease patients and 74 grouped controls (Lennette *et al.*, 1993), the cases had higher (non-significant) GMTs against EBNA-2A and EBNA-3C but not against EBNA-1 or EBNA-2B. In a later study of 61 untreated cases and 109 healthy EBV antibody-positive controls (Merk *et al.*, 1995) tested with the same assays, the cases had elevated titres against EBNA-1, EBNA-2A and EBNA-3C but not against EBNA-2B.

Chen *et al.* (1992b) developed an assay to test for immune response to recombinant full-length LMP-1. They found that 16 of 27 patients with Hodgkin's disease but only two of 26 EBV-seropositive controls and five of 22 patients with nasopharyngeal carcinoma had IgG antibodies against LMP-1. Lennette *et al.* (1995) developed a monoclonal,

Table 18. Results of case–control studies of the association between a history of infectious mononucleosis and the risk for Hodgkin's disease

Reference	Study population	No. of cases	No. of controls	Relative risk	Factors controlled
Henderson *et al.* (1979)	Population-based: Los Angeles County (USA), incident cases, 1972–73; neighbourhood controls	212	212	1.3 Nodular sclerosis, 1.5	Age, ethnicity, sex, neighbourhood
Gutensohn & Cole (1981)	Population-based: Eastern Massachusetts (USA), incident cases, 15–39 years, 1973–77; population controls	225	447	1.8 $p < 0.05$	Age, sex, family size, birth order, housing density in childhood
Gutensohn (1982)	As above, cases 40–54 years; population controls	53	106	1.3	Age, sex, family size, religion
Evans & Gutensohn (1984)	As above, cases 15–54 years; sibling controls	262	250	1.5	Family
Bernard *et al.* (1987)	Population-based: Yorkshire Health District (UK), incident cases, 1979–84; hospital controls [subjects appear to be ≥ 15 years]	248	489	1.0 Males 15–35 years, 4.9 $p = 0.04$	Sex, age, health district
Serraino *et al.* (1991)	Hospital-based: Pordenone (Italy), 1985–90, cases 15–77 years	152	613	8.2 Nodular sclerosis, 13.1 $p < 0.05$	Sex, education

Table 19. Results of case–control studies of the association between antibody titres against EBV capsid antigen and risk for Hodgkin's disease

Reference	Cases				Controls				RR
	No.	Prevalence	GMT	High titre[a]	No.	Prevalence	GMT	High titre[a]	
Goldman & Aisenberg (1970)	57	0.58	–	0.12	54	0.61	–	–	–
Johansson *et al.* (1970)	60	0.95	100	0.47	47	0.89	43	0.17	4.3
Levine *et al.* (1971)	63	–	367	0.35[b]	85	–	91	0.05	11
de Schryver *et al.* (1972)	17	–	60	0.24	63	–	23	0.17	1.5
Henle & Henle (1973b)	489	0.89	105	0.40	294	0.84	55	0.14	4.1
Henderson *et al.* (1973)	142	0.94	92	0.47	142	0.93	53	0.25	2.6
Langenhuysen *et al.* (1974)[c]	25	0.92	1580	0.88[d]	25	0.92	585	0.60	4.9
Hirshaut *et al.* (1974)	51	0.82	141	0.16[d]	45	0.89	–	0.09	1.9
Rocchi *et al.* (1975)	100	0.98	177	0.68	100	0.91	34	0.12	16
Gotlieb-Stematsky *et al.* (1975)	67	0.91	67	0.46	186	0.66	9	0.01	79
Hilgers & Hilgers (1976)	43	0.95	2420	–	43	0.95	401	–	1.9
Hesse *et al.* (1977)[e]	185	0.95	272	0.54	185	0.95	141	0.30	12
Evans *et al.* (1978)	67	1.00	146	0.31[e]	162	–	51	0.04	12
Lange *et al.* (1978)[f]	27	0.63	116[c]	–	71	0.61	101	–	–
ten Napel *et al.* (1980)[c]	15	0.94	3180	–	17	1.00	924	–	–
Mochanko *et al.* (1979)	37	1.00	69	0.27	40	0.83	44	0.07	4.6
Evans *et al.* (1980)	70	0.97	110	0.35[e]	70	0.84	30	0.03	17
Shope *et al.* (1982)[f]	15	0.73	103	0.20	24	0.58	73	0.25	0.8
Evans & Gutensohn (1984)	304	0.86	176	0.39[e]	276	0.87[g]	58	0.14	4.0
Lennette *et al.* (1993)	23	1.00	1359	–	75	1.00	303	–	–

Table 19 (contd)

Reference	Cases				Controls				RR
	No.	Prevalence	GMT	High titre[a]	No.	Prevalence	GMT	High titre[a]	
Merk *et al.* (1995)[c]	61	1.00	–	0.72[h]	109	1.00	–	0.14	16
Lenette *et al.* (1995)	113	–	878	–	30	–	199	–	–

From Evans and Gutensohn (1984), except for the last three references
RR, relative risk; GMT, geometric mean titre
[a] ≥ 1:160, unless otherwise noted
[b] > 1:640
[c] Sera collected before initiation of treatment; all other studies involve treated patients
[d] ≥ 1:640
[e] ≥ 1:320
[f] Children only
[g] Siblings of cases
[h] ≥ 1:1280

enhanced, indirect immunofluorescence assay for detecting antibodies against LMP 2A/2B. None of 113 Hodgkin's disease patients but 64% of patients with nasopharyngeal carcinoma had these antibodies.

Table 20. Results of case–control studies of the association between prevalence of antibodies against the early antigen complex of the EBV and risk for Hodgkin's disease

Reference	Cases				Controls				RR
	No.	Prevalence[a]	High titre[b]	GMT	No.	Prevalence	High titre	GMT	
de Schryver *et al.* (1972)	15	0.27	0.07	–	0	–	–	–	–
Henle & Henle (1973b)	458	0.30[c]	–	30	1718	0.03[c]	–	20	14
Rocchi *et al.* (1975)	100	0.50[c]	0.28	34	100	0.02	0	5	49
Gotlieb-Stematsky *et al.* (1975)	63	0.27	–	–	101	0	–	–	∞
Hilgers & Hilgers (1976)	43	0.91	–	52	43	0.81	–	9	2.4
Hesse *et al.* (1977)	176	0.84	0.26[d]	63	176	0.82	0.27	55	1.2
Evans *et al.* (1978)	42	0.21	–	–	11	0.09	–	–	2.7
Lange *et al.* (1978)[e,f]	17	0.53[c]	–	–	43	0.09	–	–	11
Evans & Gutensohn (1984)[g]	304	0.46	0.30[c]	13	276[h]	0.23	0.11	8	2.8
Lennette *et al.* (1993)[g]	23	0.39	–	20	75	0.04	–	5	15
Merk *et al.* (1995)[e]	61	0.25	0.11[i]	–	109	0.21	0.09	–	1.3

From Evans and Gutensohn (1984), except the last two references
RR, relative risk; GMT, geometric mean titre
[a] ≥ 1:5
[b] ≥ 1:40
[c] ≥ 1:10
[d] ≥ 1:160
[e] Sera collected before initiation of treatment
[f] Children only
[g] EA(D) only
[h] Siblings of cases
[i] ≥ 1:40

Table 21. Results of case–control studies of the association between proportion of high titres against the nuclear antigen complex of the EBV and risk for Hodgkin's disease

Reference	Cases			Controls			RR
	No.	High titre (%)	GMT	No.	High titre (%)	GMT	
Rocchi *et al.* (1975)	100	34[a]	18.9	100	0	6.2	∞
Hilgers & Hilgers (1976)	20[b]	–	186.0	20	–	169.0	–
	23[c]	–	128.0	23	–	198.0	–
Lange *et al.* (1978)[d]	28	–	13.0[e]	43[f]	–	42.8	–
Mochanko *et al.* (1979)	37	49[g]	58.2	40	14	36.2	[1.8]
Shope *et al.* (1982)[d]	15	13[g]	–	24	0	–	∞
Wutzler *et al.* (1983)	57	–	8.0	57	–	7.0	–
Lennette *et al.* (1993)	20	–	197.0	73	–	245.0	–
Merk *et al.* (1995)[h]	61	26[i]	–	109	17	–	[1.7]

Anticomplement immunofluorescence test (Reedman & Klein, 1973) used in all studies; numbers in square brackets were calculated by the Working Group.
[a] ≥ 1:40
[b] Mixed cellularity type
[c] Nodular sclerosis type
[d] Children only
[e] Before therapy
[f] Children and adults
[g] ≥ 1:80
[h] Untreated cases
[i] ≥ 1:1280

(c) *Cohort studies*

(i) *Infectious mononucleosis*

The risk for Hodgkin's disease after a diagnosis of infectious mononucleosis has been evaluated in six cohort studies (Table 22) involving nearly 42 000 young adults with serologically confirmed infectious mononucleosis. The expected number of cases of Hodgkin's disease was based on data for the general population in each study. Overall, there was about a threefold excess of Hodgkin's disease.

Miller and Beebe (1973) identified 2437 men in whom infectious mononucleosis had been diagnosed in 1944 while they were in the service of the United States Army. This cohort was traced for death through 1965. Diagnoses of Hodgkin's disease were based on information on death certificates. The two cases identified both died with Hodgkin's disease 20 years after the diagnosis of infectious mononucleosis.

Table 22. Results of cohort studies of persons with a history of infectious mononucleosis (IM) and subsequent Hodgkin's disease (HD)

Reference	Study population	No. of cases of IM	Follow-up period	No. of cases of HD observed	No. of cases of HD expected[a]	RR
Miller & Beebe (1973)	US Army during Second World War	2 437	1946–65	2	[1]	[2.0]
Connelly & Christine (1974)	Connecticut, USA	4 529	1948–68	5	[1]	[5.0]
Rosdahl *et al.* (1974)	Denmark	17 073	1940–70	17	6	2.8
Carter *et al.* (1977)	University students, USA	2 282	1949–74	3[b]	1.3	[2.3]
Muñoz *et al.* (1978)	Scotland and Sweden	9 454	1959–73	7	1.8	3.9
Kvåle *et al.* (1979)	Norway	5 840	1961–75	3[b]	[1]	[3.0]

RR, relative risk; numbers in square brackets calculated by the Working Group

[a] Expected number based on data for general population, except when taken from original paper

[b] After 12 months

Connelly and Christine (1974) identified 4529 residents of Connecticut (United States) in whom infectious mononucleosis was diagnosed between 1948 and 1964, from the records of the State Department of Health. This cohort was followed through 1968, with matching to the Connecticut Tumor Registry. Five cases (four female, one male) of Hodgkin's disease were diagnosed 3–10 years (median, 8 years) after the diagnosis of infectious mononucleosis.

Rosdahl *et al.* (1974) identified 17 073 Danes in whom infectious mononucleosis had been diagnosed at the Statens Seruminstitut in Copenhagen between 1940 and 1969. The records of the Danish Cancer Registry were screened for cancers diagnosed after an interval of 12 months to mid-1970. Seventeen of these people (one woman and 16 men) subsequently developed Hodgkin's disease between one and seven years (median, three years) after the diagnosis of infectious mononucleosis.

Carter *et al.* (1977) identified 2282 former university students in the United States in whom infectious mononucleosis was diagnosed between 1949 and 1969. Of these, three men had a subsequent diagnosis of Hodgkin's disease three, four and seven years after their infectious mononucleosis.

Muñoz *et al.* (1978) identified a cohort of 9454 persons in whom infectious mononucleosis had been diagnosed from laboratory records in either the city hospital in Aberdeen, Scotland, in 1959–1971 or at four public health laboratories in Sweden in 1952–70. The cohort was matched against local cancer registries for cancer occurring two years later than the diagnosis of mononucleosis. The seven cases (five female, two

male) of Hodgkin's disease were diagnosed 2–12 years (median, 2 years) after infectious mononucleosis.

Kvåle *et al.* (1979) identified 5840 persons with infectious mononucleosis diagnosed in nine laboratories in Norway in 1961–72 and matched them against the records of the Cancer Registry of Norway. Three male cases were diagnosed at least 12 months (one, two and four years) after diagnosis of mononucleosis.

(ii) *Serology*

Two case–control studies nested within cohorts included reports on EBV serology preceding a diagnosis of Hodgkin's disease. The first, by Mueller *et al.* (1989), consolidated the resources of five serum banks containing specimens from over 240 000 persons and identified 43 patients from whom blood had been drawn and stored for an average of 50.5 months before diagnosis of Hodgkin's disease. These were compared with 96 matched controls from whom blood had been drawn at the same time. All of the blood specimens were assayed for IgG, IgA and IgM antibodies against VCA and IgG antibodies against EA(D) and EA(R) and EBNA. As had been reported in the case–control studies, the cases had elevated IgG titres against VCA (RR, 2.6; 90% CI, 1.1–6.1), EA(D) (RR, 2.6; 90% CI, 1.1–6.1) and EA(R) (RR, 1.9; 90% CI, 0.90-4.0), with adjustment for IgM antibodies. In addition, a greater proportion of the cases than controls had elevated titres of IgA against VCA (RR, 3.7; 90% CI, 1.4–9.3; adjusted for IgM), and substantially fewer had IgM antibodies against VCA (RR, 0.22; 90% CI, 0.04–1.3). When all of the antibodies were controlled for simultaneously, the most significant findings were the prevalence of high titres of EBNA (RR, 6.7; 1.8–25) and an inverse association for IgM antibodies (RR, 0.07; 90% CI, 0.01–0.53). This finding of altered EBV antibody patterns before the diagnosis of Hodgkin's disease was generally stronger in blood specimens drawn at least three years before diagnosis than in those tested closer to the time of diagnosis and did not vary appreciably between the sexes, by age or by histological type.

Lehtinen *et al.* (1993) conducted a similar study in a cohort of 39 000 healthy Finnish adults who were followed-up for 12 years. Of these, six were subsequently found to have Hodgkin's disease. Although the data were not shown, the authors reported that the risk for Hodgkin's disease was associated with increased antibody responses to EBNA and to EA, consistent with the results of the previous study.

2.4 Nasopharyngeal carcinoma

2.4.1 *Clinical features and histopathology*

2.4.1.1 *Clinical features*

Nasopharyngeal carcinoma commonly arises from the fossa of Rosenmüller, a region of the nasopharynx rich in lymphoreticular tissue, and eustachian cushions, regardless of race or geographical area. Nasopharyngeal carcinoma may also arise in the roof of the nasopharynx and very rarely in the anterior and inferior walls of the nasopharynx (Simons & Shanmugaratnam, 1982).

Clinically, nasopharyngeal carcinoma may metastasize, particularly to cervical lymph nodes, and as a result is frequently identified only after dissemination. In several series, more than 50% of patients presented with cervical lymph node metastases (Ho, 1971; Sugano *et al.*, 1978; Levine & Connelly, 1985; Pathmanathan, 1997). The jugulodigastric lymph nodes are the most commonly affected cervical nodes (Stanley, 1997) and are found to be enlarged in 50–70% of patients. The presenting symptoms may thus be bilateral cervical node enlargement and also blood-stained nasal discharge, nasal obstruction, blood-stained sputum, tinnitus and hearing loss due to tumour involvement of the eustachian tube. Cranial nerves may be involved singly or in different combinations early or late in the disease; however, cranial nerve palsies, particularly involving cranial nerves III, IV, V and VI, IX and X are often seen. Intracranial extension of the tumour into the base of the skull may give rise to frequent headaches, which are occasionally unilateral, central or retro-orbital in distribution. The neurological signs and symptoms reflect the severity and extent of tumour invasion.

2.4.1.2 *Histopathology*

The microscopic appearance of nasopharyngeal carcinoma or of a metastatic nasopharyngeal carcinoma in lymph node material may be diagnostic and provide confirmation of a clinical diagnosis. When a biopsy sample from an early lesion shows no carcinoma cells, particularly in a patient with suggestive serological results (see below), additional curettings of the nasopharynx should be done and the tissue examined.

The WHO histopathological classification of nasopharyngeal carcinoma (Shanmugaratnam & Sobin, 1991) has been adopted by several investigators. It comprises (1) keratinizing squamous-cell carcinoma, (2) differentiated non-keratinizing carcinoma and (3) undifferentiated carcinoma. Keratinizing squamous-cell carcinomas are further divided into well, moderately or poorly differentiated squamous-cell carcinomas, depending on the presence of intercellular bridges and/or evidence of keratinization (pearl formation). Differentiated non-keratinizing carcinomas have organized cellular patterns but show no evidence of keratinization. The cells have vesicular nuclei with prominent nucleoli, well-defined cell margins, and show a stratified or pavemented pattern. A plexiform pattern may be seen, but the syncytial pattern of undifferentiated carcinomas is not apparent. Undifferentiated carcinomas include lymphoepitheliomas. They are composed of malignant epithelial cells arranged in a syncytial fashion with heavy infiltration of non-malignant lymphocytes. The malignant epithelial cells have vesicular nuclei and prominent nucleoli, and the cell margins are indistinct, consistent with the types described by Schminke (1921) and Regaud (1921). Occasionally, spindle-shaped tumour cells with hyperchromatic nuclei may be present.

2.4.2 *Epidemiology*

Nasopharyngeal carcinoma is a disease with a remarkable racial and geographical distribution. It constitutes 75–95% of all malignant tumours occurring in the nasopharynx in low-risk populations and virtually all of those in high-risk populations (Ho, 1971; Sugano *et al.*, 1978; Levine & Connelly, 1985).

2.4.2.1 *Descriptive epidemiology*

(*a*) *International patterns*

Nasopharyngeal carcinoma is a rare malignancy in most parts of the world, where the age-standardized incidence rate for people of either sex is generally less than 1 per 100 000 persons per year (Parkin *et al.*, 1997). Table 23 lists the handful of populations that are known to deviate from this low-risk pattern, together with the age-standardized (world population) incidence rates for men and women separately. The overall incidence of nasopharyngeal carcinoma is elevated in China, with substantial variation between regions. In general, the incidence increases from the north to the south. Whereas the rates in Chinese men in the northernmost provinces are about 2–3/100 000 person-years, those residing in the southernmost province of Guangdong have rates of 25–40/100 000 person-years (National Cancer Control Office, Nanjing Institute of Geography, 1979; Yu *et al.*, 1981; Parkin *et al.*, 1997). High rates approaching those observed in southern China are seen among Inuits and other natives of the Arctic region (Albeck *et al.*, 1992; Nutting *et al.*, 1993). Intermediate to moderately high rates of nasopharyngeal carcinoma (3–10/100 000 person-years) are seen among many indigenous peoples of Southeast Asia, including Thais, Vietnamese, Malays and Filipinos (Parkin *et al.*, 1997). In Sabah, Malaysia, rates similar to those observed among the Inuits have been reported for the native Kadazans (Rothwell, 1979). Intermediate to moderately high rates of nasopharyngeal carcinoma are also observed among Arabs in Kuwait and Algeria (Parkin *et al.*, 1997). Reviews of hospital series indicate that the rates of nasopharyngeal carcinoma are increased in the mainly Arab populations of Tunisia, Morocco, the Sudan and Saudi Arabia (Muir, 1971; Cammoun *et al.*, 1974; Hidayatalla *et al.*, 1983; Al-Idrissi, 1990). Migrants to Israel from North Africa (Morocco, Algeria and Tunisia) have intermediate rates of nasopharyngeal carcinoma which persist in their offspring born in Israel (Parkin & Iscovich, 1997).

The proportions of the histological types of nasopharyngeal carcinoma appear to vary with geographical location and race. In the low-risk areas of Japan, type-1 nasopharyngeal carcinoma accounts for 12% of cases, whereas type 3 predominates in high-risk areas of Taiwan (Sugano *et al.*, 1978). In sub-Saharan Africa, which is a relatively low-risk area for nasopharyngeal carcinoma, type-3 nasopharyngeal carcinoma is also the predominant histopathological type (Cammoun *et al.*, 1974). Further details on the pathology of nasopharyngeal carcinomas are given in the proceedings edited by Muir and Shanmugaratnam (1967) and de Thé and Ito (1978).

(*b*) *Migration*

Most Chinese living outside China originate from the provinces of Guangdong and Fujian in the south-east (Ho, 1959), a region with a high incidence of nasopharyngeal carcinoma (National Cancer Control Office, Nanjing Institute of Geography, 1979). Southern Chinese migrants, irrespective of the country to which they migrate, continue to have high rates of nasopharyngeal carcinoma (Worth & Valentine, 1967; King & Haenszel, 1973; Armstrong *et al.*, 1979; Gallagher & Elwood, 1979; Lee *et al.*, 1988;

Table 23. Populations at increased risk for nasopharyngeal cancer

Population	Age-standardized (world) incidence[a]		Reference
	Male	Female	
Chinese, Hong Kong	24.3	9.5	Parkin *et al.* (1997)
Chinese, Taipei	8.1	3.2	Chen *et al.* (1988a)
Chinese, Shanghai	4.5	1.8	Parkin *et al.* (1997)
Chinese, Tianjin	1.8	0.6	Parkin *et al*. (1997)
Inuits, Greenland	12.7	9.2	Albeck *et al.* (1992)
Inuits, Athabascans, Aleuts, Alaska	11.9	5.6	Nutting *et al.* (1993)
Thais, Chiang Mai	2.6	1.5	Parkin *et al.* (1997)
Vietnamese, Hanoi	10.3	4.8	Parkin *et al.* (1997)
Malays, Singapore	6.5	2.0	Parkin *et al.* (1997)
Filipinos, Manila	7.6	3.7	Parkin *et al.* (1997)
Kadazans, Sabah	15.9	8.7	Rothwell (1979)
Kuwaitis, Kuwait	2.3	0.6	Parkin *et al.* (1997)
Algerians, Sétif	8.0	2.7	Parkin *et al.* (1997)
Israeli Jews born in Morocco, Algeria or Tunisia	2.8	1.3	Steinitz *et al.* (1989)

[a] Per 100 000 person-years

Parkin *et al.*, 1997), although succeeding generations of such immigrants in countries such as the United States (King & Haenszel, 1973; Buell, 1974; Yu *et al.*, 1981) and Australia (Worth & Valentine, 1967) show continually declining rates. In contrast, Singapore-born Chinese do not have lower rates of nasopharyngeal carcinoma than Chinese born in China (Lee *et al.*, 1988), perhaps because Chinese in Southeast Asia generally adhere to their traditional culture and customs while those in western countries gradually adopt the occidental way of life.

There have been reports that low-risk racial groups born and raised in high-risk areas have an increased risk for nasopharyngeal carcinoma. Buell (1973) reviewed deaths due to nasopharyngeal carcinoma in California, United States, and observed that white men born in the Philippine Islands or China had a higher risk than other white men, and Jews in Israel who were born in North Africa had higher rates of nasopharyngeal carcinoma than other Jews (Steinitz *et al.*, 1989), the increase persisting in their children (Parkin & Iscovich, 1997). Men of French origin who were born in North Africa had a significantly higher rate of nasopharyngeal carcinoma than French men born in France (Jeannel *et al.*, 1993).

(*c*) *Sex and age*

In virtually all of the populations studied, the rates are higher among males than females. In most populations, the male:female ratio is 2–3:1 (Parkin *et al.*, 1997). The

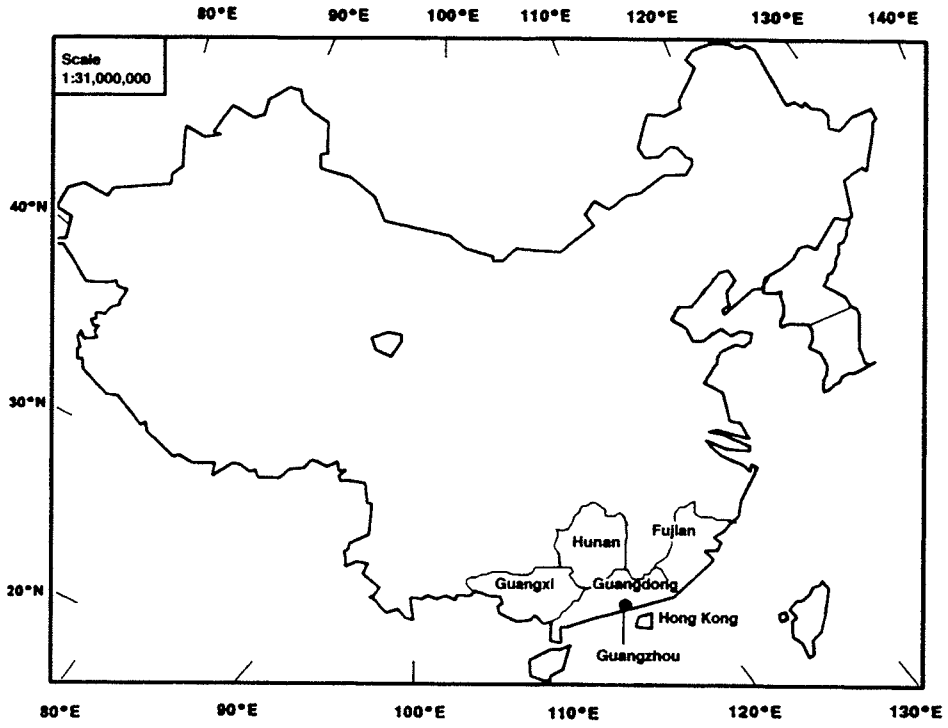
age distribution, however, shows interesting differences by population. In high-risk southern Chinese, the incidence in people of each sex increases steadily with age, reaching a peak at 45–54, and shows a definite decline at older ages (Armstrong *et al.*, 1979; Yu *et al.*, 1981; Parkin *et al.*, 1997). The rates in China as a whole show no such decline after the age of 55 years; and the increase continues to at least 70–74 (National Cancer Control Office, Nanjing Institute of Geography, 1979). The distribution of age-specific rates in the low-risk populations studied, at least after age 20 years, is similar to the distribution of the overall rates in China (Balakrishnan, 1975; Burt *et al.*, 1992; Parkin *et al.*, 1997).

A peak in incidence is observed in adolescents of each sex in a number of populations at low to moderate risk for nasopharyngeal carcinoma. In the United States, a minor peak in the age group 10–19 years is seen in blacks (Burt *et al.*, 1992; Parkin *et al.*, 1997). In Sabah, Malaysia, Kadazan men have a secondary peak between the ages of 15 and 24 years (no data were available for Kadazan women; Rothwell, 1979). In India, the age distribution of 666 consecutive cases of nasopharyngeal carcinoma showed a peak at age 13–22 years, regardless of sex (Balakrishnan, 1975). When Balakrishnan (1975) pooled the incidence rates of nasopharyngeal cancer in 48 population groups published by Doll *et al.* (1970), he found a definite mode in young people of each sex between the ages of 10 and 19 years, which was not seen when similarly pooled data for cancers of the nose and nasal sinuses were plotted against age.

(*d*) *Race and ethnicity*

The high risk for nasopharyngeal carcinoma among Chinese is mainly confined to those residing in the southern provinces of Guangdong, Guangxi, Hunan and Fujian (see Figure 9; National Cancer Control Office, Nanjing Institute of Geography, 1979). The several distinct racial and ethnic groups that reside in this high-risk region have different rates of nasopharyngeal carcinoma. The highest rates are observed among the Tankas, a sub-ethnic group of Cantonese who inhabit the Pearl River Delta basin in central Guangdong. One feature that distinguishes the Tankas from other Cantonese is that they are seafaring people (either fishermen or sea transporters), who live on houseboats moored along the banks of the many branches of the Pearl River. The rates of nasopharyngeal carcinoma among the Tankas are twice those among land-dwelling Cantonese (Ho, 1978; Li *et al.*, 1985). In turn, the land-dwelling Cantonese (who comprise 98–99% of Cantonese) have a twofold higher rate of nasopharyngeal carcinoma than the Hakka and Chiu-Chau dialect groups, who live in north-east Guangdong (Yu *et al.*, 1981; Li *et al.*, 1985). The people of Fujian Province are culturally similar to the Chiu-Chau people in Guangdong Province, and so are their rates of nasopharyngeal carcinoma (National Cancer Control Office, 1980). It is interesting to note that the Hakkas (who rarely intermarry with other dialect groups) originated from northern China more than 500 years ago (Ho, 1959), but their rates resemble those of their Chiu-Chau neighbours and not those of their low-risk ancestors in the north. Even after they migrate to other parts of Southeast Asia, the Cantonese continue to have a twofold higher risk for nasopharyngeal carcinoma than the Hakkas, Chiu-Chaus and Fujianese (Armstrong *et al.*, 1979; Lee *et al.*, 1988).

Figure 9. Map of China, showing provinces where the risk for nasopharyngeal carcinoma is high



From National Cancer Control Office, Nanjing Institute of Geography (1979)

Two distinct racial groups inhabit the Autonomous Region of Guangxi. While the Zhuang people in western Guangxi have a rate of nasopharyngeal carcinoma that is one-fifth of that in Cantonese, the Han (the predominant race in China) people of eastern Guangxi, who are ethnically close to the Cantonese in Guangdong, have similar rates. The areas of Hunan Province that border both Guangxi and Guangdong to the north, not surprisingly have high rates of nasopharyngeal carcinoma. In addition, the Tujia and Miao minorities who inhabit the mountainous region of western Hunan have rates of nasopharyngeal carcinoma that approach those of the Cantonese (National Cancer Control Office, 1980).

In summary, the geographical variation in the incidence of nasopharyngeal carcinoma within southern China closely parallels the distribution of racial and ethnic groups inhabiting the region. The relatively high rates observed among the Hakkas who originated from low-risk northern China argues against genetic predisposition as a major cause of the varying risk patterns among these population groups. As these ethnic groups differ, however, in their customs and food habits, environmental factors inherent in their cultures may be responsible for the differences in susceptibility to nasopharyngeal carcinoma.

In Malaysia and Singapore, large numbers of Chinese and Indians live alongside the native Malays. As mentioned earlier, the Chinese and Malays have high rates; however, Indians in Southeast Asia have very low rates of nasopharyngeal carcinoma, comparable to those seen in whites in the United States (Armstrong *et al.*, 1979; Parkin *et al.*, 1997).

Different rates of nasopharyngeal carcinoma have also been reported among different ethnic groups inhabiting different parts of sub-Saharan Africa (Clifford, 1965; Schmauz & Templeton, 1972; Hidayatalla *et al.*, 1983).

(*e*) *Socioeconomic status*

Among the high-risk southern Chinese, individuals in the lower social strata have higher rates of nasopharyngeal carcinoma than those in the higher social strata (Geser *et al.*, 1978; Armstrong *et al.*, 1978; Yu *et al.*, 1981). Similar information for low-risk populations is scanty. Among white men living in rural or semi-rural counties in the United States, an inverse association is seen between years of schooling and the rate of mortality from nasopharyngeal carcinoma, while no clear trend is apparent among those living in predominantly urban counties (Hoover *et al.*, 1975).

(*f*) *Urbanization*

No difference in the risk for nasopharyngeal carcinoma has been noted between urban and rural residents in south China, or among Chinese in the Malaysian state of Selangor (Armstrong *et al.*, 1979). In metropolitan Hong Kong, local-born Cantonese have rates similar to those of people born and raised in the rural regions of Guangdong Province (Yu *et al.*, 1981). Urban residents in some low-risk populations, however, seem to have higher rates of nasopharyngeal carcinoma than their rural counterparts. In the United States, the mortality rate from this cancer in counties that are 100% urban is about twice that in counties that are 100% rural (Hoover *et al.*, 1975). In a comparison of the rates in urban and rural residents in 11 populations in Australia, Europe and Japan, seven populations had similar rates, while the urban rates were 1.3–2 times higher than those in rural areas in four populations (Muir *et al.*, 1987).

(*g*) *Time trends*

Early records showed that nasopharyngeal carcinoma was common among southern Chinese well over 50 years ago (Ho, 1978). Examinations of cancer registries in Malaysia between 1968 and 1977 and in Singapore between 1968 and 1987 showed little change in incidence in these southern Chinese populations (Armstrong *et al.*, 1979; Lee *et al.*, 1988, 1992). Similar data in Hong Kong, however, indicated a monotonic decline in the rates of nasopharyngeal carcinoma in people of each sex between 1974 and 1992 (the latest year for which complete incidence data were available). The male and female rates in 1974–77 were 32.9 and 14.4, respectively, while the corresponding figures in 1988–92 were 24.3 and 9.5, respectively (see Table 24). This decrease in the incidence of nasopharyngeal carcinoma in Hong Kong Chinese parallels a reduction in the use of Chinese-style salted fish (a human carcinogen, see IARC, 1993, and section 2.4.3.1(*a*)) since the mid- to late 1940s, as Hong Kong developed economically (Geser *et al.*, 1978; Yu *et al.*, 1986).

Data from the Connecticut Tumor Registry (United States) between 1935 and 1974 show relatively stable rates over the 40-year period (Levine *et al.*, 1980b). Similarly, an analysis of data collected from the nationwide Surveillance, Epidemiology and End

Table 24. Time trends in average annual incidence rates of nasopharyngeal carcinoma (per 100 000) in Hong Kong

Period	Average annual incidence	
	Males	Females
1974–77	32.9	14.4
1978–82	30.0	12.9
1983–87	28.5	11.2
1988–92	24.3	9.5

Based on data from *Cancer Incidence in Five Continents* (Waterhouse *et al.*, 1982; Muir *et al.*, 1987; Parkin *et al.*, 1992, 1997)

Results programme in the United States during 1973–86 showed no evidence of a change in incidence rates during the 14-year period (Burt *et al.*, 1992). A review of data on Canadian Inuits over a 25-year period (1949–74) also indicated no appreciable variation in rates over time (Schaefer *et al.*, 1975). In contrast, the rates for Chinese Americans have been declining steadily since 1950, such that the mortality rate from this cancer in men was halved between 1950–54 and 1970–79, from 12 to 6 per 100 000 person-years (Fraumeni & Mason, 1974; Levine *et al.*, 1987). This trend is likely to be the result of increased representation of local-born Chinese in the age groups at high risk for nasopharyngeal carcinoma as this lower-risk population ages, and increased migration from Taiwan and other intermediate- to low-risk regions in China since the 1950s. Prior to that time, virtually all Chinese Americans originated from Guangdong Province (Chinn *et al.*, 1969; Lai, 1988).

(*h*) *Correlation with age-specific prevalence of EBV infection*

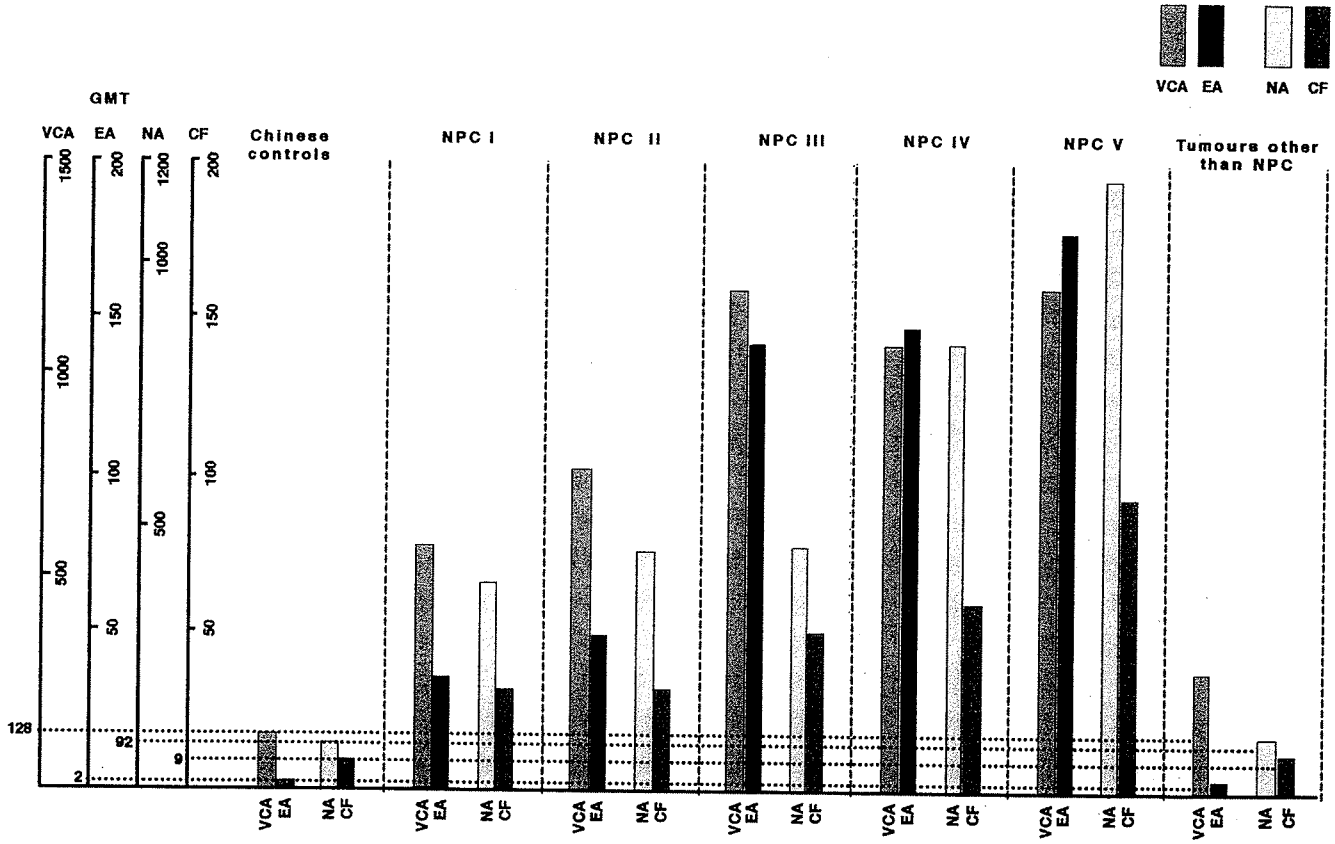
Throughout China, there is little variation in the prevalence of infection or the age at primary infection with EBV (Zeng, 1985), although a more than 20-fold difference in risk exists within the country. Virtually all Chinese children are infected by the age of three to five, and no difference in serological profile has been observed between Chinese populations with drastically different rates of incidence of nasopharyngeal carcinoma (Gu & Zeng, 1978; Zeng, 1985). de Thé *et al.* (1975) compared the age-specific prevalence of antibodies to EBV VCA in three populations at low risk for nasopharyngeal carcinoma (Singapore Indian, Ugandan and French Caucasian) with that in Singapore Chinese, who have the highest incidence of nasopharyngeal carcinoma in the world (Parkin *et al.*, 1997). In comparison with Singapore Chinese, the age at primary infection with EBV is earlier in Ugandans and Singapore Indians and later in French Caucasians; yet the high risk for nasopharyngeal carcinoma is unique to Singapore Chinese.

2.4.2.2 *Case series*

(*a*) *Antibodies in sera and throat washings*

The association between nasopharyngeal carcinoma of the undifferentiated type and EBV was first revealed by Old *et al.* (1966). As seen in Figure 10, titres of IgG antibodies to VCA, EA and soluble complement-fixing antigens increase with clinical stage of disease (Henle *et al.*, 1970; de Thé *et al.*, 1975). IgA antibodies to VCA and EA were later found to be an outstanding feature of nasopharyngeal carcinoma (Wara *et al.*, 1975; Henle & Henle, 1976b; Ho *et al.*, 1976; Desgranges *et al.*, 1977; Pearson *et al.*, 1978b).

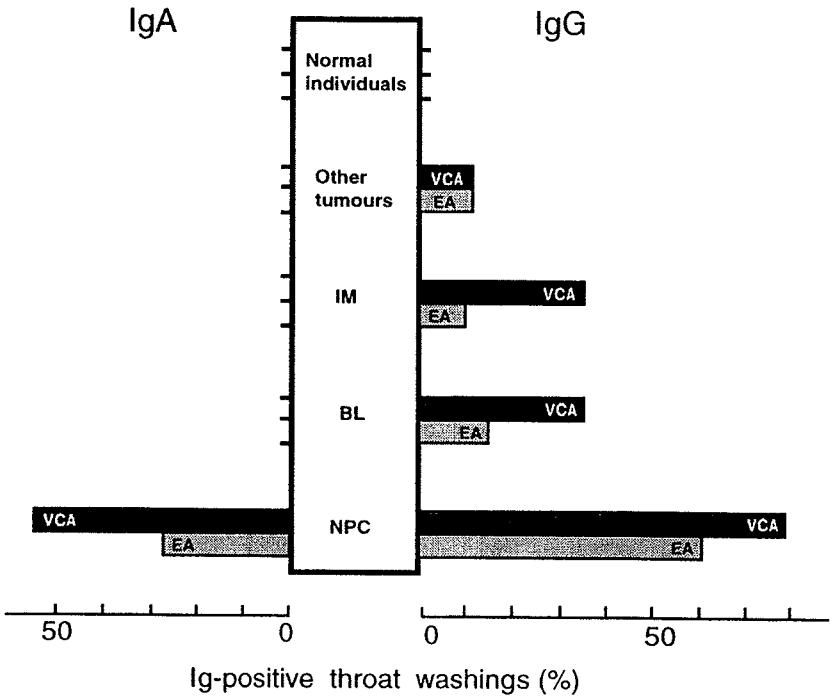
Figure 10. Titres of IgG antibodies to EBV in Chinese nasopharyngeal carcinoma (NPC) patients at different stages of the disease and, for comparison, in normal Chinese controls and Chinese patients with other tumours



From de Thé *et al.* (1975b)
GMT, geometric mean titre; VCA, viral capsid antigen; EA, early antigen; NA, nuclear antigen; CF, complement-fixing antigens

Figure 11 shows the prevalence of IgG and IgA antibodies to VCA and EA in throat washings from patients with nasopharyngeal carcinoma and with other cancers. The titres of both complement fixing antibodies to a soluble EBNA (Sohier & de Thé, 1971) and antibodies to EBNA have been found to be elevated in nasopharyngeal carcinoma patients (de Thé *et al.*, 1973; Baskies *et al.*, 1979).

Figure 11. Titres of IgA and IgG antibodies to EBV in throat washings from patients with nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), infectious mononucleosis (IM) or other tumours and from normal individuals



From Desgranges and de Thé (1978)
VCA, viral capsid antigen; EA, early antigen

Several studies have indicated that the EBV serological profile also differs between patients with different histological types of nasopharyngeal carcinoma. In one of the first studies of these histological subsets, patients with squamous-cell carcinoma were found to have lower EBNA titres but similar titres of IgG to VCA and EA (Shanmugaratnam *et al.*, 1979). Krueger *et al.* (1981) showed elevated titres of IgA to VCA and of IgG to EA and VCA in patients with type-2 or type-3 nasopharyngeal carcinoma, while two patients with type-1 squamous-cell carcinomas had normal titres of IgG to VCA. In a study of Malaysian patients of Chinese, Malay and other ethnic origins, patients with any form of nasopharyngeal carcinoma had elevated IgA titres to VCA (Sam *et al.*, 1989). EBV serology is useful in the identification of patients with occult nasopharyngeal carcinoma (Ho *et al.*, 1976), as shown by elevated titres of anti-IgA VCA; however, histopathological characterization of the tumour is essential for diagnosis, prognosis and treatment.

(*b*) *Nucleic acid markers in carcinoma cells*

Nearly all cases of types-2 and -3 nasopharyngeal carcinoma had detectable EBV DNA sequences, as ascertained by DNA/DNA or c-RNA/DNA hybridization and DNA/-DNA reassociation kinetics in biopsy samples (zur Hausen *et al.*, 1970; Wolf *et al.*,

1973; Nonoyama & Pagano, 1973; Desgranges *et al.*, 1975; Pagano *et al.*, 1975). EBV EBNA was detected in touch smears of nasopharyngeal carcinomas (de Thé *et al.*, 1973; Huang *et al.*, 1974; Klein *et al.*, 1974; Zeng *et al.*, 1981).

Early studies by hybridization kinetics analysis revealed that the viral DNA found in Burkitt's lymphoma is homologous to that in nasopharyngeal carcinomas and that the latter have a relatively high copy number of the EBV genome (zur Hausen *et al.*, 1970; Nonoyama & Pagano, 1973). In another study, viral DNA and EBNA were detected in malignant epithelial cells rather than in the abundant infiltrating lymphoid cells (Wolf *et al.*, 1973). All samples of type-3 nasopharyngeal carcinoma from endemic areas and from areas of intermediate or low incidence contained EBV DNA (Desgranges *et al.*, 1975; Pagano *et al.*, 1975; Raab-Traub *et al.*, 1987). The detection of EBV DNA and EBERs has been useful in identifying carcinomas that have metastasized to lymph nodes when the primary tumour has not been identified (Ohshima *et al.*, 1991; Chao *et al.*, 1996). Type-1 nasopharyngeal carcinoma has been associated with a low copy number of EBV (Raab-Traub *et al.*, 1987): in some studies, EBV was not found in more than half of the cases of type 1 nasopharyngeal carcinoma from nonendemic areas, whereas cases from endemic areas all contained EBV (Pathmanathan *et al.*, 1995a).

Clonal EBV infection was demonstrated in biopsy samples from nasopharyngeal carcinomas in China and the United States (Raab-Traub & Flynn, 1986; Raab-Traub *et al.*, 1987; Pathmanathan *et al.*, 1995b), and also in dysplastic lesions of the nasopharynx in individuals with elevated IgA titres in China, suggesting involvement of EBV prior to the carcinomatous state (Pathmanathan *et al.*, 1995b).

Viral replication is minimal in tumour cells, but the virus can be isolated afer grafting into nude mice (Trumper *et al.*, 1977).

(*c*) *Viral gene expression in tumour specimens*

Transcriptional expression of EBV latent genes in nasopharyngeal carcinoma cells has been studied by northern hybridization (Raab-Traub *et al.*, 1983; Gilligan *et al.*, 1990b, 1991; Brooks *et al.*, 1992; Busson *et al.*, 1992a; Chen *et al.*, 1992c; Karran *et al.*, 1992), by RT-PCR and, in some cases, by in-situ hybridization (Wu *et al.*, 1991; Cochet *et al.*, 1993).

BARFO, *LMP-2*, *EBER* and *EBNA-1* are always expressed in nasopharyngeal carcinoma cells (Fåhraeus *et al.*, 1988; Young *et al.*, 1988; Brooks *et al.*, 1992; Gilligan *et al.*, 1991; Sbih-Lammali *et al.*, 1996), whereas LMP-1 is expressed in a variable proportion of tumour cells (Fåhraeus *et al.*, 1988; Young *et al.*, 1988). The early genes *BALF 5*, *BZLF*, *BMFRI*, *EA(D)* and *BHRF* (*EA(R)*) are occasionally detected in a few cells. Early proteins such as ribonucleotide reductase, BZLF1, BMRF1 (EA(D)) and BHRF-1 (EA(R)) can be detected with monoclonal antibodies (Luka *et al.*, 1988; Lung *et al.*, 1989; Cochet *et al.*, 1993). The presence of EA(D), encoded by *BMRF1*, was not confirmed (Young *et al.*, 1989a), and the site of EA production, which is at the origin of the strong humoral response to that antigen by nasopharyngeal carcinoma patients, remains an open question.

2.4.2.3 *Case–control studies*

(*a*) *Based on pre-diagnostic serological tests*

Lanier *et al.* (1980a) compared the results of serological tests on seven native Alaskan patients with histologically confirmed nasopharyngeal carcinoma and eight controls matched for sex, age, race and residence for whom frozen sera could be found. Several sera were collected for each subject. The oldest samples were collected 22–120 months before diagnosis of nasopharyngeal carcinoma in cases and 3–90 months after the date of diagnosis of the cases in controls. IgA antibodies to VCA were detected in one case 22 months before and three months after diagnosis but in none of the controls. Two patients who showed no IgA antibodies to VCA on two occasions before diagnosis had raised titres at the time of diagnosis and in subsequent tests. All of the cases and controls had detectable titres of anti-EBNA: GMT, [60] in cases, [21] in controls; median titre, [80] in cases, [20] in controls.

(*b*) *Based on serological tests at time of diagnosis*

These studies are summarized in Table 25.

Patients with advanced nasopharyngeal carcinoma, whether Cantonese Chinese in Hong Kong, Maghrebian Tunisians or Caucasians in France, had higher IgG and IgA titres to VCA and EA than patients with other tumours or than normal subjects (de Thé *et al.*, 1978b).

Pearson *et al.* (1983b) compared the IgG and IgA anti-VCA antibody patterns of sera from 124 consecutive cases of nasopharyngeal carcinoma in the United States and in 278 sera obtained from relatives of the cases or of patients with benign diseases or cancer of the head and neck other than nasopharyngeal carcinoma. All cases and 90% of controls had detectable levels of IgG to VCA; IgA antibodies to VCA were detected in 69% of the cases (84% of undifferentiated carcinomas) and 9% of the controls, and IgG antibodies to EA were found in 76% of cases (86% of undifferentiated carcinomas) and 29% of controls.

Two case–control studies in Chinese populations examined the association between the prevalence of anti-VCA IgA antibodies in nasopharyngeal carcinomas and in population controls. Zheng *et al.* (1994a) reported an odds ratio associated with infection of 55 (95% CI, 11–280) adjusted for sex and age and foods associated with nasopharyngeal carcinoma. Chen *et al.* (1987) also found a significant increase in the odds ratio with increasing anti-VCA IgA titres. The odds ratio for positive versus negative subjects, adjusted for sex, age, marital status and consumption of foods associated with nasopharyngeal carcinoma, was 39 (statistically significant).

2.4.2.4 *Cohort studies*

Chan *et al.* (1991) identified seven cases of undifferentiated or poorly differentiated nasopharyngeal carcinoma in four Asians and three Caucasians (six male and one

Table 25. Case–control studies of nasopharyngeal carcinoma and EBV serology at time of diagnosis

Reference and region	Ethnicity	No of cases/No. of controls	Morphology	EBV marker	Measure	Results	Comments
de Thé *et al.* (1978b) Hong Kong, France, Tunisia	Chinese, Causasian, North African	132/150 selected cases of advanced nasopharyngeal carcinoma; controls in same age range	[92%] undifferentiated or poorly differentiated	IgG/VCA IgG/EA	Geometric mean titre	Significantly higher in cases	
Pearson *et al.* (1983b) Northern USA	Mainly Caucasian	124 /278 Controls: age-matched relatives, head-and-neck tumours other than nasopharyngeal carcinoma, benign disorders of head and neck	Undifferentiated, 85 Non-keratinizing, 11 Squamous, 26	IgG/VCA IgA/VCA IgG/EA	Positive versus negative Positive versus negative Positive versus negative Positive for both IgG EA and IgA VCA	Positive: 100% cases, 90% controls 69% cases (84% undifferentiated), 9% controls 76% cases (86% undifferentiated), 29% controls 62% of undifferentiated and non-keratinizing, 7% of squamous, 1% of normal	Crude odds ratio [23], 95% CI [13–40] [32] [18–57]
Chen *et al.* (1988b) Taiwan	Chinese	205/205 neighbourhood controls matched on sex and age	Histologically verified	IgA/VCA Anti-EBV DNase	Positive versus negative < 2 2 ≥ 4	Odds ratio, 39, $p < 0.01$ 1 12 62, $p < 0.01$	Adjusted for matching variables and dietary factors
Zheng *et al.* (1994a) China	Chinese	203/163 (blood available), neighbouhood controls matched on sex and age	Histologically confirmed but type not reported	IgA/VCA	Positive versus negative	Odds ratio, 55 (95% CI, 11–280)	Adjusted for matching variables and some dietary factors

Numbers in square brackets were calculated by the Working Group.
VCA, viral capsid antigen; EA, early antigen; CI, confidence interval

female) that developed in a cohort of over 240 000 individuals in Norway and the United States and for whom sera had been stored in four banks. Two controls per case were selected from the cohort and matched on bank, storage duration, age, sex and race. The intervals between serum collection and diagnosis of nasopharyngeal carcinoma were 26, 76, 122, 124, 134, 153 and 154 months. The GMTs of IgA antibodies to VCA, anti-EA(D), anti-EA(R) and anti-EBNA were similar in cases and controls. The odds ratio associated with high titres of IgA to VCA was 1.0 (95% CI, 0.1–11) and that for high anti-EBNA titres was 0.7 (95% CI, 0.1–5.3).

2.4.2.5 *Mass serological surveys*

These studies are summarized in Table 26.

Two screening surveys for EBV positivity in sera were conducted in Guangxi Autonomous Region, China. In the first, 148 029 subjects living in rural areas of Zangwu County were screened for IgA antibodies to VCA. Among the first 56 584 examined, 117 (0.2%) had positive results. These people then underwent thorough clinical investigation, which led to the diagnosis of 18 cases of nasopharyngeal carcinoma; two additional cases were diagnosed in the 10 months after the intervention (Zeng *et al.*, 1980). The final number of antibody-positive subjects in the cohort was 3533 (prevalence, 2.4%), and 55 nasopharyngeal carcinomas were found in this group (Zeng *et al.*, 1983a). Thus, the prevalence of IgA antibodies to VCA in the 91 445 subjects recruited after the interim report (Zeng *et al.*, 1980) was 3.7%. In the second survey, individuals aged 40 years or more and resident in Wuzhou City in Zangwu County were examined. Among 20 726 people screened, 5.5% had IgA antibodies to VCA; 18 cases of nasopharyngeal carcinoma (1.6%) were detected among the latter (Zeng *et al.*, 1985; Zeng, 1987). Similar prevalences of antibody-positive subjects and nasopharyngeal carcinoma cases had been reported in a preliminary analysis of the first 12 932 individuals screened (Zeng *et al.*, 1982). Thirty-five additional cases of nasopharyngeal carcinoma were identified retrospectively in the cohort of antibody-positive individuals during the 10 years after the survey; however, the number of person-years of observation was not available. Four nasopharyngeal carcinomas were seen in subjects who had no IgA antibodies to VCA (Zeng *et al.*, 1993).

In a third survey, conducted in Taiwan (Chen *et al.*, 1989), anti-EBV DNase activity was found in 1250 of 22 596 (5.5%) Government employees and 1176 of 9869 (11.9%) residents of a high-risk area. When those with antibodies were referred for clinical investigation, three and 11 nasopharyngeal carcinoma cases were detected among those who complied with referral in the two groups, corresponding to prevalences of 0.6 and 1.3%, respectively. EBV-negative individuals were not screened for nasopharyngeal carcinoma, and no long-term follow up of the cohort has been performed.

Table 26. Detection of EBV in mass serological surveys and association with nasopharyngeal carcinoma

Reference and region	No. screened	EBV detection	Presence of antibodies		Cases of nasopharyngeal carcinoma in EBV-positive cases		Incidence in EBV-positive cases
			No.	%	No.	%	
Zeng *et al.* (1979, 1980, 1983a); rural Zangwu County, China	Interim analysis: 56 584 Final cohort: 148 029	IgA VCA	117 3 533	0.2 2.4	18 55	15.4 1.6	Two additional cases in 10-month follow-up
Zeng *et al.* (1982, 1985); Zeng (1987); Wuzhou City, Zangwu County	Interim analysis: 12 932 Final cohort: 20 726	IgA VCA	680 1 136	5.3 5.5	13 18	5.3 1.6	35 additional cases in 10-year follow-up (Zeng *et al.*, 1993)
Chen *et al.* (1989); Taiwan		Anti-EBV DNAse					
Government employees	22 596		1 250	5.5	3/477[a]	0.6	477 who complied with referral
High-risk area	9 869		1 176	11.9	11/829[a]	1.3	829 who complied with referral

[a] Number of people attending a specific clinic at the National Taiwan University Hospital

2.4.3 *Cofactors*

2.4.3.1 *Dietary factors*

(*a*) *Cantonese-style salted fish*

The higher rates of nasopharyngeal carcinoma among the boat-dwelling Tankas than among the land-dwelling Cantonese in Hong Kong was first noted by Ho (1967), who also observed that salted fish is the principal source of supplementary food in the diet of these people, which consists mainly of rice. He subsequently suggested that Cantonese-style salted fish be investigated as a 'possible etiological factor' for nasopharyngeal carcinoma (Ho, 1971). He made two additional observations: the salting process is inefficient and the product, aged for several days to weeks, becomes partially putrefied, liberating a pungent odour that is offensive to those who were not raised in the southern Chinese culture (Ho, 1972). Later, he recognized that salted fish mixed with soft rice is a popular weaning food in southern China (Ho, 1976).

Eleven case–control studies have been conducted in various Chinese populations with distinct risks for nasopharyngeal carcinoma to investigate the possible association with the consumption of salted fish. Five were conducted in high-risk Cantonese populations (Geser *et al.*, 1978; Henderson & Louie, 1978; Yu *et al.*, 1986, 1989a; Zheng *et al.*, 1994a), three were carried out in populations with about half the incidence rate of Cantonese (Chen *et al.*, 1988b; Yu *et al.*, 1988; Zheng *et al.*, 1994b), one study was conducted in the relatively low-risk population of Tianjin in northern China (Ning *et al.*, 1990), and two studies were conducted in various Chinese populations in Southeast Asia (Armstrong *et al.*, 1983; Lee *et al.*, 1994b). One epidemiological study addressed the relationship between consumption of Cantonese-style salted fish and nasopharyngeal carcinoma risk in a non-Chinese population (Sriamporn *et al.*, 1992).

Henderson and Louie (1978) in the United States interviewed 74 Chinese patients with nasopharyngeal carcinoma and 110 Chinese control subjects. The patients represented incident cases identified from the population-based cancer registries of Los Angeles County and the San Francisco Bay area. The controls were clinic or hospital patients who were frequency matched to the cases on race, age, sex and socioeconomic status; the subjects were all asked about how often they ate salted fish. A statistically significant association with risk for nasopharyngeal carcinoma was seen (p for linear trend = 0.02), and the odds ratio for consumption two or more times per week relative to no consumption was 3.1.

Geser *et al.* (1978) conducted a hospital-based case–control study in Hong Kong involving 150 patients with nasopharyngeal carcinoma and 150 age- and sex-matched hospital controls. Salted fish intake was not determined for the study subjects, but older women in the households of the patients and controls were interviewed, when possible, about weaning practices. This information was collected from 108 mothers of nasopharyngeal carcinoma patients and 103 mothers of controls. The only food given to babies during and after weaning for which a statistically different prevalence was found between case and control households was salted fish (odds ratio, 2.6; $p < 0.01$).

In Hong Kong, Yu *et al.* (1986) interviewed 250 patients with nasopharyngeal carcinoma under the age of 35 and an equal number of age- and sex-matched friends about their dietary habits three years before diagnosis of the index case and at 10 years of age. Furthermore, 182 mothers of cases and 155 mothers of control subjects were interviewed about the dietary habits of the subjects during weaning, between the ages of one and two years and at the age of 10 years. All of the cases were histologically confirmed at the four hospitals in Hong Kong in which over 90% of new nasopharyngeal carcinoma cases are diagnosed. Salted fish consumption at all periods was significantly related to the risk for this tumour, and increasing frequency of intake was consistently associated with increasing risk. The association with salted fish was stronger when exposure occurred during childhood as compared with adulthood (i.e. three years before interview). Exposure during weaning was associated with an odds ratio of 7.5 (95% CI, 3.9–15); the odds ratio for weekly or more versus less than monthly consumption at age 10 was 38 (95% CI, 14–100). No other dietary or environmental factor studied was significantly associated with nasopharyngeal carcinoma after adjustment for salted fish intake, and the odds ratios for salted fish consumption remained highly significant after all other potential risk factors had been taken into account. It was estimated that over 90% of the cases of nasopharyngeal carcinoma in young people in Hong Kong could be attributed to childhood consumption of Cantonese-style salted fish.

Yu *et al.* (1989a) interviewed 306 patients with histologically confirmed nasopharyngeal carcinoma who lived in Guangzhou, China, and were under the age of 50 years and an equal number of controls matched for age, sex and neighbourhood. Subjects were asked about their dietary pattern three years before diagnosis of the index case and at the age of 10. The mothers of 110 cases under the age of 45 years and of 139 of their matched controls were interviewed about their own consumption of salted fish during the index pregnancy and during lactation and about the subjects' intake of salted fish during weaning, between the ages of one and two years, and at the age of 10. Exposure to salted fish, whether in adulthood or in childhood, was significantly associated with an increased risk for nasopharyngeal carcinoma. The association was strongest for exposure during weaning (odds ratio, 2.1; 95% CI, 1.2–3.6); exposures at all other times were no longer significantly related to the risk for nasopharyngeal carcinoma after adjustment for exposure during weaning. Childhood exposure to salted fish remained a highly significant risk factor after adjustment for other dietary and non-dietary risk factors identified in this study.

Zheng *et al.* (1994a) conducted a hospital-based case–control study in Guangzhou, China, comprising 205 histologically confirmed cases of nasopharyngeal carcinoma under the age of 55 years and an equal number of friends who were individually matched to the cases by age and sex. Subjects were asked about their dietary habits during the past seven years and at the age of 10. Furthermore, the mothers of 151 cases and 195 controls were interviewed about the subjects' dietary habits during the first three years of life and at the age of 10. Salted fish consumption during all of the periods studied was significantly associated with risk for nasopharyngeal carcinoma. The association was strongest for consumption during the first three years of life ($p < 0.001$): the odds ratio for weekly or daily intake relative to less than monthly was 13 (95% CI, 5.2–21). The results

were unchanged after adjustment for the presence of IgA antibodies against EBV VCA. It was estimated that salted fish intake could explain 73% of the cases of nasopharyngeal carcinoma occurring in this Cantonese population.

In a case–control study in Taipei, Taiwan, involving 205 histologically confirmed cases from a single hospital and 205 neighbourhood controls individually matched to the cases by age and sex, Chen *et al.* (1988b) collected information on the frequency of intake of a number of preserved foods before the age of 20, by interview. Individuals who ate salted fish 10 or more times per month had an odds ratio of 1.5 relative to less than monthly intake, which was not statistically significant. Childhood exposure to salted fish was not examined in this study.

Yu *et al.* (1988) interviewed 128 mothers of 231 eligible patients with nasopharyngeal carcinoma under the age of 45 in Yulin Prefecture, Guangxi Autonomous Region, China, in order to examine the relationship between exposures during childhood and subsequent development of nasopharyngeal carcinoma. They also interviewed 174 mothers of 231 individually matched population controls. The mothers were asked about their consumption of salted fish during the index pregnancy and lactation and about the subjects' intake of salted fish during weaning, between the ages of one and two and at the age of 10. Salted fish was not a common weaning food in this population: only 6.3% of controls were exposed. Nonetheless, exposure to salted fish during this early period of life was associated with a 2.6-fold increase in the risk for nasopharyngeal carcinoma (95% CI, 1.2–5.6). The mother's consumption of salted fish during the pregnancy and lactation was also significantly related to risk (p for linear trend = 0.003 and 0.01, respectively). Exposure at the age of 10 was less strongly related, the odds ratios for monthly and weekly intake as compared to less than monthly consumption being 1.5 in both instances and not statistically significant.

Zheng *et al.* (1994b) conducted a case–control study in Zangwu County in eastern Guangxi, China, which involved 88 incident cases of nasopharyngeal carcinoma and twice as many population controls individually matched to the cases by age, sex and neighbourhood of residence. Subjects were asked about their dietary habits during weaning, before the age of two, between the ages of two and 10 and one year before diagnosis of nasopharyngeal carcinoma. Salted fish intake at any of the three times during childhood was significantly related to the risk for nasopharyngeal carcinoma (during weaning, odds ratio, 2.4; $p = 0.01$). Consumption one year before diagnosis of the tumour was relatively rare (2.3% in cases and 0.6% in controls) and was not significantly related to risk.

Ning *et al.* (1990) studied 100 histologically confirmed cases of nasopharyngeal carcinoma in the Han population in people under the age of 65 identified from a population-based cancer registry covering the Tianjin Metropolitan Area in northern China, and three times as many population controls individually matched to the cases by age, sex and neighbourhood of residence. Questions were asked about the frequency of intake of selected foods, including salted fish, at or before the ages of 10, 20, 30, 40 and 50 years. In this relatively low-risk population, in which salted fish is not a common dietary item, any exposure to salted fish was a significant risk factor for nasopharyngeal

carcinoma (odds ratio, 2.2; 95% CI, 1.3–3.7). Four characteristics of exposure to salted fish contributed independently to the increased risk: (1) low age at first exposure; (2) long duration of consumption; (3) frequent consumption at age 10; and (4) cooking by steaming as opposed to frying, grilling or boiling. The last factor could be determined in this population because, unlike the southern Chinse who almost always steam salted fish, consumers in Tianjin use a variety of methods. It was estimated that 40% of nasopharyngeal carcinoma cases occurring in this low-risk region could be attributed to the consumption of salted fish.

Armstrong *et al.* (1983) conducted a case–control study among Malaysian Chinese resident in 27 census districts surrounding (and including) metropolitan Kuala Lumpur, in central Selangor. The 100 cases were all histologically confirmed and identified at the only radiotherapy centre in Malaysia. The 100 control subjects were selected from the neighbourhoods in which the cases lived and were individually matched to the index case by sex and age. Subjects were asked about their intake of salted fish during childhood, during adolescence and at the time of interview. Since there was evidence that the diet of many of the patients had changed since manifestation of clinical cancer, the investigators discarded the information on intake at the time of interview which, for a number of the cases, was several years after the diagnosis. Salted fish intake during childhood and adolescence were both significantly related to the risk for nasopharyngeal carcinoma, with a stronger association for exposure during childhood. The odds ratio was 3.0 ($p = 0.04$) for any consumption during childhood and 17 (95% CI, 2.7–110) for daily consumption.

In Singapore, Lee *et al.* (1994b) compared 200 Chinese patients in whom nasopharyngeal carcinoma had been diagnosed consecutively at a major general hospital; the 406 controls were patients at the same hospital who were frequency matched to the cases by ethnicity, age and sex. Information on dietary habits one year previously was collected from all cases and controls, and information on diet during infancy and at the age of 10 was collected from the mothers of cases or controls, if they were available for interview, or from the subjects themselves. A total of 64 mothers of cases and 103 mothers of controls were interviewed, for a participation rate of 28%. The results for diet during infancy were considered by the authors to be inconclusive, and the Working Group considered that the information on diet at the age of 10 is also uninterpretable because information was obtained from both subjects and mothers and is likely to be of variable reliability. No significant relationship was found between salted fish intake one year before diagnosis and the risk for nasopharyngeal carcinoma (p for linear trend = 0.6), although the highest frequency category was associated with an excess fourfold risk (odds ratio, 4.4; 95% CI, 0.7–26).

Two types of salted fish are eaten by the population in northeast Thailand: home-made salted freshwater fish and Cantonese-style salted marine fish available on markets. Sriamporn *et al.* (1992) examined current consumption of both kinds of salted fish in a hospital-based case–control study involving 120 histologically confirmed cases of nasopharyngeal carcinoma and an equal number of hospital controls individually matched to the cases by age and sex. Intake of Thai-style salted fish was not related to the risk for nasopharyngeal carcinoma, but consumption of Chinese-style salted fish showed a clear,

statistically significant dose–response relationship with risk, weekly consumers having an odds ratio of 2.5 (95% CI, 1.2–5.2) relative to non-consumers.

In summary, the epidemiological data strongly support the hypothesis that Cantonese-style salted fish is a nasopharyngeal carcinogen in humans. The studies suggest that age at exposure is an important co-determinant of risk, earlier age at exposure being associated with a higher risk for disease. Experimental data to support the carcinogenicity of Cantonese-style salted fish is summarized in section 4.5.2.

(*b*) *Other types of salted fish*

There is preliminary evidence that early exposure to other types of salted fish may be responsible for at least some of the increase in risk for nasopharyngeal carcinoma in the native peoples of Southeast Asia and of the Arctic region. Armstrong and Armstrong (1983) studied 13 Malay cases of nasopharyngeal carcinoma and 50 Malay population controls of comparable age and sex distribution in a small case–control study conducted in Selangor, Malaysia. Five cases and four controls had eaten salted fish daily during childhood. The excess was not statistically significant.

In the Philippines, West *et al.* (1993) studied 104 predominantly Filipino cases of nasopharyngeal carcinoma from a single hospital, 104 hospital controls from the same hospital and 101 community controls, who were matched to the cases by age and sex; the hospital controls were also matched to the cases on type of hospital ward (private versus public), and the community controls were further matched to the cases on neighbourhood of residence. The two sets of controls were combined in the analysis. Individuals with the highest frequency of current intake of salted fish had a nonsignificantly increased risk for nasopharyngeal carcinoma relative to those with the lowest frequency (crude rate, 60%; adjusted rate, 30%). Childhood consumption of salted fish was not investigated in this study.

Lanier *et al.* (1980b) interviewed 13 nasopharyngeal carcinoma patients and 13 controls in Alaska who were individually matched to the cases by age, sex, race (Inuit, Indian, Aleut) and village of residence. The same questionnaire was administered to 17 patients with other head-and-neck tumours and their similarly matched control subjects. More nasopharyngeal carcinoma patients than controls had eaten salted fish as children, while no such association was observed for the patients with other head-and-neck cancers.

(*c*) *Other preserved foods*

Early exposure to other preserved foods has also been shown to be related to the risk for nasopharyngeal carcinoma in Chinese populations and was addressed in some of the studies described in section 2.4.3.1(a). In Yulin, China, where salted fish is a relatively rare food item, a variety of preserved foods other than salted fish eaten during childhood have significant, independent effects on the risk (Yu *et al.*, 1988). These include salted ducks' eggs (odds ratio, 5.0; $p = 0.03$), salted mustard greens (odds ratio, 5.4; $p = 0.03$) and *chung choi* (a salted root; odds ratio, 2.0; $p = 0.003$) eaten during weaning, and dried fish (p for linear trend = 0.002), fermented black-bean paste (p for linear trend = 0.009) and fermented soya-bean paste (p for linear trend = 0.007) eaten between the ages of one

and two. In the Cantonese population of Guangzhou, China, childhood exposure to mouldy bean curd (p for linear trend = 0.07), salted shrimp paste (p for linear trend = 0.04), *chan pai mui* (p for linear trend = 0.01) and *gar ink gee* (p for linear trend = 0.03) (the last two are preserved plums) was independently related to the risk for nasopharyngeal carcinoma after adjustment for intake of salted fish (Yu *et al.*, 1989a). In Taiwan, exposure before the age of 20 to fermented bean products (odds ratio for > 10 times/month versus less than once a month, 1.8; $p < 0.05$) and smoked meat (odds ratio for > 1/month versus less frequently, 3.3; adjusted for all other risk factors; $p < 0.05$) were independently associated with the risk for nasopharyngeal carcinoma after adjustment for IgA antibody titres against EBV VCA (Chen *et al.*, 1988b). In Tianjin, China, exposure to salted shrimp paste at the age of 10 years was related to risk independently of salted fish intake (odds ratio for weekly/daily versus monthly frequency, 3.2; $p = 0.007$) (Ning *et al.*, 1990). In Singapore, Lee *et al.* (1994b) noted that consumption of five preserved foods one year before cancer diagnosis was significantly associated with an increased risk. These were *belachan* (salted shrimp paste; p for linear trend = 0.04), salted soya beans (fermented soya-bean paste; p for linear trend = 0.002), tinned pickled vegetables (p for linear trend = 0.004), *szechuan chye* (a salted tuber; p for linear trend = 0.008) and salted mustard greens (*kiam chye*; p for linear trend = 0.007). The effects of salted soya beans, *szechuan chye* and *kiam chye* remained statistically significant in a multivariate model that included other risk factors for nasopharyngeal carcinoma. While the levels of childhood exposure to most of these foods were low in the series studied, *chung choi* was given at weaning in 60% of cases in Yulin (Yu *et al.*, 1988), salted shrimp paste was consumed at the age of 10 by 28% of cases in Tianjin (Ning *et al.*, 1990), and 63% of the cases in Taiwan consumed fermented bean products before the age of 20 (Chen *et al.*, 1988b).

Jeannel *et al.* (1990) conducted a case–control study among Tunisians, who are at intermediate risk for nasopharyngeal carcinoma. Eighty histologically confirmed incident cases identified at the only cancer hospital in Tunisia and 160 population controls individually matched to the cases by age, sex and neighbourhood of residence were interviewed about dietary habits in the year preceding the cancer diagnosis and during childhood. The intake of several preserved food products during childhood and/or adulthood was found to be significantly associated with the risk for nasopharyngeal carcinoma after adjustment for socioeconomic status. They were *touklia* (a stewing mixture of red and black peppers, paprika, caraway seed and/or coriander seed, salt and olive or soya-bean oil), *quaddid* (dried mutton preserved in olive oil), pickled vegetables, pickled olives and *harissa* (a mixture of red pepper, garlic, caraway seed, salt and olive oil). After adjustment for each other and for other potential confounders, only childhood exposure to *touklia*, *quaddid* and *harissa* were significant risk factors for nasopharyngeal carcinoma in Tunisia. The odds ratio for childhood consumption of *touklia* was 8.6 (95% CI, 1.7–44), that for consumption of *quaddid* more than once a month was 1.9 (95% CI, 1.0–3.7) and that for consumption of *harissa* more than once a month was 4.2 (95% CI, 1.1–17).

Studies of the presence of carcinogens, genotoxins, and EBV-activating substances in samples of foods associated with nasopharyngeal carcinoma are described in section 4.5.2.

(*d*) *Deficits of fresh vegetables and fruit*

In an uncontrolled study of 24 patients under the age of 25 with nasopharyngeal carcinoma in Hong Kong, Anderson *et al.* (1978) noted that 'all families felt that vegetables and fruits were bad for babies, and the children had been fed accordingly.' Similar findings were reported in several case–control studies conducted among Chinese. In both Hong Kong (Yu *et al.*, 1986; Ning *et al.*, 1990) and Guangzhou (Yu *et al.*, 1989a), patients with nasopharyngeal carcinoma had a lower intake of fresh vegetables and fruits during weaning, between the ages of one and two and at the age of 10 than controls, and many of the differences were statistically significant. Whereas in Hong Kong the protective effects of fresh vegetables and fruit were no longer significant after adjustment for salted fish intake, in Guangzhou the effects were not due to different consumption of salted fish and other preserved foods by cases and controls (Yu *et al.*, 1989a). In Tianjin (Ning *et al.*, 1990), fewer patients had eaten carrots at the age of 10 than controls. Increased frequency of consumption of garlic, the only other specific vegetable on the questionnaire, also resulted in decreased risks for nasopharyngeal carcinoma, although the effect was not statistically significant. In contrast, Yu *et al.* (1988) did not find a negative association between childhood exposure to fresh vegetables and fruit and the risk for nasopharyngeal carcinoma in Yulin, and Jeannel *et al.* (1990) reported no association between fruit or vegetable intake and nasopharyngeal carcinoma risk in Tunisia.

Clifford (1972) measured serum carotene levels in 17 male African patients with nasopharyngeal carcinoma and 53 male controls and reported a significantly lower level in the cases. In Singapore, Lee *et al.* (1994b) found that the dietary levels of vitamin C, vitamin E and β-carotene one year before diagnosis of nasopharyngeal carcinoma were lower among cases than controls and significantly so for vitamins C and E.

2.4.3.2 *Other environmental factors*

(*a*) *Fumes, smoke and dust*

Dobson (1924) proposed that exposure to smoke from wood fires inside chimneyless houses was the cause of nasopharyngeal carcinoma in southern Chinese, and Clifford (1972) also noted the presence of wood fires in chimneyless houses among tribal groups in Kenya with moderately elevated rates of nasopharyngeal carcinoma.

The relationship between domestic exposure to smoke and risk for nasopharyngeal carcinoma among southern Chinese has been investigated in several case–control studies. Zheng *et al.* (1994b) reported an odds ratio of 5.4 [95% CI, 1.5–20] for use of wood fuel during the year before cancer diagnosis in a case–control study in eastern Guangxi involving 88 cases of nasopharyngeal carcinoma and 176 controls matched for age, sex and neighbourhood. In contrast, Yu *et al.* (1988) observed no association between use of wood fuel and risk in a county adjacent to that studied by Zheng *et al.* (1994b). When lifetime histories of cooking fuels used were collected from each study subject in case–control studies in Hong Kong (Yu *et al.*, 1986) and Guangzhou (Yu *et al.*, 1990), China, no significant association was found with smoke from burning wood.

The role of occupational exposure to smoke, dust and fumes has been examined. A case–control study in Los Angeles and San Francisco (United States) involved 156 histologically confirmed cases of nasopharyngeal carcinoma and 267 hospital or clinic controls matched to the cases by race (whites, blacks, Hispanics, Chinese and other Asians), sex and age (Henderson *et al.*, 1976). Cases were identified through the population-based cancer registries in both metropolitan areas. All interviews were administered in person, and a lifetime occupational history was obtained, including information on usual exposure to fumes, dust, smoke, chemicals or heat in each job. Occupational exposures to fumes (odds ratio, 2.0; $p = 0.006$), dust (odds ratio, 1.5; $p = 0.07$), smoke (odds ratio, 3.0; $p = 0.008$), chemicals (odds ratio, 2.4; $p = 0.006$) and heat (odds ratio, 1.6; $p = 0.05$) were all positively related to the risk for nasopharyngeal carcinoma, but only exposure to smoke showed a clear duration–response relationship (odds ratio for 10 years or less, 1.5; odds ratio for more than 10 years, 7.5; $p < 0.05$).

In the study of Armstrong *et al.* (1983), described on p. 182, a complete occupational history was obtained from each subject by personal interview. Subjects were specifically asked if they had had regular exposure to a specific dust, smoke or chemical fume. Occupational exposure to smoke (odds ratio, 6.0; $p = 0.006$) and dust (odds ratio, 4.0; $p < 0.001$), irrespective of type, was significantly associated with the risk for nasopharyngeal carcinoma, and a clear duration–response relationship was seen for men and women which was independent of salted fish intake.

In the study of Chen *et al.* (1988b), described on p. 181, subjects were asked whether they had been exposed occupationally to dust or smoke. Exposure to smoke (odds ratio, 1.7; $p < 0.05$) was significantly associated with risk for nasopharyngeal carcinoma in univariate but not in a multivariate analysis that included other risk factors. Exposure to dust was not related to increased risk (odds ratio, 1.1).

In Guangzhou, China, Yu *et al.* (1990) interviewed 306 patients under the age of 50 with histologically confirmed nasopharyngeal carcinoma and an equal number of controls matched for age, sex and neighbourhood to obtain a complete occupational history from each respondent. For each job held for six months or longer, the subjects were asked to indicate whether they had been exposed to dust, smoke or chemical fumes and to name the substances involved. To avoid recall bias, a specialist who was unaware of the case or control status of the subjects assessed these exposures on the basis of job title, activity and industry. Occupational exposure to dust was not related to increased risk, on the basis of the subjects' responses (odds ratio, 1.2) or the expert judgement (odds ratio, 0.9); however, exposure to smoke was significantly associated with the risk for nasopharyngeal carcinoma (subjects: odds ratio, 2.1; 95% CI, 1.3–3.5; expert: odds ratio, 1.6; 95% CI, 1.1–2.5). A clear duration–response relationship was seen in both assessments. Exposure to chemical fumes was significantly related to risk, depending on the duration of exposure (odds ratio, 1.7; 95% CI, 1.1–2.4) when based on the subjects' recall but not when based on the expert judgement (odds ratio, 1.0; 95% CI, 0.7–1.4).

In the study of West *et al.* (1993), described on p. 183, lifetime occupational histories were recorded after interview with each subject. An industrial hygienist who was unaware of the case–control status of the subjects then classified each job as either likely

or unlikely to involve exposure to dust, solvents or exhaust fumes. The investigators found all three exposures to be significantly associated with risk for nasopharyngeal carcinoma. People first exposed to dust 35 or more years before cancer diagnosis had an odds ratio of 4.7 (95% CI, 1.8–13) relative to no exposure; the comparable odds ratios were 2.6 (95% CI, 1.1–6.3) for exposure to solvents and 2.8 (95% CI, 1.1–7.0) for exposure to exhaust fumes. As the three exposures were highly correlated, a combined exposure index was used in the final multivariate model. The index maintained an independent effect on risk after adjustment for other factors.

A number of epidemiological studies have suggested that woodworkers are at increased risk for nasopharyngeal carcinoma. Besides wood dust (see IARC, 1995a), these workers are exposed to various chemicals that are applied to the wood, including pesticides, phenols, chlorophenols and asbestos. Demers *et al.* (1995) conducted a pooled analysis of five cohort studies of mortality among furniture, plywood and wood-model workers in the United Kingdom and the United States. A total of 28 704 subjects, 7665 of whom died, were studied. A significant excess of nasopharyngeal cancer was noted: nine deaths were observed when 3.8 were expected on the basis of general population rates (standardized mortality ratio (SMR), 2.4; 95% CI, 1.1-4.5). This excess risk was seen among both furniture and plywood workers, and in those with either low or high probability of exposure to wood dust.

Kawachi *et al.* (1989) used data from the New Zealand Cancer Registry on male patients aged 20 years or more in whom nasopharyngeal carcinoma was diagnosed between 1980 and 1984, to identify possible cancers related to wood-working, since the current or most recent occupation of the patient at the time of cancer diagnosis is recorded in the Registry. Twenty-four cancer sites were examined, compared with all other cancer patients and for each site. Five cases of nasopharyngeal cancer were found among woodworkers (three carpenters and two forestry/logging workers), which represented a nonsignificant excess relative to controls (odds ratio, 2.5; 95% CI, 0.9–6.6).

(*b*) *Formaldehyde*

The possible role of formaldehyde (see IARC, 1995b) in development of nasopharyngeal carcinoma has been examined in three large-scale historical cohort studies of exposed workers (Blair *et al.*, 1986, 1987; Stayner *et al.*, 1988; Gardner *et al.*, 1993) and four case–control studies in diverse populations (Olsen *et al.*, 1984; Vaughan *et al.*, 1986a,b; Roush *et al.*, 1987; West *et al.*, 1993).

Blair *et al.* (1986) evaluated the mortality of 26 561 workers employed in 10 facilities where formaldehyde was produced or used in the United States. About 600 000 person–years of follow-up were available. There were seven deaths from cancer of the nasopharynx in the cohort, when 2.2 were expected on the basis of the mortality rates of the general population ($p < 0.05$). The risk increased with increasing cumulative exposure to formaldehyde among people who were also exposed to particulates (Blair *et al.*, 1987). On the basis of five cases, the SMRs were 190 for cumulative exposure of < 0.5 ppm–years, 400 for 0.5–< 5.5 ppm–years and 750 for ≥ 5.5 ppm–years (this trend was not statistically significant). A closer examination of the data revealed that three of the five deaths from nasopharyngeal carcinoma had occurred in people who had worked at the

plants for less than a year, and that the deaths at one plant out of the 10 studied were responsible for the observed excess (Collins *et al.*, 1988).

Gardner *et al.* (1993) studied the mortality experience of workers in the United Kingdom exposed to formaldehyde in six factories where this chemical was manufactured or used. The cohort consisted of 7660 men who were first employed between 1920 and 1964 and 6357 men first employed between 1965 and 1982. The cohort was followed-up from 1 January 1941 to 31 December 1989. There were no cases of nasopharyngeal cancer, while 1.3 were expected.

In Denmark, Olsen *et al.* (1984) compared the occupational exposures of 266 patients with nasopharyngeal carcinoma with those of 2465 controls with other cancers. Exposure to 12 specific substances or procedures, including formaldehyde, was assessed by industrial hygienists who were unaware of the case or control status of the cancer patients. No significant association was found between exposure to formaldehyde and risk for nasopharyngeal carcinoma, in either men (odds ratio, 0.7; 95% CI, 0.3–1.7) or women (odds ratio, 2.6; 95% CI, 0.3–21.9).

Vaughan *et al.* (1986a,b) conducted a case–control study of 27 cases of nasopharyngeal carcinoma and 552 population controls identified through the random digit dialling method in western Washington State (United States). Occupational exposures and other information were obtained by telephone interviews; however, one-half of the interviews were conducted with next-of-kin. A job–exposure matrix was used to determine if study subjects had had occupational exposure to formaldehyde. A modest, statistically non-significant association was found between duration of exposure to formaldehyde and risk for nasopharyngeal carcinoma. The odds ratios for 1–9 and ≥ 10 years of exposure were 1.2 (95% CI, 0.5–3.1) and 1.6 (95% CI, 0.4–5.8), respectively, relative to no exposure. A history of living in a mobile home (which is believed to be associated with exposure to formaldehyde from the particle-board and plywood commonly used) was positively and significantly related to increased risk, with odds ratios of 2.1 (95% CI, 0.7–6.6) for 1–9 years of living in a mobile home and 5.5 (95% CI, 1.6–19) for ≥ 10 years, relative to no residence in a mobile home. The odds ratio for those with a history of exposure to formaldehyde on the job and living in a mobile home, relative to those with neither exposure, was 6.7 (95% CI, 1.2–39).

Roush *et al.* (1987) compared 173 deaths from nasopharyngeal carcinoma with 605 control subjects selected from the Connecticut (United States) death certificate files. City directories and death certificates were used to reconstruct the occupational status of each study subject 1, 10, 20, 25, 30, 40 and 50 years before death. An industrial hygienist who was unaware of the case or control status then assigned each combination of job, industry and year to give two exposure scores: four degrees of probability of exposure (unexposed, possibly exposed, probably exposed, definitely exposed) and three levels of exposure (zero, low, high). The odds ratio for probable exposure at some level for most of a working life and probable exposure to high levels ≥ 20 years before death was 2.3 (95% CI, 0.9–6.0).

In the study of West *et al.* (1993), described on p. 183, early occupational exposure to formaldehyde was significantly related to the risk for nasopharyngeal carcinoma. First

exposure before the age of 25 was associated with an odds ratio of 2.7 (95% CI, 1.1–6.6) relative to no exposure, and the excess risk persisted after adjustment for other risk factors for nasopharyngeal carcinoma.

(*c*) *Tobacco*

The relationship between cigarette smoking (see IARC, 1986) and nasopharyngeal carcinoma has been examined in a number of case–control studies and one cohort study in diverse populations. Although the results of earlier studies, many of which were based on either small sample sizes, very young cases or hospital controls, are equivocal, more recent data from better designed studies are consistent in showing that cigarette smoking is a risk factor for nasopharyngeal carcinoma, regardless of race. Three- to fivefold increased risks were observed among the heaviest smokers in China, the Philippines and the United States (Yu *et al.*, 1990; Nam *et al.*, 1992; Chow *et al.*, 1993; West *et al.*, 1993; Zhu *et al.*, 1995).

In the study of Yu *et al.* (1990), described on p. 186, subjects and their spouses were asked about lifetime use of cigarettes and water pipes. A moderate but statistically significant association was found between tobacco use and nasopharyngeal carcinoma risk. Use of a water pipe and cigarettes were both related to increased risk. Most of the water-pipe users also were cigarette smokers. A lifetime exposure of 30 or more pack–years (assuming that 2.5 g of tobacco, equivalent to 2.5 cigarettes, were consumed each time a subject smoked a water pipe) resulted in a 3.7-fold increased risk for nasopharyngeal carcinoma relative to non-users, after adjustment for all other risk factors (95% CI, 1.2–12).

Nam *et al.* (1992) conducted a case–control study of white cases of nasopharyngeal carcinoma identified from the National Mortality Followback Survey, which provides information from death certificates on people who died at the age of 25 years or more in the United States in 1986. White control subjects were selected from the same database and were matched to the cases by sex and age. Those who had died from causes known to be related to cigarette or alcohol use were excluded from the pool of potential controls. Thus, 204 cases of nasopharyngeal carcinoma and 408 controls were included in the study. Proxy interviews were conducted by using a structured questionnaire, mainly with spouses. Cigarette use was related to the risk for nasopharyngeal carcinoma in both men and women, and the risk increased with increasing duration of use and with increasing number of cigarettes smoked per day. For the heaviest smokers (those with ≥ 60 pack–years of cumulative exposure), the odds ratio was 3.1 (95% CI, 1.6–6.1) in men and 4.9 (95% CI, 1.2–20.9) in women.

Chow *et al.* (1993) used information collected in 1954 and 1957 on 248 046 United States veterans and their mortality through 30 September 1980 to examine the relationship between tobacco use and the development of nasopharyngeal carcinoma. Forty-eight deaths from this cause were studied. Current smokers had a 3.9-fold increased risk for nasopharyngeal carcinoma (95% CI, 1.5–10), and the risk increased with the number of cigarettes smoked per day. There was no excess risk among people who smoked only cigars or pipes.

In the study of West *et al.* (1993), described on p. 183, no association between cigarette smoking and nasopharyngeal carcinoma was found in comparison with hospital controls, but a significant association between heavy use of cigarettes and risk was observed when the cases were compared with community controls. After adjustment for all other risk factors, the odds ratio for smokers of ≥ 31 years was 4.9 (95% CI, 1.6–15).

Zhu *et al.* (1995) carried out a case–control study in eight areas of the United States covered by population-based cancer registries. The cases were in men aged 15–39 in 1968 in whom nasopharyngeal cancer had been diagnosed in 1984–88. The controls were men selected from the general populations of the eight study areas by random-digit dialling and matched to the cases by age. A total of 113 cases and 1910 controls were included; 62% of the cases were white (including Hispanics), 11% were black and 27% were Asian. Most of the subjects were interviewed directly by telephone. Cigarette smoking was found to be related to the risk for nasopharyngeal carcinoma, and the risk increased monotonically with increasing number of cigarettes smoked per day and with increasing number of pack–years of cumulative exposure. The odds ratio for individuals with ≥ 45 pack–years of exposure was 3.9 (95% CI, 2.0–7.8) relative to nonsmokers.

Vaughan *et al.* (1996) conducted a case–control study in five locations in the United States covered by population-based cancer registries. Telephone interviews were completed with 231 cases and with 246 controls matched to the cases on age, sex and race. Most of the cases occurred in non-Hispanic whites (77%) or African Americans (10%). A statistically significant dose–response relationship was observed between cigarette smoking and risk for nasopharyngeal carcinoma, which was confined to differentiated squamous-cell carcinomas and those classified as epithelial tumours not otherwise specified; no association was observed between cigarette smoking and the 54 cases of undifferentiated non-keratinizing carcinoma.

The possible role of passive smoking in the development of nasopharyngeal carcinoma has been examined in two case–control studies, with inconclusive results. In the study of Yu *et al.* (1988), described on p. 181, mothers were asked about the smoking habits of all household members around the time of birth of the index subject and when he or she was 10 years old. The fathers of more cases than controls had smoked when the subjects were born (odds ratio, 1.5; $p = 0.05$), and the risk increased with increasing number of cigarettes smoked by the father (p for linear trend = 0.04). Very few mothers had smoked (5.5% of case mothers and 5.2% of control mothers), and the odds ratio was 1.0. More cases than controls had been exposed to tobacco smoke from other household members, but the difference was not statistically significant. Overall, the presence of a smoker in the household around the time of birth was associated with an increased risk for nasopharyngeal carcinoma (odds ratio, 2.0; $p = 0.004$). Exposure at birth and at the age of 10 was highly correlated, with a slightly stronger association for exposure at birth; there was no significant residual effect for exposure at the age of 10 after adjustment for exposure at birth. Adjustment for personal cigarette smoking did not affect these findings.

In the study of Yu *et al.* (1990), described on p. 186, in which a significantly positive association with active smoking was observed, the same set of questions as used in the

study described above was given to the mothers of cases and controls. No associations were observed. Having a spouse who smoked was also not related to the risk for nasopharyngeal carcinoma (odds ratio, 1.2; 95% CI, 0.6–2.4). The odds ratio for any domestic exposure to passive smoking was 0.9 (95% CI, 0.6–1.4). The results were unchanged when the analysis was restricted to non-tobacco users.

(*d*) *Alcohol*

The possible association between alcohol intake and the development of nasopharyngeal carcinoma has been investigated in a number of case–control studies among Chinese in and outside of China and among whites in the United States (Lin *et al.*, 1973; Henderson *et al.*, 1976; Geser *et al.*, 1978; Shanmugaratnam *et al.*, 1978; Armstrong *et al.*, 1983; Mabuchi *et al.*, 1985; Chen *et al.*, 1988b; Ning *et al.*, 1990; Nam *et al.*, 1992; Vaughan *et al.*, 1996). Only the studies of Nam *et al.* (1992) and Vaughan *et al.* (1996) found an association. Nam *et al.* (1992) relied on surrogate interviews for information on exposure, as all of the cases and controls were identified from death certificates. The 80% excess risk noted in that study after control for level of cigarette smoking could have been the result of residual confounding. Vaughan *et al.* (1996) reported a significant association between heavy alcohol use (21 or more drinks per week) and risk for nasopharyngeal carcinoma after adjustment for cigarette use. The association was confined to differentiated squamous-cell carcinoma and epithelial tumours not otherwise specified.

(*e*) *Herbal drugs*

A number of Chinese herbs have been shown to induce EBV antigens in human lymphoblastoid cell lines carrying the EBV genome (Hirayama & Ito, 1981; Zeng *et al.*, 1983b; Zeng, 1987), raising the possibility that exposure to these products may affect the risk for nasopharyngeal carcinoma. The geographical distribution of *Croton tiglium*, the seeds of which are used in Chinese herbs, has been noted to loosely parallel that of nasopharyngeal carcinoma within China (Hirayama & Ito, 1981). In fact, croton seeds are rarely used in herbal mixtures due to their extreme potency. In the studies of Yu *et al.* (1986, 1988, 1990), questions on lifetime use of croton seeds were asked, but none of the subjects reported exposure to this herb. Yu *et al.* (1990) also examined lifetime use of *Phyllanthus emblica* and *Croton crassifolius*, two EBV-inducing herbs that are commonly prescribed in Guangzhou (according to Government sales figures); no relationship with nasopharyngeal carcinoma was observed.

Use of herbal drugs in general was reported to be associated with nasopharyngeal carcinoma in three case–control studies (Lin *et al.*, 1973; Hildesheim *et al.*, 1992; Zheng *et al.*, 1994b). Lin *et al.* (1973) interviewed 343 patients with nasopharyngeal carcinoma identified in four counties in Taiwan and 1017 population controls matched to the cases by age, sex and neighbourhood of residence. Use of herbal drugs was associated with the risk for nasopharyngeal carcinoma, occasional users having an odds ratio of 1.7 and frequent users an odds ratio of 3.5 ($p < 0.001$), relative to non-users. The authors acknowledged that usage may have been related to the clinical symptoms of the tumour

and/or recall bias. [Use of herbal drugs is a part of the traditional life style that is an established risk factor for nasopharyngeal carcinoma.]

In the Philippines, Hildesheim *et al.* (1992) studied 104 predominantly Filipino cases of nasopharyngeal carcinoma (see West *et al.*, 1993, p. 183). Information on the use of herbal medicines was collected from study subjects at personal interviews. Since the prevalence of use was similar in the two groups of control subjects, they were combined for the analysis. Patients with nasopharyngeal carcinoma were significantly more likely than controls to have used herbal medicines (odds ratio, 2.5; 95% CI, 1.4–4.5), and the result was unchanged after adjustment for potential confounders. [The same methodological concerns outlined at the end of the last paragraph apply to this study.]

In the study of Zheng *et al.* (1994b), described on p. 181, subjects were asked about their use of herbal drugs during the year before cancer diagnosis and during childhood. Recent use was strongly associated with the risk for nasopharyngeal carcinoma (odds ratio, 4.5; $p = 0.006$), and the risk was reduced when childhood use was considered (odds ratio, 1.8; $p = 0.07$).

In contrast, three case–control studies conducted in southern China of the frequency of use of the most popular herbal tea in the region, 'cooling soup', during adulthood (three years before cancer diagnosis) or childhood (at the age of 10) showed no association between exposure and risk (Yu *et al.*, 1986, 1988, 1989a). Herbal medicines are almost never given to Chinese infants.

(f) *Incense and anti-mosquito coils*

Domestic exposure to burning incense and anti-mosquito coils has been postulated as a risk factor for nasopharyngeal carcinoma and has been investigated in a number of case–control studies conducted in various southern Chinese populations. In Taiwan (Lin *et al.*, 1973; Chen *et al.*, 1988b), Hong Kong (Yu *et al.*, 1986), Guangxi, Yulin prefecture (Yu *et al.*, 1988) and Guangzhou (Yu *et al.*, 1990), such exposures were not associated with the risk for nasopharyngeal carcinoma. Yu *et al.* (1986, 1988, 1990) examined the frequencies of the two exposures at birth, at the age of 10 and three years before cancer diagnosis, while Lin *et al.* (1973a) and Chen *et al.* (1988b) compared any with no exposure to the two types of smoke. In Singapore, two case–control studies of incense burning and risk for nasopharyngeal carcinoma showed no association (Shanmugaratnam *et al.*, 1978; Lee *et al.*, 1994b).

The study of Shanmugaratnam *et al.* (1978) was a hospital-based study with two sets of hospital controls: non-nasopharyngeal carcinoma patients from the same clinics and wards where the cases were recruited and medical, surgical and orthopaedic patients from a different Government hospital. The two sets of controls were not comparable to the cases with regard to socioeconomic status; the cases were less educated than the controls with ear-nose-and-throat conditions and were more educated than the 'other hospital' controls. Anti-mosquito coil burning was associated with increased odds ratios of 1.3–1.4 ($p < 0.05$) in comparison with both control groups when users were compared with non-users and those of unknown status. The frequencies of the groups with no use, any use and unknown status were not given.

In the study of West *et al.* (1993), described on p. 183, use of mosquito coils in the year before cancer diagnosis was investigated. Daily users of anti-mosquito coils had a sixfold increased risk for nasopharyngeal carcinoma relative to non-users (odds ratio, 5.9; 95% CI, 1.7–20).

(*g*) *Chinese nasal oil*

The use of Chinese nasal oil, the main ingredients of which are camphor and menthol, has been postulated as a risk factor for nasopharyngeal carcinoma. Yu *et al.* (1986, 1990) noted greater recent (three years before) use among cases than controls, which was related to the clinical symptoms of nasopharyngeal carcinoma. The more relevant exposure during childhood was examined in three case–control studies (Yu *et al.*, 1986, 1988, 1990). Although in two studies (Yu *et al.*, 1988, 1990) the mothers of more cases than controls reported use during the subjects' childhood, most of the exposures were infrequent and/or unsubstantiated by a medical condition that would suggest intense, sustained use. Several other case–control studies of Chinese have shown greater recent use in cases than controls, but none could rule out the possibility that the oil was used to treat symptoms of nasopharyngeal carcinoma (Lin *et al.*, 1973; Shanmugaratnam *et al.*, 1978; Lee *et al.*, 1994b).

2.4.3.3 *Host factors*

Case–control studies have established several associations between HLA locus A and B antigens and risk for nasopharyngeal carcinoma in southern Chinese. The presence of both A2 and BW46 antigens was associated with a twofold increased risk for nasopharyngeal carcinoma among Chinese in Singapore, Malaysia, Hong Kong and Guangzhou (Simons *et al.*, 1976, 1977, 1978, 1980; Chan *et al.*, 1983a). Interestingly, the frequency of the A2-BW46 phenotype is twice as common among Cantonese than in the Chiu Chau/Fujianese dialect group, parallelling the twofold difference in nasopharyngeal carcinoma incidence between these two ethnic groups (Simons *et al.*, 1976). Other HLA antigens that show an association with nasopharyngeal carcinoma in southern Chinese are B16 (RR, 6.0), B17 (RR, 2.1–2.3), especially in combination with B17 (RR, 2.4–2.5), A11 (RR, 0.5) and B13 (RR, 0.5) (Simons *et al.*, 1978; Chan *et al.*, 1981, 1983a; Wu *et al.*, 1989). Preliminary HLA locus DR typing in this high-risk population has shown significant differences in antigen frequencies between cases and controls (Chan *et al.*, 1981, 1983b; Wu *et al.*, 1989). A linkage study of affected pairs of siblings in southern Chinese in China, Hong Kong and Singapore suggests that a gene (or genes) closely linked to the HLA locus is associated with a 20-fold increased risk for nasopharyngeal carcinoma (Lu *et al.*, 1990). A recent study in Singapore Chinese showed that allele DRB10803 is associated with a 2.8-fold increase (95% CI, 1.0–7.5). In the same study, the investigators found a 3.5-fold increased risk (95% CI, 1.6–7.6) among individuals with allele 4 of the D6S1624 microsatellite locus located on chromosome 6 (Ooi *et al.*, 1997).

An association between HLA profile and nasopharyngeal carcinoma risk has also been reported in non-Chinese populations. B46 has been associated with the occurrence of this tumour in Thais (Chan *et al.*, 1986), and antigen B17 has been found to be posi-

tively associated with the risk of Malays (Chan *et al.*, 1985) and Australian whites (Simons & Shanmugaratnam, 1982). Other antigens shown to be associated with nasopharyngeal carcinoma in selected populations are: A29 in Kenyans and Tanzanians, B18 in Malays, A3 in Australian whites, B5 in Germans and A2 in whites in the United States (Hall *et al.*, 1982; Simons & Shanmugaratnam, 1982; Chan *et al.*, 1985; Burt *et al.*, 1994). In contrast to southern Chinese, white Americans, Europeans, Tunisians and Malays rarely have BW46, and no association with nasopharyngeal carcinoma was seen in these populations (Betuel *et al.*, 1975; Chan *et al.*, 1979b, 1985; Beigel *et al.*, 1983; Moore *et al.*, 1983).

2.4.3.4 *Familial aggregation*

Multiple cases of nasopharyngeal carcinoma occurring in first-degree relatives have been documented in diverse populations, ranging from high-risk southern Chinese and Alaskan and Greenland natives, low- to intermediate-risk Africans, to low-risk Caucasians (Stinson, 1940; Bell & Maguda, 1970; Nevo *et al.*, 1971; Ho, 1972; Williams & de Thé, 1974; Brown *et al.*, 1976; Jonas *et al.*, 1976; Lanier *et al.*, 1979; Gajwani *et al.*, 1980; Fischer *et al.*, 1984; Yu *et al.*, 1986; Schimke *et al.*, 1987; Yu *et al.*, 1990; Albeck *et al.*, 1993). Familial aggregation can be the result of shared genes, shared environments or both. Among the high-risk southern Chinese, a potent environmental factor that is strongly correlated within families — dietary exposures at weaning — has been identified, and consistent associations with certain HLA antigens imply the presence of disease susceptibility genes. Genetic studies in non-Chinese populations also suggest the involvement of hereditary factors in the development of nasopharyngeal carcinoma, and analytical studies have implicated environmental factors. Familial clustering of nasopharyngeal carcinoma is therefore likely to be a product of genetic constitution and environmental exposures.

2.5 Comparison of characteristics of Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma

Table 27 gives a comparison of the epidemiology, virological markers and sites of African Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma in areas of high risk for those tumours.

2.6 Other malignancies

2.6.1 *Lymphoepithelial carcinomas outside the nasopharynx*

The detection of EBV in virtually all undifferentiated nasopharyngeal carcinomas prompted studies into the possible association of the virus with other lymphoepithelial carcinomas. These studies concentrated initially on carcinomas morphologically similar to type-3 nasopharyngeal carcinoma, which can be distinguished from gastric carcinoma with lymphoid stroma, which may account for up to 50% of gastric carcinomas (Matsunou *et al.*, 1996). Lymphoepithelial carcinomas of the stomach are a relatively rare subtype of gastric neoplasm (Rowlands *et al.*, 1993).

Table 27. Characteristics of Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma in high-risk regions

Characteristic	African Burkitt's lymphoma	Hodgkin's disease	Nasopharyngeal carcinoma
Epidemiology			
Age	< 15 years (mode, 8–10)	Bimodal (1st mode, 15–50 years; 2nd mode, > 50 years)	> 15 years (mode, 45–54)
Sex	Males > females	Males > females	Males > females
High-risk regions	Equatorial Africa	Developed countries	Southeast Asia and south China
Period of exposure to probable carcinogens	Early	Early or post-adolescence	Early and continuous
Co-factors	Holoendemic malaria	None identified	Selected preserved foods
Virological markers			
Nucleic acids, proteins			
EBV DNA	+	+ (30–50%)	+
EBER	+	+ (30–50%)	+
EBNA	+	–	+
Serology			
VCA IgA	–	–	+
IgG	+	–	–
IgM	+	+	–
Anti-EBNA	–	++	+
Reactive stroma			
T Cells	–	++	+
B Cells	–	–	–
NK and other cells	–	+	–

EBER, EBV-encoded RNA; EBNA, EBV nuclear antigen; VCA, viral capsid antigen; Ig, immunoglobulin; NK, natural killer

An association of EBV with lymphoepithelial carcinomas arising outside the nasopharynx was initially proposed on the basis of the detection of EBV genomes in DNA extracts (Table 28). Saemundsen *et al.* (1982) found EBV genomes in DNA extracted from a lymphoepithelial salivary gland carcinoma in a Greenland Inuit. This observation was subsequently confirmed by Hamilton-Dutoit *et al.* (1991a), who also localized the viral genomes to the tumour cells by DNA in-situ hybridization in 11/11 of these carcinomas. Further studies have shown the consistent association of salivary gland lymphoepithelial carcinomas with EBV (Chan *et al.*, 1994; Leung *et al.*, 1995a; Kotsianti *et al.*, 1996; Tsai *et al.*, 1996a). Moreover, the viral episomes in these tumours have been shown to be of monoclonal origin (Leung *et al.*, 1995a). An EBV-associated lymphoepithelial carcinoma in the lachrymal sac was reported in a Chinese patient (Leung *et al.*, 1996).

Using DNA in-situ hybridization, Weiss *et al.* (1989b) detected EBV DNA in all of six nasopharyngeal lymphoepitheliomas but not in lymphoepithelial carcinomas occurring in the skin, cervix uteri, tonsil, pharynx or larynx. Of 14 lymphoepithelial carcinomas outside the nasopharynx, the only one that contained EBV was a carcinoma of the lung in a Chinese woman. This observation was confirmed by subsequent reports (Table 29; Butler *et al.*, 1989; Gal *et al.*, 1991; Pittaluga *et al.*, 1993; Wöckel *et al.*, 1995). Pittaluga *et al.* (1993) were also able to show monoclonal viral episomes in some of their cases. [The Working Group noted that the cases in these studies that contained EBV were generally in Asians.]

On the basis of PCR investigations, Burke *et al.* (1990) and Min *et al.* (1991) first suggested that lymphoepithelial gastric carcinomas are associated with EBV infection. Clonal viral episomes were subsequently detected in such cases by Southern blot hybridization (Pittaluga *et al.*, 1992). Studies with in-situ hybridization have confirmed those reports by localizing the virus to the malignant epithelial cells in such cases (Shibata *et al.*, 1991b; Niedobitek *et al.*, 1992b; Oda *et al.*, 1993; Matsunou *et al.*, 1996). Several studies have indicated that about 80% of lymphoepithelial gastric carcinomas are associated with EBV (Rowlands *et al.*, 1993; Osato & Imai, 1996; see Table 30).

The association of EBV with lymphoepithelial carcinomas arising at other anatomical sites is tenuous. Nicholls *et al.* (1994) detected EBV in one of five non-keratinizing tonsillar carcinomas among Chinese patients, but Niedobitek *et al.* (1991b) found no evidence for an association with EBV in Chinese or Malays. Mori *et al.* (1994) detected EBV in a single lymphoepithelial carcinoma of the oesophagus by PCR and EBER in-situ hybridization. Morphologically similar carcinomas of the breast, so-called medullary carcinomas, have consistently been found to be EBV-negative (Niedobitek *et al.*, 1991b; Kumar & Kumar, 1994; Lespagnard *et al.*, 1995), as were lymphoepithelial carcinomas of the skin, thyroid gland, larynx, urinary bladder, uterine cervix and vulva (Weiss *et al.*, 1989b; Carr *et al.*, 1992; Weinberg *et al.*, 1993; Martinez-Leandro *et al.*, 1994; Requena *et al.*, 1994; Axelsen & Stamp, 1995; Gulley *et al.*, 1995; MacMillan *et al.*, 1996; Shek *et al.*, 1996).

Table 28. Presence of EBV in lymphoepithelial and other carcinomas

Cancer site and reference	Ethnicity	Morphology	Method of detection of EBV	EBV-positive/ total tested
Salivary gland				
Saemundsen *et al.* (1982)	Inuit	Lymphoepithelial	ISH	1/1
		Adenocarcinoma		0/1
Hamilton-Dutoit *et al.* (1991a)	Inuit	Lymphoepithelial-like	ISH	11/11
	Non-Inuit			0/2
Chan *et al.* (1994b)	Chinese	Lymphoepithelial	ISH	5/5
		Other		0/55
Kotsianti *et al.* (1996)	NR (Greek)	Lymphoepithelial-like	ISH	1/1
Tsai *et al.* (1996a)	Chinese	Lymphoepithelial	ISH	7/7
		Adenocarcinomas		0/49
Thymus				
Dimery *et al.* (1988)	NR (USA)	Thymoma (lymphoepithelioma)	Southern blot	1/1
Teoh *et al.* (1989)	Chinese	Thymomas (lymphoepithelial-like)	NR	2/13
McGuire *et al.* (1988)	Chinese	Thymomas	Southern blot	3/3
Leyvraz *et al.* (1985)	Hispanic	Lymphoepithelial-like	*Bam*HI W	1/1
Borisch *et al.* (1990)	NR (Switzerland)	Epithelial tumours	Southern blot	0/32
Niedobitek *et al.* (1991b)	NR (Germany)	3 lymphoepithelial-like + 11 other	ISH	0/14
Mann *et al.* (1992)	NR (USA)	Carcinoma	ISH	1/7
		Thymoma		0/14
Head and neck (other than nasopharyngeal carcinoma)				
Tyan *et al.* (1993)	Chinese	Various	PCR	30/44
Weiss *et al.* (1989b)	Caucasian and black	Lymphoepithelial	ISH	0/2
		Squamous-cell		0/4

Table 28 (contd)

Cancer site and reference	Ethnicity	Morphology	Method of detection of EBV	EBV-positive/ total tested
Tonsil				
Brichácek *et al.* (1984)	NR (Czechoslovakia)	Squamous-cell carcinoma	ISH	6/7
Niedobitek *et al.* (1991b)	NR (Germany)	Squamous-cell carcinoma	ISH	0/26
		Lymphoepithelial-like		0/2
Nicholls *et al.* (1994)	Chinese	Undifferentiated carcinoma	ISH	1/5
		Squamous-cell carcinoma		0/5
Weiss *et al.* (1989b)	Caucasian	Lymphoepithelial	ISH	0/3
		Squamous-cell carcinoma		0/1
Cervix				
Martínez-Leandro *et al.* (1994)	Caucasian	Lymphoepithelial-like	ISH	0/1
Niedobitek *et al.* (1991b)	NR (Germany)	Carcinoma	ISH	0/14
Leung *et al.* (1995a)	Chinese	Lymphoepithelial-like	ISH	0/1
Payne *et al.* (1995)	NR (United Kingdom)	Pre-invasive squamous lesions	ISH	0/3
Weinberg *et al.* (1993)	Black	Lymphoepithelial	PCR and ISH	0/1
Hilton *et al.* (1993)	Caucasian	Squamous-cell and adenocarcinomas	ISH	0/24
		Intraepithelial		0/10
Landers *et al.* (1993)	Caucasian	Squamous-cell carcinoma	PCR	8/18
		CIN-III	ISH	2/25
		CIN-II		2/25
		CIN-I + normal	PCR and ISH	0/50
Se Thoe *et al.* (1993)	NR (Malaysia)	Carcinoma	ISH	5/8
Weiss *et al.* (1989b)	Caucasian	Lymphoepithelial	ISH	0/1
Vagina				
Dietl *et al.* (1994)	NR (Germany)	Lymphoepithelial	ISH	0/1

Table 28 (contd)

Cancer site and reference	Ethnicity	Morphology	Method of detection of EBV	EBV-positive/ total tested
Vulva				
Axelsen & Stamp (1995)	NR (Denmark)	Lymphoepithelial	ISH	0/1
Testis				
Rajpert-de Meyts *et al.* (1994)	NR (Denmark)	Germ-cell	PCR EBER ISH	0/19
Breast				
Niedobitek *et al.* (1991b)	NR (Germany)	Medullary carcinoma	ISH	0/9
Kumar & Kumar (1994)	NR (USA)	Lymphoepithelial-like	ISH	0/1
Labrecque *et al.* (1995)	NR (United Kingdom)	Various	PCR EBER ISH DNA ISH	19/91 6/19 12/19
Lespagnard *et al.* (1995)	NR (Belgium)	Medullary carcinoma	PCR and ISH	0/10
Thyroid gland				
Shek *et al.* (1996)	Chinese	Lymphoepithelial	ISH	0/1
Parotid gland				
Huang *et al.* (1988)	Chinese	Undifferentiated	ISH	0/1
Gallo *et al.* (1995)	Caucasian	Undifferentiated	ISH	3/7
Urinary bladder				
Gulley *et al.* (1995)	NR (USA)	Lymphoepithelial	ISH	0/11
Skin				
Weiss *et al.* (1989b)	Caucasian	Lymphoepithelial	ISH	0/4
Carr *et al.* (1992)	White	Lymphoepithelial	ISH	0/1
Requena *et al.* (1994)	NR (Spain)	Lymphoepithelial	ISH	0/1

Table 28 (contd)

Cancer site and reference	Ethnicity	Morphology	Method of detection of EBV	EBV-positive/ total tested
Oesophagus				
Mori *et al.* (1994)	NR (Japan)	Lymphoepithelial-like Other	ISH and PCR	1/1 0/29
Colon and rectum				
Yuen *et al.* (1994)	Chinese	Adenocarcinoma	ISH	0/36
Larynx				
Brichácek *et al.* (1983)	NR (Czechoslovakia)	Poorly differentiated carcinoma	ISH	3/5
MacMillan *et al.* (1996)	NR (USA)	Lymphoepithelial-like	ISH	0/8
Paranasal sinus				
Leung *et al.* (1995b)	Chinese	29 carcinomas 8 keratinizing squamous-cell carcinomas 11 transitional-cell carcinomas 4 adenocarcinomas 2 mucoepithelial carcinomas 2 adenoid cystic carcinomas 2 undifferentiated carcinomas	ISH	7/29 4/8 1/11 1/4 0/2 0/2 1/2
Lopategui *et al.* (1994)	Asian NR (western)	NR NR	ISH	7/11 0/11
Lachrymal sac				
Leung *et al.* (1996)	Chinese	Undifferentiated lymphoepithelial carcinoma	ISH	1/1

NR, not reported; ISH, in-situ hybridization; PCR, polymerase chain reaction; CIN, cervical intraepithelial neoplasia; EBER, EBV-encoded RNA

Table 29. Presence of EBV in lung carcinomas

Reference	Ethnicity	Morphology	EBV detection method	EBV-positive/ total tested
Weiss *et al.* (1989b)	Asian Caucasian	Lymphoepithelial-like carcinoma	ISH	1/1 0/3
Pittaluga *et al.* (1993)	Chinese	Lymphoepithelial-like carcinoma Other carcinomas	ISH	5/5 0/132
Conway *et al.* (1996)	Caucasian, Hispanic	Adenocarcinoma Pleural mesothelioma	ISH	0/80 0/50
Ferrara & Nappi (1995)	Caucasian	Lymphoepithelial-like carcinoma	ISH	0/2
Wöckel *et al.* (1995)	Caucasian	Lymphoepithelial-like carcinoma	ISH and PCR	0/1
Butler *et al.* (1989)	Chinese Caucasian	Lymphoepithelial-like carcinoma	ISH	1/1 1/3
Gal *et al.* (1991)	Chinese	Lymnphoepithelial-like carcinoma	ISH and PCR	1/1
Wong *et al.* (1995)	Chinese	167 carcinomas	ISH	9/167 (all 9 lympho-epithelial-like)

ISH, in-situ hybridization

2.6.2 *Other carcinomas*

2.6.2.1 *Stomach*

EBV was first detected in gastric adenocarcinomas by Shibata and Weiss (1992), in 22 of 138 cases (16%) in the United States by PCR and DNA and EBER in-situ hybridization. This observation prompted several groups to study gastric adenocarcinomas in other areas. Studies in Europe showed a generally lower proportion of EBV-associated gastric adenocarcinomas, ranging between about 2 and 8% (Rowlands *et al.*, 1993; Ott *et al.*, 1994; Selves *et al.*, 1996b). Interestingly, in one study, no difference in the prevalence of EBV was found between tumours from the United Kingdom and from Japan (Rowlands *et al.*, 1993). A number of studies were carried out in Japan where the incidence of gastric carcinoma is very high. In several large studies comprising well over 2000 cases, EBV was detected in about 7% of cases (Tokunaga *et al.*, 1993; Fukayama *et al.*, 1994; Imai *et al.*, 1994a; Osato & Imai, 1996). Shin *et al.* (1996) found that 12 of 89 consecutive gastric tumours in Korean patients were EBV-positive; nine of these EBV-infected carcinomas were of the lymphoepithelial lymphoma type. These studies are summarized in Table 30.

Table 30. Presence in EBV in gastric carcinomas

Reference	Ethnicity	Morphology	EBV detection method	EBV-positive/ total tested
Burke *et al.* (1990)	Filipino	Lymphoepithelial-like	PCR	1/1
	NR (USA)	Adenocarcinoma		0/1
Min *et al.* (1991)	White	Lymphoepithelial-like	ISH and PCR	3/3
Shibata *et al.* (1991b)	Japanese	Lymphoepithelial-like	PCR	6/7
	Caucasian (USA)			1/1
Pittaluga *et al.* (1992)	Chinese	Lymphoepithelial-like	ISH	1/1
Shibata & Weiss (1992)	NR (USA)	Adenocarcinoma	ISH and PCR	22/138
Oda *et al.* (1993)	Japanese	Lymphoepithelial-like	ISH and PCR	13/14
		Other	ISH	2/8
Rowlands *et al.* (1993)	NR (UK) and Japanese	Intestinal	ISH	1/81
		Diffuse		0/44
		Mixed		2/28
		Unclassified		0/21
		Selected lympho-epithelial-like		6/6
Fukayama *et al.* (1994)	Japanese	Lymphoepithelial-like	ISH and PCR	6/6
		Other		2/66
Mori *et al.* (1994)	Japanese	Lymphoepithelial-like	ISH and PCR	2/2
		Other		0/29
Ott *et al.* (1994)	NR (Germany)	Lymphoepithelial-like	ISH	4/4
		Other		3/35
Tokunaga *et al.* (1993)	Japanese	Carcinoma	ISH	69/999
		Lymphoepithelial-like		8/9
Yuen *et al.* (1994)	Chinese	Intestinal	ISH	6/52
		Lymphoepithelial-like		1/3
		Diffuse		0/14
		Mixed		0/5
Harn *et al.* (1995)	Chinese	Lymphoepithelial-like	ISH and PCR	1/1
		Other	ISH	5/54
Blasco *et al.* (1996)	NR (Argentina)	Lymphoepithelial-like	ISH	1/1
Gulley *et al.* (1996)	NR (USA)	Carcinoma	ISH	11/95
		Other		3/4
Matsunou *et al.* (1996)	Japanese	Lymphoepithelial-like	ISH	22/26
		Other (synchronous)		4/4
Selves *et al.* (1996b)	NR (France)	Intestinal	ISH	1/22
		Lymphoepithelial-like		4/6
		Diffuse		0/21
		Mixed		0/10
Shin *et al.* (1996)	Korean	Adenocarcinoma	ISH	12/89
		Lymphoepithelial-like		9/10
		Normal gastric		0/37

Table 30 (contd)

Reference	Ethnicity	Morphology	EBV detection method	EBV-positive/ total tested
Vasef *et al.* (1996)	NR (USA) (1 patient with 2 tumours)	Lymphoepithelial-like N-Cell lymphoma in MALT	ISH and PCR	1/1 1/1

NR, not reported; ISH, in-situ hybridization; PCR, polymerase chain reaction; MALT, mucosa-associated lymphoid tissue

EBV has been detected in both intestinal and diffuse gastric adenocarcinomas. No difference in EBV positivity was found in carcinomas in different locations in the stomach (Rowlands *et al.*, 1993; Tokunaga *et al.*, 1993; Fukayama *et al.*, 1994). The frequency of EBV positivity in gastric adenocarcinomas was 9.2% in men and 3.1% in women in a study of 999 cases in Japan (Tokunaga *et al.*, 1993) (see also Table 30).

Viral genomes in EBV-associated gastric carcinomas have consistently been found to be monoclonal (Pittaluga *et al.*, 1992; Imai *et al.*, 1994a; Gulley *et al.*, 1996). While Fukayama *et al.* (1994) reported the detection of EBV in shed gastric epithelial cells by in-situ hybridization, evidence of viral infection of normal gastric mucosa was not found in other studies with similar methods (Rowlands *et al.*, 1993; Tokunaga *et al.*, 1993; Gulley *et al.*, 1996). Gulley *et al.* (1996) reported the detection of EBV in dysplastic gastric mucosa adjacent to areas of EBV-positive adenocarcinomas.

2.6.2.2 *Other sites*

Examination of adenocarcinomas and squamous-cell carcinomas arising at other anatomical sites for the presence of EBV has produced mostly negative results (see Table 28).

Squamous-cell carcinomas of the oesophagus and colorectal adenocarcinomas were shown not to contain EBER in two studies (Mori *et al.*, 1994; Yuen *et al.*, 1994). The number of cases investigated, however, was small and an association of the virus with a small proportion of such carcinomas cannot be excluded. In addition, since EBER transcription may be suppressed in well-differentiated squamous-cell tumours, this technique is inappropriate for detecting EBV in these tumours.

Brichácek *et al.* (1984) found that six of seven squamous-cell carcinomas of the tonsils contained EBV, but this finding was not confirmed in subsequent studies with EBV DNA and EBER in-situ hybridization (Niedobitek *et al.*, 1991b; Nicholls *et al.*, 1994). Brichácek *et al.* (1983) also reported the detection of EBV in a small group of poorly differentiated laryngeal carcinomas by DNA in-situ hybridization, in contrast to the absence of the virus in lymphoepithelial carcinomas at this site (MacMillan *et al.*, 1996).

In studies of carcinomas of the salivary glands (Chan *et al.*, 1994b) and lungs (Wong *et al.*, 1995; Conway *et al.*, 1996), no association with EBV was demonstrated.

Carcinomas of the paranasal sinus constitute a wide morphological spectrum of tumours including undifferentiated carcinomas, adenocarcinomas and squamous-cell carcinomas (Leung *et al.*, 1995b). The group of undifferentiated sinonasal carcinomas includes some cases with morphological features similar to those of nasopharyngeal carcinoma. In two studies, EBV was detected in at least some sinonasal carcinomas arising in Asian patients (Lopategui *et al.*, 1994; Leung *et al.*, 1995b). The EBV-positive cases included undifferentiated carcinomas, squamous-cell carcinomas and adenocarcinomas. None of the undifferentiated sinonasal carcinomas arising in western patients was EBV-positive (Lopategui *et al.*, 1994).

EBV was detected by DNA PCR in 19 of 91 cases of breast carcinoma. Of the 18 cases found to contain EBV by PCR, six were also shown to be EBV-positive by EBER in-situ hybridization, and 12 of 18 tested were EBV-positive by *Bam* HI W in-situ hybridization (Labrecque *et al.*, 1995). [The Working Group noted that it was not clear whether some cases were positive by both methods.]

Fuelled by a report suggesting that the cervix may be a site of EBV shedding (Sixbey *et al.*, 1986), several groups examined the possible association of EBV infection with cervical carcinoma. Using in-situ hybridization with biotinylated probes, Landers *et al.* (1993) found EBV DNA in 8% of grade-II cervical intraepithelial neoplasia (CIN), in 8% of CIN III and in 43% of cervical carcinomas. A similar finding was reported by Se Thoe *et al.* (1993). Other studies in which EBV DNA or EBER in-situ hybridization was used consistently failed to detect the virus in CIN lesions or in invasive cervical carcinomas (Niedobitek *et al.*, 1991b; Hilton *et al.*, 1993; Payne *et al.*, 1995).

EBV DNA was detected by Southern blot hybridization in some thymomas, i.e. benign epithelial tumours of the thymus, and in thymic carcinomas, both often showing a prominent reactive lymphoid cell infiltrate (Leyvraz *et al.*, 1985; Dimery *et al.*, 1988; Katzin *et al.*, 1988; McGuire *et al.*, 1988; Teoh *et al.*, 1989). These tumours were mainly obtained from Asian patients. Subsequent studies by PCR and in-situ hybridization of thymic epithelial tumours in western patients provided no convincing evidence of an association with EBV (Borisch *et al.*, 1990; Niedobitek *et al.*, 1991b; Mann *et al.*, 1992).

2.6.3 *Smooth-muscle tumours*

Immunosuppressed transplant recipients and HIV-infected patients are at increased risk for smooth-muscle tumours, both benign leiomyomas and malignant leiomyosarcomas (Mueller *et al.*, 1992; Lee *et al.*, 1995b). The increase is observed primarily in immunosuppressed children; cases in adults are observed rarely. The tumours that occur in this situation have frequently been found to contain EBV.

McClain *et al.* (1995) tested five leiomyosarcomas and two leiomyomas from five HIV-infected children aged less than nine years and one HIV-infected adult, aged 24, by EBER-1 in-situ hybridization. Essentially all cells in all seven tumours showed positive signals. In contrast, none of seven smooth-muscle tumours (three leiomyosarcomas and four leiomyomas) from seven HIV-negative children (under 15) were EBV-positive. EBV clonality was assessed in two of the HIV-associated cases of leiomyosarcoma: one

showed biclonality; the other consisted of two tumours at different sites, each of which contained a different clone of EBV.

Lee *et al.* (1995b) tested six smooth-muscle tumours from three children under six years of age who had undergone immunosuppression for liver allotransplantation. Most cells in all six tumours were shown to contain EBV by EBER-1 in-situ hybridization; all three tumours contained monoclonal EBV. Southern blot analysis of one tumour suggested that the EBV DNA was associated with host sequences, indicating viral integration. When tested by immunohistochemistry, all six tumours were LMP-negative but three of three tumours tested were EBNA-2-positive.

These results were corroborated by additional case reports of EBV-positive smooth muscle tumours in AIDS patients (van Hoeven *et al.*, 1993; Prévot *et al.*, 1994) and transplant recipients (Timmons *et al.*, 1995; Kingma *et al.*, 1996; Morel *et al.*, 1996), although an EBV-negative case was described in one transplant recipient (van Gelder *et al.*, 1996).

2.6.4 *Other tumours*

Conway *et al.* (1996) reported the absence of the virus in a series of mesotheliomas. When PCR and EBER in-situ hybridization were used, no evidence of EBV was found in testicular germ-cell tumours (Rajpert-de Meyts *et al.*, 1994).

3. Studies of Cancer in Animals

3.1 EBV in non-human species

Considerable efforts have been made to develop suitable animal models to study EBV infection and pathogenesis; however, such attempts have had little success, because Old World primates carry their own EBV-like virus and cross-reacting antibody (Kieff, 1996). New World primates do not carry EBV homologues, however, and at least five species appear to be infectable by EBV, i.e. the cotton-topped tamarin (*Saguinus oedipus oedipus*), white-lipped marmoset (*Saguinus fuscicollis*) and common marmoset (*Callithrix jacchus*), owl monkey (*Aotus trivirgatus*) and squirrel monkey (*Saimiri sciureus*). The most susceptible non-human primate for the carcinogenicity of EBV is the cotton-topped tamarin. This is, however, an endangered species and not widely available for use in laboratories. When common marmosets are infected with EBV, only some animals develop an infectious mononucleosis-like syndrome (Wedderburn *et al.*, 1984).

3.1.1 *Infection of non-human primates with EBV*

3.1.1.1 *New World primates*

The special susceptibility of cotton-topped tamarins to EBV is not well understood. The response to EBV infection varies from occult infection to benign lymphoproliferation to malignant lymphoma (Shope *et al.*, 1973; Deinhardt *et al.*, 1975; Werner *et al.*, 1975; Miller *et al.*, 1977; Rabin *et al.*, 1977; Johnson *et al.*, 1983; Cleary *et al.*, 1985;

Niedobitek *et al.*, 1994). The lymphomas induced in cotton-topped tamarins are morphologically similar to EBV-associated lymphomas in humans (Rabin *et al.*, 1977; Cleary *et al.*, 1985) and are frequently observed in the mesenteric nodes and small intestine. Each tumour nodule in an animal has a dominant clonotype, but many different clones are found in different locations.

Shope *et al.* (1973) induced malignant lymphomas in four of eight cotton-topped tamarins inoculated with cell-free EBV extracted from a lymphoblastoid cell line (B95-8) originally transformed *in vitro*. The first tumour developed after 222 days.

Epstein *et al.* (1973) reported that one of three owl monkeys inoculated with EB3 cells from a Burkitt's lymphoma (frozen and thawed) developed lymphoproliferative disease without evidence of true lymphoma. Antibodies to VCA were detected in the serum obtained from this animal.

Leibold *et al.* (1976) inoculated EBV-transformed squirrel monkey lymphocytes (6–12 $\times$ 10^8 cells) by intraperitoneal or subcutaneous injection into three squirrel monkeys. All three animals developed multiple undifferentiated malignant lymphomas and died 8–10 days after inoculation. Necropsy specimens showed the presence of EBV DNA and contained 7–21 genomes per cell. Lymphoblastoid cell lines were established from the tumour tissue and were found to express EBNA and EA.

Werner *et al.* (1975) showed that inoculation of concentrated EBV from a transforming (Kaplan) mononucleosis cell line resulted in malignant lymphoproliferation in one of three cotton-topped tamarins six weeks after inoculation, whereas inoculation of non-transforming EBV (P3HR-1) did not result in lymphoproliferation. The tumour induced by the Kaplan cell line was a lymphosarcoma and contained EBV DNA. The tumour-bearing animals produced anti-VCA antibodies.

Sundar *et al.* (1981) reported that one of six white-lipped marmosets inoculated with a transforming EBV (B95-8) developed diffuse malignant lymphoma within 18 weeks. EBV-DNA was detected in the pathologically enlarged mandibular lymph nodes, and the animal had an anti-VCA antibody titre of 1:320.

In a study in which eight cotton-topped tamarins were inoculated with sufficient quantities of transforming EBV (B95-8), multiple tumours were induced in each animal within 14–21 days which were identified histologically as large-cell lymphomas. The tumour cells contained multiple copies of the EBV genome. Cell lines derived from the tumours showed no chromosomal abnormalities. Hybridization of tumour DNA with immunoglobulin gene probes revealed that the tumours were oligo- or monoclonal in origin; in each animal, individual tumours arose from different B-cell clones (Cleary *et al.*, 1985).

Horizontal transmission of EBV in common marmosets was demonstrated when two animals experimentally infected with EBV (from M81-transformed cells) were paired with two animals seronegative to EBV. The seronegative animals seroconverted after four to six weeks, and the presence of EBV DNA was demonstrated in their peripheral blood lymphocytes (Cox *et al.*, 1996).

3.1.1.2 *Old World primates*

Levine *et al.* (1980a) inoculated 42 rhesus monkeys (*Macaca mulatta*), four chimpanzees (*Pan troglodytes*) and one cynomolgus monkey (*Macaca fascicularis*) with non-transforming EBV (P3HR-1). None of the animals developed any clinical manifestation or tumour within three to eight years. [The Working Group noted that the lack of response in this study could be due either to the use of a non-transforming EBV strain or the presence in these primates of their own EBV-like herpesvirus (see section 3.2).]

3.1.2 *Transformation of monkey cells by EBV* in vitro

Desgranges *et al.* (1976) transformed peripheral blood mononuclear cells of common marmosets with concentrated EBV (HKLY-28 virus) obtained from a nasopharyngeal carcinoma lymphoblastoid cell line. The monkey cells produced more EBV than the original human cell line, and the transforming activity of the EBV obtained from monkey cells was similar to that of EBV from B95-8 cell lines.

Ablashi *et al.* (1977) showed that squirrel monkey peripheral blood mononuclear cells infected with transforming EBV (B95-8) were immortalized and that these cells contained EBV DNA.

Griffin and Karran (1984) showed that specific EBV DNA fragments could immortalize a subpopulation of epithelial cells of the African green monkey (*Cercopithecus aethiops*). EBV-related sequences were observed in all the established cell lines.

3.1.3 *Rodent models for EBV infection and pathogenesis*

3.1.3.1 *Severe combined immunodeficiency (SCID) mouse model*

The SCID mouse model has been widely used to study lymphomas induced by EBV *in vivo*, EBV protein expression and specific therapies against EBV-induced lymphoproliferation (e.g. anti-CD40 and anti-CD23 monoclonal antibodies) (Garnier *et al.*, 1993; Funakoshi *et al.*, 1995; Katano *et al.*, 1996). SCID mice lack mature B and T cells but can be immunologically reconstituted to a certain extent by xenografts of human leukocytes. If the lymphocytes are derived from human EBV-seropositive donors, the animals frequently develop multiple foci of proliferating EBV-positive human B cells within 10–16 weeks. The SCID mouse lesions are composed of immunoblasts and plasmacytoid cells.

In SCID mice grafted with EBV-positive human peripheral blood mononuclear cells, Okano *et al.* (1990) detected EBV-positive lymphoproliferative lesions that expressed LMP and B-lymphocyte activation antigen (CD23) and adhesion molecules (ICAM-1 and CD18). The authors suggested that these lymphoid lesions in SCID mice were comparable to EBV-immortalized lymphoblastoid cells and not to malignant lymphomas like Burkitt's lymphoma.

SCID mice were grafted with peripheral blood lymphocytes from immunodeficient patients with X-linked lymphoproliferative syndrome. Mice receiving lymphocytes from EBV-seronegative patients were then infected with B95-8 EBV. The mice developed EBV-induced oligoclonal or polyclonal disease comparable to that occurring in both

immunodeficient patients and normal immunocompetent donors. The lesions were diffuse, monomorphic and human B-cell-related, and activated associated antigens were present. Analysis of the sera revealed that the B-cell populations were oligoclonal or polyclonal; however, phenotypic and cytogenetic analysis did not reveal the monoclonality generally observed in Burkitt's lymphoma (Nakamine *et al.*, 1991; Purtilo *et al.*, 1991).

Walter *et al.* (1992) compared the local growth of a Burkitt's lymphoma with the disseminated, invasive growth of EBV-immortalized lymphoblastoid cells in SCID mice. The lymphoma grew progressively but was locally restricted, whereas the lymphoblastoid cells were both locally invasive and disseminated into lymphoid tissue.

Katano *et al.* (1996) studied the lymphoid tumours induced in SCID mice after grafting of human EBV-infected B cells. Molecular analysis of the tumours revealed expression of EBV LMP-1 molecules at quantitatively different levels in the individual tumours.

[The Working Group noted that the SCID mouse model is limited to the study of lymphoproliferative disorders.]

3.1.3.2 *Nude mouse model*

Ablashi *et al.* (1978) inoculated EBV-positive P3HR1 cells (1.0×10^6 cells in 0.2 mL medium) into 30 athymic nude mice. All animals developed lymphoid tumours after an average latent period of 36 days. The tumours were poorly differentiated lymphomas of varied cell size and shape. The nuclei were variably hyperchromatic and were reminiscent of those of Burkitt's lymphoma. EBNA was detected in 80% of these cells and in 90% of the cells used for the inoculum, but VCA was detected in only 2% of the cell lines obtained from the tumour and in 13% of cells in the inoculum.

Yang *et al.* (1982) inoculated cells from 36 biopsy samples of nasopharyngeal carcinoma into BALB/c nude mice. In three mice, the inoculum grew to detectable tumours with metastases within 20–43 days. The tumours were then passaged 6–15 times over a period of 12–22 months into other groups of nude mice, with an average take of 67–73%. Touch smears from the tumours showed the presence of EBNA. Only two of four of the cell cultures derived from tumours expressed VCA after induction with 5-iodo-2′-deoxyuridine. Co-cultivation of epithelial cells with human cord blood mononuclear cells provoked transformation of the latter, and the transformed cells produced IgM.

3.2 EBV-like viruses isolated from non-human primates

The *Gammaherpesvirinae* subfamily of Herpesviridae is subdivided into two genera: *Lymphocryptovirus* (e.g. HHV4 or EBV) and *Rhadinovirus* (e.g. ateline herpesvirus-2) (Roizman *et al.*, 1992). For further details of the classification of herpesviruses that infect animals, see the Introduction.

Lymphocryptoviruses (so-called gamma-1) include EBV-like viruses isolated from Old World primates such as chimpanzees (*Pan troglodytes*), baboons (*Papio hamadryas*) and orangutans (*Pongo pygmaeus*). The gamma-1 lymphocryptoviruses are B lympho-

tropic, transforming, generally restricted in their host range and similar to each other in genomic organization (Kalter, 1994). Their genomes are collinearly homologous to that of EBVand are more closely related to that of the saimiri herpesvirus-1, a member of the *Rhadinovirus* genus, than to genomes of the beta- or alphaherpesviruses.

Non-human primate rhadinoviruses (gamma-2 herpesviruses) are discussed in the monograph on KSHV/HHV8.

Lymphocryptoviruses cross-react antigenically with EBV and can transform or immortalize cells. These viruses are transmitted horizontally and infect their host at the juvenile stage, inducing the production of specific antibodies (Deinhardt & Deinhardt, 1979). The first isolation of a lymphocryptovirus in non-human primates was reported by Landon *et al.* (1968), who established a permanent lymphoblastoid cell line from a healthy chimpanzee in which they detected a herpesvirus. A similar virus was reported in chimpanzees (Gerber *et al.*, 1976b), which was B-lymphotropic and transformed human and other primate cells. Since the first isolation of *Herpesvirus pan* (pongine herpesvirus 1) from chimpanzees (Gerber *et al.*, 1977), the following primate lymphocryptoviruses have been reported: *Herpesvirus gorilla* (pongine herpesvirus 3; Neubauer *et al.*, 1979a), *Herpesvirus pongo* (pongine herpesvirus 2) from orangutans (Rasheed *et al.*, 1997), *Herpesvirus papio* (cercopithecine herpesvirus 12) from baboons (Falk *et al.*, 1976), *Herpesvirus cercopithecus* (cercopithecine herpesvirus 14) from African green monkeys (Böcker *et al.*, 1980) and herpesviruses from rhesus monkeys (*Macaca mulata*; Rangan *et al.*, 1986), cynomolgus monkeys (*M. fascicularis*; Feichtinger *et al.*, 1992; Li *et al.*, 1994) and *M. arctoides* (Lapin *et al.*, 1985).

The double-stranded DNA of these viruses has a molecular mass of about 110×10^6, and the genome of cercopithecine herpesvirus 12 (baboons) and pongine herpesvirus 1 (chimpanzees) is about 170 kb. The virion has a diameter of 118–220 nm. The viral morphogenesis is similar to that of EBV. The natural host range is limited to the family or order of its host. DNA sequence homology among primate gamma-1 herpesviruses is approximately 40%. The DNAs of cercopithecine herpesvirus 12 and pongine herpesvirus 1 show extensive collinear homology (Lee *et al.*, 1980, 1981; Kieff, 1996).

3.2.1 Herpesvirus papio *(cercopithecine herpesvirus 12)*

Agrba *et al.* (1975) reported the establishment of two lymphoblastoid cell lines (SPG-1, KMPG-1) from bone marrow and spleen of hamadryas baboons with malignant lymphomas in the Sukhumi colony. Herpesvirus particles were detected in these cell lines by electron microscopy. Upon further investigation, other herpesvirus types (HSV-1, HSV-2, cytomegalovirus) were ruled out. The virus was later named *Herpesvirus papio* (HVP). Increased antibody titres to this virus were demonstrated in baboons which developed lymphoma (Voevodin *et al.*, 1983).

A similar virus was isolated in the United States from several baboon colonies (*Papio hamadryas*, *P. ambis*, *P. cynocephalus*) by Falk *et al.* (1976). Antibodies that reacted with viral antigens in both HVP and EBV cell lines (P3HR1, B95-8) were demonstrated. All of the established cell lines were B cell-derived, and 5–15% contained antigens (see also Falk, 1979).

HVP is indigenous to Africa and is restricted to that continent; however, animals bearing the virus have been transported throughout the world. Although no specific seasonal variation in infection is known in the wild, peak infection in the Sukhumi colony occurred in the autumn and spring (Kalter, 1994).

3.2.1.1 *Cell lines, persistence and transformation*

To date, the cell lines established from peripheral blood mononuclear cells or other tissues of HVP-infected tumour-bearing animals have been B lymphocytes (Falk, 1979), and infected monkey and human cord blood mononuclear cells exhibit B-cell markers; however, the markers are not uniform (Deinhardt & Deinhardt, 1979). HVP transforms lymphocytes of several species of non-human primates (*Macaca mulatta*, *M. arctoides*, *Hylobates lar*, *Saguinus* sp., *Callithrix jacchus*, *Saimiri sciureus*) as well as human cord blood mononuclear cells *in vitro*. The presence of the virus can be demonstrated by the presence of antigens (Ohno *et al.*, 1978; Falk, 1979). The nuclear antigen of HVP is similar to EBNA. In a comparative study, all EBNA-positive sera reacted with HVP nuclear antigen, but none of the sera containing this antigen reacted with EBNA.

3.2.1.2 *Prevalence of infection with* Herpesvirus papio

Voevodin *et al.* (1985), using an assay to detect anti-VCA to HVP, showed that the prevalence of antibody in two groups of baboons, one with a high prevalence of lymphoma and the other lymphoma-free, was 80–90% in both groups, in contrast to the results of previous studies in which complement fixation was used (Voevodin *et al.*, 1979). Newly imported baboons had a significantly lower prevalence (35%). Moreover, the prevalence of infection was age-dependent, increasing during the first years of life, reaching the highest rate (100%) at five years of age, staying stable up to 18 years of age and decreasing subsequently. Infection in newly imported animals increased with age up to 71%. Nevertheless, when antibodies to early, capsid and nuclear antigens were compared in baboons in the Sukhumi colony, animals with lymphoid disease had higher frequencies and higher titres than did control animals (Neubauer *et al.*, 1979b).

3.2.1.3 *Molecular biology*

The organization of the genome of HVP shows similar unique features to that of EBV DNA (see section 1.1) and shows 40% homology with EBV DNA (Lee *et al.*, 1980). Replication of HVP involves both *cis*- and *trans*-acting functions, analogous to those found in EBV (Pesano & Pagano, 1986). For more information, see Lee *et al.* (1980, 1981) and Kieff (1996).

It has been shown that non-producer HVP cell lines have two or three viral genome equivalents per cell in an apparently integrated form, whereas in HVP producer cell lines the HVP-free DNA fraction is composed of both linear and circular DNA molecules that are similar in length to the circular DNA of EBV in human cell lines, as shown by electron microscopy (Falk *et al.*, 1979).

Howe and Shu (1988) showed that two small RNAs of HVP hybridize to EBERs, and two genes of HVP RNAs, *HVP-1* and *HVP-2*, are located together in a small unique

region at the left end of the viral genome and are transcribed by RNA polymerase III in a rightward direction, like the *EBER*s. There is considerable similarity between *EBER-1* and *HVP-1* RNA, except for an insert of 22 nucleotides which increases the length of *HPV-1* RNA to 190 nucleotides. *HVP-2* RNA and *EBER-2* are less similar, but both are about 170 nucleotides long. Like EBERs, *HVP* RNAs are abundant in HVP-transformed cells and are efficiently bound to cellular proteins. EBNA-1 and EBNA-2 of EBV do not cross-react well with HPV nuclear antigen, and cloning of *EBNA-2* of the HVP homologue showed significant nucleotide and amino acid divergence, as predicted by serological studies. The HVP *EBNA-2* homologue does not retain the acidic transactivating domain of EBV *EBNA-2*. This gene, as in EBV, is important in B-cell immortalization and the resultant alterations in B-cell growth pattern which regulate expression from specific viral and cellular promoters. Ling *et al.* (1993b) showed that, in comparison with the *EBNA-2* of EBV, all of which are either type 1 or type 2, the HVP *EBNA-2* gene falls into neither type 1 or type 2. The divergence of *EBNA-2* of HVP from that of EBV is thus ideally suited for comparative studies. Similarities between the EBV and the HVP *LMP-2* gene indicate that the latter is important in infection *in vivo* (Franken *et al.*, 1995).

These molecular biological studies with HVP provide not only an understanding of the action and function of various viral and cellular genes but also a unique opportunity to study their role in pathogenesis, because they can infect, replicate and cause B-cell lymphoproliferation in various non-human primates.

3.2.1.4 *Pathogenesis and immune response to* Herpesvirus papio

HVP is a potent transforming agent, since it can efficiently transform human and non-human primate peripheral blood mononuclear cells. Falk *et al.* (1976) established three lymphoblastoid cell lines from the splenic lymphocytes of a lymphomatous baboon (*Papio hamadryas*) in which they could identify the presence of HVP. Adult or newborn marmosets of three species (*Saguinus oedipus oedipus*, *S. fascicollis*, *Callithrix jacchus*) were inoculated with lymphoblastoid cells derived from this baboon (Deinhardt *et al.*, 1978). All six adult animals developed moderate leukocytosis, relative lymphocytosis beginning 7–16 days after inoculation. Three of the animals, two of which also had developed large tumours at the site of inoculation, developed marked generalized lymphadenopathy and died 13–22 days after inoculation. None of the eight newborn marmosets died after inoculation; they developed only minimal lymph-node swelling during the first two to three weeks after inoculation, without haematological abnormalities.

Gerber *et al.* (1977) inoculated two HVP-seronegative baboons (*Papio cynocephalus*), two rhesus monkeys (*Macaca mulatta*) and two cotton-topped tamarins (*Saguinus oedipus oedipus*) either intraperitoneally, intravenously or subcutaneously, with a total of 3.0 ml of HVP with a transforming titre of 10^{3-5} units derived from a spontaneously transformed baboon lymphoid cell line. The animals were kept for six months. Both baboons seroconverted, and HVP was excreted by the oropharyngeal route. Seroconversion was seen in the rhesus monkeys but not the tamarins, and neither species excreted detectable

levels of HVP from the oropharynx. None of the inoculated animals had detectable disease or palpable tumours.

3.2.2 *Gamma-1 herpesvirus from cynomolgus monkey (*Macaca fascicularis*)*

Fujimoto *et al.* (1990) established a B-lymphoblastoid cell line from lymph nodes of an apparently healthy cynomolgus monkey (*Macaca fascicularis)* in which EBV-like herpesvirus was demonstrated. The cell-free supernatant from the cell line transformed the lymphocytes of another cynomolgus monkey.

Feichtinger *et al.* (1992) inoculated cynomolgus monkeys with simian immunodeficiency virus type 1, and about 40% of the animals developed B-cell lymphoma. A herpesvirus termed HVMF1 was detected in all tumours tested, and lymphoma tissue and two established cell lines reacted with EBV-specific DNA probes (Li *et al.*, 1993, 1994). Homology to EBV was found in *EBNA-2*, *EBNA-5*, *EBERs* and *oriP*, and proteins that cross-reacted with EBNA-1 and EBNA-2 were identified by western blotting. On the basis of the close homology with EBV, the investigators proposed that malignant B-cell lymphoma in cynomolgus monkeys experimentally infected with simian immunodeficiency virus could be used as a model for EBV-associated lymphomagenes is in immunodepressed persons.

3.2.3 *Rabbit model of malignant lymphoma induced by EBV-like virus from* Macaca arctoïdes

An EBV-like herpesvirus isolated from *Macaca arctoïdes* was successfully used to induce malignant lymphoma in rabbits (*Oryctolagus cuniculus*). Sixteen out of 32 infected rabbits became ill between days 21 and 143 after inoculation, and in 15/16 animals with clinical signs, lymphoproliferative disease was shown by histological examination. The disease consisted either of malignant lymphoma or lymphoid hyperplasia of the spleen and parenchymal organs (Wutzler *et al.*, 1995).

3.3 Other models of relevance to EBV

3.3.1 *Murid herpesvirus 4*

Murid herpesvirus 4, or MHV-68, is a B-lymphotropic gammaherpesvirus which on phylogenetic grounds was classified as a gamma-2 herpesvirus. It induces B-lymphoproliferative disease in mice and has therefore been proposed as a model for EBV infection. As it is a gamma-2 herpesvirus, it is discussed in the monograph on KSHV/-HHV8.

3.3.2 *Marek's disease*

Marek's disease virus (MDV), first described by Josef Marek in 1907, has clearly played a major role in comparative herpesvirology and oncology (Calnek, 1986). MDV is a lymphotropic herpesvirus which, after an early cytolytic infection, induces T-cell lymphomas in chickens, its natural host. The lymphoma cells are latently infected with MDV, but the viral contribution to the transformed phenotype is not fully understood.

The integration pattern of MDV DNA suggests the clonal nature of tumour formation, and MDV-transformed cell lines established *in vitro* maintain the integration pattern of primary lymphomas (Delecluse *et al.*, 1993b).

Malignant lymphomas occur in a variety of organs and tissues, including the spleen, gonads, liver, lungs, heart, mesentery, bursa of Fabricius, thymus, adrenal gland, pancreas, proventriculus, intestine, skeletal muscle and skin. The various strains of MDV have different organ distributions. Marek's disease is also characterized by the formation of lesions in peripheral nerves, the spinal roots and/or the root ganglia. The affected peripheral nerves show loss of cross-striations, enlargement, discolouration and sometimes oedema. Affected spinal root ganglia become enlarged and appear translucent and somewhat discoloured (Calnek & Witter, 1991).

MDV is transmitted through direct or indirect contact between chickens. The fully infectious virus becomes airborne when it is shed from the feather follicle epithelium during skin keratinization (Calnek *et al.*, 1970). Both diseased and apparently unaffected chickens can continually shed MDV continuously for many weeks (Kenzy & Cho, 1969).

Propagation of the causative agent in cell culture (Churchill & Biggs, 1967) was followed by transmission of the disease with the cell culture-propagated virus (Churchill & Biggs, 1968). Eventual transmission of the disease with cell-free infectious herpesvirus particles isolated from the feather follicles of infected chickens was the definitive study that dispelled doubt that MDV is the causative agent of Marek's disease (Calnek *et al.*, 1970).

Marek's disease is the first, most important system in which a herpetic affliction and/or a neoplastic condition has been successfully controlled by vaccination. Vaccination against Marek's disease was first demonstrated in 1969 with a serotype-1 strain attenuated by passage in cell culture (Churchill *et al.*, 1969). Herpesvirus of turkeys, isolated in 1969 (Kawamura *et al.*, 1969), provides good protection against Marek's disease (Okazaki *et al.*, 1970).

Three avian herpesviruses share common antigen determinants: Marek's disease herpesvirus 1 (gallid herpesvirus 2), Marek's disease herpesvirus 2 (gallid herpesvirus 3) and turkey herpesvirus 1 (maleagrid herpesvirus 1) (Witter *et al.*, 1970).

A major step in MDV virology was made by Buckmaster *et al.* (1988), who showed that MDV and turkey herpesvirus sequences bear greater similarity to varicella-zoster virus (human herpesvirus 3), an alphaherpesvirus, than to EBV sequences, and that the MDV and turkey herpesvirus genomes are collinear with that of varicella-zoster virus. Therefore, even if some biological aspects of Marek's disease resemble EBV infection in humans, it cannot be considered virologically to be a true model for human EBV infection because MDV is clearly an alphaherpesvirus.

4. Other Data Relevant to an Evaluation of Carcinogenicity and Its Mechanisms

4.1 Growth transformation

4.1.1 *Role of EBV*

In view of the role of viral gene products in the development of non-Hodgkin's lymphoma, it is clear that an important factor is the viral gene functions that have been shown to be involved in growth transformation of normal resting human B lymphocytes (Henle *et al.*, 1967; Pope *et al.*, 1968). Growth transformation by EBV is regarded as an in-vitro equivalent of the lymphoproliferation occurring in EBV-infected individuals in the absence of a functional immune system. Growth transformation by EBV *in vitro* is defined as the induction of immortalization. EBV-infected, proliferating B lymphocytes are similar to lymphocytes activated in response to antigens, mitogens or treatment with IL-4 and anti-CD40 (Banchereau & Rousset, 1991), resulting in expression of a similar repertoire of activation markers and adhesion molecules (Åman *et al.*, 1986; Hurley & Thorley-Lawson, 1988; Alfieri *et al.*, 1991). EBV-infected tumour cells in non-Hodgkin's lymphomas *in vivo*, however, probably also have genetic and epigenetic changes, in addition to the phenotype of the immortalized B lymphocyte studied *in vitro*.

In EBV-transformed B cells, only a small number of viral genes is expressed (see section 1.1.5 for nomenclature), including those for six nuclear antigens, EBNA-1, -2, -3A, -3B, -3C and EBNA-LP, the genes for three membrane proteins, LMP-1, LMP-2A and LMP-2B (two splice variants of LMP-2), *BHRF1*, *BARF0* and two small nuclear non-polyadenylated RNAs (EBERs). *BHRF1* encodes a viral homologue of Bcl-2 (Cleary *et al.*, 1986) which is highly expressed during the lytic cycle. *BHRF1* has also been reported to be transcribed during latency in EBV-immortalized cells (Austin *et al.*, 1988; Oudejans *et al.*, 1995a), while the BHRF1 protein has not been detected in latently infected cells. *BARF0*, a reading frame contained in a set of transcripts first described in nasopharyngeal carcinomas passaged in nude mice (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b, 1991), has recently been shown to encode a 30-kDa protein in lymphoblastoid cell lines and nasopharyngeal carcinoma samples (Fries *et al.*, 1997).

4.1.2 *Minimal set of transforming genes*

The transforming potential of EBV is maintained within about one-third of the viral genome (Mark & Sugden, 1982; Kempkes *et al.*, 1995a; Robertson & Kieff, 1995). Genetic analyses of recombinant viruses have also shown that not all of the genes expressed in growth-transformed cells are required for initiation and maintenance of transformation. Neither the *EBER*s (Swaminathan *et al.*, 1991) nor *EBNA-3B* (Tomkinson & Kieff, 1992a), *BARF0* (Robertson *et al.*, 1994), *EBNA-LP* (Hammerschmidt & Sugden, 1989) or *LMP-2* is required for transformation (Longnecker *et al.*, 1992; Kim & Yates, 1993). Viral *IL-10*, a gene of the lytic cycle with suspected B-cell growth factor activity, is also not required (Swaminathan *et al.*, 1993); however, *EBNA-LP* and *LMP-2* were

shown to significantly improve the outgrowth of transformed cells (Mannick *et al.*, 1991; Allan *et al.*, 1992; Brielmeier *et al.*, 1996). *EBNA-1*, *EBNA-2*, *EBNA-3A*, *-3C* and *LMP-1* are essential for initiation of transformation (Cohen *et al.*, 1989; Kaye *et al.*, 1993; Tomkinson *et al.*, 1993). *EBNA-1* (Yates *et al.*, 1984, 1985), *EBNA-2* (Kempkes et al., 1995b) and *LMP-1* (Sandberg et al., 1997) are also definitely required for maintenance of transformation, whereas *EBNA-3A* is not (Kempkes *et al.*, 1995a). Information on the role of *EBNA-3C* in the maintenance of transformation is not available. The minimal set of viral genes required for the initiation and maintenance of growth transformation is still unknown, however, and the question of whether the combination of *EBNA-1*, *EBNA-2*, *EBNA-3A*, *EBNA-3C* and *LMP-1* is also sufficient for transformation has not yet been addressed experimentally. Functional redundancy among the non-essential genes might permit the deletion of one but not several simultaneously.

4.1.3 *Growth transformation* in vitro *and induction of lymphoproliferation* in vivo

It is not known whether induction of B-cell proliferation by EBV in an immunocompromised host *in vivo* requires the same set of viral genes as EBV-induced B-cell transformation *in vitro*, or a larger or smaller set of viral genes. The genes required for growth transformation *in vitro* are obviously most likely to be involved in the induction of lymphoproliferation *in vivo* and therefore represent the main focus here. It should be kept in mind, however, that the genes required *in vitro* are not necessarily those required *in vivo*. Proliferating cells may receive signals from their surroundings that may bypass the requirement for an active viral oncogene. Alternatively, genes that are unnecessary for growth transformation i*n vitro* may be absolutely essential for the induction of lymphoproliferation *in vivo*. Given the complexity of the virus–host interaction, which has evolved over millions of years, this is the more likely possibility. The complete life cycle of the virus is influenced by its interaction with the immune system of the host *in vivo* and therefore plays an important role in the development of EBV-associated malignancies. Control of latency *in vivo*, reactivation, re-entry into the lytic cycle, reinfection of new target cells and interference by the immune system at any of these levels are therefore of key importance for EBV-induced lymphoproliferation *in vivo*. These events not only implicate the viral genes involved in growth transformation and their cellular targets but also reflect the complexity of the virus–host interaction *in vivo*, which can be studied only in a model system of a related primate virus and its natural host.

4.1.4 *Viral transcription pattern after infection of human primary B lymphocytes by EBV*

Infection of B cells with EBV is mediated through CD21, the receptor for complement component C3d (Fingeroth *et al.*, 1984). Binding of the virus triggers a signalling cascade initiated from the CD21 molecule, which activates the cell and gives it the competence to respond to the growth-promoting action of *EBNA-2* and *EBNA-LP* (Hutt-Fletcher, 1987; Sinclair *et al.*, 1994). The viral genome is circularized within about 16–20 h after infection (Hurley & Thorley-Lawson, 1988) and gives rise to large transcripts initiated in the W promoter of the large internal repeats (Woisetschlaeger *et al.*, 1990).

After expression of *EBNA-1* and *EBNA-2*, transcription switches to the C promoter and gives rise to multiply spliced transcripts that code for EBNA-LP, EBNA-2, EBNA-3A, -3B, -3C and EBNA-1 (Schlager *et al.*, 1996; Woisetschlaeger *et al.*, 1990). *OriP*, to which EBNA-1 binds, acts not only as an origin of replication (Yates *et al.*, 1984) but also as an EBNA-1-dependent enhancer of the C promoter (Sugden & Warren, 1989) and the *LMP-1* promoter (Gahn & Sugden, 1995). C promoter transcription occludes the W promoter and brings the viral genome under its own transcriptional control (Puglielli *et al.*, 1996). Expression of the *LMPs* is under transcriptional control of *EBNA-2*. *LMP-1* and *LMP-2B* are transcribed from a bi-directional promoter that responds to *EBNA-2* (Laux *et al.*, 1994a; Johannsen *et al.*, 1995), whereas *LMP-2A* is transcribed from a separate promoter controlled by *EBNA-2* (Zimber-Strobl *et al.* , 1993).

4.1.5 *Viral proteins involved in growth transformation*

4.1.5.1 *EBNA-1*

EBNA-1 is the only viral protein expressed in growth-transformed cells that binds directly to DNA in a sequence-specific manner through its DNA binding and dimerization domain in the C terminus (Ambinder *et al.*, 1991; Bochkarev *et al.*, 1995). EBNA-1 binds to the plasmid origin of replication (*oriP*) which consists of multiple tandem repeats of EBNA-1 binding sites followed by two symmetrical binding sites (Rawlins *et al.*, 1985; Reisman *et al.*, 1985). Cooperative binding of EBNA-1 to the multiple binding sites on *oriP* is required, and sufficient, for episomal replication of the viral genome. Furthermore, *oriP* acts as an EBNA-1-dependent enhancer and plays a crucial role in the regulation of viral transcription from both the C and the *LMP-1* promoter in growth-transformed cells (Sugden & Warren, 1989; Gahn & Sugden, 1995). Because of their role in episomal replication and transcription regulation, EBNA-1 and *oriP* are of prime importance for EBV-induced proliferation of lymphocytes *in vitro* and *in vivo*.

Apart from its role in regulation of viral replication and transcription, EBNA-1 might also affect cellular replication and transcription if binding sites for this protein exist in the cellular genome. Comparison of EBV-negative Burkitt's lymphoma cell lines with EBV-positive group I Burkitt's lymphoma cell lines expressing EBNA-1, however, indicates that the growth pattern and cellular phenotypes are identical (Rowe *et al.*, 1987a; Falk *et al.*, 1993).

EBNA-1 can bind RNA *in vitro* through arginine/glycine (RGG) motifs (Snudden *et al.*, 1994). EBNA-1 also activates expression of the lymphoid recombinase genes (RAGs) through an as yet unidentified mechanism (Srinivas & Sixbey, 1995). Activation of the RAGs could promote chromosomal rearrangement and translocations and possibly also facilitate viral integration. This may indicate that EBNA-1 can activate expression of critical cellular genes and affect cellular growth control. EBNA-1 is also expressed in the permissive EBV infection, hairy leukoplakia, indicating that it contributes to replicative EBV infection (Murray *et al.*, 1996).

Evidence that EBNA-1 may itself have oncogenic potential is provided by the finding that lymphomas developed in two transgenic mouse lines expressing EBNA-1 under the

control of the immunoglobulin heavy-chain intron enhancer (Wilson *et al.*, 1996a). Moreover, EBV-positive EBNA-1-expressing Akata cell clones were reported to be able to clone in soft agar and to induce tumours in nude mice (Shimizu *et al.*, 1994).

Another hallmark of EBNA-1 is an array of glycine–alanine repeats located in the N-terminal part of the protein. These repeats were shown to prevent presentation of MHC class I-restricted CTL epitopes (Levitskaya *et al.*, 1995), which represents a novel, unique mechanism for bypassing recognition by cytotoxic T cells and maintaining viral latency.

4.1.5.2 *EBNA-LP*

EBNA-2 and *EBNA-LP* are the first viral genes expressed after infection of primary human B cells with EBV (Rooney *et al.*, 1989; Allday *et al.*, 1989). *EBNA-LP* appears to be important at least for initiation of B-cell transformation, since mutant virus lacking the C-terminal exons has a severely reduced transformation potential, and cells infected with mutant virus require a feeder layer for outgrowth (Mannick *et al.*, 1991). Ectopic expression of *EBNA-LP* in these cells restored the growth behaviour (Allan *et al.*, 1992).

EBNA-LP was reported to bind to *p53* and *Rb* (Szekely *et al.*, 1993), but there is no evidence that the function of *p53* and *Rb* is modified by such binding (Allday *et al.*, 1995; Inman & Farrell, 1995). *EBNA-LP* was recently described as localizing to the nuclear compartment defined by accumulation of the promyelocytic leukaemia gene product in normal cells (Dyck *et al.*, 1994; Weis *et al.*, 1994; Szekely *et al.*, 1996), the main function of which is again unknown. A role for *EBNA-LP* in cell cycle regulation is suggested by the finding that, with *EBNA-2*, it can induce cyclin D2 and cell cycle activation in primary human B cells pretreated with gp340 (Sinclair *et al.*, 1994) and that *EBNA-LP* phosphorylation is dependent on the cell-cycle stage (Kitay & Rowe, 1996a). Recent studies suggest that *EBNA-LP* greatly enhances *EBNA-2*-induced *trans*-activation of *LMP-1* expression through interaction with the *EBNA-2* acidic *trans*-activation domain (Harada & Kieff, 1997). Expression of *EBNA-LP* in transgenic mice has no effect on development or tumour incidence; the animals die of heart failure, probably due to a toxic effect of EBNA-LP itself (Huen *et al.*, 1993).

4.1.5.3 *EBNA-2*

EBNA-2 has long been suspected to play a crucial role in growth transformation, because a 6.6-kb deletion in the P3HR1 viral strain, encompassing the boundary of the large internal repeats to the long unique region (Ragona *et al.*, 1980; Bornkamm *et al.*, 1980, 1982; Jeang & Hayward, 1983), is associated with loss of transforming capacity, while infectivity is maintained (Miller *et al.*, 1974). A recombinant transforming virus lacking the deletion was observed after either superinfection of Raji cells with P3HR1 virus (Fresen *et al.*, 1980; Skare *et al.*, 1985) or by reconstituting the deletion in the P3HR1 virus with cloned fragments spanning the deletion and harbouring the *EBNA-2* gene (Cohen *et al.*, 1989; Hammerschmidt & Sugden, 1989). Analysis of the virus harboured by the parental Jijoye line from which P3HR1 had been obtained by single cell cloning (Hinuma *et al.*, 1967) revealed the surprising finding that the *EBNA-2* genes of

Jijoye and B95-8 share only about 50% sequence homology (Dambaugh *et al.*, 1984; Adldinger *et al.*, 1985). Although strain differences between EBV types 1 (B95-8 prototype) and 2 (Jijoye and AG876 prototypes; Dambaugh *et al.*, 1984) were later also found in other genes (Sample *et al.*, 1990), the difference in the transforming ability of the two strains (Rickinson *et al.*, 1987) could be pinpointed to the differences in the *EBNA-2* genes (Cohen *et al.*, 1992).

EBNA-2 expressed from the SV40 promoter in transgenic mice induced tubular kidney hyperplasia which advanced to adenocarcinoma (Törnell *et al.*, 1996).

EBNA-2 is a *trans*-activator of many cellular (*CD23*, *CD21*, *c-fgr*) and viral genes (*LMP-1*, *LMP-2*, *Cp*) but cannot bind to DNA directly. A cellular protein that mediates binding of *EBNA-2* to its response element was first described in the *LMP-2A* promoter (Zimber-Strobl *et al.*, 1993) and later shown also to have binding sites in the C and *LMP-1* promoters (Ling *et al.*, 1993a; Grossman *et al.*, 1994; Laux *et al.*, 1994b; Yalamanchili *et al.*, 1994). This protein turned out to be recombination signal binding protein Jκ (RBP-Jκ) (Grossman *et al.*, 1994; Henkel *et al.*, 1994; Waltzer *et al.*, 1994; Zimber-Strobl *et al.*, 1994), a ubiquitously expressed protein which is highly conserved in evolution, with homologues in species as distant as *Drosophila* (suppressor of hairless, *Su(H)*) (Furukawa *et al.*, 1992; Schweisguth & Posakony, 1992) and *Caenorhabditis elegans* (Christensen *et al.*, 1996). Its name is misleading since it proved not to bind to the immunoglobulin Jκ sequence (Tun *et al.*, 1994). The genetics of *Drosophila* provided a clue to its physiological function. RBP-Jκ/Su(H) is a DNA-binding protein and a downstream target of a cellular receptor called Notch (Fortini & Artavanis-Tsakonas, 1994), which in mammals is part of a family of related proteins. Ligand binding activates Notch, and subsequent processing and transport of Notch to the nucleus converts the repressor RBP-Jκ (Hsieh & Hayward, 1995; Waltzer *et al.*, 1995) into an activator and turns on target genes (Jarriault *et al.*, 1995; Hsieh *et al.*, 1996). EBNA-2 and activated Notch interact with similar regions in the RBP-Jκ molecule and fulfil similar functions (Hsieh *et al.*, 1996; Sakai & Honjo, 1997). EBNA-2 may therefore be regarded as a functional homologue of a constitutively active Notch receptor. By analogy with other viral oncoproteins, it seems likely that *EBNA-2* has functions additional to activation of RBP-Jκ. Binding of EBNA-2 to RBP-Jκ, although necessary, is not sufficient for *trans*-activation of EBNA-2-regulated promoters. In the *LMP-1* promoter, binding of PU.1 is additionally involved in *trans*-activation by EBNA-2 (Laux *et al.*, 1994b; Johannsen *et al.*, 1995), and other proteins involved are still to be identified. The region in EBNA-2 that interacts with RBP-Jκ has been mapped to amino acids 318–327 (Yalamanchili *et al.*, 1994; Ling & Hayward, 1995), a region highly conserved among the *EBNA-2* genes of EBV types 1 and 2 and HVP (Ling *et al.*, 1993b). It is one of the three regions of *EBNA-2*, including the C-terminal *trans*-activation domain and an N-terminal domain with unknown function, that are critically required for transformation (Cohen *et al.*, 1991). Additional details on the biochemistry and interaction with basal transcriptional machinery are given in Section 1.

In view of the functional equivalence between EBNA-2 and an activated Notch receptor, Notch signalling may partially substitute for EBNA-2 function in activation of viral and cellular genes in the EBNA-2-negative forms of latency.

4.1.5.4 *EBNA-3A, -3B and -3C*

EBNA-3A, *-3B* and *-3C* differ in type 1 and type 2 strains, but these differences do not affect the transforming ability of the viruses (Sample *et al.*, 1990; Tomkinson & Kieff, 1992b). *EBNA-3A* and *-C* are required for transformation, whereas *EBNA-3B* is not (Tomkinson & Kieff, 1992a; Tomkinson *et al.*, 1993). This is quite surprising, since *EBNA-3B* is a major target for recognition of latently infected cells by cytotoxic T cells (Moss *et al.*, 1996) and might be counterselected if it were not an essential gene for the viral life cycle *in vivo*. The EBNA-3C and -3B proteins have been reported to participate in transcriptional regulation of the *LMP-1*, *CD21* and *CD23*, and *CD40*, *CD77* and *vimentin* genes, respectively (Wang *et al.*, 1990a; Allday *et al.*, 1993; Silins & Sculley, 1994). EBNA-3A, -3B and -3C proteins were recently shown to inhibit transcriptional activation of EBNA-2-responsive promoters (Le Roux *et al.*, 1994) by preventing RBP-Jκ and EBNA-2/RBP-Jκ complexes from binding to their cognate RBP-Jκ binding sites (Waltzer *et al.*, 1996; Robertson *et al.*, 1996; Zhao *et al.*, 1996). EBNA-3 proteins are thus believed to counterbalance and finely tune the action of EBNA-2, and it is likely that they have other functions. EBNA-3C has been described as countering the action of the cyclin-dependent kinase inhibitor p16 INKLA and inactivating the retinoblastoma protein (Rb) functionally, like the viral proteins HPV E7 and adenovirus E1A (Parker *et al.*, 1996).

4.1.5.5 *LMP-1*

LMP-1 is the only known EBV gene which, acting alone, can transform rodent fibroblasts (Wang *et al.*, 1985). *LMP-1*-expressing rodent fibroblasts grow in low concentrations of serum, acquire anchorage-independent growth in soft agar and become tumorigenic. In primary B cells, *LMP-1* induces DNA synthesis and up-regulation of *CD23*, *CD21* and *CD54* (Peng & Lundgren, 1992).

LMP-1 is highly toxic if expressed at high levels (Hammerschmidt *et al.*, 1989; Floettmann *et al.*, 1996). When expressed at lower levels, it induces a number of phenotypic and functional changes such as up-regulation of adhesion molecules and activation markers (e.g. *CD23*, *CD39*, *CD40*, *CD54* and *CD58*; Wang *et al.*, 1988b, 1990b), restoration of immunological function, i.e. coordinated up-regulation of peptide transporters and HLA class-I and class-II molecules (Cuomo *et al.*, 1990; de Campos-Lima *et al.*, 1993b; Zhang *et al.*, 1994a; Rowe *et al.*, 1995) and induction of cyclin D2 (Arvanitakis *et al.*, 1995) and stress-response genes that prevent apoptosis, such as *bcl-2* and *A20* (Henderson *et al.*, 1991; Laherty *et al.*, 1992; Rowe *et al.*, 1994). Most of these functions are usually attributed to the ability of *LMP-1* to induce NFκB (Hammarskjold & Simurda, 1992), but this is not the only signalling pathway to which *LMP-1* contributes (Miller *et al.*, 1997). The molecular link between *LMP-1* and induction of the expression of genes such as *bcl-2* is in fact still missing (Martin *et al.*, 1993; Rowe *et al.*, 1994). When expressed in epithelial cells, LMP-1 inhibits cell differentiation in keratinocyte raft cultures *in vitro* (Dawson *et al.*, 1990) and induces morphological transformation and aberrant keratin expression (Fåhraeus *et al.*, 1990b) and severe epidermal hyperplasia in transgenic mice (Wilson *et al.*, 1990).

LMP-1 is absolutely required for both the initiation and maintenance of B-cell transformation by EBV (Kaye *et al.*, 1993). Genetic experiments have shown that the first transmembrane domain and the neighbouring amino acids of the cytoplasmic N-terminal domain are required for B-cell transformation (Kaye *et al.*, 1993), while the function of the 155 C-terminal amino acids can be complemented by co-cultivation of the cells on a fibroblast feeder layer (Kaye *et al.*, 1995). Constitutive *LMP-1* expression in EBV-transformed cells carrying a conditional *EBNA-2* gene has provided evidence that *LMP-1* in the absence of functional *EBNA-2* promotes survival of the cells without maintaining proliferation, similar to the stimulation of the endogenous CD40 receptor by CD40 ligand, which is even more effective in promoting cell survival than LMP-1 (Zimber-Strobl *et al.*, 1996). The functional similarity between LMP-1 and CD40 is corroborated by the fact that the two proteins recruit the same types of signalling molecules on the cytoplasmic side of the membrane. These molecules, which are known as tumour necrosis factor (TRAF) receptor-associated factors mediate activation of NFκB (Mosialos *et al.*, 1995). Deletion of the TRAF interaction domain within the LMP-1 molecule abolishes B-cell transformation by EBV (Izumi *et al.*, 1997).

4.1.5.6 *LMP-2A and -2B*

A clue to the function of LMP-2A has been provided by the observations that (i) it has eight tyrosine motifs that can be phosphorylated and interact with other kinases and adapter proteins; (ii) it is a substrate for members of the src family of kinases expressed in B cells (fyn, lyn) and is stably phosphorylated on tyrosine (Longnecker *et al.*, 1991; Burkhardt *et al.*, 1992); (iii) it is associated with another stably phosphorylated tyrosine kinase (syk) (Miller *et al.*, 1995a); (iv) it shares the sequence motif $(YXXL/I)_2$ with signal transducing subunits of antigen receptors in B and T cells (Reth, 1989; Beaufils *et al.*, 1993); and (v) it inhibits anti-immunoglobulin-mediated Ca^{2+} mobilization, PLCγ2 activation and anti-immunoglobulin-induced reactivation of the lytic cycle, which can be bypassed by TPA with Ca^{2+} ionophore (Miller *et al.*, 1994b, 1995a). These data are consistent with a model in which LMP-2A sequesters the receptor-associated tyrosine kinase, blocking its autophosphorylation and downstream signalling events (Miller *et al.*, 1995a).

Consistent with this model, *LMP-2A* and *-2B* proved not to be required for B-cell transformation by EBV *in vitro* (Longnecker *et al.*, 1992, 1993a,b), even though they contribute to the efficiency of transformation (Brielmeier *et al.*, 1996). Maintenance of latency and prevention of entry into the lytic cycle due to inhibition of the B-cell receptor may, however, be a more important issue for the virus *in vivo* than *in vitro*.

4.1.6 *Cellular genes induced during growth transformation by EBV*

Normal cells are induced to proliferate by signalling molecules (soluble molecules such as hormones and growth factors or matrix or cell-borne molecules) that interact with specific receptors, deliver a signal to the cell, transmit it into the nucleus and convert the cell to a new transcriptional programme and a proliferative response. As seen with oncogenes, malignant growth is a consequence of genetic changes which, on the one hand,

abrogate negative control mechanisms and, on the other, render the signalling process constitutive. This is achieved by shortcuts introduced into the signal transduction pathway at some level between the membrane and the nucleus.

Since different proliferative signals have to converge into a common programme leading to entry into S phase, the proteins that control these checkpoints, such as Rb and p53, are frequent targets for transforming viruses like papillomaviruses, SV40 and adenoviruses, which abrogate the cellular control mechanisms. In contrast, there is no evidence for a direct functional interaction between EBV latency proteins and either Rb or p53; however, it is likely that EBV indirectly affects these pathways. Recent data suggest that EBNA-3C overcomes the action of cyclin-dependent kinase inhibitor p16 and in this way affects cellular cycle control mechanisms (Parker *et al.*, 1996). In addition, *LMP-1* expression inhibits p53-mediated apoptosis (Okan *et al.*, 1995; Fries *et al.*, 1996).

In comparison with resting B cells, EBV-transformed lymphoblastoid cell lines show dramatic changes in their gene expression programme. In fact, EBV appears to mimic physiological B-cell activation brought about by antigen and helper T cells, and up-regulates many kinds of cell-surface molecules that might be important in signalling and growth regulation. Among the cellular genes induced by EBV are those that code for growth factors such as IL-5 (Paul *et al.*, 1990), IL-6 (Tanner & Tosato, 1992; Tanner *et al.*, 1996), IL-10 (Burdin *et al.*, 1993; Nakagomi *et al.*, 1994), TNFα and lymphotoxin (Estrov *et al.*, 1993; Gibbons *et al.*, 1994), thioredoxin (Wakasugi *et al.*, 1990), receptors such as the transferrin receptor, CD21, CD23, the TNFα receptor (Gibbons *et al.*, 1994; Wei *et al.*, 1994), TRAF-1 (Mosialos *et al.*, 1995), a protein related to the p40 subunit of the IL-12 receptor (Devergne *et al.*, 1996), G-protein-coupled receptors (Dobner *et al.*, 1992; Birkenbach *et al.*, 1993), activation markers (CD39), adhesion molecules (CD44, CD48, CD54, CD58) and molecules involved in cytoskeleton formation such as vimentin and actin-bundling protein (Birkenbach *et al.*, 1989; Mosialos *et al.*, 1994; Wang *et al.*, 1990b; Yokoyama *et al.*, 1991). It seems likely that these molecules participate in the induction and maintenance of growth transformation by EBV by establishing autocrine loops (only convincingly shown for lymphotoxin) or cell–cell-mediated cross-stimulatory pathways.

It should be borne in mind, however, that there is currently no way of testing such hypotheses. A conditional knock-out system for cellular genes (e.g. conditional induction of antisense RNA or ribozymes) is urgently required to elucidate the role of individual cellular genes for the maintenance of growth transformation.

4.2 Burkitt's lymphoma

4.2.1 *Molecular abnormalities in relation to the tumour-cell precursor*

4.2.1.1 *Translocation of the* c-myc *oncogene*

The discovery of non-random chromosomal translocations associated with Burkitt's lymphoma (Manolov & Manolova, 1972; Zech *et al.*, 1976; Bernheim *et al.*, 1981) paved the way to an understanding of the genetic derangements that are a central component of its pathogenesis.

From the observations that a chromosomal breakpoint on chromosome 8, band q24 is common to all three of the observed translocations in Burkitt's lymphoma and that the breakpoints are located on chromosomes 14, 2 and 22, at the heavy- and light-chain immunoglobulin loci (Croce *et al.*, 1979; Lenoir *et al.*, 1982; Malcolm *et al.*, 1982; McBride *et al.*, 1982), a strategy for identifying a potential oncogene on chromosome 8q24 was devised. Probes from the immunoglobulin regions were used to clone adjacent DNA fragments, which were soon shown to contain *c-myc* (Dalla-Favera *et al.*, 1982; Taub *et al.*, 1982; Adams *et al.*, 1983). Each of the chromosomal translocations results in the juxtaposition of *c-myc* to immunoglobulin sequences — either *c-myc* is translocated distal to heavy-chain sequences on chromosome 14q32, or light chain sequences from chromosomes 2p11 (kappa) or 22q12 (lambda) are translocated distal to *c-myc* on chromosome 8 (reviewed by Magrath, 1990; Lüscher & Eisenman, 1990). The *c-myc* gene is known to be important in the control of cell proliferation and cell death (Askew *et al.*, 1991; Evan *et al.*, 1992).

The juxtaposition of *c-myc* to immunoglobulin genes in a B-cell lymphoma suggests that the latter genes themselves have an important role to play in pathogenesis. Firstly, they are probably relevant to the genesis of the translocations, because recombinational events occur in immunoglobulin genes during B-cell ontogeny. The physiological breakage and relegation of the DNA strand to the regions in which the chromosomal breakpoints occur is unlikely to be due to chance; either the chromosomes are more readily broken in areas of the DNA strand that are exposed to recombinases, or translocations are actually created by the same recombinases that are responsible for VDJ rearrangements. Secondly, the transcriptional deregulation of the *c-myc* gene, which results from the translocation, is due to its juxtaposition to transcriptional control elements within the immunoglobulin locus (Madisen & Groudine, 1994; Polack *et al.*, 1993; Hörtnagel *et al.*, 1995). The net consequence of the translocation appears to be that *c-myc* is regulated as if it were an immunoglobulin gene, i.e. it is constitutively expressed in these immunoglobulin-synthesizing tumour cells. Thirdly, structural changes within the regulatory and coding regions of *c-myc*, reminiscent of somatic mutations in the hypervariable regions of the immunoglobulin genes, invariably accompany the translocation (Rabbitts *et al.*, 1984; Pelicci *et al.*, 1986; Magrath, 1990; Zajac-Kaye *et al.*, 1990; Bhatia *et al.*, 1995; Raffeld *et al.*, 1995).

Because the *c-myc*–immunoglobulin translocation is the central element in the pathogenesis of Burkitt's lymphoma, one possible role for cofactors is to increase the likelihood that a translocation will occur. On the basis of evidence derived by Southern blot that the J region of the immunoglobulin heavy chain locus involved in the translocation is not rearranged in a large fraction of Burkitt's lymphomas, it has been surmised that the translocation occurs early in B-cell differentiation (Bhatia *et al.*, 1995). This assumption is consistent with the notion that precursor B cells in the process of, or just about to, rearrange their immunoglobulin genes are particularly susceptible to translocation. By extrapolating from mouse models, it can be calculated that 10^{10} new B cells are generated daily in humans (Opstelten & Osmond, 1983). An environmental agent that increases the rate of B-cell ontogeny could simultaneously increase the frequency of occurrence of translocations, even if the latter is a purely stochastic event. Both EBV and malaria cause

B-cell hyperplasia and thus could influence the development of Burkitt's lymphoma in this way (Lenoir & Bornkamm, 1987). When both agents are present, as in African infants, the risk of developing a translocation is likely to be increased. Other agents, such as phorbol esters present in medicinal plants, have been proposed to increase the risk for chromosomal translocations additionally (Osato *et al.*, 1990; van den Bosch *et al.*, 1993).

EBV cannot only induce cell proliferation and thus increase on a statistical basis the likelihood that a *c-myc*–immunoglobulin translocation will arise, but it may also directly promote immunoglobulin gene recombination. Such gene recombination in lymphoid cells is dependent upon expression of the recombination activating genes *RAG-1* and *RAG-2*. To minimize the risk of aberrant recombination, the expression of these genes is limited to a narrow window in the development of B cells. EBV infection of sporadic Burkitt's lymphoma-negative cells and transfection of *EBNA-1* have been shown to up-regulate *RAG* expression (Kuhn-Hallek *et al.*, 1995; Srinivas & Sixbey, 1995). Expression of *LMP-1* and of *RAG-1* were, however, found to be mutually exclusive. The possibility that inappropriate *RAG* expression increases the likelihood of aberrant immunoglobulin gene recombination is supported by the observation that EBV-transformed, fetal liver-derived precursor B cells frequently develop translocations involving the immunoglobulin heavy-chain locus (Altiok *et al.*, 1989). B Lymphocytes that have undergone aberrant recombination are normally eliminated by apoptosis. Precursors to Burkitt's lymphoma cells with aberrant recombinations may be illegitimately rescued from apoptosis by EBV-encoded *LMP-1* if the malignant stage involves type II or type III latency phenotypes.

If the role of EBV in the pathogenesis of Burkitt's lymphoma were confined to the likelihood that a *myc* translocation occurs, it would follow that once such a translocation has developed the viral genome is no longer necessary.

4.2.1.2 *The Burkitt's lymphoma-cell phenotype resembles that of a germinal-centre cell*

While there is evidence that the chromosomal translocations occur in precursor B cells, the immunophenotype of Burkitt's lymphoma is that of a germinal-centre cell, indicating that differentiation must have occurred after the translocation. This may be an explanation for the essentially invariable expression of surface immunoglobulin, since differentiating B cells that fail to express a functional immunoglobulin molecule are destined to undergo apoptosis. The need for genetic changes, or possibly the expression of EBV genes that permit a translocation-bearing cell to avoid apoptosis, is likely to be an essential component of lymphomagenesis.

Consistent with the hypothesis that Burkitt's lymphoma cells have a germinal-centre phenotype is the presence of mutations in the hypervariable region of the heavy immunoglobulin chain in these cells (Chapman *et al.*, 1995; Klein *et al.*, 1995; Tamaru *et al.*, 1995), raising the possibility that exposure to antigens may be relevant to the pathogenesis of Burkitt's lymphoma. Burkitt's lymphoma cells do not, however, express activation antigens (Gregory *et al.*, 1987b) and in this respect, differ markedly from EBV-

transformed lymphoblastoid cell lines. This phenotype seems to be a consequence of the c-*myc* translocation (Polack *et al.*, 1996).

4.2.1.3 *Mutations in* p53 *in Burkitt's lymphoma*

Mutations in *p53* have been detected in up to 37% of biopsy samples from primary Burkitt's lymphomas (Gaidano *et al.*, 1991; Bhatia *et al.*, 1992). The incidence of *p53* mutation is independent of the geographic origin of the tumour, the chromosomal translocation or the association with EBV. The specific mutations in *p53* in Burkitt's lymphoma are distinct from those found in non-lymphoid tumours, as most are clustered in codons 213–248. The frequency of *p53* mutation is higher in cell lines, most of which are derived from recurrent tumours, more than 70% of which harbour mutations (Gaidano *et al.*, 1991; Farrell *et al.*, 1991; Bhatia *et al.*, 1992). This finding may indicate that *p53* mutation contributes to tumour progression. One consequence of the absence of *LMP-1* expression in Burkitt's lymphoma (Rowe *et al.*, 1987a) is the lack of its protective effects on *p53*-mediated apoptosis (Okan *et al.*, 1995; Fries *et al.*, 1996). Mutations in *p53* are rare in EBV-associated malignancies in which *LMP-1* is expressed (Spruck *et al.*, 1992; Sun *et al.*, 1992; Fåhraeus *et al.*, 1988).

4.2.2 *EBV infection in Burkitt's lymphoma*

Although chromosomal translocations affecting *c-myc* and the immunoglobulin loci are always present in Burkitt's lymphoma, EBV is not invariably found. About 95% of the cases in endemic areas of Africa and Papua–New Guinea contain EBV, but the prevalence in Burkitt's lymphoma outside the endemic areas varies with underlying disease and geographic region from 15 to more than 90% (see section 2.1). Both EBV type 1 and type 2 may be associated with Burkitt's lymphoma (Zimber *et al.*, 1986). A definitive conclusion about the pathogenetic role of EBV in Burkitt's lymphoma cannot be reached solely on the basis of epidemiological observations; molecular studies provide a number of additional insights and are the most likely source of unequivocal evidence for a pathogenetic role of the virus.

4.2.2.1 *EBV is monoclonal in Burkitt's lymphoma*

One requirement that must be fulfilled if EBV is to subserve a pathogenetic role in Burkitt's lymphoma is that the virus must not only be present in the tumour cells but must also have entered the cell before its final conversion to a malignant lymphoma. Since malignant tumours originate from a single cell which contains all of the necessary genetic abnormalities, EBV should be present in all tumour cells and should also be monoclonal, i.e. all tumour cells should be derived from the same EBV-infected cell. The detection of a homogeneous number of terminal repeats in EBV episomes in Burkitt's lymphoma tumour cells showed that the virus is indeed clonal and indicated that the tumour developed from a single EBV progenitor cell (Raab-Traub & Flynn, 1986; Neri *et al.*, 1991).

4.2.2.2 *Integration of viral DNA in Burkitt's lymphoma cells*

Many oncogenic viruses contribute to tumorigenesis by insertional mutagenesis, e.g. by activation of proto-oncogenes and/or inactivation of tumour suppressor genes. Integration of the EBV genome has been observed in several Burkitt's lymphoma-derived cell lines (Henderson *et al.*, 1983; Delecluse *et al.*, 1993a); however, no preferential integration site has, so far, been identified. Moreover, integration is not a *sine qua non*, since EBV-positive Burkitt's lymphoma cells may (rarely) lose the viral genomes when grown *in vitro* (Takada *et al.*, 1995), and EBV-positive and EBV-negative cells co-exist in a Burkitt lymphoma line (Trivedi *et al.*, 1995).

4.2.2.3 *Expression of EBV genes in EBV-associated Burkitt's lymphoma*

If EBV exerts a direct oncogenic effect, one or more viral genes should be expressed in tumour cells. This criterion is also fulfilled, although the pattern of expression of EBV proteins is markedly different in Burkitt's lymphoma and in lymphoblastoid cell lines. This suggests that there are basic differences in the mechanisms whereby EBV transforms B cells on the one hand and contributes to the development, or maintenance, of Burkitt's lymphoma on the other. Although the full range of latency genes is expressed in EBV-transformed cell lines, only *EBNA-1* and the *EBERs* are invariably expressed in Burkitt's lymphoma cells (Rowe *et al.*, 1986, 1987a; Niedobitek *et al.*, 1995). Rare cells within a tumour may express other EBV genes, such as *EBNA-2*, *LMP* and *BZLF1* (Niedobitek *et al.*, 1995), and may even enter the lytic cycle (Gutiérrez *et al.*, 1993).

The differences in latent gene expression in lymphoblastoid cell lines and Burkitt's lymphoma are regulated at the level of promoters in these cell types. In lymphoblastoid cell lines, all of the EBNA proteins are encoded from spliced transcripts derived from a common long precursor initiated from promoters in the W and/or C *Bam*HI fragments of the EBV genome, whereas in Burkitt's lymphoma *EBNA-1* is transcribed from the Q promoter (Schaefer *et al.*, 1995). Q Promoter-initiated transcripts that encode *EBNA-1* are negatively regulated by *EBNA-1* (Sample *et al.*, 1992).

A recent study revealed that transgenic mice that express *EBNA-1* under the control of immunoglobulin enhancer develop B-cell lymphomas (Wilson *et al.*, 1996c). This suggests that *EBNA-1* may have additional properties beyond maintenance and regulation of the viral genome and that it contributes to the development of malignancy.

4.2.2.4 *Expression only of EBNA-1 is associated with reduced immunogenicity*

The limited expression of EBV gene products in Burkitt's lymphoma cells provides significant protection from immune recognition, as most of the CTL response is directed against EBNA-3 proteins. In addition, EBNA-1 is not processed or presented in class I MHC molecules (Levitskaya *et al.*, 1995). The EBNA-1 protein contains a simple repeat of glycine and alanine. Transfer of this repeat element to a heterologous protein sequesters that protein from processing and expression within MHC class I. This unique property enables an EBV-infected cell that expresses only *EBNA-1* to escape immune recognition.

Burkitt's lymphoma cells not only lack EBV-derived immunogenic proteins, they also express low levels of HLA class-I molecules (Masucci *et al.*, 1987; Andersson *et al.*, 1991; Gavioli *et al.*, 1992; Imreh *et al.*, 1995), adhesion molecules, including LFA-1 (CD11a), ICAM-1 (CD54) and LFA-3 (CD58), and co-stimulatory molecules, B7-1 and B7-2 (CD80 and CD86), that participate in antigen presentation, while CD10, CD38 and CD77 are un-regulated (Gregory *et al.*, 1988, 1990; Billaud *et al.*, 1990; Ling *et al.*, 1989; Magrath, 1990; Martel-Renoir *et al.*, 1995). As a consequence, Burkitt's lymphoma cells are virtually invisible to the immune system, as evidenced by the fact that the cells are unable to elicit an allostimulatory T-cell response (Cuomo *et al.*, 1990). The phenotype of Burkitt's lymphoma cells is a consequence of high *c-myc* expression and non-expression of the viral proteins involved in growth promotion, except for EBNA-1 (Polack *et al.*, 1996).

4.2.2.5 *The proliferation programme driven by c-myc–immunoglobulin is incompatible with expression of EBNA-2 and LMP-1 in the type-III latency programme*

After the discovery of the chromosomal translocations in Burkitt's lymphoma cells, it was suspected that, in analogy to the well-documented cooperation of oncogenes (Hunter, 1991), EBV viral gene products would cooperate with an activated *c-myc* gene to promote a fully malignant phenotype. It was therefore not anticipated that *EBNA-2* and *LMP-1* would have adverse effects on the proliferation of Burkitt's lymphoma cells and even abrogate their tumorigenic potential (Torsteinsdottir *et al.*, 1989; Cuomo *et al.*, 1992; Floettmann *et al.*, 1996). This can be explained, at least in part, by the fact that *EBNA-2* inhibits the transcriptional regulatory elements of the immunoglobulin heavy chain locus. *EBNA-2* down-regulates IgM expression, and, in cells in which *c-myc* transcription is driven by translocation into the immunoglobulin heavy-chain locus, it down-regulates *c-myc* expression concomitantly (Jochner *et al.*, 1996).

Thus, while *EBNA-2* is absolutely required for proliferation of EBV-infected normal cells, switching on the function of *EBNA-2* inhibits proliferation of Burkitt's lymphoma cells with the t(8;14) translocation (Kempkes *et al.*, 1996). In cells with the variant t(2;8) translocation, *EBNA-2* does not affect *c-myc* expression directly but slows proliferation in an indirect manner (Polack *et al.*, 1996). *EBNA-2* expression and proliferation mediated by constitutive activation of c-*myc* are thus mutually exclusive.

EBV-positive Burkitt's lymphoma cells tend to change their phenotype and switch to the type III latency programme, expressing *EBNA-2* and *LMP-1*, when explanted into tissue cultures, which at first sight seems contradictory to what is stated above. Burkitt's lymphoma cells in type I latency are highly susceptible to apoptosis and evolve variants resistant to apoptosis *in vitro* which have switched to the type III latency programme (Gregory *et al.*, 1991). Under these conditions, however, the cells proliferate significantly more slowly (Falk *et al.*, 1993) and express lower levels of *c-myc* RNA and protein than Burkitt's lymphoma cells in type-I latency (Jochner *et al.*, 1996), due to the effects of *EBNA-2* on heavy-chain-regulated *c-myc* (Bornkamm *et al.*, 1988; Spencer &

Groudine, 1991), while the expression of *EBNA-2* and *LMP-1* seems sufficiently high to protect the cells from apoptosis.

4.2.2.6 *Contribution of the viral strategy for latent persistence to lymphomagenesis*

The different type I latency programmes together represent a strategy necessary for the virus to maintain a permanent reservoir of viral genomes in B cells in normal EBV-infected individuals. This strategy also contributes to lymphomagenesis (see section 4.2.1 above; Rowe *et al*, 1992; Chen *et al.*, 1995a; Miyashita *et al.*, 1995). Under physiological conditions, the type-I latency programme allows maintenance of EBV DNA without risk of elimination by CTL, as discussed elsewhere. This programme, which under physiological conditions appears to operate only in non-proliferating B cells, is maintained in Burkitt's lymphoma cells.

4.2.2.7 *EBNA-1 subtypes*

Recently, subtypes of EBNA-1 that differ in their amino-acid sequence have been identified (Bhatia *et al.*, 1996). These differences do not seem to reflect geographical regions. Interestingly, a subtype marked by alanine at position 487 is the commonest form in normal individuals but is rarely found in tumours. In contrast, the subtype marked by leucine at 487 but containing several additional amino-acid substitutions is frequently found in Burkitt's lymphomas and not in normal individuals (Gutiérrez *et al.*, 1997). This subtype, *V-leu*, differs only at position 487 from another subtype, *V-pro*, which is present in normal lymphocytes but only in association with other variants. These data suggest that the subtypes arise *in vivo* and that mutations in *EBNA-1* may be relevant to the pathogenesis of Burkitt's lymphoma.

4.2.3 *Effects of malaria on B-cell activation and EBV infection*

While epidemiological evidence for a role of malaria as a predisposing factor (see section 2.1.4.1) is strong, it will be important to identify the specific mechanism whereby malaria influences the pathogenesis of Burkitt's lymphoma. To date, the mechanism has not been identified, although experimental evidence derived from studies in mice provide paradigms that may be applicable to Burkitt's lymphoma.

In a study conducted by Jerusalem (1968), 64% of mice infected with *Plasmodium berghei* eventually developed malignant lymphoma, while Osmond *et al.* (1990) showed that infection of mice with *P. bergei yeollii* increased the proportion of immature B cells (pro-B cells) in the bone marrow and decreased the pool of mature B cells. It is this expansion of precursor B cells that is likely to be responsible for the increased susceptibility of *Plasmodium*-infected mice to the development of lymphoma.

In a study of concurrent infection, 10 of 12 adult mice infected with *P. berghei yeollii* and Moloney leukaemia virus developed lymphoma, in comparison with one of 11 infected only with Moloney virus and none of 10 infected with malaria alone (Wedderburn, 1970). Similar findings were reported by Salaman *et al.* (1969). Moloney virus does not contain an oncogene and activates oncogene expression by a process of promoter insertion. The combination of precursor B-cell hyperplasia and genetic lesions

induced by Moloney virus is likely to account for the increased frequency of lymphomas in the doubly infected mice.

A similar situation may apply to African children. If malarial infection in humans also expands pro-B- and pre-B-cell populations, the risk for developing Burkitt's lymphoma may be increased, as the pool of cells that could develop a *c-myc*–immunoglobulin translocation is larger. EBV infection may well add an additional potentially tumorigenic lesion, although the molecular pathways involved are unlikely to include promoter insertion.

While expansion of precursor B-cell populations in children in regions where malaria is hyperendemic has not been examined, there is evidence that the B-cell system is hyperactive during the course of infection. Children with malaria have very high serum levels of IgG and IgM, most of which are not due to anti-plasmodial antibodies and plateau after the age of five to six years (McGregor, 1970). Turnover of IgG is as much as seven times greater in adults from holoendemic malarious regions than among those from control regions (Cohen *et al.*, 1961; Cohen & McGregor, 1963).

Chronic malaria, like HIV infection, leads to a shift in the helper T-cell response towards T_h2 cells (von der Weid & Langhorne, 1993). T_h2 cytokines, such as IL-10, suppress the humoral arm of the immune response, support the early steps of B-cell immortalization by EBV (Burdin *et al.*, 1993) and suppress CTL function. As a consequence, the number of B lymphocytes latently infected with EBV increases, while the ability of T cells to suppress the outgrowth of EBV-infected lymphoblastoid cells is impaired (Gunapala *et al.*, 1990; Moss *et al.*, 1983a; Whittle *et al.*, 1984; Lam *et al.*, 1991).

In addition, EBV seroconversion is known to occur much earlier in populations of low socioeconomic status or those who live in crowded conditions than in populations in which families are smaller and have more spacious accommodation (Niederman *et al.*, 1968; Hinuma *et al.*, 1969; Henle & Henle, 1970, 1979). In developing countries, seroconversion occurs in the majority of children by the age of three years (Diehl *et al.*, 1969; Kafuko *et al.*, 1972), occurring earlier in rural areas than in urban zones (Biggar *et al.*, 1981), and is usually not associated with a clinical syndrome (Diehl *et al.*, 1969). It is possible, as originally suggested by de Thé (1977), that early infection with EBV predisposes to the development of EBV-associated Burkitt's lymphoma because of differences in the immune system of infants and older individuals and, in the context of African children, because of the presence of coincident B-cell hyperplasia and immunosuppression caused by malaria.

4.2.4 *Plant products*

Phorbol esters interact with protein kinase C and are potent inducers of cell differentiation. They can induce the differentiation of Burkitt's lymphoma-derived cell lines (Benjamin *et al.*, 1984; Ho *et al.*, 1987; Osato *et al.*, 1990). Several authors have demonstrated that extracts of *Euphorbia tirucalli* and related plants, or even extracts of soil or reservoir water near places where *Euphorbia* plants are growing, can induce the expression of EA and VCA in Raji cells (Ito *et al.*, 1981). Lin *et al.* (1982) showed that

TPA, a phorbol ester, increases viral replication, as measured by an increase in genome copies. This potential activation of EBV replication could result in an increased number of lymphocytes that become infected with EBV.

Similar extracts have been reported to increase the outgrowth of EBV-transformed B cell lines and to decrease EBV-specific T-cell killing (Osato *et al.*, 1990; Imai *et al.*, 1994b). In addition, of potentially considerable interest, Osato *et al.* (1990) observed that dual exposure of B lymphocytes to EBV and deoxyphorbol ester, the active principle of *Euphorbia*, increased the numbers of chromosomal translocations and deletions involving chromosome 8. Thus, the presence of these tumour-promoting compounds in the environment could influence both EBV infection and the genesis of chromosomal abnormality.

4.2.5 *Genetic disposition*

Although familial Burkitt's lymphoma has been reported (see section 2.1.2), genes that could account for an increased risk for the development of Burkitt's lymphoma have not been identified, although it is probable that genetic determinants of the response to relevant cofactors such as malaria, e.g. the presence of sickle-cell trait (Williams, 1966; Pike *et al.*, 1970; Nkrumah & Perkins, 1976), may influence the risk for development of this tumour in Africa. Similarly, inherited immunodeficiency syndromes, such as X-linked lymphoproliferative disorder (Provisor *et al.*, 1975; Purtilo, 1976), are associated with a markedly increased risk for the development of non-Hodgkin's lymphomas (Harrington *et al.*, 1987), which sometimes contain *c-myc*–immunoglobulin gene translocations and have the histological appearance of Burkitt's lymphoma (Egeler *et al.*, 1992).

4.2.6 *Burkitt's lymphoma in AIDS patients*

Burkitt's lymphoma arising in the context of AIDS is associated with EBV infection in only 30–40% of cases, which is greater than that observed in sporadic Burkitt's lymphoma (Ernberg & Altiok, 1989; Gaidano & Dalla-Favera, 1995). This percentage is, however, much lower than that in other non-Hodgkin's lymphomas (see section 4.3.1.3).

In comparison with other non-Hodgkin's lymphomas, AIDS-associated Burkitt's lymphoma develops earlier in the progress of the disease and in less severely immunocompromised individuals. While the characteristics of endemic Burkitt's lymphoma may also apply to AIDS-related disease, there are some important differences: (i) EBV is found in only a proportion of AIDS-related Burkitt's lymphomas, (ii) the patients are much older than those with endemic Burkitt's lymphoma, and (iii) the pattern of *c-myc* translocation is different, involving the switch region rather than the VDJ region.

The clinical, molecular and viral features of AIDS-related Burkitt's lymphoma indicate that its pathogenesis is similar to that of sporadic Burkitt's lymphoma in immunocompetent individuals; however, in the context of AIDS, these lymphomas occur at increased incidence. This suggests the contribution of an AIDS-related factor. That this factor is unlikely to be simply immunosuppression is indicated by the fact that Burkitt's lymphomas occur only rarely in transplant patients and then only in association with rela-

tively low immunosuppression (Niedobitek *et al.*, 1997b). Chronic immune stimulation by malaria has been suggested to play a role in the pathogenesis of endemic Burkitt's lymphoma, and stimulation by e.g. schistosomiasis may be involved in the development of cases in other regions (Araujo *et al.*, 1996). Indeed, it has been proposed that HIV-induced polyclonal B-cell proliferation may contribute to the development of AIDS-related Burkitt's lymphoma (Kalter *et al.*, 1985; Seigneurin *et al.*, 1987; Gaidano & Dalla-Favera, 1995).

For a discussion of lymphomas in AIDS patients, see section 4.3.1.3.

4.3 Other Non-Hodgkin's lymphomas and lymphoproliferative conditions

4.3.1 *Immunosuppressed patients*

The most conspicuous features of human infections with EBV are their ubiquitous distribution in the adult population and that they are mostly asymptomatic. Equally impressive is the high frequency of EBV-positive lymphomas occurring in patients with severe immunosuppression caused by e.g. inherited immune defects and iatrogenic or HIV-associated immunosuppression. These facts together serve as the strongest evidence for a direct oncogenic effect of EBV infection in B cells, when physiological immune regulation and natural defence barriers have broken down.

4.3.1.1 *Primary immune defects due to genetic abnormalities with EBV-positive lymphoproliferation as one consequence*

Patients with X-linked lymphoproliferative syndrome suffer from a number of serious complications after primary infection with EBV. The genetic basis of this syndrome is described in Section 1 (Skare *et al.*, 1993). Other relevant primary immune defects that may involve EBV-related complications are Wiskott-Aldrich syndrome, common variable immunodeficiency and ataxia telangectasia. These genetic disorders result in different types of defects affecting the immune system, in particular T-cell or NK-cell maturation, but also B-cell maturation in common variable immunodeficiency (Purtilo, 1991; Sander *et al.*, 1992; Nakanishi *et al.*, 1993). Lymphoproliferative disorders in these patients encompass a disease spectrum ranging from polyclonal lymphoid hyperplasia at one end to overtly malignant monoclonal high-grade non-Hodgkin's lymphoma at the other (for a review, see Niedobitek *et al.*, 1997c). In particular, in the Wiskott-Aldrich and X-linked lymphoproliferative syndromes, many of the lymphoproliferations are polyclonal (Falk *et al.*, 1990; Nakanishi *et al.*, 1993). Most cases of non-Hodgkin's lymphoma arising in association with primary immune defects are of the B-cell phenotype and are EBV-positive (Falk *et al.*, 1990; Purtilo, 1991; Sander *et al.*, 1992; Nakanishi *et al.*, 1993). Whereas the pattern of EBV gene expression in most polyclonal lymphoproliferations is similar to that in lymphoblastoid cell lines (type-III latency), monoclonal lesions often display a type-I latency. In some cases, EBNAs have been expressed in the absence of detectable LMP-1. Whether this represents a new form of EBV latency or merely reflects technical problems is currently uncertain. The recent detection of a similar phenotype in EBV-associated smooth-muscle tumours (Lee *et al.*,

1995b) suggests that the EBNA-positive/LMP-1 phenotype indeed represents a novel form of stable EBV latency.

Efforts are being made to clone the *XLP* gene and to characterize its function (see Section 1). This will eventually afford a better understanding of the role of EBV in the development of lymphoproliferation in these patients. In general terms, the frequent occurrence of virus-associated lymphoproliferation in patients with genetic immune defects illustrates the significance of intact immune regulation in maintaining the EBV–host balance. Notably, however, immunosuppression does not appear to be associated with nasopharyngeal carcinoma, an epithelial tumour. The range of lymphoproliferations observed may reflect progression from polyclonal lymphoblastoid cell line-like lesions to monoclonal lymphomas as one of the pathogenetic mechanisms of EBV-positive non-Hodgkin's lymphomas (see discussion on post-transplant lymphoproliferative disorders below). In situations with type II or type III latency in the lymphoproliferative tissue, it is conceivable that EBV contributes to driving cell proliferation or, at least, contributes to extended cell survival.

4.3.1.2 *Post-transplant lymphoproliferative disorders*

Post-transplantation lymphoproliferative disorders are a major complication in allograft recipients, occurring in 1–20% of patients (Nalesnik & Starzl, 1994). The incidence tends to be lowest for renal transplant recipients and highest for lung transplant patients (Nalesnik & Startzl, 1994; Montone *et al.*, 1996a; Swinnen, 1996), which may reflect more intensive use of immunosuppressive therapy in the latter. The incidence of post-transplant lymphoproliferative disorders in bone-marrow transplant patients is generally low, although it may be up to 24% in recipients of mismatched T cell-depleted marrow (Shapiro *et al.*, 1988). More recently, similar lesions have been reported in patients being treated for rheumatoid disease with methotrexate (Kamel *et al.*, 1993, 1994). The lymphoproliferations in these patients appear to be morphologically and biologically similar to post-transplant lymphoproliferative disorders and are thus not discussed separately in this context. The vast majority of such cases have been shown to be of B-cell origin and to be associated with EBV infection (Locker & Nalesnik, 1989; Swerdlow, 1992; Craig *et al.*, 1993); however, EBV-negative non-Hodgkin's lymphomas of B- and T-cell phenotypes occur occasionally in transplant recipients (Nalesnik & Starzl, 1994). These may represent sporadic lymphomas developing independently of iatrogenic immunosuppression, and are not discussed here.

The general consensus is that post-transplant lymphoproliferative disorders developing in recipients of solid-organ transplants are of host origin, while those occurring in bone-marrow transplant patients are of donor origin (Zutter *et al.*, 1988; Chadburn *et al.*, 1995a; Weissmann *et al.*, 1995); however, exceptions in both directions have been reported. Thus, Zutter *et al.* (1988) demonstrated that two of 12 cases of post-transplant lymphoproliferative disorder arising in bone-marrow recipients were of host origin, in keeping with the reported persistence of EBV-positive B cells of host origin in bone-marrow transplant patients (Gerhartz *et al.*, 1988). More surprisingly perhaps, Larson

et al. (1996) and Mentzer *et al.* (1996) showed recently that post-transplant lymphoproliferative disorders of donor origin may develop in recipients of solid organs.

For reasons as yet unknown, post-transplant lymphoproliferative disorder frequently develops in the gastrointestinal tract and in the transplanted organ (Guettier *et al.*, 1992; Opelz & Henderson, 1993; Palazzo *et al.*, 1993; Mentzer *et al.*, 1996; Randhawa *et al.*, 1996). Histologically, these conditions represent a spectrum ranging from benign polyclonal, polymorphic lymphoproliferations at one end to frankly malignant monoclonal, monomorphic lymphomas at the other (Swerdlow, 1992; Craig *et al.*, 1993). Typically, B cells in these lymphoproliferations express a broad spectrum of virus-encoded latent proteins, including EBNA-1, EBNA-2 and LMP-1 (Young *et al.*, 1989a; Thomas *et al.*, 1990). This form of EBV latency (type III) is similar to that seen in lymphoblastoid cell lines *in vitro* and, accordingly, these cells usually display a lymphoblastoid-like pattern of cellular gene expression, including lymphocyte activation and adhesion molecules (Thomas *et al.*, 1990). A large proportion of cases of post-transplant lymphoproliferative disorder also show lytic infection, usually in a small subset of cells (Patton *et al.*, 1990; Rea *et al.*, 1994; Montone *et al.*, 1996b). Whether this is sufficient justification for the inclusion of acyclovir in the treatment of these conditions, as often advocated, is uncertain (Zutter *et al.*, 1988; Patton *et al.*, 1990; Davis *et al.*, 1995). It seems likely that entry into the lytic cycle is simply a reflection of the underlying immune defect.

It is generally held that the above-mentioned morphological spectrum of post-transplant lymphoproliferative disorder represents an evolutionary process (Craig *et al.*, 1993). Thus, it is believed that the pathogenesis of the condition starts with EBV-driven polyclonal B-cell proliferation; the acquisition of additional genetic changes then leads to the emergence of dominant B-cell clones and eventually to fully developed malignant lymphomas which are morphologically indistinguishable from lymphomas arising in immunocompetent individuals. This scheme is based on the demonstration in studies of immunoglobulin gene rearrangement and analysis of the structure of the viral terminal repeat sequences that some polymorphic cases are polyclonal. Histologically monomorphic large-cell lymphomas, however, have been shown to be monoclonal using both approaches. In addition, in one group of polymorphic lesions minor clonal components are detectable on the background of polyclonal B-cell proliferations (Locker & Nalesnik, 1989). Furthermore, only monomorphic, monoclonal cases of post-transplant lymphoproliferative disorder contain alterations involving oncogenes or tumour suppressor genes, e.g. *c-myc* translocations or mutations of the N-*ras* and *p53* genes (Delecluse *et al.*,1995b; Knowles *et al.*, 1995). In a recent study, 24 separate lesions in one patient were all clonally distinct (Chadburn *et al.*, 1995b). In a series of 11 patients with recurrent post-transplant lymphoproliferative disorders, the recurrent lesions were either relapses of the original disease or clonally distinct. In some cases, progression from polymorphic disease to frankly malignant lymphoma, including Hodgkin's disease and T-cell lymphomas, was observed. Significantly, no primarily monomorphic case recurred as polymorphic disease, further supporting the notion that the disease progresses from early polymorphic polyclonal lesions to monomorphic monoclonal disease (Wu *et al.*, 1996).

This concept of the pathogenesis of post-transplant lymphoproliferative disorder is also supported by the frequent regression of lesions in response to a reduction in immu-

nosuppressive therapy (Starzl *et al.*, 1984). The evidence described above would imply that early polyclonal lesions are more susceptible to this approach than late monoclonal lesions. Indeed, it has been observed that cases that develop less than one year after transplantation have a much higher response rate than those occurring more than one year later (Armitage *et al.*, 1991). Similarly, polyclonal cases appear to respond better than monoclonal lesions; however, this distinction is not absolute, and a trial of reduced immunosuppression has been recommended even in patients with monoclonal disease (Nalesnik *et al.*, 1992). Lucas *et al.* (1996) demonstrated recently that the frequency of EBV-specific CTL precursors in the peripheral blood of bone-marrow recipients returned to normal in nine of 13 patients within six months after transplantation. In this series, one patient with low precursor CTL levels four months after transplantation developed post-transplant lymphoproliferative disorder, further underlining the significance of suppressed T-cell immunity in its pathogenesis. Regression of lymphoproliferative lesions has also been reported in patients with rheumatoid disease after withdrawal of methotrexate therapy, further reflecting the similarity of some of these lesions to post-transplant lymphoproliferative disorder (Kamel *et al.*, 1993; Salloum *et al.*, 1996; Thomason *et al.*, 1996).

Studies to identify transplant patients who are at risk of developing post-transplant lymphoproliferative disorder have defined certain risk factors. Individuals who are EBV-negative before transplantation and undergo seroconversion after transplantation appear to be particularly prone to development of the condition (Ho *et al.*, 1985; Walker *et al.*, 1995). Further risk factors are the duration and severity of immunosuppression, including use of OKT3, CDw52 and anti-LFA1 antibodies (Opelz & Henderson, 1993; Nalesnik & Starzl, 1994; Gerritsen *et al.*, 1996; Walker *et al.*, 1995). More direct approaches to monitoring the risks of individual patients include quantitative PCR assays to measure the viral DNA load in peripheral blood and techniques for counting the number of virus-carrying B cells in blood or allograft biopsy samples (Randhawa *et al.*, 1992; Crompton *et al.*, 1994; Riddler *et al.*, 1994; Savoie *et al.*, 1994; Kenagy *et al.*, 1995).

Several recent studies have indicated a certain heterogeneity of post-transplant lymphoproliferative disorders. Yoshizawa *et al.* (1993) isolated a cell line from a case which resolved in response to reduced immunosuppression. This cell line, SUBL, displayed a pre-B-cell phenotype and genotype and carried a t(2;3)(p11;q27) translocation, indicating that factors other than proliferation of mature B cells can also give rise to the condition. Furthermore, some EBV-positive T-cell lymphomas have been reported (Borisch *et al.*, 1992b; Kumar *et al.*, 1993; Waller *et al.*, 1993) in which the virus was found by in-situ hybridization to be present in only rare neoplastic cells; thus, the role of EBV in the pathogenesis of these tumours remains uncertain (Borisch *et al.*, 1992b). The virus is consistently detected in the rare cases of Hodgkin's disease that occur in the context of transplantation (Garnier *et al.*, 1996), reflecting the detection of EBV in most cases of HIV-associated Hodgkin's disease (Uccini *et al.*, 1990; Boiocchi *et al.*, 1993b; Herndier *et al.*, 1993).

The analysis of EBV gene expression patterns in post-transplant lymphoproliferative disorders shows considerable variability both between and within lesions. Thus, not all cases display type-III latency (Rea *et al.*, 1994; Delecluse *et al.*, 1995a; Oudejans *et al.*,

1995a). Particularly in monomorphic lymphomas, EBV latent protein expression may be restricted to type-I or type-II latency (Rea *et al.*, 1994; Delecluse *et al.*, 1995a). Moreover, even in those cases in which both EBNA-2 and LMP-1 (indicative of type-III latency) can be identified, expression of these proteins is often seen in variable subsets of cells (Thomas *et al.*, 1990; Rea *et al.*, 1994; Delecluse *et al.*, 1995a; Oudejans *et al.*, 1995b). Thus, a relatively small proportion of cells may display type-III latency and others show type-I or type-II latency. In addition, cells in which EBNA-2 is expressed in the absence of LMP-1 have been observed, as also seen in infectious mononucleosis (Oudejans *et al.*, 1995b; Niedobitek *et al.*, 1997b).

4.3.1.3 *AIDS*

In the general population, the incidence of non-Hodgkin's lymphomas is increasing rapidly, but they still account for only about 4% of all cancers (Parkin *et al.*, 1992). In contrast, non-Hodgkin's lymphomas are very common in HIV-infected individuals, primarily at extranodal sites. Particularly common are primary central nervous system lymphomas, which account for 1.6% of non-Hodgkin's lymphomas in the HIV-negative population (Krogh-Jensen *et al.*, 1994). In a recent Norwegian study, the results of 153 autopsies of AIDS patients were evaluated, representing 73% of all AIDS patients who died. The overall accumulated cancer incidence was 35%. In this series, more cases of primary central nervous system lymphoma (19 cases) than systemic non-Hodgkin's lymphoma (12 cases) were identified (Goplen *et al.*, 1997). In a study in the United States, central nervous system lymphomas constituted only some 20% of all AIDS-related non-Hodgkin's lymphomas (Beral *et al.*, 1991). In larger surveys, 1–4% of AIDS patients had lymphomas (for review, see IARC, 1996). These figures are likely to be underestimates because of the usually low autopsy rates for AIDS patients. Morphologically, AIDS-related non-Hodgkin's lymphomas fall into two broad groups: diffuse large B-cell non-Hodgkin's lymphomas, which often show a prominent immunoblastic component, and Burkitt's lymphoma and Burkitt's-like lymphoma. As lymphomas in AIDS patients have been reviewed recently (IARC, 1996), we focus here only on the EBV-related lymphomas and mechanistic aspects.

(*a*) *Viral factors*

The two histopathological types of AIDS-related non-Hodgkin's lymphoma show striking differences in their relationship to EBV, suggesting different pathogenetic mechanisms. Most diffuse large B-cell non-Hodgkin's lymphomas, particularly those with immunoblastic differentiation, and all AIDS-related central nervous system lymphomas are EBV-positive (MacMahon *et al.*, 1991; for review, see IARC, 1996). Diffuse large B-cell lymphomas have been reported to occur relatively late in AIDS patients (Gaidano & Dalla-Favera, 1995), and more advanced depression of the immune system is a risk factor for their development (Pedersen *et al.*, 1991). The exceedingly rare primary effusion lymphomas fall into the group of diffuse large B-cell non-Hodgkin's lymphomas on morphological grounds; however, they appear to be a special case because of the unusual site of presentation and because the tumour cells often harbour KSHV/HHV8 in addition to EBV (Cesarman *et al.*, 1995). This suggests that the two

viruses cooperate in the pathogenesis of this disease, but the nature of the co-infection is at present uncertain. More recently, primary effusion lymphomas harbouring KSHV/-HHV8 only have also been described (Chang *et al.*, 1994), and thus the role of EBV in the development of this special type of non-Hodgkin's lymphoma remains uncertain.

Regardless of histological type, most AIDS-related non-Hodgkin's lymphomas appear to be monoclonal, both with respect to their antigen receptor genes and to the EBV episomes (Ballerini *et al.*, 1993; Delecluse *et al.*, 1993c; Shibata *et al.*, 1993). This situation differs from that in lymphoproliferative processes occurring in transplant patients (see section 4.3.1.2), which are frequently polyclonal; however, Delecluse *et al.* (1993c) demonstrated that rare cases may be polyclonal and thus resemble post-transplant lymphoproliferative disorder.

(*b*) *Disturbances of immunity as cofactors*

As EBV-positive, AIDS-related B-cell lymphomas consistently lack the HIV genome, a direct contribution of HIV to tumorigenesis beyond suppression of the immune system is unlikely (Knowles, 1993; IARC, 1996). Shiramizu *et al.* (1994) reported rare cases of HIV-containing tumours, but this work requires confirmation.

As discussed below, HIV may contribute by inducing severe immunosuppression, leading to a loss of EBV-specific T-cell immunity, or by chronic stimulation of the B-cell system. The relative risk for AIDS-related non-Hodgkin's lymphoma increases with duration of HIV infection (Rabkin *et al.*, 1992) and to a certain extent with immune suppression (Muñoz *et al.*, 1993). T Cell-mediated immunosurveillance is known to play an important part in controlling the proliferation of EBV-infected B lymphocytes in humans (for review, see Rickinson *et al.*, 1992; Masucci & Ernberg, 1994). The spontaneous outgrowth of EBV-positive cells in peripheral blood from AIDS patients is greater than that in immunocompetent individuals (Birx *et al.*, 1986; Ragona *et al.*, 1986; Rinaldo *et al.*, 1986). Similarly, the number of EBV-carrying lymphocytes detected in peripheral lymphoid tissues is greater in HIV-infected individuals than in non-immunocompromised controls (Niedobitek *et al.*, 1992a). This evidence indicates that disturbances in EBV-specific immunity are a pathogenetic factor in the development of some EBV-associated, AIDS-related non-Hodgkin's lymphomas. The location to the central nervous system of one predominant group of AIDS-associated lymphomas may reflect the fact that this site is less accessible for immunosurveillance (MacMahon *et al.*, 1991).

The absence of those viral latent genes that are commonly recognized by cytotoxic T cells from AIDS-associated Burkitt's lymphomas is in keeping with the generally better immune system of these patients in comparison with those with EBV-associated diffuse large-cell non-Hodgkin's lymphomas. It also suggests that the pathogenetic mechanisms leading to the development of these two lymphoma types are different (Knowles, 1996); however, a few AIDS-related cases of non-Hodgkin's lymphoma fall between the two morphological categories, diffuse large B-cell non-Hodgkin's lymphoma and Burkitt's lymphoma, and these cases carry *c-myc* translocations and may thus represent evolution of Burkitt's lymphoma *in vivo* (Delecluse *et al.*, 1993c). This process may be similar to the shift of Burkitt's lymphoma cells to the lymphoblastoid cell line-like phenotype observed *in vitro*. That this may also occur *in vivo* is suggested by the detection of iso-

lated cells expressing LMP-1 and/or EBNA-2 in cases of endemic and AIDS-related Burkitt's lymphoma (Hamilton-Dutoit *et al.*, 1993a; Niedobitek *et al.*, 1995). Such a shift could be expected to coincide with progressive deterioration of the immune system.

(*c*) *Oncogenes and genetic abnormalities as possible cofactors*

There is some evidence that disruption of the cytokine network contributes to the pathogenesis of AIDS-related non-Hodgkin's lymphoma. Thus, it has been shown that B cells from HIV-infected patients, unlike those of normal controls, constitutively express IL-6 and TNFα (for review see Gaidano & Dalla-Favera, 1995). This is of relevance, as IL-6 is known to promote the growth of EBV-positive B cells (Tanner & Tosato, 1992). IL-6 expression has been shown in endothelial cells and macrophages admixed with the neoplastic cells in biopsy samples of AIDS-related non-Hodgkin's lymphomas (Emilie *et al.*, 1992a). Moreover, EBV-positive AIDS-related Burkitt's lymphomas have been shown to express IL-10 (Emilie *et al.*, 1992b; Masood *et al.*, 1995), another cytokine which may directly or indirectly promote the growth of EBV-positive B cells (Vieira *et al.*, 1991; Herbst *et al.*, 1996b). It appears therefore, that IL-6 and IL-10 (and possibly other cytokines) can contribute to the development of AIDS-related non-Hodgkin's lymphoma through paracrine or autocrine regulatory loops.

As discussed previously, AIDS-related Burkitt's lymphomas, both EBV-positive and EBV-negative, have been consistently shown to harbour the characteristic *c-myc* translocation. These translocations have been detected in a minority of diffuse large B-cell lymphomas and cases with morphological features between large B-cell lymphoma and Burkitt's lymphoma (Ballerini *et al.*, 1993; Delecluse *et al.*, 1993c; Bhatia *et al.*, 1994). Although *p53* point mutations have been detected in 60% of Burkitt's lymphomas in AIDS patients, they were not seen in any other type of non-Hodgkin's lymphoma (for review, see IARC, 1996). Chromosomal translocations in AIDS-related non-Hodgkin's lymphomas frequently involve *bcl-6*, a proto-oncogene that affects B-cell maturation, which maps to 3q27 (Ye *et al.*, 1993). This is the region mainly affected in large-cell lymphomas, rearrangements being seen in 20% of cases. Although the rearrangements of *bcl-6* and *c-myc* occur in approximately 20% of cases of diffuse large B-cell non-Hodgkin's lymphoma, they have been reported to be mutually exclusive (Gaidano *et al.*, 1994).

Point mutations were detected in N-*ras* and K-*ras* in four of 27 AIDS-related non-Hodgkin's lymphomas investigated, but not in non-Hodgkin's lymphomas in immunocompetent hosts (Ballerini *et al.*, 1993).

Deletion of the long arm of chromosome 6 at band q27 was described in five of 13 large-cell lymphomas, some of which were EBV-positive (Pastore *et al.*, 1996). This rearrangement is seen in a wide range of non-Hodgkin's lymphomas in immunocompetent hosts (Gaidano *et al.*, 1992). Genetic abnormalities in chromosome 1q have been reported in AIDS-related cases of Burkitt's lymphoma by several groups, similar to those seen in endemic cases (Bernheim & Berger, 1988; Polito *et al.*, 1995), site 1q 21-25 being frequently involved.

Thus, AIDS-related non-Hodgkin's lymphomas, whether EBV-associated or not, frequently show genetic changes implicated in the pathogenesis of malignant lymphomas, including *c-myc* translocations, *p53*, N-*ras* and K-*ras* point mutations and deletions in the long arm of chromosome 6 (Gaidano & Dalla-Favera, 1995).

(*d*) *Pathogenesis of EBV-associated, AIDS-related non-Hodgkin's lymphoma: A scenario*

AIDS-related non-Hodgkin's lymphomas thus appear to fall into two broad categories with distinct pathogenetic pathways. Diffuse large B-cell lymphomas occur in severely immunocompromised individuals, are mostly EBV-positive and express type-II or -III latency patterns. While most of these cases are monoclonal and morphologically malignant, rare EBV-positive polyclonal lymphoproliferations have been described. It appears, therefore, that the pathogenesis of these lesions is similar to that of post-transplant lymphoproliferative disorder (Gaidano & Dalla-Favera, 1995), with an initial EBV-driven proliferation of B cells leading eventually to the outgrowth of fully malignant lymphomas. This process would depend on the acquisition of additional genetic change, e.g. *c-myc* or *bcl-6* rearrangements (Gaidano & Dalla-Favera, 1995).

The clinical, molecular and viral features of AIDS-related Burkitt's lymphoma suggest a pathogenesis similar to that of sporadic Burkitt's lymphoma. The comparatively high incidence of these lymphomas in AIDS patients points to the contribution of an AIDS-related factor independent of HIV-mediated immunosuppression. As these lymphomas have been shown to be HIV-negative (Knowles, 1993), a direct role of HIV is unlikely. The primary event in AIDS-related Burkitt's lymphoma appears to be chronic stimulation of the immune system, leading to polyclonal B-cell hyperplasia; this process may well be driven initially by HIV infection. The relatively low rate of EBV infection in these lymphomas suggests that the function of the virus can be substituted by other, as yet unidentified, factors.

4.3.2 *T-Cell lymphomas*

The relatively frequent detection of EBV in T-cell lymphomas does not fit easily into the well-established B-cell lymphotropism of the virus *in vitro*. As noted earlier (see Section 1), a subset of T cells has been shown to express CD21 or a related molecule that could act as the receptor for EBV. Interpretation of the detection of EBV in T-cell non-Hodgkin's lymphomas and an assessment of the role of the virus in the pathogenesis of T-cell lymphomas are complicated by two factors. Firstly, if EBV infection of certain T cells *in vitro* leads to a predominantly lytic infection, EBV infection of T cells may be accidental rather than part of the viral strategy to establish persistent infection. Such accidental infection of a cell type not adapted to latent EBV infection may contribute to the development of EBV-associated T-cell lymphomas. Secondly, in many cases, the virus is detected in only a small proportion of tumour cells (Anagnostopoulos *et al.*, 1996). Particularly in T-cell lymphomas, this problem is compounded by the polymorphism of the tumour cells. Thus, it may be difficult to decide if an individual cell is a tumour cell or a reactive lymphoid cell. Furthermore, the identification of EBV in neoplastic cells usually rests on the detection of the EBERs, which may not be expressed

even in the presence of EBV. Nevertheless, there seems to be good evidence to suggest that the reported detection of EBV in a fraction of cells in T-cell lymphomas reflects the actual situation (Asada *et al.*, 1994; Imai *et al.*, 1996).

The question of the significance of the virus in the pathogenesis of these lymphomas thus arises. Although the virus is present at the onset of the neoplastic process, it may subsequently be lost from the tumour cells. While there is now some evidence to suggest that this may happen *in vitro* (Takada *et al.*, 1995), it has not yet been shown to occur *in vivo*. The alternative scenario would be secondary infection of established neoplastic T cells with the virus. While this would exclude a contribution of the virus to the initial neoplastic process, it would be compatible with a contribution to the disease process, similar to the role of late *p53* gene mutations in the progression of follicular lymphomas. The frequent expression of the LMP-1 protein of EBV in T-cell lymphomas (d'Amore *et al.*, 1996) would seem to argue in favour of such a role.

EBV is often monoclonal in T-cell lymphomas containing a subset of virus-carrying tumour cells, which argues against simple superinfection of an established tumour (Ott *et al.*, 1992). Studies of EBV-associated T-cell lymphomas at primary presentation and at relapse could be informative.

In addition to the peripheral T-cell lymphomas occurring in western populations, in which subpopulations of the tumour cells often carry the virus, there are clearly some T-cell non-Hodgkin's lymphomas in which the presence of monoclonal EBV genomes is a defining characteristic of the neoplastic population. This group is exemplified by angiocentric sinonasal T-cell lymphomas, which occur predominantly in Asian populations. The role of the virus in the pathogenesis of these tumours is uncertain; however, the clonal nature of the EBV genome and its detection in virtually all tumour cells suggests a role of EBV early in tumorigenesis. While mechanisms have not been identified in lymphomatous granulomatosis, it has been shown that the EBV-infected B cells express LMP-1 (Takeshita *et al.*, 1996).

4.4 Hodgkin's disease

Over the last decade, evidence has accumulated implicating EBV in the pathogenesis of Hodgkin's disease. Up to 50% of cases in western countries (Weiss *et al.*, 1989a; Herbst *et al.*, 1991a; Armstrong *et al.*, 1992) and up to 100% in some other populations (Weinreb *et al.*, 1996b) carry the virus in the presumably neoplastic HRS cells. Sero-epidemiological studies have shown elevated antibody titres to EBV antigens in Hodgkin's disease patients both at the time of presentation and some years before the onset of the disease (Mueller *et al.*, 1989; Mueller, 1997). Furthermore, patients with infectious mononucleosis have a fourfold increase in risk for developing Hodgkin's disease (Muñoz *et al.*, 1978). More direct evidence linking EBV with Hodgkin's disease has accumulated (see also section 1.2.2). Thus, EBV DNA has been detected in tumour biopsies and has been localized to malignant HRS cells by in-situ hybridization (Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989; Weiss *et al.*, 1989a). Significantly, it has been shown by EBER in-situ hybridization that the virus is present in virtually all tumour cells in EBV-associated Hodgkin's disease (Wu *et al.*, 1990; Herbst *et al.*, 1992). This finding

is consistent with the detection of monoclonal EBV episomes in cases of Hodgkin's disease by Southern blot hybridization and indicates that the EBV infection took place before initiation of clonal cellular proliferation (Weiss *et al.*, 1987; Anagnostopoulos *et al.*, 1989). In agreement with this idea, it has been shown that patients with multifocal Hodgkin's disease have the virus in all affected tissues and that the virus persists in recurrent disease (Coates *et al.*, 1991a; Boiocchi *et al.*, 1993a; Brousset *et al.*, 1994; Vasef *et al.*, 1995).

Further evidence suggesting a role for EBV in the pathogenesis of Hodgkin's disease comes from the analysis of EBV gene expression in HRS cells. Transcriptional studies by RT-PCR have shown that *EBNA-1* expression in Hodgkin's disease tissues is driven exclusively by the F promoter; *LMP-1* and usually *LMP-2A* and *LMP-2B* are also expressed, in addition to *EBER*s (Deacon *et al.*, 1993). Immunohistochemical studies have now confirmed that EBNA-1, LMP-1 and LMP-2A are indeed expressed by the HRS cells in EBV-associated Hodgkin's disease, while EBNA-2 is consistently not detectable (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a; Delsol *et al.*, 1992; Grässer *et al.*, 1994; Niedobitek *et al.*, 1997a). This pattern of EBV latent gene expression is consistent with type-II latency. These studies indicate that the viral genome is actively expressed in HRS cells and thus reveal that EBV is not merely a silent passenger in these cells.

The main function of *EBNA-1* is maintenance of the viral episome (Yates *et al.*, 1984); however, a recent study of *EBNA-1*-transgenic mice suggests that this gene may also contribute to the development of lymphoid tumours (Wilson *et al.*, 1996a). Whether *EBNA-1* expression in HRS cells contributes to the pathogenesis of Hodgkin's disease remains uncertain.

LMP-2A expression is thought to block the induction of the lytic viral cycle after cross-linking of the B-cell receptor complex, by acting as a dominant negative inhibitor of Src and Syk kinases (Longnecker & Miller, 1996). In line with this scheme and with the frequent detection of LMP-2A in HRS cells (Niedobitek *et al.*, 1997c), it has been shown that EBV lytic cycle proteins are expressed only infrequently in HRS cells (Pallesen *et al.*, 1991c; Brousset *et al.*, 1993). Several studies suggest that *LMP-2A* and *LMP-2B* are not required for EBV-induced B-cell transformation (Longnecker *et al.*, 1992, 1993a,b); however, recent work by Brielmeier *et al.* (1996) indicates that *LMP-2A* enhances the efficiency of transformation and that this effect is not dependent on prevention of entry into the lytic cycle. Thus, *LMP-2A* expression may contribute to the malignant phenotype of HRS cells in EBV-associated Hodgkin's disease.

Of particular relevance is the detection of LMP-1 in HRS cells in virtually all cases of virus-associated Hodgkin's disease (Herbst *et al.*, 1991a; Pallesen *et al.*, 1991a; Delsol *et al.*, 1992) and the ability of LMP-1 to induce expression of activation markers such as CD23, CD30 and CD70 (Wang *et al.*, 1988b, 1990a; Isaacson *et al.*, 1992; Hamilton-Dutoit *et al.*, 1993a); however, these antigens are strongly expressed in HRS cells of both EBV-positive and EBV-negative cases.

There is growing evidence to suggest that EBV infection modulates the phenotype of Hodgkin's disease. Thus, EBV is preferentially associated with the development of mixed cellularity Hodgkin's disease. Moreover, the presence of EBV in HRS cells has

been shown to correlate with increased expression of lymphocyte activation antigens and decreased expression of the CD20 B-cell antigen (Bai *et al.*, 1994). Of particular relevance is the association of EBV infection with the expression of T_h2 cytokines such as IL-10 and IL-6 in HRS cells (Herbst *et al.*, 1996b; Klein *et al.*, 1996). Thus, although definite proof of an etiological role of EBV in the pathogenesis of Hodgkin's disease has yet to be attained, the available evidence strongly implicates the virus as a cofactor in the pathogenesis and morphogenesis of a significant proportion of cases.

It has been observed that cases of Hodgkin's disease arising in the setting of iatrogenic or HIV-induced immunosuppression are almost invariably EBV-associated. Moreover, regression of EBV-positive cases of Hodgkin's disease has been reported after restoration of the immune system (Berger & Delecluse, 1993), providing further evidence for an etiological role of EBV in the pathogenesis of Hodgkin's disease.

4.5 Nasopharyngeal carcinoma

4.5.1 *EBV infection*

4.5.1.1 *Molecular and biochemical studies*

EBV-positive nasopharyngeal carcinoma cells cannot yet be cultivated *in vitro*, and only a small proportion of tumour samples can be propagated in nude mice. Therefore, EBV infection in nasopharyngeal carcinoma has been characterized primarily by analysis of viral nucleic acids and proteins in samples obtained at biopsy and tumours passaged in nude mice (Raab-Traub *et al.*, 1983; Busson *et al.*, 1988). EBV episomes have been detected in nasopharyngeal carcinoma samples by electron microscopy (Kaschka-Dierich *et al.*, 1976) and by restriction enzyme analyses based on identification of the ends of the EBV genome (Raab-Traub & Flynn, 1986). Clonal EBV episomes are detected in such samples (Raab-Traub & Flynn, 1986), indicating that every copy of the viral genome within each cell is identical and the EBV episomes in every cell within the tumour are identical. The detection of homogeneous EBV molecules indicates that the tumour cells are a clonal expansion of a single cell that was infected with EBV.

In addition, in some tumour samples, particularly from endemic areas, faint ladder arrays of fragments representing linear genomes are also detected. This suggests that in occasional cells within the tumour the virus may be reactivated from latent infection. It is likely that this sporadic replication is the source of the antigenic stimulus that provokes the elevated antibody response to viral replicative antigens.

As episomal EBV DNA is present in multiple copies in nasopharyngeal carcinoma, it is difficult to determine whether EBV is also integrated; however, evidence of EBV integration was detected by pulse field analysis in four of 17 samples of nasopharyngeal carcinoma (Kripalani-Joshi & Law, 1994). Although integration may promote changes in cellular or viral expression that could contribute to tumour development, it is probably not a necessary event for development of this tumour.

4.5.1.2 *EBV expression*

EBV expression in nasopharyngeal carcinoma is different in several striking respects from that detected in transformed lymphocytes. The first studies of viral expression in samples of nasopharyngeal carcinomas revealed that sequences from the *Eco*RI-*Dhet* and the *Bam*HI K fragments were consistently transcribed (Raab-Traub *et al.*, 1983). These sequences were subsequently shown to encode the LMPs and EBNA-1, respectively, Surprisingly, the sequences that encode EBNA-2, a viral protein essential for transformation of lymphocytes, are not transcribed in nasopharyngeal carcinoma.

The pattern of gene expression suggested by transcriptional studies has for the most part been confirmed by the identification of specific viral proteins with monoclonal antibodies. LMP-1 and EBNA-1 have been detected, while EBNA-2, -3A, -3B, -3C and -LP are usually not found (Fåhraeus *et al.*, 1988; Young *et al.*, 1988). LMP-1 is not always detected in all cells or in all tumours on immunoblots, and EBNA-2 has not been identified in tumour samples, possibly due to a lack of suitable reagents (Stewart & Arrand, 1993). The BZLF1 protein has been detected in a few cells in some nasopharyngeal carcinomas, and spliced *BZLF1* mRNA and other mRNAs encoding lytic cytoproteins can also be detected by RT-PCR (Cochet *et al.*, 1993; Martel-Renoir *et al.*, 1995).

As the mRNAs that encode EBNAs all initiate at the W or C promoter in lymphocytes, it was of interest to determine the structure of the *EBNA-1* mRNA in nasopharyngeal carcinomas. Intriguingly, it was shown to initiate from sequences within *Bam*HI Q at a previously unidentified promoter, Q (Smith *et al.*, 1993; Tsai *et al.*, 1995). The Q promoter is complex and is apparently both autoregulated and governed by interferon regulatory factors (Sample *et al.*, 1992; Sung *et al.*, 1994).

In nasopharyngeal carcinoma, two different mRNAs encode LMP-1: the well-characterized, 2.8-kb *LMP-1* mRNA and a second, 3.7-kb mRNA (Gilligan *et al.*, 1990a). The 3.7-kb mRNA initiates from a promoter within the terminal repeat unit, which is transactivated *in vitro* by the SP1 transcription factor (Sadler & Raab-Traub, 1995b). Consistent transcription of *LMP-2* has been detected in nasopharyngeal carcinomas by northern blot analysis and RT-PCR (Busson *et al.*, 1992a). One study suggested that the *LMP-2B* or *TP2* transcript is preferentially expressed in nasopharyngeal carcinoma in comparison with matched B-cell lines (Smith & Griffin, 1991). Antibodies to LMP-2 were found highly specifically in patients with nasopharyngeal carcinoma (Frech *et al.*, 1993; Lennette *et al.*, 1995), suggesting that this protein is expressed in the tumour *in vivo*. In nasopharyngeal carcinoma, *EBNA-2*, *-3A*, *-3B*, *-3C* and *-LP* are not expressed, yet the promoters for *LMP-1* and *LMP-2* are apparently active, as the genes are transcribed. It is presently unclear how expression of these genes is regulated in the absence of the EBNA-2 and EBNA-3 proteins. Several studies have shown that methylation of the viral genome is an important regulatory element. The viral genome in nasopharyngeal carcinoma cells is extensively methylated, with the exception of *oriP* and regulatory elements for the *LMP-1* and *LMP-2* promoter (Ernberg *et al.*, 1989; Minarovits, 1991, 1994b; Hu *et al.*, 1991b). It is particularly striking that although the coding sequences for *LMP-1* are methylated, the promoter region is methylated only in those tumours in which the LMP-1 protein is not detected.

In addition to these RNAs, which are known to encode protein, a family of intricately spliced mRNAs was originally identified in samples of nasopharyngeal carcinomas (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b, 1991). Sequence analysis of cDNA revealed that the RNAs are 3′ co-terminal but differentially spliced (Smith *et al.*, 1993; Sadler & Raab-Traub, 1995a). The various RNAs contain different exons, forming novel ORFs at least one of which has been shown to encode a protein (Gilligan *et al.*, 1991; Fries *et al.*, 1997) that has been detected in most nasopharyngeal carcinoma samples. Although these RNAs are most abundant in nasopharyngeal carcinoma, they have also been identified in transformed lymphocytes and in Burkitt's lymphoma (Brooks *et al.*, 1993a).

The most abundant RNAs in EBV-infected cells are small nuclear RNAs transcribed by RNA polymerase III (Lerner *et al.*, 1981; Arrand & Rymo, 1982). These *EBERs* are present at approximately 10^6 copies per cell but are not necessary for lymphocyte transformation (Swaminathan *et al.*, 1991). They are, however, expressed in all of the malignancies associated with EBV and presumably contribute in some way to the maintenance of latency *in vivo* (Wu *et al.*, 1991). Interestingly, expression of the *EBERs* seems to be down-regulated during differentiation. Thus, samples of nasopharyngeal carcinoma that show differing degrees of differentiation lack EBER expression in differentiated areas (Pathmanathan *et al.*, 1995a). Extensive screening showed the presence of EBERs in 4 of 5 (80%) subtype 1, 71 of 73 (97%) subtype 2 and 71 of 73 (97%) subtype 3 tumours. In the subtype 1, EBER expression was largely confined to the basal area and was not detected in regions of differentiation (Tsai *et al.*, 1996b). The EBERs are also not detected in the permissive EBV infection, hairy leukoplakia (Gilligan *et al.*, 1990b).

These findings indicate that nasopharyngeal carcinoma represents a latent infection with EBV involving consistent expression of specific viral genes in a pattern distinct from that found in transformed B lymphocytes. The properties of the viral proteins expressed in nasopharyngeal carcinoma are reviewed in sections 1 and 4.1 This unique state of latency is also found in several other malignancies linked to EBV, including Hodgkin's disease and T-cell lymphoma, and has been called type II latency (Rickinson & Kieff, 1996; see Section 1).

4.5.1.3 *Phenotype and cellular gene expression*

The presence of an intense lymphoid stroma is a characteristic feature of undifferentiated carcinomas of the nasopharyngeal type; the functional role of this stroma has been a matter of debate. Recent studies have suggested that in undifferentiated carcinomas the stroma is required to support tumour cell growth (Busson *et al.*, 1987; Agathanggelou *et al.*, 1995). The difficulty in establishing nasopharyngeal carcinoma xenografts in nude mice may reflect loss of the infiltrating stroma (Busson *et al.*, 1987).

Expression of many markers, including potential factor receptors, usually expressed in B cells has been described in primary nasopharyngeal carcinoma samples. These include CD23, CD24, Ia antigen and Cdw70 (Ebbers *et al.*, 1985; Billaud *et al.*, 1989; Rousselet & Tursz, 1992; Niedobitek *et al.*, 1992c; Karran *et al.*, 1995). The expression of adhesion molecules is also altered, with elevated expression of ICAM-1 (CD54) and reduced expression of LFA3 (CD58) in comparison with EBV-immortalized B cells

(Busson *et al.*, 1992b). These effects are probably due to expression of *LMP-1* in nasopharyngeal carcinoma cells.

Interestingly, clinical analysis of nasopharyngeal carcinomas with and without LMP-1 expression suggested that the LMP-positive tumours grew more rapidly but that the LMP-1-negative tumours were more likely to recur and had an increased tendency to metastasize (Hu *et al.*, 1995). This difference may be due to the fact that B7 (CD80/CD87) is expressed in LMP-1-positive but not in LMP-1-negative tumours (Agathanggelou *et al.*, 1995).

4.5.1.4 *EBV infection and transformation of epithelial cells* in vitro

Although EBV readily infects and transforms B lymphocytes, human epithelial cells cannot easily be infected or transformed; however, monkey epithelial cells were transformed by transfer of specific EBV DNA fragments cloned into cosmid vectors (Griffin & Karran, 1984). The fragments that had transforming ability included the *Bam*HI A region of the genome which has since been shown to be expressed in nasopharyngeal carcinomas (Hitt *et al.*, 1989; Gilligan *et al.*, 1990b). Some attempts to infect human epithelial cells directly have resulted in morphological transformation but not permanent transformation or EBNA expression (Desgranges & de Thé, 1977; Huang *et al.*, 1978a). In another study of epithelial cells infected *in vitro*, evidence of EBV replication was detected only in wild-type strains obtained directly from throat washings, suggesting distinct biological properties of some strains (Sixbey *et al.*, 1983).

The difficulty of infecting epithelial cells may be due to differences in expression of the EBV receptor, CD21 or CR2. Evidence for expression of the receptor is conflicting, depending on the monoclonal antibody used and the source of epithelium (Young *et al.*, 1989c; Sixbey *et al.*, 1987). Transfer of CD21 into an epithelial cell line facilitated infection, and a high level of infected cells was detected early after infection with the Akata strain of EBV (Li *et al.*, 1992). The genome was rapidly lost from these cells, and some cells spontaneously entered the replicative cycle; however, cell clones could be established that retained the EBV genome in episomal form and expressed *EBNA-1* and *LMP-1* (Knox *et al.*, 1996). The stably infected clones seemed to be impaired in their ability to differentiate or to allow viral replication. Clones that lacked EBV were also impaired in differentiation, suggesting that impairment in differentiation influences the ability of EBV to establish a latent, transforming infection.

The mode of viral entry may also influence the outcome of infection. It has been shown that secretory IgA facilitates the entry of EBV into epithelial cells (Sixbey & Yao, 1992). The cellular organization also modulates EBV infection, such that EBV is transcytosed by EBV-specific IgA directly through the epithelium in polarized cultures, but EBV replicative gene products are detected in unpolarized cultures (Gan *et al.*, 1997).

In some epithelial cell lines, LMP-1 has been reported to inhibit differentiation and cause morphological transformation, with decreased cytokeratin expression (Dawson *et al.*, 1990; Fåhraeus *et al.*, 1990a; Niedobitek *et al.*, 1992d). Decreased expression of E-cadherin and increased invasive ability has also been described in epithelial lines expressing LMP-1 (Fåhraeus *et al.*, 1992). In transgenic mice in which LMP-1 was

expressed in skin, epithelial hyperplasia and altered expression of keratin 6 were observed (Wilson *et al.*, 1990).

LMP-1 expression induces expression of the epidermal growth factor receptor, specifically in epithelial cells, and elevated levels of epidermal growth factor receptor are also detected in nasopharyngeal carcinoma (Zheng *et al.*, 1994c; Miller *et al.*, 1995b). As discussed in section 4.1, two domains have been identified in the carboxy terminus of *LMP-1* that can activate NFκB (Huen *et al.*, 1995). A recent study indicated that the *TRAF* interacting domain is responsible for induction of expression of the epidermal growth factor receptor (Miller *et al.*, 1997). This suggests that *LMP-1* activates two distinct signalling pathways: one that activates NFκB and a second pathway mediated by *TRAF* activation that induces expression of genes like *EGFR* and also activates NFκB.

Several EBV replicative proteins have been shown to have different effects in epithelial cells and lymphoid cells or to have transforming properties in rodent cell lines. Although only *BZLF1* can disrupt latency in lymphoid cell lines, in infected epithelial cell lines such as NPC-KT and D98/HR1, the immediate early gene *BRFL1* can also disrupt latency (Zalani *et al.*, 1996). In addition, *BZLF1* has been shown to interact with *p53* (Zhang *et al.*, 1994b), the p65 component of NFκB (Gutsch *et al.*, 1994), and induce expression of TGFβ. The EBV early gene, *BHRF1*, is homologous to the *bcl2* gene and can block apoptosis induced by various agents (Henderson *et al.*, 1993). *BHRF1* can also complement transformation by the adenovirus *E1A* gene (Theodorakis *et al.*, 1996). Another early gene encoded by the *BARF1* ORF can transform rodent fibroblasts and induce tumorigenicity in the EBV-negative Louckes Burkitt's lymphoma cell line (Wei *et al.*, 1994). As expression of EBV replicative gene products occurs in only a few percent of nasopharyngeal carcinoma cells *in vivo*, it is unlikely, however, that they contribute to malignant growth.

4.5.1.5 *Detection of EBV infection in normal, premalignant and malignant nasopharyngeal tissues*

The site and state of infection in normal nasopharynx and in populations at high risk for developing nasopharyngeal carcinoma have been investigated in several studies. In patients with infectious mononucleosis, EBV DNA and mRNA encoding EA were detected in sloughed epithelial cells (Lemon *et al.*, 1978; Sixbey *et al.*, 1984). This suggested that the nasopharyngeal epithelial cells were the source of the infectious virus that is detected in saliva during infectious mononucleosis. Evidence of EBV replication has also been detected in epithelial cells in parotid tissue, where high copy numbers of EBV genomes were detected by in-situ hybridization (Wolf *et al.*, 1984). A recent study also showed evidence of EBV replication in epithelial cells adjacent to an EBV-positive T-cell lymphoma (Wen *et al.*, 1997). The epithelial cells had high copy numbers of EBV DNA and were BZLF1-positive but EBER-negative, while the malignant lymphocytes were positive for EBER and LMP-1 expression. In contrast, studies of normal nasopharyngeal mucosa have shown evidence of EBV only in lymphocytes in which EBER-positive and BZLF1-positive cells were occasionally detected (Tao *et al.*, 1995). EBV-infected lymphocytes were detected in nasal polyps, and some were positive for BZLF1

expression (Tao *et al.*, 1996). Similarly, Karajannis *et al.* (1997) detected isolated EBV-positive B cells but no EBV-positive epithelial cells in throat washings from patients with infectious mononucleosis, suggesting that lymphocytes are the source of virus secreted into the oropharynx in reactivated infection.

Comprehensive screening of a large Chinese population resulted in the identification of 1267 individuals with IgA to VCA. Of the 203 from whom a biopsy sample was taken, 46 had early nasopharyngeal carcinoma and an additional 12 had detectable nasopharyngeal carcinoma within 12 months. EBV was detected by Southern blot hybridization in 14 samples from individuals without detectable nasopharyngeal carcinoma (Desgranges *et al.*, 1982), but it was not determined whether the tissues were normal or whether the patients had occult nasopharyngeal carcinoma.

Attempts to culture transformed or EBV-infected cells taken at biopsy resulted in the establishment of EBV-positive lymphoid lines from nasopharyngeal tissues from patients with and without nasopharyngeal carcinoma and from adenoidal tissue from other patients; however, epithelial cell lines were not established (de Thé *et al.*, 1970; Takimoto *et al.*, 1989). Further, attempts led to the development of cell lines in which EBV genomes were initially detected but were lost over time (Gu *et al.*, 1983; Lin *et al.*, 1993b). It is unclear whether these cell lines represent the tumour cells.

EBV markers for EBNA or latent or replicative genes were not detected in samples of normal tissue from patients without histological evidence of nasopharyngeal carcinoma, and were found only in samples with nests of tumour cells (Huang *et al.*, 1978b; Sam *et al.*, 1993). Subsequent screening showed EBV in all biopsy samples of nasopharyngeal carcinoma, but only the genome was detected in a subset of cells from carcinoma *in situ* (Yeung *et al.*, 1993). Samples from carcinomas *in situ* with microinvasion had a higher proportion of EBV-positive cells. An extensive screening of 5326 biopsy samples from the ear-nose-and-throat clinic at the University of Malaya resulted in the detection of nasopharyngeal carcinoma in 1744 samples; 56 samples had carcinoma *in situ* and invasive carcinoma, and only 11 samples showed dysplasia or carcinoma *in situ* without adjacent invasive carcinoma. In these samples, EBER and LMP-1 were detected in all cells, and analysis of the EBV termini revealed clonal EBV (Pathmanathan *et al.*, 1995a,b).

Some studies have suggested that EBV is detected only in discrete areas within a nasopharyngeal carcinoma, while in other studies homogeneous infection is detected throughout the samples (Wu *et al.*, 1991; Lin *et al.*, 1994; Pathmanathan *et al.*, 1995a,b). This variation may reflect differences in the method of detection or the state of infection. EBV DNA or EBER expression has been looked for as an indicator of the presence of EBV in many studies, but EBV DNA may not be detected in cells with a very low copy number and EBER expression may vary, as EBERs are not expressed in the permissive infection, hairy leukoplakia, or within areas of differentiation in tumours of mixed histological type (Gilligan *et al.*, 1990a; Pathmanathan *et al.*, 1995a,b). [The Working Group noted that the role of EBV in primary infection of epithelial cells is controversial.]

Taken together, these studies indicate that EBV infection of epithelial cells is rare. It may occur during primary infection but, in normal asymptomatic infection, EBV is

harboured in lymphoid cells. The rare event that leads to the development of nasopharyngeal carcinoma may enable establishment of a latent EBV infection in epithelium. The changes that predispose the cells to this event may have already induced changes in growth properties, which were originally suggested by Lenoir and de Thé (1978). The impaired differentiation observed by Knox *et al.* (1996) may have a correlate *in vivo* in the dysplasia described by Yeung *et al.* (1993). Furthermore, the study of Pathmanathan *et al.* (1995b) suggests that the combination of EBV oncogene expression and pre-existing cellular changes results in the rapid proliferation of an infected epithelial cell, which rapidly develops into malignant, invasive carcinoma (Raab-Traub, 1992a,b).

4.5.1.6 *Strain variation*

Many of the cancers associated with EBV, and particularly nasopharyngeal carcinoma, occur in distinct populations with endemic patterns of incidence. As a possible contributing factor is the prevalence of specific variants of EBV in distinct geographical locations with unique biological or pathological properties, strain variation has been studied continuously (Bornkamm *et al.*, 1980; Raab-Traub *et al.*, 1980). Most biological studies of EBV have involved virus obtained from Burkitt's lymphomas (HR1, W91, Akata, AG876) or from a patient with infectious mononucleosis (B95-8); one viral strain was obtained from a nasopharyngeal carcinoma biopsy sample that was co-cultured with lymphocytes (Crawford *et al.*, 1979). Viral replication could be induced in some of the lymphoid cell lines, and these studies revealed that virus from nasopharyngeal carcinomas is both replication and transformation competent. This isolate, the MABA strain, was further compared by restriction enzyme analysis and shown to be largely identical to other EBV strains from cases of infectious mononucleosis and Burkitt's lymphoma (Bornkamm *et al.*, 1980; Polack *et al.*, 1984b). EBV DNA in nasopharyngeal carcinoma biopsy samples was also shown to be similar to that in laboratory strains (Raab-Traub *et al.*, 1987). A second nasopharyngeal carcinoma strain was obtained by direct fusion of nasopharyngeal carcinoma tumour cells with a human adenoidal epithelial cell line (Takimoto *et al.*, 1985). This cell line, NPC-KT, produces virus that can transform lymphocytes and can spread during cell-to-cell fusion (Sato *et al.*, 1989; Yoshizaki *et al.*, 1994).

Specific genetic variations in the viral genome have been looked for by restriction enzyme polymorphism, and specific strains were found to be prevalent in different populations. Lung *et al.* (1990) and Lung and Chang (1992) first described a common Chinese variant marked by loss of the *Bam*HI site between the W1′ and I1′ fragments (designated *C* variant), frequently with an additional *Bam*HI site in the F fragment (*f* variant). This *Cf* variant was detected in 86% of nasopharyngeal carcinoma biopsy samples from southern China, while healthy subjects usually had the *C* variation without the *f* polymorphism. The *f* variant was more frequently detected in the nasopharyngeal carcinoma biopsy samples than in peripheral blood lymphocytes or throat washings (Lung *et al.*, 1992). The *C* variation was also prevalent in Japanese populations; however, this variant lacked the *f* polymorphism (Tamura *et al.*, 1993). A polymorphism at an *Xho*I site was also detected in most Chinese samples (Hu *et al.*, 1991a; Miller *et al.*, 1994b).

Abdel-Hamid *et al.* (1992) performed similar analyses on epithelial and lymphoid malignancies from endemic and non-endemic regions, including Chinese, Mediterranean and American populations, determining the restriction enzyme polymorphisms and EBV type. The prevalent strain in Southeast Asia was a variant of EBV-1, which had the *Bam*HI and *Xho*I polymorphisms described above. In nasopharyngeal carcinomas from areas of middle or low incidence, a distinctive EBV-1 subtype, originally identified in the nasopharyngeal carcinoma-derived MABA strain, was found in approximately 50% of the tumours, and an additional 25% had the Chinese polymorphisms. In the Alaskan Inuit population, a new variant of EBV-2, with the polymorphisms from the right end of the genome that distinguish the Chinese EBV-1 strain, was identified in nasopharyngeal carcinomas and carcinomas of the parotid gland. In contrast, the standard EBV-1 genome, lacking the above-mentioned polymorphisms, was detected in lymphomas from central Africa (Burkitt's lymphoma), Egypt and the United States.

The presence of specific restriction enzyme polymorphisms suggested that EBV strains might have consistent sequence variation. As EBV *LMP-1* is essential for transformation and is considered to be the EBV oncogene, many studies have focused on strain variation in the *LMP-1* gene. The sequence of a Chinese isolate, Cao, revealed several amino-acid changes and deletion of 10 amino acids within LMP-1 (Hu *et al.*, 1991a). The sequence of 50 Taiwanese isolates revealed that a particular strain was prevalent in Taiwan and had greater transforming ability in BALB/c 3T3 cells than LMP-1 from B95-8 (Chen *et al.*, 1992a). A similar comparison of the transforming ability of B95-8 and Cao in the RHEK epithelial cell line showed that both increased proliferation, with higher colony-forming efficiency and decreased differentiation, marked by decreased involucrin expression. The B95-8 and Cao transformants differed morphologically, and only the Cao transformants induced tumours in nude mice (Hu *et al.*, 1993; Zheng *et al.*, 1994c).

Sample *et al.* (1994) found that the *LMP-1* sequence in the prototype EBV-1 and EBV-2 strains were nearly identical. A comprehensive analysis of *LMP-1* sequence variation in nasopharyngeal carcinoma isolates from several geographical regions revealed that consistent base-pair changes, including the 10-amino acid deletion, marked a Chinese EBV-1 strain. In contrast, the Alaskan strain had many of the same base-pair changes but was undeleted and represented EBV-2. This study also showed that the number of small-repeat elements within *LMP-1* can vary, such that viral replication could produce progeny with differing numbers of the small *LMP-1* repeat (Miller *et al.*, 1994a). In a comparison of the EBV strain in nasopharyngeal carcinoma, T-cell lymphoma, normal nasopharynx and throat washings in Taiwan, the Chinese prototype strain was detected in 48/48 nasopharyngeal carcinoma samples, 23/25 samples of normal nasopharynx and 37/44 throat washings, indicating the widespread prevalence of this strain in Taiwan (Chang *et al.*, 1995).

Many studies have focused on the small deletion in *LMP-1*. The effect of the deletion on BALB/c 3T3 cell transformation was analysed by producing chimaeric proteins (Li *et al.*, 1996). It was found that the enhanced transforming ability of the Chinese strain in BALB/c 3T3 cells was transferred with the carboxy terminus that included the 10-amino acid deletion. Deletion of the 10 amino acids in B95-8 LMP-1 resulted in the ability to

induce transformation in BALB/c 3T3 cells and tumorigenicity, while insertion of the 10 amino acids into the Chinese strain eliminated transformation and tumorigenicity. As B95-8 *LMP-1* itself transforms in rodent fibroblasts, the significance of these differences in the transforming ability of *LMP-1* in cell lines remains unclear.

The presence or absence of the deleted form of *LMP-1* and multiple sequence changes in various disease states have also been evaluated (Knecht *et al.*, 1996). Some studies suggest that strains with deleted *LMP-1* are found in more aggressive tumours, and others indicate that these changes reflect the prevalence of a given strain within a population (Knecht *et al.*, 1993; Cheung *et al.*, 1996: Khanim *et al.*, 1996). In epithelial tumours, specific amino-acid changes have been detected in EBNA-1 which are suggested to influence its biological properties (Snudden *et al.*, 1995).

Trivedi *et al.* (1994) studied the effect of *LMP-1* on the immunogenicity of mouse mammary carcinoma cells. Immunization with B95-8 *LMP-1*-transfected tumour cells protected against challenge with B95-8 LMP-1-positive tumour cells, whereas immunization with Cao LMP-1 did not. This difference could be due to an inability of this particular mouse strain to present the MHC class-I or class-II epitopes of the Cao strain. It is possible that similar differences in immune response occur in distinct human populations.

These studies suggest that different EBV strains predominate in different geographical areas and that some variants may be detected preferentially in nasopharyngeal carcinoma. The prevalence of these distinct strains in epithelial malignancies could reflect an epithelial cell tropism in which particular variants of EBV preferentially establish latent infection in epithelial cells, or it may reflect differences in immunogenicity. It is also possible that strains that encode *LMP-1* with increased transforming properties have increased pathogenicity *in vivo.*

4.5.2 *Contribution of environmental and genetic factors*

4.5.2.1 *Dietary cofactors*

(*a*) *Experiments in rodents*

Groups of 10 male and 10 female Wistar WA rats were fed salted fish for six months and observed for one to two years. Four female rats developed carcinomas in the nasal or paranasal regions, while none was observed in the six control rats (Huang *et al.*, 1978c).

Salted fish purchased weekly in streetside markets in Hong Kong was fed to 111 male and 110 female Wistar-Kyoto rats aged 21 days, immediately after weaning in order to recreate the human experience. The animals were randomized by sex into one of three experimental groups: groups of 37 males and 37 females were fed a powdered diet consisting of one part steamed salted fish and three or five parts certified rat chow for the first 18 months; 37 males and 36 females were given rat chow only throughout the experiment. After 18 months, all rats were given rat chow pellets for the remainder of their lifespan. Four malignant nasal cavity tumours developed among the treated rats: the first three tumours (one undifferentiated carcinoma, one moderately differentiated squamous-cell carcinoma and one spindle-cell carcinoma) all occurred in the group at the

high dose; the fourth tumour was a spindle-cell tumour (not otherwise specified) in a rat at the low dose. No tumours of the upper respiratory tract were observed among control rats, consistent with the rarity of reports of such spontaneous tumours in rats. The rate of tumour occurrence among treated rats was statistically different from the base rate of zero ($p = 0.02$). The levels of salted fish fed to the rats were close to the range of human consumption, i.e. the amounts fed to Cantonese babies during weaning, as determined in a survey of mothers in Guangzhou, China (Yu *et al.*, 1989b).

In a two-year experiment, 16 pregnant Sprague-Dawley rats were randomized into four groups. Four rats were fed a diet consisting of 10% by weight steamed salted fish (purchased in Guangzhou, China) throughout pregnancy and lactation, and the 41 offspring continued to be fed the diet after weaning. Four pregnant rats were fed standard rat chow during pregnancy and lactation, but their 41 offspring were fed the 10% fish diet after weaning. Rats in the third group received a diet consisting of 5% salted fish by weight, as for the first group. Rats in the last group served as controls, both the mothers and the 40 offspring being fed a standard rat chow diet throughout the experiment. Seven nasal tumours were observed among the rats fed salted fish, with four in group 1 (one squamous-cell carcinoma, one poorly differentiated carcinoma, one fibrosarcoma and one adenocarcinoma), two in group 2 (one squamous-cell carcinoma and one rhabdomyosarcoma) and one soft-tissue sarcoma in group 3; no respiratory-tract tumours were seen in the controls. The difference in the occurrence of nasal tumours in the four experimental groups was statistically significant (p for linear trend = 0.04) (Zheng *et al.*, 1994d).

(*b*) *High-risk populations*

Low levels (subparts per million) of several volatile nitrosamines, including *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosodi-*n*-propylamine, *N*-nitrosodi-*n*-butylamine and *N*-nitrosomorpholine, have been detected in samples of Chinese salted fish (Huang *et al.*, 1981; Tannenbaum *et al.*, 1985). Most of these volatile nitrosamines can induce nasal tumours in animals (Haas *et al.*, 1973; Pour *et al.*, 1973; Althoff *et al.*, 1974; Lijinsky & Taylor, 1978). Bacterial mutagens were detected in Chinese salted fish that had been exposed to a nitrosating agent under simulated gastric conditions (Tannenbaum *et al.*, 1985; Weng *et al.*, 1992). Directly acting genotoxic substances have also been found in extracts of this food (Poirier *et al.*, 1989). In addition, samples of Chinese salted fish were found to contain substance(s) capable of activating EBV in latently infected Raji cells (Shao *et al.*, 1988; Poirier *et al.*, 1989).

Low levels (parts per billion) of several volatile nitrosamines have been detected in samples of Chinese mustard green, *chung choi* and fermented soya-bean paste and in Tunisian *quaddid* and *touklia* (Poirier *et al.*, 1987). *N*-Nitrosodimethylamine was detected in *quaddid*, *touklia*, salted mustard greens and *chung choi*; *N*-nitrosopiperidine in *touklia* and salted mustard greens; and *N*-nitrosopyrrolidine in *quaddid*, *touklia*, salted mustard greens, *chung choi* and fermented soya-bean paste. Directly acting genotoxic substances have been found in samples of Chinese salted shrimp and fermented soya-bean paste and in Tunisian *quaddid*, *touklia* and *harissa* (Poirier *et al.*, 1989). In addition, samples of *harissa* and *quaddid* were shown to contain substances capable of activating

EBV in latently infected Raji cells (Shao *et al.*, 1988; Poirier *et al.*, 1989). These substances have been characterized as including macromolecular lignin which can activate the *ZEBRA* EBV latent gene (Bouvier *et al.*, 1995).

Zheng *et al.* (1993) compared the levels of urinary nitrate and four nitrosamino acids in 77 healthy subjects in two villages in southern China in which there is a 10-fold range in the incidence of nasopharyngeal carcinoma. The five urinary metabolites were measured in 12-h samples of urine collected in the absence of treatment, after ingestion of L-proline in the urine of subjects from the high-risk village or after ingestion of L-proline in combination with vitamin C. The total urinary nitrosamino acids in the urine of subjects from the high-risk village was significantly increased above the baseline level after proline treatment, while no difference from background levels was noted after treatment with both proline and vitamin C. No such variation in nitrosamino acid levels was found in subjects in the low-risk village. The mean urinary nitrate levels were significantly higher among subjects in the high-risk than the low-risk village in both treated and untreated urine samples. These results demonstrate a higher potential for endogenous nitrosation in subjects living in areas of high risk for nasopharyngeal carcinoma and suggest the presence of nitrosation inhibitors in the diet of southern Chinese living in areas at relatively low risk for nasopharyngeal carcinoma. These results also support the hypothesis that dietary precursors of nitroso compounds are involved in the pathogenesis of nasopharyngeal carcinoma.

4.5.2.2 *Genetic factors*

The role of HLA-related genes in nasopharyngeal carcinoma is discussed in section 2.4.3. Several HLA types have been associated with development of the disease, and familial aggregation has been linked to the HLA region (Chan *et al.*, 1983b; Lu *et al.*, 1990). A recent study revealed that the CTL presentation of LMP-2 was restricted to HLA A2.1 (Murray *et al.*, 1992b), and an inverse association between risk for nasopharyngeal carcinoma and the HLA A2 type has been described (Burt *et al.*, 1994). It has also been suggested that patients with nasopharyngeal carcinoma have impaired T-cell immunity to EBV in general (Tamura *et al.*, 1992); however, if impaired T-cell immunity and HLA-restricted presentation contribute to the development of nasopharyngeal carcinoma, an elevated incidence of this tumour would be expected in patients with HIV, which has not been observed to date (Melbye *et al.*, 1996).

High-risk families in which multiple cases of nasopharyngeal carcinoma have occurred have been described in both high- and low-risk populations (for details, see section 2.4.3.3). Familial clustering of nasopharyngeal carcinoma is likely to be the result of genetic constitution and environmental exposures.

Genetic changes characteristic of other malignancies, such as *c-myc* rearrangement, *p53* mutation, *Rb* alterations or *ras* mutations, would not necessarily contribute to altered growth, and such mutations have not been detected in nasopharyngeal carcinomas in Chinese, American or Arab populations (Effert *et al.*, 1992; Spruck *et al.*, 1992; Sun *et al.*, 1993; Nasrin *et al.*, 1994; Sun *et al.*, 1995). Unmutated p53 protein is, however, detected at high levels in nasopharyngeal carcinomas and *p53* expression has been shown

to be induced by NFκB, a transcription factor that is activated by *LMP-1* (Niedobitek *et al.*, 1993b; Chen & Cooper, 1996). In studies to investigate whether an EBV protein interferes with some aspect of *p53* function and eliminates a selection for inactivating mutations, it was found that the EBV BZLF1 protein binds p53 and inhibits p53-dependent transcriptional *trans*-activation, however, *BZLF1* is expressed in rare cells in nasopharyngeal carcinomas and would be unlikely to influence the selection of inactivating mutations in *p53* in the majority of nasopharyngeal carcinoma cells (Zhang *et al.*, 1994b). In latent infections, EBV does not interfere with the ability of *p53* to arrest cells in G1 after DNA damage by inducing expression of the *p21* cyclin kinase inhibitor. Two studies have shown, however, that LMP-1 inhibits p53-mediated apoptosis induced by serum withdrawal (Okan *et al.*, 1995; Fries *et al.*, 1996). This property could be due to induction of *bcl2*, which has been described in lymphoid cell lines (Henderson *et al.*, 1991); but elevated *bcl2* expression is not linked to EBV infection in epithelial tumours (Lu *et al.*, 1993) and *bcl2* was not induced in epithelial cell lines expressing LMP-1 that were protected from apoptosis (Fries *et al.*, 1996). Specific protection from p53-mediated apoptosis was conferred by the A20 protein, which is induced by LMP-1 expression and protects against apoptosis induced by tumour necrosis factor or serum withdrawal. Protection against p53-mediated apoptosis is likely to be responsible for the lack of *p53* mutations in EBV-associated cancers.

Recent studies have identified areas of loss of heterozygosity on several chromosomes, including the regions 3p24 and 9p21 (Huang *et al.*, 1991, 1994). Deletions at 3p24 have also been detected in nasopharyngeal carcinomas in Indian populations (Kumari *et al.*, 1995). The *p16* gene at 9p21 is completely deleted in some samples of nasopharyngeal carcinoma (Lo *et al.*, 1995). Although *p16* is not deleted in the C15 tumour passaged in nude mice, it is not expressed (Sun *et al.*, 1995). The *p16* gene is a critical regulator of cell-cycle progression through G1, and mutations in the *Rb* gene, amplification of cyclins or inactivation of cyclin-dependent kinases can all inactivate this component of cellular control. Deletion of *p16* and repression of *p16* expression in nasopharyngeal carcinomas suggests that this pathway is affected in this tumour, as in many others. The identification of other specific genes in the regions that show chromosomal loss may help in finding the critical cellular genes that contribute to the development of nasopharyngeal carcinoma in high-risk populations or a gene that is affected by mutagenic environmental factors.

4.6 Other malignancies, including lymphoepithelial carcinomas

Latent EBV infection has been detected in gastric lymphoepithelial carcinoma, and EBV is also found in a subset of gastric adenocarcinomas in all geographic areas in which it has been studied (Shibata & Weiss, 1992; Rowlands *et al.*, 1993; Selves *et al.*, 1996a). Clonal EBV is detected in the tumours, while immunohistochemical studies suggest that EBNA-1 is expressed in the absence of detectable EBNA-2 or LMP-1 in both adenocarcinomas and lymphoepithelial carcinomas (Rowlands *et al.*, 1993; Fukayama *et al.*, 1994; Imai *et al.*, 1994a; Ott *et al.*, 1994; Murray *et al.*, 1996; Selves *et al.*, 1996b). This suggests a type-I EBV latency rather than the type-II latency found in

nasopharyngeal carcinomas. These results were confirmed recently at the transcriptional level by RT-PCR, which showed that EBNA-1 is expressed from the Q promoter, as in types-I and -II latency (Sugiura *et al.*, 1996). Consistent with the results of previous immunohistochemical studies, *EBNA-2* and *LMP-1* mRNA were not detectable; however, transcripts derived from the *Bam*HI A fragment of the viral genome were detected in all cases, and a proportion of cases had detectable levels of *LMP-2A* mRNA (Sugiura *et al.*, 1996). In a preliminary immunohistochemical study with new LMP-2A-specific monoclonal antibodies, no staining was detected in EBV-positive gastric carcinomas (Niedobitek *et al.*, 1997c). This suggests that carcinomas may represent a form of type-I latency, with possible additional expression of LMP-2A in a proportion of cases.

Expression of the BZLF1 protein and of *BZLF1* transcripts has also been reported in a few scattered cells in a proportion of gastric carcinomas (Niedobitek *et al.*, 1992b; Rowlands *et al.*, 1993), while Sugiura *et al.* (1996) did not detect lytic cycle RNA transcripts by RT-PCR. This suggests that viral replicative genes are expressed in a very small fraction of tumour cells; however, it is presently unclear whether this leads to the production of infectious virions. In a single case, Niedobitek *et al.* (1992b) reported the absence of detectable gp350 expression in a lymphoepithelial carcinoma of the stomach.

Multiple other types of epithelial cancer have also been linked to EBV. Most are rare undifferentiated carcinomas with a prominent lymphoid stroma (lymphoepithelial carcinomas), reminiscent of nasopharyngeal carcinoma. EBV has been associated with lymphoepithelial carcinoma of the parotid gland (Saemundsen *et al.*, 1982; Saw *et al.*, 1986; Huang *et al.*, 1988; Raab-Traub *et al.*, 199l), which develops at relatively high incidence among Inuit populations and in Chinese (Saw *et al.*, 1986; Huang *et al.*, 1988). Other epithelial salivary-gland tumours have been shown to be EBV-negative (Wen *et al.*, 1997). In a Japanese study, EBV was detected in lymphoepithelial carcinoma samples but not in benign lymphoepithelial lesions (Nagao *et al.*, 1996), suggesting that EBV infection is the pivotal step to malignancy. Like nasopharyngeal carcinoma, undifferentiated parotid tumours contain clonal EBV and express EBERs, LMP-1 and *Bam*HI A transcripts (Raab-Traub *et al.*, 1991).

Other rare cancers are linked to EBV infection. These include a subset of undifferentiated sinonasal carcinomas in Italian and Chinese populations (Gallo *et al.*, 1995; Leung *et al.*, 1995b), lymphoepithelial cholangiocarcinoma (Hsu *et al.*, 1996) and smooth-muscle tumours in HIV-infected children and transplant recipients (Morel *et al.*, 1996). The smooth-muscle tumours express *EBER-1* RNA and *EBNA-2* but not *LMP-1* (Lee *et al.*, 1995a). In all samples, identification of the EBV termini showed that the infection was non-permissive and clonal with regard to EBV. Genes expressed in latency type II are found in most tumours, although the detection is variable and some tumours, such as gastric carcinoma, may express only *EBNA-1*. The detection of EBV in tumours of diverse cell types indicates that EBV may gain entry into different cell types under specific circumstances and can potently affect cell growth. The clonality of EBV suggests that establishment of a latent transforming infection is the rare event that triggers cancer development.

4.7 Immune responses and EBV-associated malignancies

A common denominator of virus-associated tumours is the persistence in the malignant cells of all or parts of the viral genetic material and the continued expression of viral proteins that are the potential targets of tumour-specific rejection. The various patterns of viral gene expression identified in EBV-associated malignancies (see Table 3) and the diverse cellular origin of the malignant cells themselves could provide different types of challenge to the host immune system. Thus, failures or specific changes in the host immune response are likely to play different roles in the pathogenesis of these tumours. Characteristic changes in the pattern of antibodies to the latent antigens and to antigens associated with the productive cycle occur in patients with EBV-associated tumours. These antibody patterns reflect the load of infectious virus and/or viral antigen-expressing cells, and their role in limiting the growth of virus-infected cells is uncertain. With the exception of immunosuppressed patients who develop EBV-associated lymphomas, little is known about the status of cell-mediated virus-specific immune responses in patients with EBV-carrying tumours.

4.7.1 *B-Cell lymphoma and other tumours associated with severe immunosuppression*

The nine viral antigens that are typically detected in lymphoblastoid cell lines are also expressed in B-cell lymphomas arising in immunosuppressed patients (see Table 3). The phenotypic similarity of these lymphomas to lymphoblastoid cell lines suggests that the lymphomas represent virus-derived lesions growing out opportunistically in the absence of immune control. Indeed, regression has been reported after cessation of immunosuppressive therapy in renal transplant recipients (Starzl *et al.*, 1984), and passive transfer of blood lymphocytes or activated EBV-specific CTL cultures *in vitro* has been successfully used in the treatment of lymphomas arising in bone-marrow recipients (see section 1.5.3; Papadopoulos *et al.*, 1994; Rooney *et al.*, 1995). Immunosuppression could also play a role in the pathogenesis of EBV-positive leiomyosarcomas, since these tumours appear to express at least one highly immunogenic viral antigen (see Table 3).

4.7.2 *Burkitt's lymphoma*

Although transient impairment of EBV-specific responses has been demonstrated during acute malaria (Whittle *et al.*, 1984), normal levels of EBV-specific CTL precursors were demonstrated in a single study of the inhibition of autologous B-cell transformation in Burkitt's lymphoma patients (Rooney *et al.*, 1997). Even so, disturbance of the antiviral response is likely to play an important role in the pathogenesis of endemic Burkitt's lymphoma. EBV infection early in life and recurrent episodes of severe malaria were shown to increase the risk for the lymphoma in African children (reviewed by Magrath, 1990). Malarial infection is associated with chronic B-cell stimulation and follicular hyperplasia, which is likely to favour the establishment of a large pool of latently infected B lymphocytes from which cytogenetically altered cells may arise during the rapid proliferative phase of follicular reactions. A similar mechanism could also explain the high incidence of Burkitt's lymphoma in HIV-positive patients, in whom chronic stimulation of the B-cell compartment is a common feature in the pre-AIDS

stage. EBV-positive Burkitt's lymphoma lines that have maintained *in vitro* the phenotypic characteristics of the tumour *in vivo* are insensitive to lysis by EBV-specific MHC class I-restricted CTLs (Rooney *et al.*, 1995). This observation is consistent with the finding that the viral antigens that are recognized by these effectors are down-regulated and only EBNA-1 is expressed in the tumour. EBNA-1 may be protected from MHC class I-restricted rejection responses by an inhibitory effect of the glycine–alanine repeat on antigen processing (Levitskaya *et al.*, 1995). MHC class II-restricted EBNA-1-specific CTLs have been demonstrated *in vitro* (Khanna *et al.*, 1996), but the capacity of these cells to recognize the tumour is not firmly established. In addition to down-regulation of viral antigens, Burkitt's lymphoma cells consistently show low expression of adhesion molecules and MHC class I-restricted antigens and a selective loss of certain class-I alleles, with HLA A11 as the most consistent example (Masucci *et al.*, 1987; Andersson *et al.*, 1991). These features are characteristic of both EBV-positive and EBV-negative tumours and may therefore represent a phenotypic feature of the Burkitt's lymphoma precursor (Torsteinsdottir *et al.*, 1989). At least some may be specifically induced by the constitutive activation and overexpression of *c-myc* that characterizes this tumour (Polack *et al.*, 1996). More recently, down-regulation of the transporters associated with antigen presentation (Khanna *et al.*, 1995; Rowe *et al.*, 1995) and defects of antigen processing (Frisan *et al.*, 1996) were reported to be consistently associated with the Burkitt's lymphoma-cell phenotype.

4.7.3 *Hodgkin's disease*

The role of specific immunosuppression in the pathogenesis of Hodgkin's disease has been discussed extensively(Slivnick *et al.*, 1990). A decrease in EBV-specific responses, as assessed by the regression assay, was suggested in earlier studies; however, recent reports clearly demonstrate the presence of EBV-specific CTL precursors in the blood of Hodgkin's disease patients, independently of the EBV status of the tumour (Dolcetti *et al.*, 1995; Frisan *et al.*, 1995). A comparative study of EBV-specific responses in patients with EBV-positive and EBV-negative Hodgkin's disease failed to demonstrate virus-specific cytotoxicity within the tumour-infiltrating lymphocytes of six EBV-positive cases, while EBV-specific effectors were readily detected (Frisan *et al.*, 1995). The detection of CTLs in the blood of patients with EBV-positive Hodgkin's disease suggests that virus-specific reactivity is selectively suppressed in the tumour. The finding of multiple sequence variations in the *LMP-1* gene in EBV isolates from cases of Hodgkin's disease and nasopharyngeal carcinoma supports the possibility that the tumour cells present altered peptides that act as antagonists of EBV-specific responses. Alternatively, production of IL-10 in the LMP-1-positive tumours (Herbst *et al.*, 1996b) may mediate local inhibition of CTL responses.

4.7.4 *Nasopharyngeal carcinoma*

Nasopharyngeal carcinoma arises in apparently immunocompetent individuals, but the frequency is not increased in patients with recognized forms of immunosuppression. Lymphocytes from nasopharyngeal carcinoma patients maintain the capacity to inhibit

the proliferation of autologous virus-infected cells in regression assays, although a significant impairment in virus-specific T-cell immunity is shown in newly diagnosed patients when compared with either long-term survivors or controls (Moss *et al.*, 1983b). It is therefore quite surprising that the continuous expression of at least two highly immunogenic viral antigens, LMP-1 and LMP-2, does not lead to rejection. A characteristic feature of tumours expressing LMP-1 is the presence of abundant lymphoid infiltrate, which may be indicative of an on-going reaction to viral and/or tumour antigens. Indeed, LMP-1 was shown to increase the stimulatory capacity of EBV-negative Burkitt's lymphoma lines in allogeneic mixed-lymphocyte cultures (Cuomo *et al.*, 1990), to up-regulate the expression of adhesion molecules (Wang *et al.*, 1990) and to enhance the presentation of endogenous and exogenous antigens (de Campos-Lima *et al.*, 1993b; Rowe *et al.*, 1995). It is not known whether LMP-1 has similar effects on the immunogenic phenotype of epithelial cells, and the sensitivity of these cells to EBV-specific CTLs has not been investigated. Several surface molecules expressed in undifferentiated nasopharyngeal carcinoma cells, such as CD70, CD80 and CD86, may be involved in the induction of T-cell activation, and T cells with an activated phenotype have been detected in the tumour; however, their role in the pathogenesis of the disease remains unclear (Agathenanggelou *et al.*, 1995). A study of the expression of the T-cell receptor repertoire of tumour-infiltrating lymphocytes from nasopharyngeal carcinomas showed a significantly lower representation of certain V families (Vα10, Vα11, Vα13, Vα14, Vβ14 and Vβ20) than in control biopsies. Some of these differences were also observed in peripheral blood (Chen *et al.*, 1995b). Of particular interest is the observation that the frequencies of Vα17 ($p = 0.01$) and Vα18 were significantly lower ($p = 0.04$) in HLA B46-positive nasopharyngeal carcinoma patients than in B46-positive controls. It is presently unknown whether specific EBV peptides are present through HLA B46. Taken together, these results suggest that the expression of certain HLA alleles (see section 1.1.5) and the absence of certain T-cell receptor Vα/β families is important in the pathogenesis of nasopharyngeal carcinoma.

An additional clue is provided by comparisons of the immunogenicity of a nasopharyngeal carcinoma-derived LMP-1 with LMP-1 from the standard EBV laboratory strain B95.8 in a transfectant mammary carcinoma model in mice. While transfectants expressing the B95.8-derived LMP-1 became immunogenic and were rejected by syngeneic animals, the nasopharyngeal carcinoma-derived polypeptide failed to induce rejection (Trivedi *et al.*, 1994). Conceivably, the tumour-derived protein lacks antigenic epitopes that are relevant for rejection or fails to induce a phenotypic shift that would increase immunogenicity.

5. Summary of Data Reported and Evaluation

5.1 Virus–host interactions

Epstein-Barr virus (EBV) is a gamma-1 herpesvirus found throughout all human populations, with a prevalence of over 90% in adults. Primary infection usually occurs in early childhood and is asymptomatic, whereas delayed primary infection may cause a

self-limiting lymphoproliferative disease, infectious mononucleosis. Infection results in the establishment of a life-long carrier state characterized by the persistence of antibodies to several viral gene products and the secretion of infectious virus in saliva. Saliva is the usual vehicle of transmission. EBV can induce growth transformation of human and primate B-lymphocytes *in vitro*, and it causes malignant lymphomas in certain New World primates. B-cell growth transformation is initiated by binding of the virus to the complement receptor CD21 and is associated with the expression of a restricted set of viral genes which encode two non-translated small RNAs (EBER-1 and -2), six nuclear antigens (EBNA-1, EBNA-2, EBNA-3A, -3B, -3C and EBNA-LP) and three latent membrane proteins (LMP-1, -2A and -2B). Three forms of latent infection, referred to as latency types I–III, have been demonstrated in EBV-carrying B-cell lines and EBV-carrying tumour biopsy samples. In type-I latency, only EBNA-1 is expressed, while in type-II latency EBNA-1 is expressed together with LMP-1, -2A and -2B; in type III latency, the latent membrane proteins are expressed with all six EBNAs. Only a small fraction of latently infected B lymphocytes spontaneously enters the productive cycle, which is characterized by the co-ordinate expression of immediate early genes that encode viral transactivators, early genes that encode enzymes involved in viral replication and late genes that encode structural proteins.

Cellular and perhaps humoral immune responses contribute to the control of primary EBV infection and mediate the transition to asymptomatic persistence of the virus in healthy carriers. The humoral responses include the production of neutralizing antibodies directed to the CD21-binding protein gp350/320 and antibodies directed against lytic and latent gene products. Cells expressing type-III latency, which are detected during primary infection, are promptly eliminated by $CD8^+$ major histocompatibility class (MHC) I-restricted cytotoxic T lymphocytes which recognize epitopes from all the latent viral proteins except EBNA-1. Accordingly, only EBNA-1 appears to be expressed in the latently infected B cells that persist in healthy virus carriers. The failure of this protein to elicit cytotoxic T-lymphocyte responses could be instrumental in allowing persistence of virus-infected cells in immunocompetent hosts. $CD4^+$ MHC class II-restricted effectors could also play a role by producing growth inhibitory cytokines.

Few drugs are available that prevent viral replication without significant toxicity. Acyclovir and a number of related compounds have been used successfully to reduce viral replication but with no significant effect on persistent infection. Prophylactic, post-infection and therapeutic EBV vaccination strategies are currently being developed with recombinant subunit viral proteins and live recombinant virus vectors. The success of this endeavour will depend on a better understanding of the EBV life cycle and the immune responses generated by natural infection in humans.

5.2 Human carcinogenicity

Since the large majority of the world's population is latently infected with EBV, the mere presence of the virus in tumour tissue is insufficient evidence for its etiological role. In particular, since B lymphocytes are a normal reservoir for latent infection, the presence of EBV-infected lymphocytes in tumour tissue may be either incidental or an

effect of the tumour rather than its cause. Therefore in addition to the usual criteria (stated in the Preamble) by which epidemiologists judge the causality of association, for EBV, other factors should be considered: (1) the proportion of EBV-positive cases in a given tumour entity, (2) the proportion of tumour cells that carry the virus in any given case, (3) the monoclonality of EBV in the tumour (suggesting the presence of latent infection prior to expansion of the malignant clone); and (4) the expression of EBV proteins.

5.2.1 *Burkitt's lymphoma*

Early case–control studies indicated that African patients with Burkitt's lymphoma had much higher titres of antibodies to EB viral capsid antigen and early antigen than normal subjects. Moreover, a large cohort study in the West Nile district of Uganda showed that patients who developed Burkitt's lymphoma had significantly higher titres of antibodies to viral capsid antigen than control children between seven months and six years before diagnosis.

The viral DNA is present in Burkitt's lymphoma cells in monoclonal form. The expression of viral proteins is almost entirely restricted to EBNA-1. The frequency of this association between EBV and Burkitt's lymphoma varies geographically. *c-myc* immunoglobulin gene translocations are invariably seen in Burkitt's lymphoma.

The importance of malaria as a cofactor in the development of Burkitt's lymphoma in Africa was demonstrated by the coincidence of the distribution of hyperendemic and holoendemic malaria and Burkitt's lymphoma in different geographic areas, in different population subgroups and over time and by the reduction in the frequency of both diseases after chloroquine prophylaxis.

The importance of EBV in the causation of Burkitt's lymphoma varies in different regions and population groups, but appears to be greatest when infection occurs in the early years of life. The evidence suggests that EBV is an important pathogenic factor for the development of Burkitt's lymphoma.

5.2.2 *Non-Hodgkin's lymphomas*

EBV has particular importance in non-Hodgkin's lymphomas occurring in immunosuppressed individuals, who are at increased risk for these malignancies. Non-Hodgkin's lymphomas in transplant recipients and in patients with congenital immunodeficiency are nearly always EBV-positive. In HIV-positive subjects, EBV is uniformly associated with primary central nervous system lymphoma and is frequently associated with systemic lymphoma (although cases of all histological subtypes can be EBV-positive or EBV-negative).

EBV is strongly associated with some uncommon types of non-Hodgkin's lymphoma, on the basis of frequent EBV positivity, a high prevalence of EBV in tumour cells, EBV monoclonality in tumours and expression of EBV proteins. Sinonasal angiocentric T-cell lymphoma appears to be a malignant proliferation of EBV-infected T cells. A fraction of other peripheral T-cell lymphomas may be EBV-related, although EBV-positive and

EBV-negative cases are not distinguishable on the basis of histology or site of involvement.

The associations with lymphomas occurring in immunosuppressed individuals and sinonasal angiocentric T-cell lymphoma indicate a causal role of EBV in these forms of non-Hodgkin's lymphoma. The evidence for other types of non-Hodgkin's lymphoma is as yet inadequate.

5.2.3 *Hodgkin's disease*

In multiple specimens of Hodgkin's disease from case series, molecular evidence of clonal EBV genome with specifically restricted expression of latent viral proteins in the Reed-Sternberg cells is found in 30–50% of cases. EBV genome status appears to be uniform in involved nodes within patients and over time in those patients studied longitudinally.

The consistency of the finding of clonal EBV and the expression of LMP-1 in about half of Hodgkin's disease cases in many patient populations throughout the world argues strongly against a passenger role for the virus in these cases. Seroepidemiological findings in multiple case–control studies and two cohort studies show that patients with Hodgkin's disease can be distinguished by an altered antibody profile to EBV. The evidence indicates that EBV is a causal factor in the etiology of Hodgkin's disease.

5.2.4 *Nasopharyngeal carcinoma*

Nasopharyngeal carcinoma is a rare malignancy in most populations, although very high rates are seen in populations in southern China and more moderate rates in Inuit populations, in other parts of Southeast Asia and in North Africa. The incidence of nasopharyngeal carcinoma bears no relationship to age at infection (on the basis of the prevalence of antibodies to EBV in children and adolescents) either between or within countries.

One cause of nasopharyngeal carcinoma in high-risk populations identified previously is Chinese-style salted fish, a carcinogen in Group 1 (IARC, 1993). Other preserved foods and cigarette smoking have also been implicated.

Elevated immunoglobulin A antibodies to Epstein-Barr viral capsid and early antigens are a well-established feature of undifferentiated nasopharyngeal carcinoma. EBV DNA and viral products are regularly detected in malignant cells but not in normal nasopharyngeal epithelium.

The consistency of the molecular evidence strongly implicates EBV as a causative factor in the etiology of nasopharyngeal carcinoma: all undifferentiated nasopharyngeal carcinomas are EBV-positive, they are monoclonal with regard to EBV, and virtually all cells in each tumour also contain EBV DNA and/or EBV proteins.

5.2.5 *Other tumours*

EBV has been detected in the vast majority of gastric lymphoepithelial carcinomas and in a high proportion of lymphoepithelial carcinomas of the lung and salivary gland.

A smaller proportion of gastric adenocarcinomas is also EBV-associated. EBV DNA has been detected occasionally in epithelial tumours at a wide variety of other anatomical sites. An etiological role for EBV in lymphoepithelial and adenocarcinomas has not been conclusively established.

Smooth-muscle tumours in immunosuppressed individuals uniformly contain EBV, indicating a possible causal role for the virus in this setting.

5.3 Studies of cancer in animals

The available studies in non-human primates and rodents on the pathogenesis of EBV showed that New World primates (cotton-topped tamarins, owl monkeys and squirrel monkeys), which are naturally free of EBV-like viruses, can be infected by EBV. The pathogenesis of transforming EBV in such non-human primates varied from latent infection, to benign lymphoproliferation, to malignancy in lymphoid tissues; non-transforming EBV failed to induce tumours. Old World primates, which carry their own EBV-like viruses, did not develop clinical manifestations or tumours when inoculated with EBV. SCID mice engrafted with EBV-positive B cells developed tumours that expressed EBV proteins. The experimentally induced tumours were either oligoclonal or monoclonal.

Non-human primate lymphocryptoviruses from a variety of Old World primates have close homology to EBV. Each species of Old World primate may carry its own EBV-like herpesvirus. These herpesviruses are B-lymphotropic and transform human and monkey B cells. *Herpesvirus papio* is the only one that has been widely studied. It is commonly distributed in species of baboons and is horizontally transmitted; animals become infected early in life, and the virus becomes latent. *Herpesvirus papio* does not apparently induce tumours in the natural host or in experimentally infected non-human primates. *Herpesvirus papio* and EBV-like herpesviruses from apes (gorillas, chimpanzees and orangutans) and monkeys (rhesus, African green and cynomolgus) share antigenic cross-reactivity among themselves and with EBV. Experimental infection of such animals with EBV is therefore difficult.

A lymphoblastoid cell line derived from a lymph node of a cynomolgus monkey contained an EBV-like transforming herpesvirus. In another study, a herpesvirus (HVMF-1) isolated from cynomolgus monkeys was found to share antigenic and molecular properties with EBV. HVMF-1 remained latent in cynomolgus monkeys after infection. When such latently infected monkeys were inoculated with simian immunodeficiency virus 1, some animals developed malignant B-cell lymphomas that contained EBV-like virus. This experimental lymphoma may offer a good model for the study of EBV tumorigenesis in patients with AIDS.

5.4 Other relevant data

EBV infection of primary B lymphocytes *in vitro* efficiently induces cell immortalization into permanent cell lines. This is the only system available for the identification, characterization and functional analysis of virally encoded proteins associated with

immortalization. Altogether, 13 viral genes have been found to be expressed in the immortalized lymphocytes. The phenotype of the EBV-transformed B lymphocyte suggests that the effect of viral protein expression mimics that of antigen-driven lymphocyte activation. The immortalization-associated viral proteins regulate maintenance of episomal viral DNA and viral gene expression, drive cellular proliferation and block apoptosis.

5.4.1 *Burkitt's lymphoma*

The experimental evidence strongly suggests that malaria and early EBV infection are both important factors in increasing the risk for Burkitt's lymphoma in young African children.

Cell proliferation in Burkitt's lymphoma is driven by constitutive *c-myc* activation due to translocations involving the proto-oncogene and one of the immunoglobulin loci. The translocation is the consequence of an aberrant recombination process, which can occur in conjunction with VDJ rearrangement or immunoglobulin switching. EBV-driven cell proliferation, immune dysregulation caused by malaria, including chronic T-helper-2 stimulation of B cells, and chronic antigen exposure all contribute to the B-cell hyperplasia seen in people at risk for endemic Burkitt's lymphoma. Chronic B-lymphocyte hyperplasia increases the target cell population for aberrant recombination. The results of studies in experimental animals support a role for malaria as a cofactor in tumorigenesis.

Similar considerations apply to the pathogenesis in patients with AIDS, although the cofactor malaria is replaced by HIV infection leading to immune dysregulation. The resulting B-cell hyperplasia increases the risk for translocations at the premalignant stage. As in other Burkitt's lymphomas, *c-myc*–immunoglobulin translocations are invariably present in AIDS-associated Burkitt's lymphoma, whereas the prevalence of EBV infection varies. The EBV-positive cases express a type I latency programme.

The Burkitt's lymphoma cell has the phenotype of a germinal-centre cell. The constitutive *c-myc* expression prevents its potential entry into a resting state. The EBV type I latency programme and down-regulation of MHC class I ensure immune evasion.

5.4.2 *Non-Hodgkin's lymphomas and lymphoproliferation*

The risk of acquiring EBV-positive B-cell lymphomas increases dramatically in patients who are immunosuppressed due to primary genetic disorders, immunosuppressive therapy in transplant recipients and patients with AIDS. These lymphomas include non-Hodgkin's lymphomas of the diffuse, large B-cell type and, less frequently, of the Burkitt's lymphoma type. In these disorders, a range of conditions has been described, from polyclonal lymphoproliferation to oligoclonal and monoclonal malignancies. This may reflect a progressive situation, with an initial EBV-driven proliferation leading eventually to the outgrowth of fully malignant lymphoma. In the predominant large B-cell lymphoma types, types-II and -III latency are the most commonly detected patterns of EBV gene expression. It is therefore conceivable that the viral transforming genes drive cell proliferation and contribute to subversion of apoptosis in proli-

feration or malignancies. In the development of monoclonal conditions, additional cellular genetic changes have been observed at variable frequencies, which contribute to tumour progression. These include activation of autocrine and paracrine loops with the cytokines interleukin-6 and interleukin-10. The complex immunosuppression seen in these conditions is instrumental in pathogenesis and progression.

The T-cell lymphomas present a heterogeneous situation. EBV is detected at high frequency in many types of T-cell lymphomas. Monoclonality has been demonstrated in particular in sinonasal angiocentric T-cell lymphoma, providing one piece of evidence for a role of EBV in their pathogenesis. In other types, EBV gene expression is seen in only a minority of the cells in the tumour. There are few experimental data on the role of EBV in T-cell malignancies, including how and when EBV enters such cells and the effects of EBV transformation-associated proteins on T-cell proliferation and survival.

5.4.3 *Hodgkin's disease*

In-situ hybridization has disclosed the presence of the virus in virtually all tumour cells in EBV-positive cases, consistent with the detection of monoclonal EBV genomes in DNA extracted from most Hodgkin's disease tissues. These findings indicate that EBV infection of Hodgkin-Reed-Sternberg cells takes place before clonal expansion. The presence of EBV and the expression of type II-latency genes in these cells has been correlated with increased expression of lymphocyte activation antigens and with decreased expression of CD20 B-cell antigen. Of particular relevance is the association of EBV infection with the expression of cytokines such as interleukins 6 and 10 and in Hodgkin-Reed-Sternberg cells. Although Hodgkin's disease patients with EBV-positive tumours show a good, EBV-specific cytotoxic T-lymphocyte response in blood lymphocytes, the tumour-infiltrating lymphocytes lack EBV-specific activity and may thus be suppressed by the tumour cells. The available evidence strongly implicates the virus as a factor in the pathogenesis of EBV-positive Hodgkin's disease.

5.4.4 *Nasopharyngeal carcinoma*

EBV is detected in all undifferentiated nasopharyngeal carcinomas, and viral DNA is present in a monoclonal form in every malignant cell. The tumour cells show a type-II pattern of latency of gene expression; however, LMP-1 is not detected in all tumours. Some evidence has been provided for infection of nasopharyngeal carcinoma *in situ*. The viral genes expressed affect cell growth and differentiation, but tumour progression must involve one or several additional changes in cellular genes. The establishment of predominantly non-permissive infection in epithelium could be an important event that leads to the development of nasopharyngeal carcinoma.

Studies in experimental animals strongly support the epidemiological finding that a diet containing salted fish is an important cofactor, but the mechanism is unknown. Several additional somatic genetic alterations have been described in nasopharyngeal carcinoma, which may contribute to the predominance of a specific clone or to tumour progression. Cases of familial aggregation have been well documented, but susceptibility genes for this cancer have not been investigated.

5.4.5 *Other malignancies, including lymphoepithelial carcinomas*

EBV is strongly associated with some other tumours. These include lymphoepithelial carcinomas of the stomach, the salivary glands and the lungs. EBV has also been identified in a small but consistent proportion of gastric adenocarcinomas. In all cases analysed, monoclonal viral genomes have been found, indicating the presence of EBV before the onset of proliferation of the malignant cell clone. In contrast, the patterns of EBV latent gene expression are variable, type-I latency being prevalent in the gastric carcinomas and type-II latency in lymphoepithelial carcinomas at other sites. This implies different contributions of EBV to these neoplastic processes.

5.5 Evaluation

There is *sufficient evidence* for the carcinogenicity of EBV in the causation of Burkitt's lymphoma, sinonasal angiocentric T-cell lymphoma, immunosuppression-related lymphoma, Hodgkin's disease and nasopharyngeal carcinoma.

EBV is *carcinogenic to humans (Group 1)*.

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KAPOSI'S SARCOMA HERPESVIRUS/HUMAN HERPESVIRUS 8

1. Virus–Host Interactions

1.1 Taxonomy, structure and biology

1.1.1 *Taxonomy*

A new human herpesvirus was detected by Chang *et al.* (1994) in Kaposi's sarcomas associated with the acquired immune deficiency syndrome (AIDS; see IARC, 1996) by representational difference analysis. As it is detectable in virtually all cases of all the epidemiological forms of Kaposi's sarcoma, it is known as Kaposi's sarcoma-associated herpesvirus (KSHV). Recent seroepidemiological findings (see below) support a strong association between Kaposi's sarcoma and infection with this virus. In keeping with the nomenclature adopted for the two other recently discovered human herpesviruses (HHV), HHV6 and 7, however, and to allow for the fact that this virus is also associated with primary effusion lymphoma (body cavity-based lymphoma) and some cases of multicentric Castleman's disease, the formal designation human herpesvirus 8 (HHV8) was proposed by the herpesvirus subcommittee of the International Committee on the Taxonomy of Viruses. In this monograph, the term KSHV/HHV8 is used throughout to accommodate the two nomenclatures.

On the basis of phylogenetic analyses (Moore *et al.*, 1996a; Russo *et al.*, 1996), KSHV/HHV8 is a gamma-2 herpesvirus (rhadinovirus; see Table 2 in the Introduction) and represents the first 'human' member of this group. Of the rhadinoviruses of other species, KSHV/HHV8 appears so far to be most closely related to *Herpesvirus saimiri* and *Herpesvirus ateles*, two rhadinoviruses of New World monkeys, two herpesviruses of two macaque species, *Macaca nemestrina* and *Macaca mulatta*, murid herpesvirus 4 and bovine herpesvirus 4 (see Section 3).

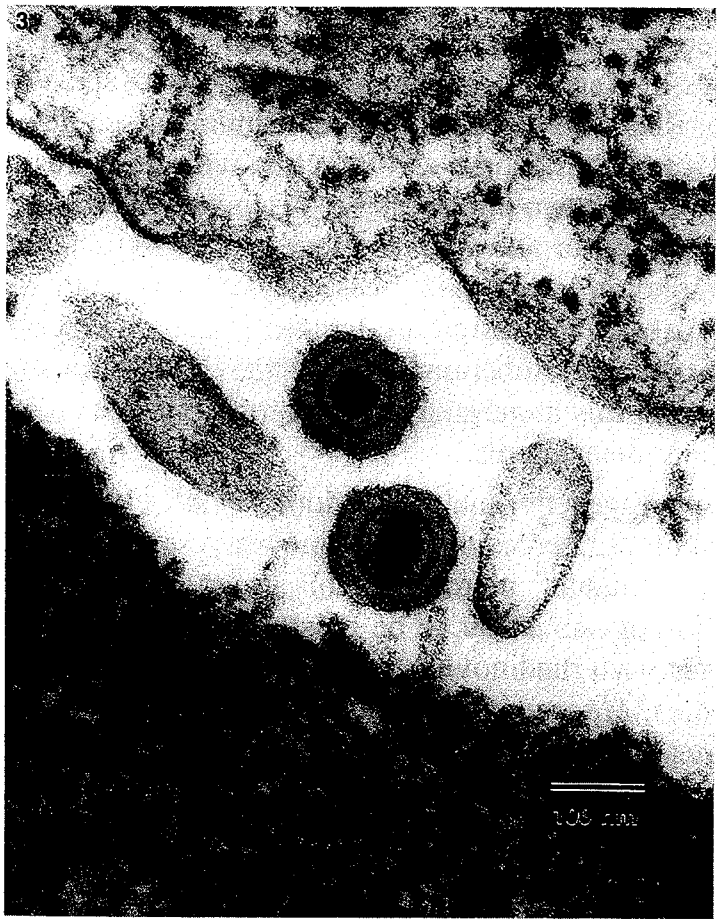
1.1.2 *Structure*

1.1.2.1 *Morphology*

KSHV/HHV8 has been shown to have some of the typical morphological characteristics of a herpesvirus in electron micrographs of KSHV/HHV8-infected primary effusion lymphoma cell lines (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996a; Said *et al.*, 1996a), a transiently infected kidney-cell line (Foreman *et al.*, 1997) and biopsy samples of Kaposi's sarcomas (Walter *et al.*, 1984; Ioachim, 1995; Orenstein *et al.*, 1997) (Figure 1), with 100–150-nm particles surrounded by a lipid envelope and an electron-dense central core (Renne *et al.*, 1996a). Capsid substructures, such as ring-shaped capsomers of approximately 9 nm in diameter arranged in linear arrays, have also been

observed in viral preparations (Arvanitakis *et al.*, 1996; Said *et al.*, 1996a). Herpesviral particles have been observed in two Kaposi's sarcoma biopsy specimens (Walter *et al.*, 1984; Orenstein *et al.*, 1997). Hexagonal intranuclear capsids of 110 nm in diameter, with or without an internal core, and mature envelope virions of 140 nm in diameter located mainly within cytoplasmic cisternae and vacuoles were seen in these samples (Orenstein *et al.*, 1997) and are likely to represent KSHV/HHV8 particles. Before the discovery of KSHV/HHV8 (Chang *et al.*, 1994), herpesvirus particles were described in short-term cultures from Kaposi's sarcoma lesions (Giraldo *et al.*, 1972); however, these were later identified as cytomegalovirus (CMV) (Giraldo *et al.*, 1980).

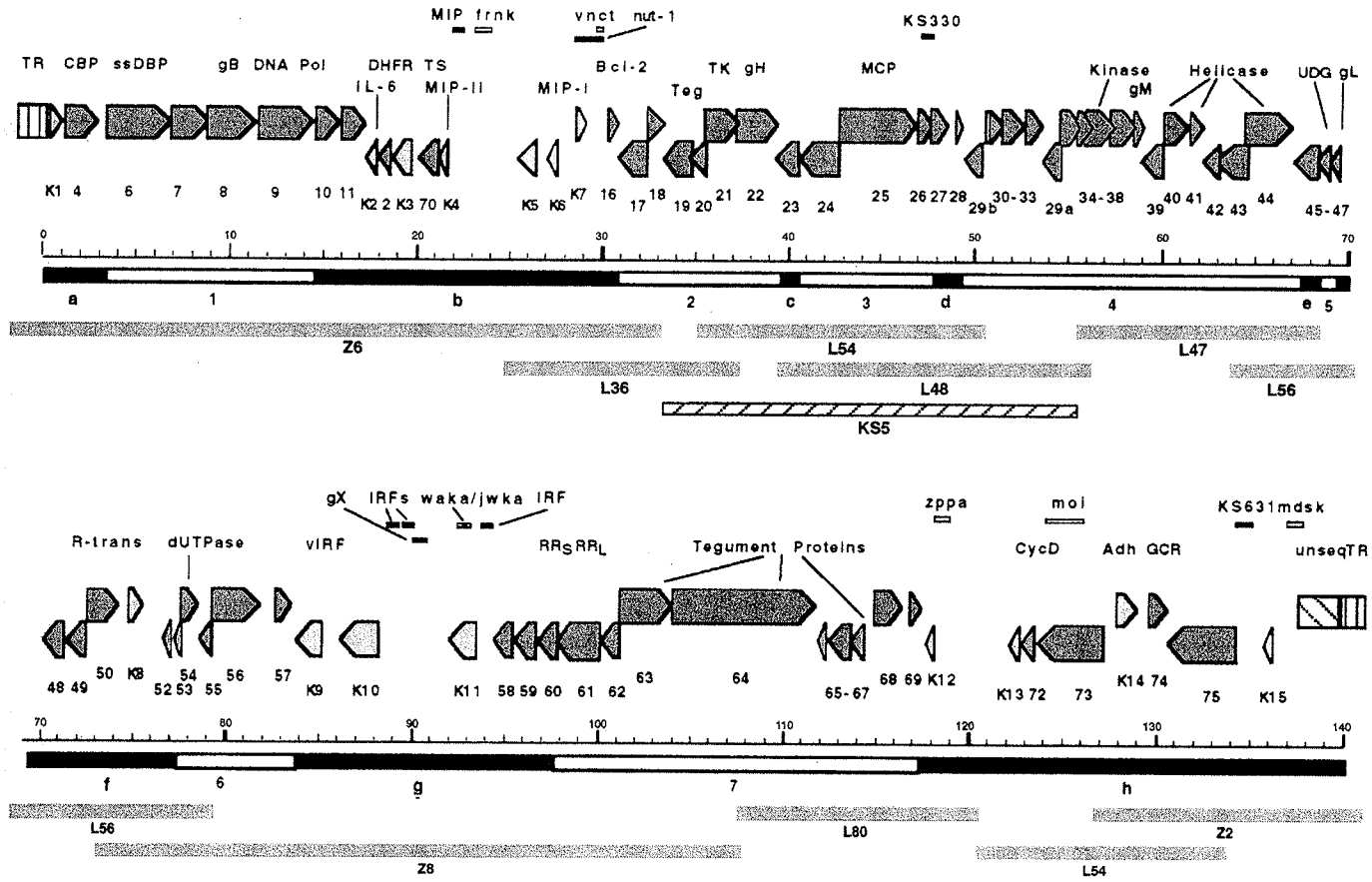
Figure 1. Electron microscopic view of KSHV/HHV8 capsid structure surrounded by a lipid envelope with an electron-dense central core representing viral DNA



1.1.2.2 *Genomic structure and properties of gene products*

The genomic structure of the virus (Russo *et al.*, 1996; Neipel *et al.*, 1997a) is similar to that of *Herpesvirus saimiri* (HVS) (Albrecht *et al.*, 1992), with a single, contiguous 140.5-kb long unique region containing all identified coding regions (Russo *et al.*, 1996; Neipel *et al.*, 1997a; Figure 2). This region is flanked on either side by a variable length terminal-repeat region composed of repeat units with a high G:C (84.5%) content, of approximately 800 base pairs.

Figure 2. Annotated long unique region and terminal repeats of the KSHV genome



From Russo *et al.* (1996)

The orientation of identified open reading frames in the long unique region is denoted by the direction of arrows, with *Herpesvirus saimiri* homologous with open reading frames as shaded areas and those not homologous as lighter areas. Seven blocks (numbered) of conserved herpesviral genes with nonconserved interblock regions (lettered) are shown under the kilobase marker. Features and putative coding regions not specifically designated are shown above the open reading frame map. Repeat regions (*frnk*, *vnct*, *waka/jwka*, *zppa*, *moi*, *mdsk*) are shown as light lines, and putative coding regions and other features not designated as open reading frames are shown as solid lines.

The size of the KSHV/HHV8 genome is calculated to be approximately 165 kb on the basis of studies of the genome banded from productive primary effusion lymphoma cells (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996b) and confirmed by Gardella gel electrophoresis (Decker *et al.*, 1996) and mapping of the whole genome (Russo *et al.*, 1996). Larger estimates made earlier (~ 270 kb) (Mesri *et al.*, 1996; Moore *et al.*, 1996a) were based on analyses of the primary effusion lymphoma-derived BC-1 cell line, which contains a large (> 30 kb) genomic duplication. This genomic duplication was also found in another cell line, independently derived from the same tumour, and may therefore have been present in the parental lymphoma (Russo *et al.*, 1996).

(*a*) *Terminal-repeat region*

The terminal-repeat region is a conserved feature of herpesviruses and is involved in packaging of the viral DNA into new virions during the lytic cycle of replication. KSHV/HHV8 has approximately 30 terminal-repeat units. In the BC-1 strain, insertions of long unique region fragments have been observed in the terminal repeats. None of the open reading frames (ORFs) present in the Epstein-Barr virus (EBV; see monograph on Epstein-Barr virus) have so far been identified within the terminal-repeat region of KSHV/HHV8. Hybridization with the terminal-repeat region used as a probe is a sensitive method for detecting viral DNA (Russo *et al.*, 1996). In EBV, circularized viral genomes in latently infected cells maintain the length of their terminal repeats, and the presence of a clonal cell population arising from a single latently infected cell can therefore be demonstrated by Southern blot analysis with restriction enzymes that do not cut into the terminal repeat (Raab-Traub & Flynn, 1986). The restriction enzyme Taq I frequently cuts within the long unique region of the KSHV/HHV8 genome but not within the terminal repeat (Russo *et al.*, 1996). The potential application of this approach to determine the clonality of Kaposi's sarcoma lesions is discussed in section 4.1.1.

The mechanism of replication of KSHV/HHV8 is probably similar to that of other herpesviruses (Roizman, 1993), in which the genome is replicated as a rolling circle, monomeric genomes being cleaved within the terminal-repeat region to form linear genomes. Sequences homologous (similar) to HVS packaging and cleaving sites are present in the terminal-repeat unit sequence (Russo *et al.*, 1996). Linear genomes are packaged into virions, as demonstrated by pulse-field and Gardella gel electrophoresis studies of viral particles (Renne *et al.*, 1996b), and most likely recircularize at the terminal repeat after entry into the recipient cell.

(*b*) *Long unique region*

The 140.5-kb KSHV/HHV8 long unique region is larger than the corresponding regions of HVS and EBV, encoding at least 81 predicted ORFs (Russo *et al.*, 1996; Neipel *et al.*, 1997a). The ORFs were named according to the corresponding HVS genes with which they share a significant level of homology. Unique genes that are not homologous with HVS have a K prefix. It is likely that additional genes and alternatively transcribed ORFs will be identified experimentally. The long unique region has blocks of genes conserved among all subfamilies of herpesviruses (Chee *et al.*, 1990), which include genes that encode herpesvirus structural proteins and replication enzymes.

Homologues to several major herpesvirus glycoproteins (gB [encoded by *ORF 8*], gH [*ORF 22*], gM [*ORF 39*] and gL [*ORF 47*]) are encoded by KSHV/HHV8; and conserved capsid proteins (major capsid protein [*ORF 25*], VP23 [*ORF 26*]), other capsid proteins encoded by *ORFs 17, 43* and *65*, tegument proteins encoded by *ORFs 19, 63, 64, 67* and *75*, replication enzymes (DNA polymerase [*ORF 9*], helicases [*ORFs 40, 41* and *44*], DNA replication proteins [*ORFs 56* and *59*]) and enzymes involved in nucleic acid metabolism (thymidylate synthetase [*ORF 70*], thymidine kinase [*ORF 21*], uracil glucosidase [*ORF 46*], dUTPase [*ORF 54*], ribonucleotide reductases [*ORFs 60* and *61*]) are found in the long unique region (Russo *et al.*, 1996; see Table 1). These conserved proteins are likely to play an important role during the lytic replication cycle of KSHV/HHV8.

Between the conserved herpesvirus gene blocks lie blocks of genes that are either found only in rhadinoviruses or are unique to KSHV/HHV8 (Russo *et al.*, 1996). The majority of genes in this category share significant sequence similarity with cellular genes and were presumably pirated at some point during the evolution of these viruses. Thus, KSHV/HHV8 encodes a complement binding protein (encoded by *ORF 4*) that is related to a family of mammalian complement regulatory proteins and a similar protein in HVS, an interleukin (IL)-6 homologue (*ORF K2*) which is unique to KSHV/HHV8, two chemokine homologues related to macrophage inflammatory protein (MIP)-1α (viral [v]-MIP-I and v-MIP-II; encoded by *ORFs K6* and *K4*), a possible third chemokine homologue (encoded by a putative *ORF K4.1*), a bcl-2 homologue (*ORF 16*), a homologue of interferon regulatory factor (v-IRF; *ORF K9*), a homologue of a D-type cyclin (v-cyclin; *ORF 72*), an adhesion molecule homologue (OX-2; *ORF K14*) and a chemokine receptor homologue (*ORF 74*) (Russo *et al.*, 1996; Cesarman *et al.*, 1996a; Neipel *et al.*, 1997a,b; Nicholas *et al.*, 1997a,b). Also in this category are several proteins of unknown function: Unique to KSHV/HHV8 are the putative type-I transmembrane protein encoded by *ORF K1* and a putative small hydrophobic protein ('kaposin') encoded by *ORF K12*. Also found in some (e.g. HVS, bovine herpesvirus 4 [BHV-4], murine herpesvirus 68 [MHV-68]), but not other (e.g. equine herpesvirus 2) rhadinoviruses, is the latency-associated nuclear antigen (LANA) protein encoded by *ORF 73* (Russo *et al.*, 1996; Neipel *et al.*, 1997a; Rainbow *et al.*, 1997). The probable function of these genes in the virus life cycle and tumour formation is discussed in section 4.1.

1.1.3 *Strain variation*

As expected for a DNA virus, different KSHV/HHV8 isolates have highly conserved genomes. Several partial and complete genomic KSHV/HHV8 sequences have recently been reported for viruses found in both Kaposi's sarcoma lesions and primary effusion lymphoma cell lines, which are highly conserved (Russo *et al.*, 1996; Moore *et al.*, 1996a; Nicholas *et al.*, 1997a; Neipel *et al.*, 1997a). A comparison of a 20-kb region sequenced from both a Kaposi's sarcoma lesion and a primary effusion lymphoma cell line showed less than 0.1% nucleotide variation (Russo *et al.*, 1996). Several groups have reported a limited degree of sequence variation in *ORF 26* (Boshoff *et al.*, 1995a; Collandre *et al.*, 1995; Huang *et al.*, 1995; Moore & Chang, 1995; Marchioli *et al.*, 1996;

Table 1. KSHV/HHV8 open reading frames (ORFs) with homology to genes in other herpesviruses

Name	Pol.	Start	Stop	Size (aa)	KSHV/HHV8 vs. HVS		KSHV/HHV8 vs. EBV			Putative function
					% Sim.	% Id.	EBV name	% Sim.	% Id.	
K1	+	105	974	289						
ORF 4	+	1 142	2 794	550						Complement binding protein
(to 4a)					45.3	31.2				
(to 4b)					46.4	34.0				
ORF 6	+	3 210	6 611	1 133	74.1	55.2	*BALF2*	65.6	42.1	Single-strand DNA binding protein
ORF 7	+	6 628	8 715	695	65.0	44.7	*BALF3*	59.9	41.3	Transport protein
ORF 8	+	8 699	11 236	845	72.5	54.9	*BALF4*	62.1	42.6	Glycoprotein B
ORF 9	+	11 363	14 401	1 012	77.6	62.1	*BALF5*	70.9	55.6	DNA polymerase
ORF 10	+	14 519	15 775	418	50.4	26.2				
ORF 11	+	15 790	17 013	407	49.4	28.9	*Raji LF2*	44.4	27.9	
K2	–	17 875	17 261	204						IL-6 homologue
ORF 02	–	18 553	17 921	210	65.8	48.4				Dihydrofolate reductase
K3	–	19 609	18 608	333						BHV-4-IE1 homologue
ORF 70	–	21 104	20 091	337	79.5	66.4				Thymidylate synthase
K4	–	21 832	21 548	94						v-MIP-II
K5	–	26 483	25 713	257						BHV-4-IE1 homologue
K6	–	27 424	27 137	95						v-MIP-I
K7	+	28 622	29 002	126						
ORF 16	+	30 145	30 672	175	50.0	26.7	*BHRF1*	46.3	22.8	Bcl-2 homologue
ORF 17	–	32 482	30 821	553	60.3	42.9	*BVRF2*	58.8	34.3	Capsid protein
ORF 18	+	32 424	33 197	257	70.6	48.4				
ORF 19	–	34 843	33 194	549	62.8	43.8	*BVRF1*	62.5	42.0	Tegument protein
ORF 20	–	35 573	34 611	320	59.6	42.7	*BXRF1*	54.7	34.6	
ORF 21	+	35 383	37 125	580	50.9	32.5	*BXLF1*	50.7	28.2	Thymidine kinase
ORF 22	+	37 113	39 305	730	53.9	35.1	*BXLF2*	48.3	26.5	Glycoprotein H
ORF 23	–	40 516	39 302	404	57.4	33.7	*BTRF1*	51.0	31.0	

Table 1 (contd)

Name	Pol.	Start	Stop	Size (aa)	KSHV/HHV8 vs. HVS		KSHV/HHV8 vs. EBV			Putative function
					% Sim.	% Id.	EBV name	% Sim.	% Id.	
ORF 24	–	42 778	40 520	752	65.8	45.6	*BcRF1*	56.4	37.7	
ORF 25	+	42 777	46 907	1 376	80.9	65.8	*BcLF1*	74.8	56.8	Major capsid protein
ORF 26	+	46 933	47 850	305	76.8	58.3	*BDLF1*	73.4	48.8	Capsid protein
ORF 27	+	47 873	48 745	290	49.6	29.6	*BDLF2*	43.3	19.6	
ORF 28	+	48 991	49 299	102	42.2	21.7	*BDLF3*			
ORF 29b	–	50 417	49 362	351	41.8	17.0	*BDRF1*	43.3	16.3	Packaging protein
ORF 30	+	50 623	50 856	77	52.1	31.0	*BDLF3.5*			
ORF 31	+	50 763	51 437	224	63.0	43.5	*BDLF4*	58.9	36.4	
ORF 32	+	51 404	52 768	454	51.7	30.1	*BGLF1*	47.0	26.6	
ORF 33	+	52 761	53 699	312	58.6	36.4	*BGLF2*	52.8	32.2	
ORF 29a	–	54 676	53 738	312	41.9	15.8	*BGRF1*	57.1	40.6	Packaging protein
ORF 34	+	54 675	55 658	327	58.9	42.7	*BGLF3*	54.8	33.0	
ORF 35	+	55 639	56 091	151	60.0	31.7	*BGLF3.5*			
ORF 36	+	55 976	57 310	444	49.4	31.1	*BGLF4*	50.0	30.2	Kinase
ORF 37	+	57 273	58 733	486	65.9	50.4	*BGLF5*	60.1	42.7	Alkaline exonuclease
ORF 38	+	58 688	58 873	61	58.6	39.7	*BBLF1*	52.5	23.0	
ORF 39	–	60 175	58 976	399	73.2	52.1	*BBRF3*	65.2	43.6	Glycoprotein M
ORF 40	+	60 308	61 681	457	51.9	28.1	*BBLF2*	47.1	23.3	Helicase-primase
ORF 41	+	61 827	62 444	205	53.4	29.2	*BBLF3*			Helicase-primase
ORF 42	–	63 272	62 436	278	55.8	38.9	*BBRF2*	52.9	33.0	
ORF 43	–	64 953	63 136	605	74.9	60.5	*BBRF1*	67.6	50.1	Capsid protein
ORF 44	+	64 892	67 258	788	75.5	61.4	*BBLF4*	67.8	51.1	Helicase-primase
ORF 45	–	68 576	67 353	407	50.2	30.7	*BKRF4*	48.9	26.2	
ORF 46	–	69 404	68 637	255	73.0	59.5	*BKRF3*	69.2	54.8	Uracil DNA glucosidase
ORF 47	–	69 915	69 412	167	53.0	29.9	*BKRF4*	53.8	24.2	Glycoprotein L
ORF 48	–	71 381	70 173	402	47.3	24.4	*BRRF2*	46.1	18.8	
ORF 49	–	72 538	71 630	302	45.4	21.2	*BRRF1*	49.8	28.0	
ORF 50	+	72 734	74 629	631	46.5	24.9	*BRLF1*	41.4	19.0	Transactivator

Table 1 (contd)

Name	Pol.	Start	Stop	Size (aa)	KSHV/HHV8 vs. HVS		KSHV/HHV8 vs. EBV			Putative function
					% Sim.	% Id.	EBV name	% Sim.	% Id.	
K8	+	74 850	75 569	239						
ORF 52	–	77 197	76 802	131	50.0	33.3	*BLRF2*	54.6	36.9	
ORF 53	–	77 665	77 333	110	59.6	36.0	*BLRF1*	58.1	40.9	
ORF 54	+	77 667	78 623	318	55.0	35.5	*BLLF3*	53.7	32.4	dUTPase
ORF 55	–	79 448	78 765	227	64.4	46.4	*BSRF1*	61.6	44.0	
ORF 56	+	79 436	81 967	843	62.5	44.3	*BSLF1*	56.6	35.4	DNA replication protein
ORF 57	+	82 717	83 544	275	56.9	31.5	*BMLF1*	45.1	22.0	Immediate-early protein
K9	–	85 209	83 860	449						v-IRF1
K10	–	88 164	86 074	696						
K11	–	93 367	91 964	467						
ORF 58	–	95 544	94 471	357	55.9	28.7	*BMRF2*	50.6	25.3	
ORF 59	–	96 739	95 549	396	54.1	32.3	*BMRF1*	50.7	28.3	DNA replication protein
ORF 60	–	97 787	96 870	305	79.3	64.6	*BaRF1*	74.8	57.3	Ribonucleotide reductase, small
ORF 61	–	100 194	97 816	792	69.4	52.4	*BORF2*	64.1	43.6	Ribonucleotide reductase, large
ORF 62	–	101 194	100 199	331	64.6	40.2	*BORF1*	57.7	34.7	Assembly/DNA maturation
ORF 63	+	101 208	103 994	927	53.1	32.1	*BOLF1*	47.0	24.5	Tegument protein
ORF 64	+	104 000	111 907	2 635	50.1	29.7	*BPLF1*	46.6	26.1	Tegument protein
ORF 65	–	112 443	111 931	170	60.4	40.3	*BFRF3*	49.4	27.8	Capsid protein
ORF 66	–	113 759	112 470	429	58.7	34.7	*BFRF2*	50.0	28.0	
ORF 67	–	114 508	113 693	271	71.8	53.0	*BFRF1*	62.8	39.5	Tegument protein
ORF 68	+	114 768	116 405	545	64.7	45.4	*BFLF1*	58.3	36.2	Glycoprotein
ORF 69	+	116 669	117 346	225	71.1	53.6	*BFLF2*	60.7	41.7	
K12	–	118 101	117 919	60						Kaposin
K13	–	122 710	122 291	139						
ORF 72	–	123 566	122 793	257	53.0	32.5				Cyclin D homologue
ORF 73	–	127 296	123 808	1 162	51.2	31.8				Immediate-early protein

Table 1 (contd)

Name	Pol.	Start	Stop	Size (aa)	KSHV/HHV8 vs. HVS		KSHV/HHV8 vs. EBV			Putative function
					% Sim.	% Id.	EBV name	% Sim.	% Id.	
K14	+	127 883	128 929	348						OX-2 membrane glycoprotein homologue
ORF 74	+	129 371	130 399	342	57.8	34.1				G-Protein coupled receptor
ORF 75	–	134 440	130 550	1 296	54.8	36.3	*BNRF1*			Tegument protein/FGARAT
K15	–	136 279	135 977	100						

From Russo *et al.* (1996)

Pol., polarity; aa, amino acid; % Sim., percentage similar; % Id., percentage identical; ss, single-stranded; IL, interleukin; v, viral; MIP, macrophage inflammatory protein; IE, immediate-early; IRF, interferon regulatory factor; FGARAT, *N*-formylglycinamide ribotide amidotransferase

Zong *et al.*, 1997). In comparison with the corresponding genes in EBV and HVS, *ORF 26* is among the more highly conserved genes and is therefore probably not a very informative locus (Moore *et al.*, 1996a). Limited sequence variation has also been found within two regions of *ORF 75*. In a combined analysis of several genomic regions, Zong *et al.* (1997) found up to 1.5% overall nucleotide variation between isolates, which can be grouped into three different main variants, provisionally termed A, B and C. The high concentration of sequences in the genome in homosexual men in the United States suggests the recent introduction of predominant strains of KSHV/HHV8; however, further sequence and phylogenetic analyses are required to confirm this conclusion.

In HVS, the greatest degree of sequence variation between different isolates is found towards the left end of the genome, in a region that encodes the *STP* and *Tip* genes, which are essential for the transformation of T cells by this virus (Albrecht *et al.*, 1992). A comparative analysis of the KSHV/HHV8 sequence shows that *ORF K1* of KSHV/-HHV8, located at the left end of the genome, may vary more than the structural genes studied so far (Russo *et al.*, 1996; Lagunoff & Ganem, 1997; Neipel *et al.*, 1997a).

1.1.4 *Host range*

Humans appear to be the natural host for KSHV/HHV8. Recent seroepidemiological data (see below) indicate that KSHV/HHV8 is more prevalent in some regions (e.g. Africa, southern Europe) than in northern Europe and the United States (Gao *et al.*, 1996a; Kedes *et al.*, 1996; Lennette *et al.*, 1996; Simpson *et al.*, 1996). Whether KSHV/-HHV8 was recently introduced into the human population or is an ancient human herpesvirus remains to be determined.

No published evidence for natural infection of animal species by KSHV/HHV8 is currently available. The tropism of KSHV/HHV8 for individual cell lineages is discussed in section 1.1.6.

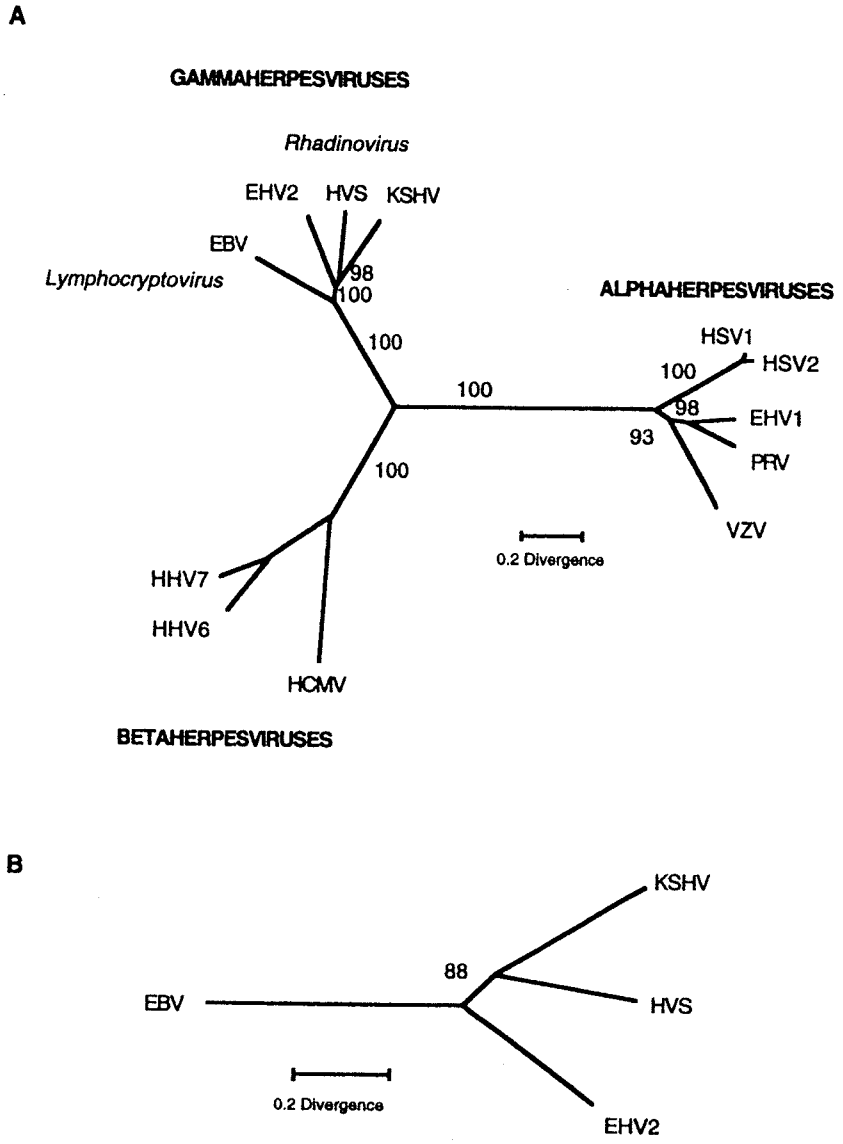
1.1.5 *Related non-human viruses*

The phylogenetic relationship of KSHV/HHV 8 to other rhadinoviruses is shown in Figure 3. As discussed in Section 3, KSHV/HHV8 is closely related to *Herpesvirus saimiri* of squirrel monkeys and to other rhadinoviruses of cattle and mice. Captive macaques belonging to two species, *M. nemestrina* and *M. mulatta*, have been shown to harbour two distinct viruses which are closely related to KSHV/HHV8 (Rose *et al.*, 1997) (see Section 3).

1.1.6 *Tropism and persistence in infected cells* in vivo

KSHV/HHV8 has been detected by the polymerase chain reaction (PCR), PCR–in-situ hybridization or conventional in-situ hybridization and immunohistochemistry, in endothelial and spindle cells of Kaposi's sarcoma lesions, in circulating endothelial cells, primary effusion lymphoma cells, B cells, macrophages, dendritic cells and prostatic glandular epithelium (Ambroziak *et al.*, 1995; Boshoff *et al.*, 1995b; Cesarman *et al.*, 1995a; Moore & Chang, 1995; Corbellino, 1996a; Li *et al.*, 1996; Moore *et al.*, 1996b; Rainbow *et al.*, 1997; Sirianni *et al.*, 1997; Staskus *et al.*, 1997; Stürzl *et al.*, 1997).

Figure 3. Phylogenetic trees of KSHV based on comparison of aligned amino-acid sequences in herpesviruses for the *MCP* gene and for a concatenated nine-gene set



From Moore *et al.* (1996a)

PRV, pseudorabies virus; VZV, varicella-zoster virus; HCMV, human cytomegalovirus; HHV6 and HHV7, human herpesviruses 6 and 7.

(A) *MCP* sequences were compared by the neighbour-joining method. The sequence is shown in unrooted form, with branch lengths proportional to divergence (mean number of substitution events per site) between the nodes bounding each branch. Comparable results (not shown) were obtained by maximum-parsimony analysis. The number of times (of 100 bootstrap samplings) that the division indicated by each internal branch was obtained is shown next to each branch; bootstrap values below 75 are not shown.

(B) Phylogenetic tree of gammaherpesvirus sequences based on a nine-gene set demonstrates that KSHV is most closely related to the gamma-2 herpesvirus sublineage, genus *Rhadinovirus*. The amino-acid sequence was used to infer a tree by the Protml maximum-likelihood method; comparable results (not shown) were obtained with the neighbour-joining and maximum-parsimony methods. The bootstrap value for the central branch is marked. On the basis of the *MCP* analysis, the root must lie between EBV and the other three species.

1.1.6.1 *Persistence and gene expression in infected endothelial cells*

Experiments with PCR–in-situ hybridization indicate that KSHV/HHV8 can infect the atypical endothelial cells lining the ectatic vascular spaces in Kaposi's sarcoma lesions and endothelial tumour (spindle) cells of fully developed, nodular Kaposi's sarcoma lesions, but is not generally present in normal endothelial cells (Boshoff *et al.*, 1995b; Li *et al.*, 1996). This has been confirmed by microdissection (Boshoff *et al.*, 1995b). More recently, in-situ hybridization (Staskus *et al.*, 1997) and immunohistochemistry with LANA (Rainbow *et al.*, 1997) have been used to confirm KSHV/HHV8 gene expression in Kaposi's sarcoma spindle cells within tumours. KSHV/HHV8 establishes a persistent infection in most of these spindle cells, as demonstrated by the expression of genes *ORF K12*, *ORF 72* and *ORF 73* (Rainbow *et al.*, 1997; Staskus *et al.*, 1997; Stürzl *et al.*, 1997). These genes are expressed in latently infected primary effusion lymphoma cell lines, and their expression is not increased by agents known to induce lytic viral replication (Cesarman *et al.*, 1996a; Renne *et al.*, 1996a). *ORF 72* encodes a homologue of mammalian D-type cyclins shown to be functionally active in phosphorylating the retinoblastoma tumour suppressor protein (Rb) in association with cyclin-dependent kinases (Chang *et al.*, 1996a; Li *et al.*, 1997). *ORF 73* encodes LANA, the function of which is still unknown (Rainbow *et al.*, 1997), and *ORF K12* may encode a small 60-amino acid, hydrophobic protein (Zhong *et al.*, 1996), also of unknown function. The KSHV/HHV8 homologue to IL-6 (encoded by *ORF K2*) is expressed during latency in primary effusion lymphoma, but is not generally expressed in Kaposi's sarcoma lesions (Moore *et al.*, 1996b).

The atypical flat endothelial cells that are found in nodular Kaposi's sarcoma and in the early stages (patch) of the disease have so far been found to express only the latent *ORF K12/T0.7* transcript (Rainbow *et al.*, 1997; Staskus *et al.*, 1997; Stürzl *et al.*, 1997); however, as *ORF K12/T0.7* transcripts are particularly abundant in both primary effusion lymphoma cell lines and Kaposi's sarcoma lesions (Renne *et al.*, 1996a; Zhong *et al.*, 1996), it is at present unclear whether *ORFs 72* and *73* are not expressed in endothelial cells or only more weakly expressed. A subpopulation (approximately 10%) of Kaposi's sarcoma spindle cells also expresses a polyadenylated nuclear RNA, *T1.1*, which may not encode a protein and is abundantly expressed in primary effusion lymphoma cell lines induced into lytic replication (Renne *et al.*, 1996a; Sun *et al.*, 1996; Zhong *et al.*, 1996; Staskus *et al.*, 1997). The distribution pattern of *T1.1*-expressing cells in Kaposi's sarcoma tissue is similar to that of a few lytically infected spindle cells, which can be defined by their expression of mRNA for the major capsid protein (encoded by *ORF 25*) (Staskus *et al.*, 1997). Expression of *T1.1* may therefore be indicative of lytic replication within Kaposi's sarcoma lesions. This suggests that a subpopulation of the spindle cells can produce KSHV/HHV8 virions, as suggested by three reports describing the presence of intranuclear herpesvirus-like particles of 120 nm in diameter or intranuclear inclusions characteristic of herpesviruses in Kaposi's sarcoma tissues (Walter *et al.*, 1984; Ioachim, 1995; Orenstein *et al.*, 1997). In addition to these viral genes, *ORF 74*, which encodes a homologue of a mammalian chemokine receptor, has been found to be expressed in

Kaposi's sarcoma lesions by reverse transcriptase (RT)-PCR (Cesarman *et al.*, 1996a), but the cell type that expresses this gene has not yet been identified.

Several independent lines of evidence therefore suggest that KSHV/HHV8 infects and persists in spindle cells *in vivo*. In contrast, all primary cell cultures established from Kaposi's sarcoma lesions lose detectable KSHV/HHV8 (Ambroziak *et al.*, 1995; Lebbé *et al.*, 1995; Aluigi *et al.*, 1996; Flamand *et al.*, 1996), and few cultures have been reported to maintain detectable KSHV/HHV8 for several passages (Lebbé *et al.*, 1995; Aluigi *et al.*, 1996). Two permanent Kaposi's sarcoma cell lines that are tumorigenic in severe combined immunodeficiency (SCID) and nude mice and contain chromosomal abnormalities (Siegal *et al.*, 1990; Lunardi-Iskandar *et al.*, 1995) also lack detectable KSHV/HHV8 (Flamand *et al.*, 1996).

1.1.6.2 *Persistence in haematopoietic cells*

The most highly expressed KSHV/HHV8 transcripts in primary effusion lymphoma cell lines are polyadenylated transcripts that encode *ORF K12* (*T 0.7*) and a nuclear untranslated RNA (*T1.1*) (Renne *et al.*, 1996a; Sun *et al.*, 1996). The *ORFs 16* (*bcl-2*), *72* (*CV-cyc*), *73* (*LANA*), *74* (*v-GCR*), *K2* (*v-IL-6*), *K4* (*v-MIP-II*), *K6* (*v-MIP-I*) and *K9* (*v-IRF*) are also expressed in primary effusion lymphoma cell lines but at lower levels (Cesarman *et al.*, 1996a; Moore *et al.*, 1996b; Rainbow *et al.*, 1997; Sarid *et al.*, 1997). A polyclonal antibody mono-specific for v-IL-6 has been used to demonstrate expression of this viral cytokine in KSHV/HHV8-infected haematopoetic cells in lymph nodes and in uninduced primary effusion lymphoma cell lines. Expression of *v-IL-6*, *v-MIP-I*, *v-MIP-II* and *v-IRF* can be induced by treatment with phorbol esters (Moore *et al.*, 1996b). Limited data are available on the persistence of KSHV/HHV8 in peripheral blood mononuclear cells (PBMC); B cells and macrophages may harbour KSHV/HHV8 genomes (Ambroziak *et al.*, 1995; Sirianni *et al.*, 1997). The presence of circular and linear KSHV/HHV8 genomes in PBMC was reported in one study, reflecting the presence of both latently and productively infected cells (Decker *et al.*, 1996).

1.1.6.3 *Presence in other tissues*

Several studies suggest the presence of KSHV/HHV8 in prostatic tissues of some infected men (Monini *et al.*, 1996a; Corbellino *et al.*, 1996b; Staskus *et al.*, 1997; see section 2.1.3), and KSHV/HHV8 is preferentially detected in semen rather than spermatocytes, suggesting secretion into seminal fluids (Monini *et al.*, 1996a; Howard *et al.*, 1997), although it cannot be excluded that KSHV/HHV8-infected mononuclear cells occasionally represent the source of KSHV/HHV8 in semen. In a survey of tissues from AIDS patients with Kaposi's sarcoma, Corbellino *et al.* (1996b) found that prostate tissues harboured the viral genome, suggesting that the prostate is a major site of infection in these patients. In-situ hybridization of prostatic glandular epithelium for a latent KSHV/HHV8 gene showed that viral gene expression is common in prostate biopsy samples from men without Kaposi's sarcoma (Staskus *et al.*, 1997), lending support to the supposition that the virus is widely disseminated in the healthy male population. Studies by PCR have not shown that prostatic tissue from men without Kaposi's sarcoma

is infected with KSHV/HHV8 (Corbellino *et al.*, 1996c; Tasaka *et al.*, 1996; Blackbourn & Levy, 1997; Rubin *et al.*, 1997).

It was suggested in one report that dorsal root ganglia in patients with AIDS and Kaposi's sarcoma harbour viral DNA (Corbellino *et al.*, 1996a).

KSHV/HHV8 has been detected in sputum, saliva, throat washing and bronchoalveolar lavage fluid, predominantly in patients with Kaposi's sarcoma (Howard *et al.*, 1995; Boldogh *et al.*, 1996; Koelle *et al.*, 1997).

1.2 Methods of detection

1.2.1 *Nucleic acids*

PCR is widely used to detect KSHV/HHV8 in clinical samples. The virus is detected consistently by PCR in biopsy samples from all epidemiological forms of Kaposi's sarcoma, including that in AIDS patients (Chang *et al.*, 1994), in persons who are not infected with human immunodeficiency virus (HIV) ('classic' Kaposi's sarcoma), in both Mediterranean countries and other geographical regions (Boshoff *et al.*, 1995a; Dupin *et al.*, 1995a; Moore & Chang, 1995; Schalling *et al.*, 1995; Chang *et al.*, 1996b; Buonaguro *et al.*, 1996; Luppi *et al.*, 1996a; Noel *et al.*, 1996), in iatrogenically immunosuppressed transplant recipients (Boshoff *et al.*, 1995a; Buonaguro *et al.*, 1996; Noel *et al.*, 1996) and in HIV-negative homosexual men (Boshoff *et al.*, 1995a; Moore & Chang, 1995). Unaffected tissues proximal to Kaposi's sarcoma lesions are more likely to have detectable viral genome than more distant tissues (Chang *et al.*, 1994; Boshoff *et al.*, 1995a; Dupin *et al.*, 1995a; Moore & Chang, 1995), suggesting that the virus is localized primarily to Kaposi's sarcoma lesions; however, it is also found in undiseased tissues, as discussed above.

The detection rate of KSHV/HHV8 DNA in all forms of Kaposi's sarcoma was about 95% in over 500 cases tested by PCR by numerous groups (for review, see Olsen & Moore, 1997; see Table 3 in section 2.1.3). A specific signal is almost always detected by PCR in DNA extracted from fresh or frozen Kaposi's sarcoma tissue samples after 30–35 amplification cycles. Detection can be enhanced by Southern blotting for the PCR product, but this does not reduce the likelihood of a false-positive result due to contamination. Formaldehyde-fixed, paraffin-embedded Kaposi's sarcoma tissue must often be tested by nested PCR in order to obtain a positive signal; this also dramatically increases the likelihood for intraexperimental contamination. It has been suggested that the PCR detection rate also depends on the histological stage of a Kaposi's sarcoma lesion and is higher in plaque and fully developed nodular lesions than in early patch lesions (Noel *et al.*, 1996). It has also been suggested that a decrease in viral DNA may precede the regression of Kaposi's sarcoma lesions in iatrogenically immunosuppressed patients, but these findings must be confirmed in larger case series (Aluigi *et al.*, 1996). As discussed in more detail in section 1.3, KSHV/HHV8 is also detected consistently in primary effusion lymphoma and some cases of multicentric Castleman's disease, as well as in lymphatic tissue, peripheral blood and semen from a proportion of KSHV/HHV8-infected individuals. The detection rates, even by nested PCR, of KSHV/HHV8 in

PBMC from patients with Kaposi's sarcoma are 50–60% (Whitby *et al.*, 1995; Moore *et al.*, 1996c; Blauvelt *et al.*, 1997; Lebbé *et al.*, 1997a; see section 2.1.3).

1.2.2 *Serology*

Immunofluorescence, western blot and enzyme-linked immunosorbent assays to detect antibodies against latent and lytic antigens of KSHV/HHV8 have been described. Most of the serological assays for KSHV/HHV8 used currently are based on B-cell lines derived from primary effusion lymphomas (Cesarman *et al.*, 1995b; Arvanitakis *et al.*, 1996; Gaidano *et al.*, 1996a; Gao *et al.*, 1996a; Renne *et al.*, 1996a; Said *et al.*, 1996a). These cell lines are latently infected with KSHV/HHV8. In the first report on immunofluorescence-based assays, the cell lines used (HBL-6, BC-1) were dually infected with KSHV/HHV8 and EBV (Moore *et al.*, 1996a), requiring absorption of EBV-specific antibodies to avoid cross-reactivity; however, lytic replication of KSHV/HHV8, but not EBV, could be induced in these cell lines with sodium butyrate, allowing the detection of antibodies to a prominent, 40-kDa, lytic (structural) antigen (Miller *et al.*, 1996). Although of limited use for determining the seroprevalence of KSHV/HHV8 in the general population, the results obtained with these early assays indicated that most individuals with AIDS-associated Kaposi's sarcoma and a much smaller proportion of HIV-infected individuals without Kaposi's sarcoma had antibodies to KSHV/HHV8 (Miller *et al.*, 1996; Moore *et al.*, 1996c).

When the nuclei of one of these dually infected cell lines (BC-1) were examined by western blot for the presence of KSHV/HHV8-specific nuclear antigens, a nuclear protein of high molecular mass (226/234 kDa) was found to react specifically with sera from Kaposi's sarcoma patients or those at increased risk for Kaposi's sarcoma (Gao *et al.*, 1996b): 80% of AIDS-associated Kaposi's sarcoma patients had antibodies to this 'latent nuclear antigen', whereas no sera from United States blood donors or HIV-infected patients with haemophilia were reactive. This antigen is not cross-reactive with EBV-specific antibodies. Thus, dually infected cell lines can be used that are readily amenable to large-scale culture.

Widespread screening of groups at risk for Kaposi's sarcoma and of the general population became possible when the first primary effusion lymphoma cell lines infected with KSHV/HHV8 alone were established. These cell lines express a latency-associated nuclear antigen (LANA), which is characterized by a typical speckled nuclear pattern (Gao *et al.*, 1996a; Kedes *et al.*, 1996). Detection of LANA by immunofluorescence assay correlates closely with reactivity to the 226/234-kDa nuclear antigen on western blots (Gao *et al.*, 1996b). The 226/234-kDa nuclear protein is encoded by KSHV/HHV8 *ORF 73*. Studies by immunoadsorption and recombinant antigens indicate that LANA is in part, and perhaps entirely, composed of the ORF 73 protein (Rainbow *et al.*, 1997). There is no homologue to the *ORF 73*-encoded nuclear protein in EBV, the most closely related known human gammaherpesvirus (Russo *et al.*, 1996).

Examination of panels of sera from populations at high and low risk for Kaposi's sarcoma suggests that antibodies to LANA predict the likelihood of Kaposi's sarcoma developing in AIDS patients. Only 0–3% of blood donors in the United States and United

Kingdom have antibodies to this latent nuclear protein (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996; Lennette *et al.*, 1996; Simpson *et al.*, 1996), but 80–90% of sera from AIDS patients with Kaposi's sarcoma and about 95% of sera from (non-immunosuppressed) 'classic' cases of Kaposi's sarcoma react with it under optimal circumstances (Gao *et al.*, 1996a; Kedes *et al.*, 1996; Simpson *et al.*, 1996). The assay is less sensitive when serum samples that have been repeatedly frozen and thawed are examined (Gao *et al.*, 1996b). Non-specific cross-reactive antibodies to cytoplasmic antigens interfere with the immunofluorescence at low serum dilutions, requiring either the isolation of whole nuclei (Kedes *et al.*, 1996) or use of diluted sera, usually 1/100–1/160 (Gao *et al.*, 1996b; Simpson *et al.*, 1996).

Lytic cycle (structural) KSHV/HHV8 antigens have also been found to react with sera from Kaposi's sarcoma patients. In addition to the 40-kDa structural protein discussed above, recognized by 67% of the sera from patients with Kaposi's sarcoma, some patients have antibodies to other lytic (structural) KSHV/HHV8 proteins of approximately 27 and 60 kDa (Miller *et al.*, 1996, 1997). While there is so far no indication that these proteins cross-react with the corresponding EBV proteins, their specificity requires further investigation. A 19-kDa capsid-related protein encoded by *ORF 65* has been used as a recombinant protein in enzyme-linked immunosorbent and western blot assays and is recognized by about 80% of sera from AIDS patients with Kaposi's sarcoma and 85–90% of those from patients with 'classic' Kaposi's sarcoma (Simpson *et al.*, 1996; Lin *et al.*, 1997). About 3–5% of blood donors in the United Kingdom and United States show reactivity to this protein. Its immunogenic determinants are located within the 80 amino acids in the carboxy terminal, and this region is 21% identical to the corresponding region in the EBV homologue *BFRF3*. Although vp19/ORF 65 is thus not recognized by most sera from EBV-positive individuals and did not react with a set of high-titre EBV-positive sera, the question of whether there may be occasional cross-reactivity with EBV is not completely resolved. Concordance between the recombinant vp19/ORF 65 antigen and latent immunofluorescence antigen is high (around 80–85%) in sera from patients with, or at risk for, Kaposi's sarcoma but lower in sera from blood donors in non-endemic regions (see below) and Africa. A combination of two or more antigen assays is likely to be required for optimal sensitivity (Simpson *et al.*, 1996).

Other recombinant lytic-phase proteins are being investigated as potential serological antigens. The minor capsid protein vp23, encoded by *ORF 26*, which was part of one of the original representational difference analysis fragments that characterize the virus, has been expressed as a recombinant protein and used as a serological antigen (André *et al.*, 1997; Davis *et al.*, 1997). Significantly more sera from AIDS patients with Kaposi's sarcoma than from HIV-negative controls react with this antigen, and no clear-cut evidence for EBV cross-reactivity was seen, despite the relatively high homology (49% amino acid identity) with its EBV homologue BDLF1. As this protein is recombinant, the antigen reacts with only about one-third of sera from AIDS patients with Kaposi's sarcoma (André *et al.*, 1997); however, a synthetic peptide from this region was reported to be recognized by 60% of sera from this group of patients (Davis *et al.*, 1997). The recombinant ORF 26 antigen was reported to react with the sera of only a few German blood donors (André *et al.*, 1997), whereas the ORF 26-derived synthetic peptide was

reactive with 20% of sera from United States blood donors (Davis *et al.*, 1997). A recombinant carboxy-terminal fragment of the major capsid protein encoded by *ORF 25* has also been investigated and shown to cross-react with high-titre EBV antibodies (André *et al.*, 1997). This observation is in line with the high homology (56% amino acid identity) of the ORF 25 protein with its EBV homologue, BclF1 (Moore *et al.*, 1996a).

Other assays involve the detection of antibodies to unknown lytic antigens. These include an immunofluorescence assay on a primary effusion lymphoma cell line treated with phorbol esters to induce the lytic replication cycle (Lennette *et al.*, 1996; Smith *et al.*, 1997; Ablashi *et al.*, 1997). With these assays, antibodies can be detected in nearly 100% of Kaposi's sarcoma patients and in 0–20% of United States blood donors. The specificity of this lytic antigen assay should be clarified. While Lennette *et al.* (1996), using a serum dilution of 1:10, found antibodies to lytic KSHV/HHV8 antigens in 20% of United States blood donors, Smith *et al.* (1997), using a 1:40 serum dilution and Evan's blue to reduce nonspecific background staining, found no antibodies in 52 blood donors. Smith *et al.* (1997) noted that sera that are reactive by immunofluorescence at a dilution of < 1:40 cannot be confirmed as reactive by radioimmunoprecipitation. The demonstration that cytoplasmic seroreactivity to primary effusion lymphoma cells infected with KSHV/HHV8 can be cross-adsorbed by EBV-containing cell lines not infected with KSHV/HHV8 (Gao *et al.*, 1996a) suggests that nonspecific reactivity in lytic antigen assays could be a major problem. Whole virion antigens may be of use in the design of competitive assays that are less sensitive to cross-reactive antibodies.

Experience with these assays suggests that LANA is a very specific antigen and can be used to detect most but not all cases of KSHV/HHV8 infection. Of the lytic KSHV/-HHV8 antigens, the recombinant vp19/ORF 65 protein gives comparable detection rates. The specificity of immunofluorescence in induced primary effusion lymphoma cells varies with the assay protocol used, and the optimal conditions for these assays should be established. While the correlation among these different assays is good for sera from Kaposi's sarcoma patients, there is considerable variation with regard to sera from blood donors in non-endemic areas.

1.2.3 *Culture* in vitro

No efficient culture system that results in a high titre of KSHV/HHV8 transmission is presently available. Some latently infected primary effusion lymphoma cell lines can be induced into lytic replication of KSHV/HHV8 by treatment with phorbol esters or sodium butyrate (Renne *et al.*, 1996a; Miller *et al.*, 1997). Some transmission of viral DNA from the BC-1 cell line to Raji, BJAB, Molt-3 and OMK cell lines and to cord blood lymphocytes (Mesri *et al.*, 1996; Moore *et al.*, 1996a) has been described; however, the replication competence of KSHV/HHV8 from BC-1 is unclear, given its large genomic duplication. KSHV/HHV8 has been directly cultured from Kaposi's sarcoma lesions in embryonal kidney 293 recipient cells (Foreman *et al.*, 1997). While 293 cells allow serial propagation of KSHV/HHV8, viral amplification is limited, requiring PCR amplification for virus detection.

1.3 Epidemiology of infection

Limited prevalence estimates in various risk and geographic groups are available from case–control studies and surveys. PCR-based estimates of prevalence in PBMC or semen are of limited value because of the low copy number of KSHV/HHV8 in these samples, which results in underestimates of the true prevalence. Measurement of KSHV/HHV8 infection in various tissues by PCR is discussed more fully in the context of case–control studies (section 2.1.3). The advantage of examining PBMC by PCR is that samples are readily acquired from various populations; serological studies to examine prevalence have the advantage that any antibodies detected may reflect both past and current viral infection. As indicated in section 1.2.2, test methods may differ in sensitivity and specificity, resulting in widely different estimates.

1.3.1 *Prevalence in peripheral blood mononuclear cells*

Several groups have attempted to detect KSHV/HHV8 in PBMC from healthy individuals, usually studied as control groups for patients with conditions linked to infection with KSHV/HHV8. When PBMC from healthy individuals in countries with a low prevalence of Kaposi's sarcoma (e.g. France, the United Kingdom, the United States; see below) were studied, no KSHV/HHV8 genomes were detected by nested PCR (Ambroziak *et al.*, 1995; Whitby *et al.*, 1995; Lefrère *et al.*, 1996; Marchioli *et al.*, 1996). In Italy, a country where 'classic' Kaposi's sarcoma is present, KSHV/HHV8 was detected in 9% of PBMC and lymphoid tissues from HIV-uninfected individuals (Bigoni *et al.*, 1996). In Uganda, where endemic Kaposi's sarcoma is common, KSHV/HHV8 was detected in 14% of patients with tumours other than Kaposi's sarcoma (Chang *et al.*, 1996b). These studies were consistent in finding a higher prevalence of KSHV/HHV8 in PBMC in populations at higher risk of developing Kaposi's sarcoma. The correlation between the detection of KSHV/HHV8 in peripheral blood of individuals and the presence of or risk for Kaposi's sarcoma is discussed in Section 2.

1.3.2 *Prevalence in semen*

On the assumption that, if KSHV/HHV8 can be sexually transmitted, the virus may be present at high copy number in semen, prevalence studies based on semen samples might result in higher rates of detection than those based on PBMC.

The presence of KSHV/HHV8 in the semen of healthy men is controversial. It is detected in some semen samples from HIV-infected patients with or without Kaposi's sarcoma, but the reported detection rates in the United Kingdom and the United States vary from 0 (only four samples tested) to 33% (Ambroziak *et al.*, 1995; Gupta *et al.*, 1996; Marchioli *et al.*, 1996; Monini *et al.*, 1996b; Howard *et al.*, 1997). The results obtained in semen samples from healthy, HIV-seronegative donors are even more controversial in view of the implications of the prevalence of KSHV/HHV8 in the general population. Samples from Italian semen donors were initially reported to be 91% positive (Monini *et al.*, 1996a) but later to be 23% positive (Monini *et al.*, 1996b), whereas not a single positive sample was found among 115 semen donors in the United Kingdom (Howard *et al.*, 1997) or in 20 in Milan, Italy (Corbellino *et al.*, 1996c). Some of these

discrepant results probably reflect regional differences in KSHV/HHV8 prevalence (as shown by serological studies) and/or selection of semen donors. A high detection rate was reported in HIV-negative semen donors in the United States (Lin *et al.*, 1995). [The Working Group noted that this rate may be flawed and requires confirmation.]

1.3.3 *Seroprevalence and geographical distribution*

The use of different serological assays for KSHV/HHV8-specific antibodies has resulted in uncertainty about the exact seroprevalence of this virus in different populations and geographical areas. Most groups agree, however, that antibodies to KSHV/-HHV8 are found in most, if not all, patients with Kaposi's sarcoma and are more common in individuals at risk for this disease than in the general population; it is also generally agreed that the virus is not as widespread in the west as, for example, EBV. Antibodies to the latent nuclear antigen are found in about 85% of AIDS patients with Kaposi's sarcoma and in more than 90% of individuals with 'classic' Kaposi's sarcoma (Gao *et al.*, 1996b; Kedes *et al.*, 1996; Simpson *et al.*, 1996). Among individuals studied in Denmark, the United Kingdom and the United States, about one-third of HIV-infected homosexual men without Kaposi's sarcoma (Simpson *et al.*, 1996; Melbye *et al.*, 1998), 8% of HIV-uninfected persons attending sexually transmitted disease clinics (10 out of 130), 0–3% of HIV-uninfected blood donors, 0–3 % of patients with haemophilia and no intravenous drug users had antibodies to this antigen (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996; Simpson *et al.*, 1996). Women in the United States, who are at low risk for AIDS-associated Kaposi's sarcoma, have correspondingly low LANA antibody titres, regardless of HIV status (Kedes *et al.*, 1997). Antibody positivity to vp19/ORF 65 shows a very similar distribution: 81% of patients with AIDS-associated Kaposi's sarcoma, 94% of those with 'classic' Kaposi's sarcoma, 31% of HIV-infected homosexual men without Kaposi's sarcoma, 2–5% of HIV-negative blood donors, 1% of patients with haemophilia and 3% of intravenous drug users (Simpson *et al.*, 1996). Therefore, the distribution of antibodies to both these antigens would suggest that KSHV/HHV8 is an uncommon infection in the general populations of those countries where it is likely to be sexually transmitted.

The phorbol ester-induced lytic immunofluorescence antigen assay may be even more sensitive (> 95%) than either vp19/ORF 65 or LANA assays. Lennette *et al.* (1996) found that the prevalence of antibodies to this antigen in the adult North American population ranged from 16 to 28%; that for patients with AIDS-associated Kaposi's sarcoma was 96–100%, that for HIV-infected homosexual men, 90%, and that for intravenous drug users, 23%. It is unclear whether the higher values represent increased sensitivity, cross-reactivity with EBV or other human herpesviruses or both. Nonetheless, results from all of these assays broadly concur in suggesting that in Europe and North America KSHV/HHV8 is markedly more common among homosexual men than in other risk groups for HIV transmission. Thus, the distribution of KSHV/HHV8 mirrors that of Kaposi's sarcoma, which has long been known to occur more frequently among HIV-infected homosexual men than among patients with haemophilia, transfusion recipients or intravenous drug users (Beral *et al.*, 1990).

Although lower seroprevalences for antibodies to vp19/ORF 65 and LANA/ORF 73 are found in the general populations of northern Europe and North America than for lytic immunofluorescence antibodies, much higher rates are found in several Mediterranean countries. In Milan, Italy, a region with a relatively low incidence of Kaposi's sarcoma, the seroprevalence among blood donors was found to be 4% by latent immunofluorescence antigen and western blot assays (Gao *et al.*, 1996a). In Greece, 12% of HIV-negative surgical patients without Kaposi's sarcoma were seropositive for ORF 65/vp19 and/or ORF 73/LANA (Simpson *et al.*, 1996), suggesting that the seroprevalence in endemic countries may be much higher than in northern Europe or the United States.

The KSHV/HHV8 seroprevalence to these two antigens is much higher (> 50%) in countries of East, Central and West Africa (Gao *et al.*, 1996a; Lennette *et al.*, 1996; Simpson *et al.*, 1996). This suggests that infection with KSHV/HHV8 may approach near universal levels in some African populations (50–70% seroprevalence). Within Europe, KSHV/HHV8 appears to be more common in regions known for their higher incidence of endemic Kaposi's sarcoma, although a careful comparison of the incidence of Kaposi's sarcoma with KSHV/HHV8 seroprevalence in southern Europe is required before definitive conclusions can be reached. These marked differences in seroprevalence within Europe may also help to explain some of the discrepant reports on KSHV/HHV8 genome prevalence detected by PCR in various populations.

Reports from other parts of the world are limited. The prevalence rates in the Caribbean and Central America have been found to be between 0% as determined by latent antigen assays and up to 29% by lytic antigen assays (Lennette *et al.*, 1996).

1.3.4 *Routes of transmission*

Serological testing is currently being used to evaluate risk factors for KSHV/HHV8 transmission. Several serological studies have indicated that, irrespective of the type of antigen used, KSHV/HHV8 infection may be more common among people attending sexually transmitted disease clinics than among blood donors (Kedes *et al.*, 1996; Lennette *et al.*, 1996; Simpson *et al.*, 1996).

A recent detailed analysis of the behavioural risk factors among Danish homosexual men revealed that variables such as promiscuity and receptive anal intercourse, but not oral–anal contact, increased the risk for KSHV/HHV8 infection (Melbye *et al.*, 1998). Furthermore, in the United States in the early 1980s, contact with homosexual men markedly enhanced the likelihood of having or acquiring antibodies to KSHV/HHV8, suggesting that KSHV/HHV8 was introduced into that community in the late 1970s or early 1980s (Melbye *et al.*, 1998). The rate of new infections decreased during the 1980s. Detailed studies will be needed to delineate the precise mechanisms by which KSHV/-HHV8 is transmitted among homosexual men and other populations at increased risk for sexually transmitted diseases. These findings suggest that KSHV/HHV8 is sexually transmitted in countries of low prevalence, consistent with the finding by PCR that infectious virus is secreted into the semen of infected men. These studies demonstrate that the behavioural risk factors that were previously shown to increase the risk for Kaposi's sarcoma (Beral *et al.*, 1990) also increase the likelihood of being infected with

KSHV/HHV8, providing further evidence that KSHV/HHV8 is indeed the postulated 'Kaposi's sarcoma agent'. To what extent sexual transmission is an important route of infection with KSHV/HHV8 in countries of high prevalence is not yet resolved. Early indications that a significant proportion of pre-adolescent children in Central Africa may have antibodies to ORF 65/vp19 and/or ORF 73/LANA suggest that the occurrence of Kaposi's sarcoma among young children in highly endemic African countries (a condition which is exceedingly rare in developed countries) occurs by vertical viral transmission in childhood in some settings (Ziegler & Katongole-Mbidde, 1996).

The route of KSHV/HHV8 transmission in Italian organ transplant recipients was investigated in one study. Parravicini *et al.* (1997) found that 10 of 11 patients who developed Kaposi's sarcoma were seropositive before receiving the allograft, in comparison with two of 17 transplant recipients who did not develop the disease. This suggests that, in KSHV/HHV8 endemic areas, most cases of transplant-associated Kaposi's sarcoma are due to reactivation of a pre-existing KSHV/HHV8 infection; however, the authors also documented one case in which transmission of KSHV/HHV8 occurred from the allograft.

In the 1960s and 1970s, a new epidemiological type of Kaposi's sarcoma associated with immunosuppression was identified among organ transplant patients, constituting up to 5% of tumours (Penn, 1979, 1983, 1988a,b; Qunibi *et al.*, 1988). It has been reported that patients who develop Kaposi's sarcoma after a renal transplant in North America are often of Jewish or Mediterranean ancestry (Harwood *et al.*, 1979). A recent study from Scandinavia found only two cases among 5000 transplant patients (Birkeland *et al.*, 1995).

1.4 Control and prevention

The role of antiviral agents in the prevention of Kaposi's sarcoma is discussed in section 4.6.

2. Studies of Cancer in Humans

Because KSHV/HHV8 was discovered only recently, few analytical data are available on its possible association with cancer in humans. Most of the available information derives from case series and case–control and cohort studies. This field of research is rapidly evolving, and the information reported below will be up-to-date for only a limited time.

2.1 Kaposi's sarcoma

A recent monograph on the evaluation of carcinogenic risks to humans dealt with Kaposi's sarcoma and human immunodeficiency viruses in detail (IARC, 1996). For completeness, the sections on the descriptive epidemiology on Kaposi's sarcoma from

that monograph have been incorporated in modified and shortened versions in the present monograph.

2.1.1 *Pathology and clinical disease*

2.1.1.1 *Epidemiological and clinical presentation*

Epidemiologically, Kaposi's sarcoma has been classified into sporadic (classic), endemic (African), epidemic (AIDS-related) and immunosuppression-associated (usually in transplant recipients) types; however, the histopathology of all of these types of Kaposi's sarcoma is identical (Templeton, 1981; Cockerell, 1991). In 1872, Dr Moriz Kaposi, a Hungarian dermatologist, first described an idiopathic, multiple pigmented sarcoma, now called 'classic' or sporadic Kaposi's sarcoma (Kaposi, 1872; Breimer, 1994). For many years, Kaposi's sarcoma was thought to be a lesion that affected predominantly elderly men of Mediterranean and eastern European origin (Dörffel, 1932; Landman *et al.*, 1984; Franceschi & Geddes, 1995). The presence of Kaposi's sarcoma was first noted in Africa in the 1920s (Williams, 1992). In the 1960s, it was reported to comprise up to 8% of malignancies, with endemic foci in parts of Africa (Oettlé, 1962; MacLean, 1963; Hutt & Burkitt, 1965; Williams, 1975). 'Endemic' Kaposi's sarcoma, like the 'classic' type, predominates in men but also occasionally affects children (Hutt & Birkitt, 1965; Williams, 1975; Ziegler & Katongole-Mbidde, 1996). The geographic distribution of endemic Kaposi's sarcoma in Africa prior to the AIDS epidemic was reported to be similar but not identical to that of Burkitt's lymphoma. In the early 1980s, a fourth variant of Kaposi's sarcoma, the 'epidemic' type, heralded the onset of the AIDS epidemic (Hymes *et al.*, 1981). Today, Kaposi's sarcoma is an AIDS-defining condition in HIV-infected individuals.

'Classic' or endemic Kaposi's sarcoma affects predominantly the skin of the lower limbs; internal organs are rarely involved. The disease typically follows an indolent course, patients surviving for an average of 10–15 years (Tappero *et al.*, 1993). Young children tend to have more severe disease than adults, the lesions often affecting the lymphatic system and internal organs rather than the skin, and shorter survival (Oettlé, 1962; Ziegler & Katongole-Mbidde, 1996). Kaposi's sarcoma in immunocompromised individuals — mainly transplant recipients and long-term users of steroids and cytotoxic drugs — often involves internal organs, lymph nodes and the face, mimicking the 'epidemic' type (Tappero *et al.*, 1993). In transplant recipients, Kaposi's sarcoma appears before most other tumours and may regress completely when immunosuppressive therapy is terminated (Penn, 1988a,b). In the epidemic form, the lesions are usually multiple, progress rapidly and may affect any area of the skin as well as internal organs. The tumours frequently begin as dusky-red or violet macules, progressing over weeks or months to plaques and raised, usually painless, firm nodules and plaques. Although the tumour may affect the legs, as seen with 'classic' Kaposi's sarcoma, lesions of the trunk, arms, genitalia and face are also common (Smith & Spittle, 1987). Lymph nodes and the oral cavity, most notably the palate, may be extensively involved. Oral Kaposi's sarcoma is often associated with involvement elsewhere in the gastrointestinal tract (Levine, 1993; Regezi *et al.*, 1993). Pulmonary Kaposi's sarcoma generally presents with short-

ness of breath and cough and is clinically difficult to distinguish from other pulmonary complications of AIDS (Levine, 1993). Median survival following a diagnosis of epidemic Kaposi's sarcoma is 14–18 months (Jacobson *et al.*, 1993; Lundgren *et al.*, 1994, 1995; Luo *et al.*, 1995).

2.1.1.2 *Histology*

The early patch-stage macular lesions contain abnormally shaped, dilated vessels surrounded by a mononuclear-cell infiltrate containing plasma cells; nuclear atypia and mitoses are rarely seen. In the plaque-stage lesions, there is proliferation of spindle-shaped cells in the superficial-to-deep dermis, with rare proliferation of spindle-shaped cells, nuclear atypia and mitoses. Spindle cells, which often surround slit-like vascular spaces, are characteristic of more advanced nodular lesions. The presence of KSHV/-HHV8 in spindle and endothelial cells and the expression of individual viral genes is discussed in section 1.1.6.1.

2.1.2 *Epidemiology*

2.1.2.1 *Incidence and geographical distribution*

The epidemiology of Kaposi's sarcoma was drastically influenced by the onset of the AIDS epidemic in the late 1970s and early 1980s. From being an exceedingly rare condition outside sub-Saharan Africa, its incidence suddenly increased dramatically among certain populations, such as homo- and bisexual men. Throughout the world, the incidence of Kaposi's sarcoma today reflects the burden of the AIDS epidemic, and as such varies considerably. Whereas the incidence appears to have reached a plateau or even a decline in parts of Europe and the United States (Dal Maso *et al.*, 1995), it is apparently rising in some African countries, such as Uganda (Wabinga *et al.*, 1993; Basset *et al.*, 1995; see Table 2).

As mentioned above, Kaposi's sarcoma represented up to 8% of all tumours in some parts of sub-Saharan Africa before the appearance of HIV infection in the 1980s. Relatively high incidence rates were reported from Israel (1970–79, 1.5/100 000 in people of each sex combined; Landman *et al.*, 1984), from Italy (1976–84, 1.05/100 000 in men, 0.27/100 000 in women; Geddes *et al.*, 1994), particularly in the south, and from Sardinia (1977–82, 1.6/100 000 in people of each sex combined; Cottoni *et al.*, 1996). Recently, high rates have also been described in two other island societies, those of Iceland and the Faeroe Islands in the North Atlantic (Hjalgrim *et al.*, 1998). Much lower age-adjusted rates are reported in Australia (1972–82, 0.065/100 000 in men, 0.029/100 000 in women; Kaldor *et al.*, 1994), England and Wales (0.014/100 000 in both men and women) and the United States (1973–79, 0.297/100 000 in men, 0.07/100 000 in women; Biggar *et al.*, 1984). On the basis of data in the Nordic cancer registries, the incidence rose among men from 0.05/100 000 in 1953–57 to 0.18/100 000 in 1978–79; in Nordic women, the corresponding rates were 0.02/100 000 and 0.08/100 000, respectively (Hjalgrim *et al.*, 1996a). Thus, in some countries, modest increases in the incidence of Kaposi's sarcoma were already occurring before the onset of the AIDS epidemic (Dictor & Attewell, 1988; Hjalgrim *et al.*, 1996a).

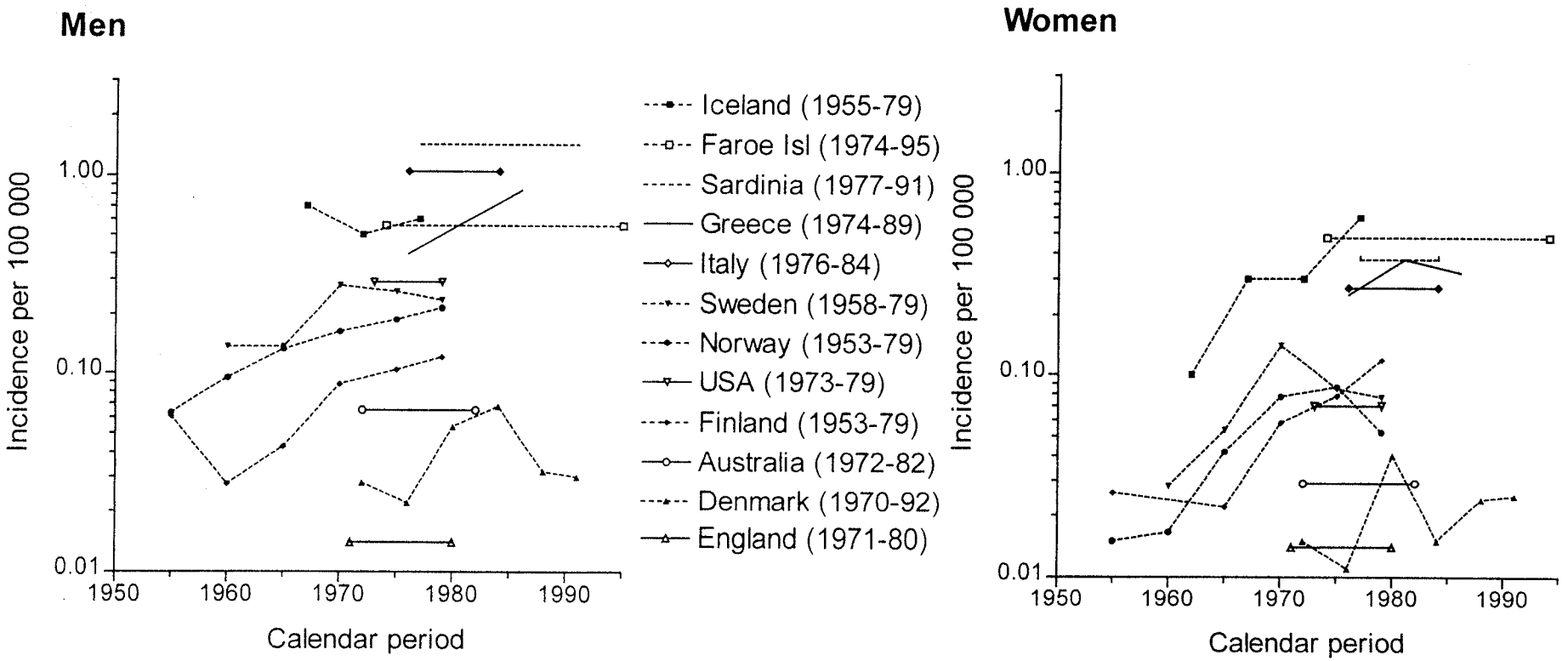
Table 2. Frequency of Kaposi's sarcoma in relation to all cancers in various areas of Africa

Reference	Location	Year(s) of study or report	Percentage of all cancers		
			Men	Women	Both
Oettlé (1962)	Former French Equatorial Africa	1953	–	–	5
	Former French West Africa	1954	–	–	1
	Ghana	1956	–	–	1
	Kenya	1948–61	–	–	2–4
	Mozambique	1958	–	–	2
	Nigeria	1934–44	–	–	2
	South Africa	1951 and 1960	–	–	1–3
	South Africa (Natal)	1957	–	–	1
	United Republic of Tanzania	1960	–	–	3
	Tunisia	1960	–	–	< 1
	Zaire	1956–57	–	–	9–13
	Zambia and Zimbabwe	1949	–	–	1
Hutt & Burkitt (1965)	Uganda	1964	–	–	4
Bayley (1984)	Zaire	1983	–	–	9
Otu (1986)	Nigeria	1986	–	–	15–20
Melbye *et al.* (1987)	Zaire	1984	16	–	–
Ngendahayo *et al.* (1989)	Rwanda	1979–86	–	–	6
Wabinga *et al.* (1993)	Uganda (registry)	1989–91	49	18	–
	Zambia				
Patil *et al.* (1992)	Children	1980–89	–	–	8.8
Patil *et al.* (1995)	Adults	1980–89	–	–	7.0
Bassett *et al.* (1995)	Zimbabwe (registry)	1990–92	23	10	–
Newton *et al.* (1996)	Rwanda (registry)	1991–93	10	3	–
Sitas *et al.* (1996)	South Africa (registry)				
	Black	1990–91	0.54	0.14	0.3
	White	1990–91	0.12	0.03	0.1

–, not reported

Studies in Australia, Denmark, the United Kingdom and the United States have shown an increased risk for Kaposi's sarcoma among persons of certain ethnicities from Central and East Africa, eastern Europe and Mediterranean countries and people of Jewish descent (Laor & Schwartz, 1979; DiGiovanna & Safai, 1981; Friedman-Birnbaum *et al.*, 1990; Grulich *et al.*, 1992; Kaldor *et al.*, 1994; Hjalgrim *et al.*, 1996b; Figure 4).

Figure 4. Reported incidence rates of 'classic' Kaposi's sarcoma



From Hjalgrim *et al.* (1998)

Dotted lines indicate world-standardized rates and solid lines the rates standardized to local populations; calendar period reflects period of observations.

2.1.2.2 *Demographic variations*

Formerly a tumour affecting predominantly the elderly (Oettlé, 1962; Templeton, 1981; Hutt, 1984, Geddes *et al.*, 1994; Hjalgrim *et al.*, 1996a), Kaposi's sarcoma has shown a substantial alteration in age distribution in recent years, in both developed and developing countries. Whereas the median age in developed countries before the AIDS epidemic was over 70 years, it is now in the late thirties.

In Europe and the United States, childhood Kaposi's sarcoma is very rare, even since the advent of the AIDS epidemic. In the early 1990s, the age-specific incidence rates in African countries such as Uganda and Zimbabwe showed a modest peak for children aged zero to four years, a decline until age 15 years and then the main peak at age 35–39 years in men and 25–29 years in women (Wabinga *et al.*, 1993; Basset *et al.*, 1995).

Studies based on registry data have found a male:female ratio of 'classic' Kaposi's sarcoma of 2–3:1 (Biggar *et al.*, 1984; Franceschi & Geddes, 1995; Hjalgrim *et al.*, 1996a). In a study based on data from the Nordic cancer registries, the male excess was primarily restricted to men over 60 years of age (Hjalgrim *et al.*, 1996a). In Africa, male:female ratios greater than 10 reported in early studies (Wahman *et al.*, 1991) have since declined to about 3:1 (Wabinga *et al.*, 1993; Basset *et al.*, 1995; Newton *et al.*, 1996).

2.1.2.3 *Behavioural factors*

Case reports suggest that Kaposi's sarcoma may occur more frequently than expected in HIV-uninfected homo- and bisexual men (Friedman-Kien *et al.*, 1990; Peterman *et al.*, 1991) and at a rate equivalent to the total number of cases diagnosed among all men under 50 years of age per year before the AIDS epidemic (Biggar *et al.*, 1984). [The Working Group noted that surveillance bias could entirely explain this observation.] Furthermore, in an analysis of 'classic' Kaposi's sarcoma in Denmark, men who had never married (used as a rough surrogate for homosexuality) were 19 times more at risk for the disease than men who had married (Hjalgrim *et al.*, 1996b); a similar analysis of data in the United States, however, showed no such difference (Biggar & Melbye, 1996).

The risk for Kaposi's sarcoma varies greatly among the different groups at risk for HIV transmission, being particularly high in homo- and bisexual men (IARC, 1996). This elevated risk is seen even among men aged 13–24 and suggests a rapid increase in risk after homosexual contact. Beral *et al.* (1990) found that 13 616 of 88 739 (15%) AIDS patients in the United States developed Kaposi's sarcoma, the proportion varying from 21% of homo- or bisexual men to 3% of heterosexuals, 2% of intravenous drug users, 3% of transfusion recipients, 1% of haemophiliacs and 1% of children infected by perinatal transmission. Furthermore, women with AIDS who were sexual partners of bisexual men were more likely to have Kaposi's sarcoma than women who were partners of intravenous drug users (Peterman *et al.*, 1993; Serraino *et al.*, 1995). Even among homo- and bisexual men, the risk for Kaposi's sarcoma is not uniform: Schechter *et al.* (1991) conducted an analysis of all AIDS-associated cases of Kaposi's sarcoma among homo- and bisexual men in Canada between 1980 and 1989 and found that the pro-

portion of cases among AIDS patients had a strong geographical association with the original centres of the AIDS epidemic in Canada. Furthermore, homosexual men born between 1945 and 1954 were more likely to present with Kaposi's sarcoma, consistent with the hypothesis of an environmental cofactor with higher levels of exposure. In another study from the same group, Archibald *et al.* (1990) found that 56% of Canadian homosexual men with AIDS who developed Kaposi's sarcoma and only 21% of those who developed AIDS but not Kaposi's sarcoma reported that they had had more than 20 sexual partners from large cities in the United States (odds ratio, 4.6; 95% confidence interval [CI], 1.6–13). Similarly, homosexual men with AIDS in the United Kingdom were more likely to have Kaposi's sarcoma if they had had sexual contact with an American (31%) or African (26%) man than if they had not (19%) ($p < 0.05$) (Beral *et al.*, 1991). Furthermore, Peterman et *al.* (1993) found that Kaposi's sarcoma was more frequently part of the AIDS definition in homosexual men from California and New York than in homosexual men from the rest of the United States.

Most analyses of the number of sexual partners of homo- and bisexual men with Kaposi's sarcoma and of those with other manifestations of AIDS (Haverkos *et al.*, 1985; Goedert *et al.*, 1987; Archibald *et al.*, 1990; Armenian *et al.*, 1993), but not all (Lifson *et al.*, 1990a,b), found that patients with Kaposi's sarcoma had had a larger number of sexual partners. Patients with this cancer have also been reported to be more likely to have a history of sexually transmitted disease (Goedert *et al.*, 1987; Armenian *et al.*, 1993). In a case–control study in New York City, United States, Kaposi's sarcoma was found to be significantly associated with receptive anal intercourse (Marmor *et al.*, 1982; Jaffe *et al.*, 1983). Several authors have subsequently reported an increased risk for Kaposi's sarcoma among HIV-positive men whose sexual practices involve faecal contact (Beral *et al.*, 1992; Darrow *et al.*, 1992). The possible association between insertive oral–anal contact and the risk for Kaposi's sarcoma remains controversial, some studies showing a possible association (Archibald *et al.*, 1990; Beral *et al.*, 1992; Darrow *et al.*, 1992) and others not (Lifson *et al.*, 1990b; Elford *et al.*, 1992; Page-Bodkin *et al.*, 1992; Armenian *et al.*, 1993; Kaldor et al., 1993). Casabona *et al.* (1991) noted that the fraction of AIDS cases with Kaposi's sarcoma was similar in southern Europe, with a relatively high incidence of 'classic' Kaposi's sarcoma, and in northern Europe, with a relatively low incidence. In Uganda, increased risk for Kaposi's sarcoma was seen in HIV-seropositive adults of each sex who had one rather than several spouses or a history of sexually transmitted diseases, and especially those who were relatively affluent, well-educated, had travelled and had spent increasing time in contact with water (Ziegler *et al.*, 1997).

In conclusion, men who develop Kaposi's sarcoma tend to be more sexually active and to have more sexual partners from epicentres of the AIDS epidemic. In conjunction with the much higher risk for Kaposi's sarcoma among homosexual men than among other HIV transmission groups, these data indicate that an infectious sexually transmitted agent (independent of HIV) is associated with Kaposi's sarcoma. Transmission of such an agent via the blood is apparently less common, since Kaposi's sarcoma occurs in only 3% of people who acquire HIV through a blood transfusion.

2.1.2.4 *Second primary malignancies after Kaposi's sarcoma*

An association between Kaposi's sarcoma and lymphomas has been suspected for many years. Both tumours occur in association with immunosuppression and can occur in the same individual. In a hospital-based cohort of 72 patients with 'classic' Kaposi's sarcoma in New York, United States, Safai *et al.* (1980) counted a total of nine lymphoid malignancies, including four non-Hodgkin's lymphomas, during 581 person–years of follow-up. This corresponds to a significantly (20-fold) increased risk for these malignancies over that of the background population. Few studies have addressed 'classic' Kaposi's sarcoma because of its infrequency. Three deaths from non-Hodgkin's lymphoma were reported among 68 patients with Kaposi's sarcoma in the United Kingdom (Grulich *et al.*, 1992). In contrast, two larger population-based studies of 492 American and 204 Italian subjects with 'classic' Kaposi's sarcoma did not confirm the suspected association (Biggar *et al.*, 1994; Franceschi *et al.*, 1996).

2.1.3 *Case series and case–control studies*

2.1.3.1 *Detection of KSHV/HHV8 DNA in tumour tissue*

Published case series and case–control studies on the detection of KSHV/HHV8 DNA in Kaposi's sarcoma tissue are summarized in Table 3. Many of these studies are small and/or included heterogeneous controls.

KSHV/HHV8 DNA is found in nearly all Kaposi's sarcoma tissues, despite differences in detection methods and in the quality or preservation of tumour material. In 28 studies in which the detection of KHSV/HHV8 DNA was described in Kaposi's sarcoma tissues (Table 3), KSHV/HHV8 was identified in 735 of 794 (91%) Kaposi's sarcoma analysed. The rates reported in one of the studies (Noel *et al.*, 1996) were very different from those in the other studies; when these results were excluded, the percentage positivity rose to 96% (686/716). The detection rate was similar whether the patients were HIV-infected (391/417; 94%) or uninfected (335/368; 91%). Furthermore, KSHV/HHV8 was found in all four epidemiological forms of Kaposi's sarcoma, with no indication of significant differences in the detection rate in the four types. With the exception of a few studies (Rady *et al.*, 1995; Gyulai *et al.*, 1996a,b), little or no evidence of KSHV/HHV8 DNA has been found in tumours other than Kaposi's sarcoma. The exceptions include primary effusion lymphomas and Castleman's disease. The recent identification of KSHV/HHV8 in bone-marrow dendritic cells of myeloma patients awaits confirmation (Rettig *et al.*, 1997; see section 2.2.3). These and other conditions potentially associated with KSHV/HHV8 are discussed in subsequent sections (see also Table 3).

A major difficulty in assessing associations with disease on the basis of detection of DNA is in selecting appropriate control tissues in order to identify differences in infection rate. In the initial description of the virus, Chang *et al.* (1994) found that 25 of 27 AIDS-associated Kaposi's sarcomas contained KSHV/HHV8 DNA in comparison with three of 27 lymphomas from AIDS patients [odds ratio, 100, $p < 10^{-7}$] (some of whom may have had Kaposi's sarcoma as a secondary malignancy), none of 29 lymphomas from non-AIDS patients and none of 49 consecutive surgical biopsy samples ($p < 10^{-5}$).

Table 3. Presence of KSHV/HHV8 DNA in Kaposi's sarcoma (KS) tissue, other tissues from Kaposi's sarcoma patients and tissues from subjects without Kaposi's sarcoma, detected by polymerase chain reaction (PCR)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Chang *et al.* (1994)	AIDS-KS	25/27			AIDS		Fresh frozen
					Lymphoma	3/27	
					Lymph node	3/12	
					Total	6/39	
					Non-AIDS		
					Lymphoma	0/29	
					Lymph node	0/7	
					Vascular tumour	0/5	
					Opportunistic infections	0/13	
					Surgical biopsy	0/49	
					Total	0/103	
Su *et al.* (1995)	AIDS-KS	4/4	None		AIDS lymph node	0/5	
	Non-AIDS KS	2/3			Benign hyperplasia	0/10	
	Total	6/7			B-Cell lymphoma	0/12	
					T-Cell lymphoma	0/10	
					Total	0/37	
Dupin *et al.* (1995a)	Classic KS	5/5	Skin, classic	3/3	Various tissues, HIV-negative	0/6	Snap-frozen
	AIDS-KS	4/4	Skin, AIDS	2/3			
	Total	9/9	Total	5/6			
Boshoff *et al.* (1995a)	Classic KS	16/17	None		Angioma/angiosarcoma	0/4	Fresh-frozen or
	Transplant KS	8/8			Skin naevi	0/3	paraffin-embedded
	AIDS-KS	14/14			Granulomatous tissue	0/4	(nested PCR)
	HIV-negative homosexual man	1/1			Total	0/11	
	Total	39/40					

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Ambroziak *et al.* (1995)	AIDS-KS, homosexual men	12/12	None		None		
	HIV-negative homosexual man	1/1					
	Total	13/13					
Moore & Chang (1995)	AIDS-KS	10/11	Skin, AIDS	1/7	Skin from healthy subjects	1/11	Fresh-frozen
	Classic KS	6/6	Skin, classic	1/5	PBMC from healthy subjects	0/10	
	HIV-negative homosexual men	4/4	Skin, HIV-negative	1/2	Total	1/21	
	Total	20/21					
Lebbé *et al.* (1995)	Immunosuppressed KS	1/1	Skin, HIV-negative	3/9	None		
	Classic KS	10/10					
	African KS	3/3					
	AIDS-KS	2/2					
	Total	16/16					
Schalling *et al.* (1995)	AIDS-KS	25/25	Pyothorax-related B-cell lymphoma-KS, HIV-positive	0/3	HIV-positive		
	African KS	18/18			Pyothorax-related B-cell lymphoma (PBMC)	0/13	
	Classic KS	3/3			Lymph node	0/8	
	Total	46/46			Total	0/21	
			Skin, HIV-negative	0/2	HIV-negative		
					Pyothorax-related B-cell lymphoma (PBMC)	0/12	
					Skin, non-KS patient	0/1	
					Haemangioma	0/1	
					Pyogenic granuloma	0/1	
					Total	0/15	

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Chang *et al.* (1996b)	AIDS-KS African KS Total	22/24 17/20 39/44	None		HIV-positive HIV-negative Total	1/7 2/15 3/22	Paraffin-embedded Negatives retested by nested PCR
Chuck *et al.* (1996)	African KS HIV-negative homosexual men Total	4/4 1/2 5/6	None		None		Fresh-frozen (endemic) or paraffin-embedded (HIV-negative homosexual men)
O'Neill *et al.* (1996)	AIDS-KS	7/7			HIV-negative	0/1	Nested PCR Fresh-frozen or paraffin-embedded
Buonaguro *et al.* (1996)	African KS Classic KS Immunosuppressed KS AIDS-KS Total	12/12 28/28 2/2 19/19 61/61	Skin, HIV-negative	9/13	Reduction mammoplasty Penile carcinoma biopsies Xeroderma pigmentosum skin cancer Xeroderma pigmentosum autologous normal skin PBMC of HIV-positive patients Total	0/3 0/4 0/5 0/5 0/15 0/32	Snap-frozen
Cathomas *et al.* (1996)	AIDS-KS Classic KS Transplant KS Total	9/9 12/12 1/1 22/22	None		Other skin lesions, HIV-positive Other skin lesions, HIV-negative Total	0/4 0/10 0/14	Paraffin-embedded Nested PCR

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Gaidano *et al.* (1996b)	AIDS-KS	35/35	AIDS, skin (PCR; only 3/6 by Southern blot)	6/6	Hodgkin's disease Primary effusion lymphoma Other non-Hodgkin's lymphoma Persistent generalized lymph-adenopathy Anogenital neoplasia Total	0/3 3/3 0/28 0/15 0/14 3/63	Fresh-frozen (a few paraffin-embedded)
Jin *et al.* (1996a)	AIDS-KS Classic KS Total	5/5 12/12 17/17	None		Haemangiosarcoma Haemangioma Lymphangioma Lymphangiomatosis Pyogenic granuloma Haemangiopericytoma Kimura's disease Lymphangiomyomatosis Total	0/15 0/75 0/15 0/2 0/25 0/3 0/2 0/1 0/138	Paraffin-embedded
Dictor *et al.* (1996)	Classic KS AIDS-KS Total	35/40 14/14 49/54	None		Endothelial lesions	0/86	Paraffin-embedded
Marchioli *et al.* (1996)	AIDS-KS Classic KS African KS HIV-negative homosexual men Total	28/28 7/8 7/10 2/2 44/48	HIV-positive Serum Plasma	 3/28 0/13	Normal PBMC Normal skin Paediatric lymphomas Adult lymphomas Carcinomas Total	0/163 0/10 0/8 0/37 0/12 0/230	Fresh-frozen and paraffin embedded

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Luppi *et al.* (1996a)	Classic KS AIDS-KS Total	15/22 3/4 18/26	None		Normal PBMC Normal salivary glands Normal saliva samples Hyperplastic tonsils Total	0/13 0/9 0/6 2/11 2/39	Paraffin-embedded
McDonagh *et al.* (1996)	KS	9/9	None		Angiosarcoma Haemangioma Haemangiopericytoma Total	7/24 1/20 0/6 8/50	Fresh-frozen and paraffin-embedded
Corbellino *et al.* (1996a,b)	AIDS-KS	7/7	HIV-positive Lymphoid tissue Prostate glands Uninvolved skin Bone marrow Paravertebral sensory lumbar ganglion	 7/7 5/5 3/5 2/3 7/7	AIDS patients HIV-positive Paravertebral sensory lumbar ganglion HIV-negative Paravertebral sensory lumbar ganglion	0/6 0/4 0/3	Snap-frozen
Lebbé *et al.* (1997a)	Classic KS African KS Castleman's disease HIV-negative homosexual men Immunosuppressed/ transplant KS Total	16/16 3/3 1/1 3/3 2/2 25/25	Skin, classic Skin, African Skin, HIV-negative homosexual men Skin, induced Total	10/13 2/3 1/3 1/1 14/20	HIV-negative Dermatology biopsies Reduction mammaplasties Total	 0/10 0/5 0/15	Fresh-frozen[a] Nested PCR
Huang *et al.* (1997)	AIDS-KS HIV-negative	12/12 2/2	HIV-positive Normal skin HIV-negative Normal skin	 5/12 1/2	HIV-positive Intravenous drug users (PBMC) HIV-negative Healthy (PBMC)	 0/5 0/5	Fresh frozen

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Albini *et al.* (1996a)	AIDS-KS African KS Classic KS Transplant KS	59/59 21/21 32/33 6/7	Skin, HIV-positive Skin, HIV-negative	0/3 3/9	HIV-positive Skin Other tissue Lymphoma HIV-negative Lymphoma	 0/4 0/2 2/10 1/34	Fresh or paraffin embedded
Uthman *et al.* (1996)	AIDS-KS Classic KS	23/23 5/5	None		HIV-positive Skin lesions HIV-negative Leiomyoma Melanoma Basal-cell carcinoma Pityriasis rosea Molluscum contagiosum Psoriasis vulgaris Viral warts Pseudolymphoma Total	 0/28 0/3 0/8 0/11 0/4 0/6 0/6 0/8 0/7 0/53	Nested PCR
Decker *et al.* (1996)	AIDS-KS	5/5	None		HIV-negative Allograft (PBMC)[b] Healthy (PBMC)[b]	 4/5 3/5	Fresh tissue

Table 3 (contd)

Reference	KS tissue	KSHV/ HHV8 (positive/ total)	Other tissue from KS patients	KSHV/ HHV8 (positive/ total)	Tissue from subjects without KS	KSHV/ HHV8 (positive/ total)	Comments
Li *et al.* (1996)	AIDS-KS Classic KS	6/6 3/3	None		HIV-negative Skin Verrucea vulgaris Total	 0/3 0/2 0/5	Fresh tissue
Noel *et al.* (1996)	AIDS-KS HIV-negative	41/61 8/17	Cutaneous and others HIV-negative	1/19	HIV-negative Cutaneous and others	 1/26	Paraffin-embedded

Modified from Olsen and Moore (1997)
PBMC, peripheral blood mononuclear cells; HIV, human immunodeficiency virus; PCR, polymerase chain reaction
[a] Positivity rate dependent on amount of added template DNA
[b] Testing of multiple samples from same individual

Similarly, Boshoff *et al.* (1995a) found KSHV/HHV8 in 39 of 40 Kaposi's sarcoma lesions of all types but in none of 11 pathologically similar tissues (angioma/angiosarcoma, skin naevi and granulomatous tissues). In a case–control analysis of Kaposi's sarcoma tissues from HIV-positive and HIV-negative persons and skin and PBMC from HIV-negative persons, Moore and Chang (1995), who were unaware of the case or control status of the subjects, found viral DNA in 20 of 21 Kaposi's sarcoma lesions and in only one of 21 control tissues (odds ratio, 400; 95% CI, 19–17 000). Jin *et al.* (1996a) and Dictor *et al.* (1996) compared Kaposi's sarcomas from HIV-positive and HIV-negative persons with a wide variety of tissues resembling Kaposi's sarcoma, including those of endothelial origin and angiogenic and skin tumours. They found viral DNA in 88–100% of 71 Kaposi's sarcoma lesions and none of 224 control tissues. Table 3 also gives a partial list of studies in which control tissues from nearly all organ systems were examined by PCR. Overall, 34 of 1128 (3%) tissues not from Kaposi's sarcomas contained KSHV/HHV8 DNA. Several of these samples were primary effusion lymphomas (described in section 2.2.1), which are also associated with KSHV/HHV8 (Chang *et al.*, 1994; Gaidano *et al.*, 1996b), and the results in two studies (Decker *et al.*, 1996; McDonagh *et al.*, 1996) accounted for nearly half of all the positive findings.

The amount of viral DNA detected by Southern blot in Kaposi's sarcoma lesions averages from undetectable to an estimated 10–20 viral genome copies per cell equivalent. Similarly, KSHV/HHV8 DNA is readily detectable by PCR in DNA extracted from fresh tissue, whereas nested PCR is often required to obtain positive results from fixed, paraffin-embedded tissue. These conditions probably play a role with respect to the differences in positivity rate observed by different investigators.

Several groups reported a higher detection rate of KSHV/HHV8 by PCR in late plaque or nodular stages than in early or patch-stage Kaposi's sarcoma (Luppi *et al.*, 1996a; Noel *et al.*, 1996).

2.1.3.2 *Detection of KSHV/HHV8 DNA in peripheral blood mononuclear cells*

The rate of detection of HHV8 in PBMC from Kaposi's sarcoma patients varies widely (Table 4); however, most of the larger studies suggest that about 50% of PBMC samples from Kaposi's sarcoma patients give positive results when tested by nested PCR under standard conditions, e.g. using 100–500 ng of PBMC DNA (Whitby *et al.*, 1995; Bigoni *et al.*, 1996; Lefrère *et al.*, 1996; Moore *et al.*, 1996c; Lebbé *et al.*, 1997a). When assaying for the presence of KSHV/HHV8 DNA in PBMC, it is important to use sufficient DNA to detect a low copy number of viral DNA (Decker *et al.*, 1996; Blackbourn *et al.*, 1997).

As shown in Table 4, 42% (161/386) of HIV-positive patients and 53% (47/89) of HIV-negative patients with Kaposi's sarcoma had detectable KSHV/HHV8 DNA in their PBMC. Albini *et al.* (1996a) reported an exceptionally low positivity rate (3/54) for KSHV/HHV8 in PBMC from HIV-infected Kaposi's sarcoma patients. If these exceptional results are excluded, the positivity rate among HIV-infected patients in the remaining studies was 48% (158/332). In a study of Mediterranean Kaposi's sarcoma patients, Brambilla *et al.* (1996) reported a particularly high concordance for patients with

Table 4. Detection of KSHV/HHV8 DNA in peripheral blood mononuclear cells (PBMC)

Reference	KS type	KSHV/HHV8 (positive/ total)	Control PBMCs	KSHV/HHV8 (positive/ total)	Odds ratio	95% CI	Comments
Collandre *et al.* (1995)	AIDS-KS	2/10	HIV-positive, no KS	0/9			PCR Southern blot
Ambroziak *et al.* (1995)	AIDS-KS	7/7	HIV-positive, no KS	0/6			
	HIV-negative KS	3/3	HIV-negative, no KS	0/14			
	Total	10/10	Total	0/20			
Whitby *et al.* (1995)	AIDS-KS	24/46	HIV-positive, no KS	11/143			Nested PCR
			Oncology patients	0/26			
			Blood donors	0/134			
			Total	11/303			
Moore *et al.* (1996c) and correction by Parry & Moore (1997)	AIDS-KS	11/21	Homo- or bisexual AIDS patients, no KS	3/23	7.3	1.4–47.9	Nested PCR
			Haemophiliac AIDS patients, no KS	0/19	21.8	2.4–978	
			Total	3/42			
Marchioli *et al.* (1996)	AIDS-KS	46/99	HIV-positive	0/64			PCR Southern blot
	HIV-negative homosexual men	0/2	HIV-negative	0/163			
			Total	0/227			
	Total	46/101					
Humphrey *et al.* (1996)	AIDS-KS	34/98	HIV-positive, no KS	12/64	2.3	1.0–5.3	PCR Southern blot
			HIV-negative, no KS	0/11			
			Total	12/75			
Decker *et al.* (1996)	AIDS-KS	8/9	Allograft patients	4/5			Multiple samples tested to obtain positive results in controls
			Healthy donors	3/5			
			Total	7/10			

Table 4 (contd)

Reference	KS type	KSHV/HHV8 (positive/ total)	Control PBMCs	KSHV/HHV8 (positive/ total)	Odds ratio	95% CI	Comments
Lebbé *et al.* (1997a)	Classic KS	9/18	Blood donors	0/20			PCR Southern blot Nested PCR (32% of KS subjects positive with unnested PCR)
	African KS	2/3					
	Castleman's disease	0/1					
	HIV-negative homosexual men	1/4					
	Immunosuppressed or transplant KS	0/2					
	Total	12/28					
Albini *et al.* (1996a)	AIDS-KS	3/54	Healthy donors	0/4			
	Classic KS	1/6					
	Transplant	1/2					
	Total	5/62					
Brambilla *et al.* (1996)	Classic KS		None				
	Stage I	9/16					
	Stage II	1/5					
	Stage III	6/11					
	Stage IV	8/8					
	Total	24/40					
Heredia *et al.* (1996)	AIDS-KS	2/2	HIV-positive	0/2			
	HIV-negative KS	6/8	HIV-negative	0/8			
	Total	8/10	Total	0/10			
Howard *et al.* (1995)	AIDS-KS	11/17	AIDS, no KS	0/6			Nested PCR
Huang *et al.* (1997)	AIDS-KS	3/12	None				
Lefrère *et al.* (1996)	AIDS-KS	10/11	AIDS, no KS	1/14			
			HIV-positive (asymptomatic)	1/45			
			HIV-negative	0/20			

HIV, human immunodeficiency virus; PCR, polymerase chain reaction

disseminated Kaposi's sarcoma, 100% of whom had detectable KSHV/HHV8 DNA in their PBMC.

Under the same conditions, KSHV/HHV8 is only rarely detected in PBMC from blood donors or other subjects without Kaposi's sarcoma (Table 4; Ambroziak *et al.*, 1995; Whitby *et al.*, 1995; Bigoni *et al.*, 1996; Lefrère *et al.*, 1996; Moore *et al.*, 1996c; Blackbourn *et al.*, 1997).

2.1.3.3 *Detection of KSHV/HHV8 DNA in other tissues*

Similarly high rates of KSHV/HHV8 positivity have been observed in uninvolved skin of Kaposi's sarcoma patients (Moore & Chang, 1995; Albini *et al.*, 1996a; Buonaguro *et al.*, 1996; Corbellino *et al.*, 1996b; Huang *et al.*, 1997), with no significant difference between those who are HIV-infected and uninfected (Table 3). In contrast, skin biopsy samples from non-Kaposi's sarcoma patients very rarely have detectable KSHV/HHV8.

KSHV/HHV8 is also detected to varying degrees in other tissues (e.g. semen, serum, prostate glands, bone marrow) from Kaposi's sarcoma patients (Table 5; see also section 1.1.6). Corbellino *et al.* (1996a) detected KSHV/HHV8 DNA by nested PCR in all of seven paravertebral sensory lumbar ganglia from Kaposi's sarcoma patients and in none of similar materials from patients without this tumour. Howard *et al.* (1995) detected KSHV/HHV8 by nested PCR in bronchoalveolar lavage fluid from 11 of 14 HIV-positive men with both cutaneous and pulmonary Kaposi's sarcoma, but in none of six men with only cutaneous manifestations and in one of 19 HIV-positive men with no evidence of either cutaneous or pulmonary Kaposi's sarcoma; the last case presented three months later with pulmonary manifestations of Kaposi's sarcoma.

2.1.3.4 *Serology*

Serological assays have been developed to detect antibodies to either a LANA (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996) and/or a defined (Miller *et al.*, 1996; Simpson *et al.*, 1996) or undefined (Lennette *et al.*, 1996; Ablashi *et al.*, 1997) structural ('lytic') antigen of KSHV/HHV8 (Rickinson, 1996). The exact prevalence of KSHV/HHV8 infection in northern Europe and the United States, as measured by these assays, is still controversial and ranges from 0–20%. The available evidence indicates considerable geographical variation. Infection with KSHV/HHV8 seems to be widespread in several African countries (50–70%) and more common in some Mediterranean countries than in northern Europe or the United States (for a more detailed discussion of currently available serological assays and KSHV/HHV8 prevalence, see section 1.2.2).

Studies in which the association between Kaposi's sarcoma and KSHV/HHV8 antibodies was analysed are summarized in Table 6. Irrespective of differences in the assays used, most of the rates reported were more than 80% seropositivity in all epidemiological types of Kaposi's sarcoma.

Most studies in which antibodies to LANA were measured by immunofluorescence or western blotting (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996; Simpson *et al.*, 1996) are consistent in detecting antibodies in 80–90% of Kaposi's sarcoma patients but in only

Table 5. Detection of KSHV/HHV8 in semen and prostate tissue

Reference (country or region)	KS type	Positive/ total	Control/other populations	Positive/ total	Method
Semen					
Ambroziak *et al.* (1995) (USA)	AIDS-KS	0/4	–	–	PCR hybridization detection of amplicons
Monini *et al.* (1996a) (Italy)	–	–	Semen donors	5/10[a] 30/33[b]	Nested PCR
Corbellino *et al.* (1996c) (Italy)	–	–	Semen donors	0/20	Nested PCR
Monini *et al.* (1996b) (Italy)	AIDS-KS	1/5	AIDS without KS Semen donors	0/10 3/13	Nested PCR PCR
Marchioli *et al.* (1996) (USA)	AIDS-KS Classic KS	4/31 0/2	–	–	PCR[a]
Gupta *et al.* (1996) (USA)	AIDS-KS	2/14	AIDS without KS	0/10	Nested PCR; sampling at two times
Howard *et al.* (1997) (United Kingdom)	AIDS-KS	3/15	AIDS without KS Semen donors	3/9 0/115	Nested PCR
Huang *et al.* (1997) (USA)	AIDS-KS Classic KS	3/12 0/2	AIDS without KS HIV-positive intravenous drug users HIV-negative	0/4 0/5 0/7	PCR hybridization and in-situ PCR detection of amplicons
Viviano *et al.* (1997) (Sicily)	AIDS-KS	1/1	AIDS without KS HIV-negative	1/10 6/45	Nested PCR

Table 5 (contd)

Reference (country or region)	KS type	Positive/ total	Control/other populations	Positive/ total	Method
Prostate tissue					
Monini *et al.* (1996a) (Italy)	–	–	Benign hyperplasia and carcinoma	7/16	Nested PCR
Corbellino *et al.* (1996c) (Italy)	AIDS-KS	5/5	–	–	
Corbellino *et al.* (1996c) (Italy)	–	–	AIDS without KS HIV-negative	0/20 0/8	Nested PCR
Tasaka *et al.* (1996, 1997) (Italy and USA)	–	–	Prostate biopsy	0/52	Nested PCR
Monini *et al.* (1996b) (Italy)	–	–	Hyperplastic prostate biopsy	2/7	Nested PCR
Lebbé *et al.* (1997b) (France)	–	–	Benign hyperplasia and carcinoma	0/19	Nested PCR
Staskus *et al.* (1997) (USA)	–	–	Prostate biopsy	12/16	In-situ hybridization
Rubin *et al.* (1997) (USA)	–	–	Prostate biopsy	0/45	PCR[a]

Modified from Blackbourn & Levy (1997)
[a]Blinded analysis
[b]Unblinded analysis

Table 6. Serological studies of KSHV/HHV8 infection

Reference	Assay	KS type	No. positive/ total	Control population	Antibodies to LANA (No. positive/total)	Antibodies to lytic antigen (No. positive/total)
Gao *et al.* (1996a,b)	BC-1 LANA Western blot	USA		AIDS without KS, US homosexual men	7/40	ND
		AIDS (homosexuals)	32/40	HIV-positive US haemophiliacs	0/20	ND
		Italy		Ugandan cancer patients		
		AIDS	11/14	HIV-positive	25/35	ND
		Classic	11/11	HIV-negative	29/47	ND
		Uganda		Blood donors		
		AIDS	16/18	USA	0/122	ND
		Endemic	1/1	Italy	4/107	ND
	BCP-1 LANA IFA	USA		US EBV-positive	0/69	ND
		AIDS	35/40	AIDS without KS, US homosexual men	12/40	ND
		Italy		HIV-positive US haemophiliacs	0/20	ND
		AIDS	10/14	Ugandan cancer patients		
		Classic	11/11	HIV-positive	18/35	ND
		Uganda		HIV-negative	24/47	ND
		AIDS	14/18	Blood donors		
		Endemic	1/1	USA	0/122	ND
				Italy	4/107	ND
				US EBV-positive	0/69	ND
Kedes *et al.* (1996)	BCBL-1 LANA IFA	AIDS	37/45	HIV-positive US haemophiliacs	9/300	ND
		Classic	1/1	HIV-positive US transfusion recipients	2/44	ND
				Sexually transmitted disease clinic attendees		
				HIV-positive (bi/homosexual)	13/37	ND
				HIV-positive (heterosexual)	0/9	ND
				HIV-negative	10/130	ND
				US blood donors		
				HIV-positive	41/138	ND
				HIV-negative	2/141	ND
Kedes *et al.* (1997)	BCBL-1 LANA IFA	AIDS (women)	2/2	HIV-positive women	12/302	ND
				HIV-negative women	1/84	ND

Table 6 (contd)

Reference	Assay	KS type	No. positive/ total	Control population	Antibodies to LANA (No. positive/total)	Antibodies to lytic antigen (No. positive/total)
Lennette *et al.* (1996)	BCBL-1 LANA IFA	US (AIDS and a few classic)	47/91	HIV-positive, US homosexual men	19/94	87/94
				HIV-positive male intravenous drug users	0/13	3/13
		African endemic	28/28	HIV-positive women	0/33	7/33
	BCBL-1 TPA induced lytic IFA	US (AIDS and a few classic)	87/91	Children < 16 years	0/263	10/263
				Adults > 16 years	0/174	33/174
		African endemic	28/28	US blood donors	0/44	9/44
				US women	0/54	15/54
				Haemophiliacs	0/83	10/83
				Various tumours (Dominican Republic, Sweden, Malaysia and Netherlands)	0/147	19/147
				EBV-positive patients	0/40	8/40
				Rheumatoid arthritis	0/20	5/20
				Zimbabwe	4/37	12/37
				Nigeria	3/52	29/52
				Zaire	4/16	13/16
				Uganda	9/82	63/82
				The Gambia	11/45	38/45
				Ivory Coast	4/7	7/7
				Haiti	0/52	15/52
				Dominican Republic	0/40	5/40
				Guatemala	0/20	2/20

Table 6 (contd)

Reference	Assay	KS type	No. positive/ total	Control population	Antibodies to LANA (No. positive/total)	Antibodies to lytic antigen (No. positive/total)
Simpson *et al.* (1996)	ORF 65 ELISA	AIDS (USA, UK)	46/57	HIV-positive homosexual men	10/33	
		AIDS (Uganda)	14/17	HIV-positive women with sexually transmitted diseases	3/15	
		Classic (Greece)	17/18	Haemophiliacs		
				HIV-positive	0/26	
				HIV-negative	ND	
	BCP-1 LANA IFA	AIDS (US, UK)	84/103	Intravenous drug users		
		Classic (Greece)	17/18	HIV-positive	0/38	2/38
				HIV-negative	0/25	0/25
				HIV-negative homosexual men with sexually transmitted diseases	8/65	ND
				Heterosexual men	4/75	ND
				Heterosexual women	2/26	ND
				Children with rash or fever	0/24	ND
				Blood donors		
				UK	4/150	3/174
				USA	0/117	6 /117
				Greek age-/sex-matched controls	3/26	3/26
				Ugandan controls		
				HIV-positive	18/34	16/34
				HIV-negative	9/17	7/54
Miller *et al.* (1996)	Sodium butyrate-induced BC-1 western blot	AIDS	32/48	HIV-positive US homosexual men	ND	7/54
	Sodium butyrate-induced BC-1 IFA	AIDS	31/48	HIV-positive US homosexual men	ND	7/54
Davis *et al.* (1997)	ORF 26 peptide ELISA	AIDS	21/35	HIV-negative blood donors	ND	6/30
				HIV-positive homosexual men	ND	2/8
				HIV-positive haemophiliacs	ND	6/24

Table 6 (contd)

Reference	Assay	KS type	No. positive/ total	Control population	Antibodies to LANA (No. positive/total)	Antibodies to lytic antigen (No. positive/total)
Lin *et al.* (1997)	Recombinant whole ORF 65 western blot	AIDS	42/47	HIV-positive US adults	ND	11/54
				HIV-positive children	ND	0/12
				HIV-negative children	ND	0/10
				Haemophiliacs	ND	0/25
				Autoimmune patients	ND	0/25
				Children with acute illness	ND	0/25
				Healthy adults	ND	3/28
				Nasopharyngeal cancer patients (China)	ND	0/25
Smith *et al.* (1997)	BCBL-1 TPA-induced lytic IFA with Evans blue counterstain	AIDS	7/7	HIV-positive	ND	6/18
				US blood donors	ND	0/52

Modified from Olsen & Moore (1997)
ND, not determined; LANA, latency-associated nuclear antigen; IFA, immunofluorescent assay; ELISA, enzyme-linked immunosorbent assay; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

30% of HIV-positive homosexual or bisexual men and less than 2% of HIV-positive patients with haemophilia or blood donors in the United Kingdom or the United States. Simpson *et al.* (1996) also found no LANA-reactive sera among 38 HIV-positive intravenous drug users. Gao *et al.* (1996a,b), in a nested case–control study within the cohort of a multicentre study on AIDS, compared 40 AIDS patients with Kaposi's sarcoma and 40 randomly selected AIDS patients without Kaposi's sarcoma, matched for $CD4^+$ count. The odds ratio for an association between LANA positivity and Kaposi's sarcoma was 16. A similar comparison of 45 AIDS patients with Kaposi's sarcoma and 37 HIV-positive homosexual and bisexual men showed an odds ratio for LANA positivity of 8.5 (Kedes *et al.*, 1996). Lennette *et al.* (1996), who found a lower LANA antibody detection rate among Kaposi's sarcoma patients (52%) than in other studies, obtained an odds ratio of 4.2 for a similar comparison.

In view of the rarity of Kaposi's sarcoma among HIV-infected haemophilia patients, the virtual absence of LANA antibodies in this group (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996; Simpson *et al.*, 1996) is an important finding. In these studies, a total of 346 HIV-positive persons with haemophilia from three defined cohorts were investigated.

The association between antibodies to KSHV/HHV8 structural proteins and Kaposi's sarcoma has also been examined. Miller *et al.* (1996) compared antibodies to a 40-kDa structural antigen by western blot in AIDS patients with Kaposi's sarcoma (67%) and in HIV-positive homosexual men (13%), giving an odds ratio of 13. For a recombinant capsid-related protein, vp19/ORF 65, Simpson *et al.* (1996) found 81% antibody reactivity among AIDS patients with Kaposi's sarcoma and 31% in HIV-positive homosexual men (odds ratio, 9.2). In contrast, Lennette *et al.* (1996), using an immunofluorescence assay for low titres of antibodies to undefined structural proteins, found positive results in 96% of AIDS patients with Kaposi's sarcoma and in 93% of HIV-positive homosexual men in the United States (odds ratio, 1.8). A comparison of antibody positivity for both LANA and structural antigens in cases of AIDS plus Kaposi's sarcoma and in blood donors and other groups at risk for HIV infection (haemophilia patients and intravenous drug users) yielded much higher odds ratios.

2.1.4 *Temporal associations*

The long-term consequences of infection with KSHV/HHV8 have been examined in a limited number of studies of few exposed individuals. These studies have addressed only the association between Kaposi's sarcoma and prior exposure to KSHV/HHV8, determined by either PCR or serology.

In a cohort study, Whitby *et al.* (1995) followed (for a median of 30 months) 143 HIV-positive patients who did not have Kaposi's sarcoma at the time their first or only blood sample was taken. Of the 11 men who initially had detectable KSHV/HHV8 in their PBMC by nested PCR, six (54%) developed Kaposi's sarcoma, whereas only 12 of 132 (9%) men who were KSHV/HHV8-negative developed the disease (odds ratio, 7.0; 95% CI, 2.8–13).

In a nested case–control study, Moore *et al.* (1996c) and Parry and Moore (1997) compared the detection of KSHV/HHV8 by PCR in paired samples of PBMC drawn

from 21 HIV-infected patients before and after a diagnosis of Kaposi's sarcoma with that in paired samples from 23 high-risk, HIV-infected homosexual men who later developed AIDS. Nine of the 21 Kaposi's sarcoma patients and one of the 23 homosexual controls were KSHV/HHV8-positive at the time the initial sample was taken (odds ratio, 17; 95% CI, 1.8–755). Overall, 11/21 patients and 3/23 controls (at any sample) had evidence of KSHV/HHV8 before the onset of Kaposi's sarcoma (odds ratio, 7.3; 95% CI, 1.4–48).

Lenette *et al.* (1996) analysed 13 pairs of sera collected before and after diagnosis of Kaposi's sarcoma. No clear association between seroconversion to KSHV/HHV8 (latent or lytic antibodies) and the development of Kaposi's sarcoma was found after a median interval of about 12 months.

In a longitudinal study of 40 patients who developed AIDS-associated Kaposi's sarcoma over a period of 13–103 months, 11 patients (28%) showed positive results at all visits, and 21 seroconverted to KSHV/HHV8 6–75 months before a diagnosis of Kaposi's sarcoma (Gao *et al.*, 1996b). The median duration of positivity for antibodies to LANA before the diagnosis was 33 months (Figure 5). The LANA antibody titres remained constant between seroconversion and Kaposi's sarcoma development, which the authors suggested was inconsistent with seroconversion to LANA reflecting reactivation of a pre-existing KSHV/HHV8 infection (Gao *et al.*, 1996a; Figure 6). In a subsequent study based on an indirect immunofluorescence assay for LANA on the EBV-negative KSHV/HHV8-infected cell line BCP-1, similar results were obtained (Gao *et al.*, 1996a).

2.2 Lymphoproliferative disorders

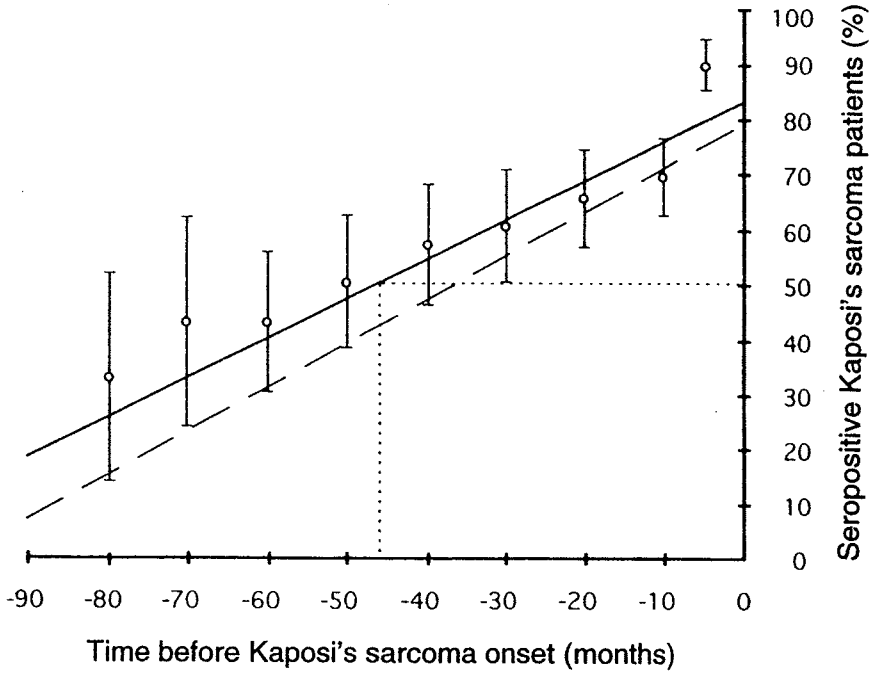
2.2.1 *Primary effusion lymphomas*

2.2.1.1 *Pathology and clinical presentation*

Another neoplastic condition associated with KSHV/HHV8 is primary effusion lymphoma. AIDS-related lymphomas presenting as primary malignant lymphomatous effusions in body cavities were first recognized in the late 1980s (Knowles *et al.*, 1989; Walts *et al.*, 1990; Karcher *et al.*, 1992). This lymphoma is a rare, distinct subtype of non-Hodgkin's lymphoma that has morphological features shared by large-cell immunoblastic lymphomas and anaplastic large-cell lymphoma (Ansari *et al.*, 1996; Carbone *et al.*, 1996a; Cesarman *et al.*, 1996b). Primary effusion lymphoma is defined by distinctive clinical, immunophenotypic and molecular genetic features (Cesarman *et al.*, 1995a). It presents predominantly as malignant effusions in the pleural, pericardial or peritoneal cavities, usually without significant tumour mass or lymphadenopathy; however, lymphomatous infiltration of serosal surfaces adjacent to the site of the primary malignant effusion is sometimes seen (Komanduri *et al.*, 1996).

Morphologically, the cells bridge the features of large-cell immunoblastic and anaplastic large-cell lymphomas. They are usually large and irregularly shaped, with abundant cytoplasm and variably chromatic and pleomorphic nuclei. One or more prominent nucleoli are usually present, and mitotic features are abundant (Ansari *et al.*, 1996).

Figure 5. Prevalence of seropositivity for BCP-1 immunofluorescence and for latent nuclear antigen in 39 homosexual AIDS patients before onset of Kaposi's sarcoma



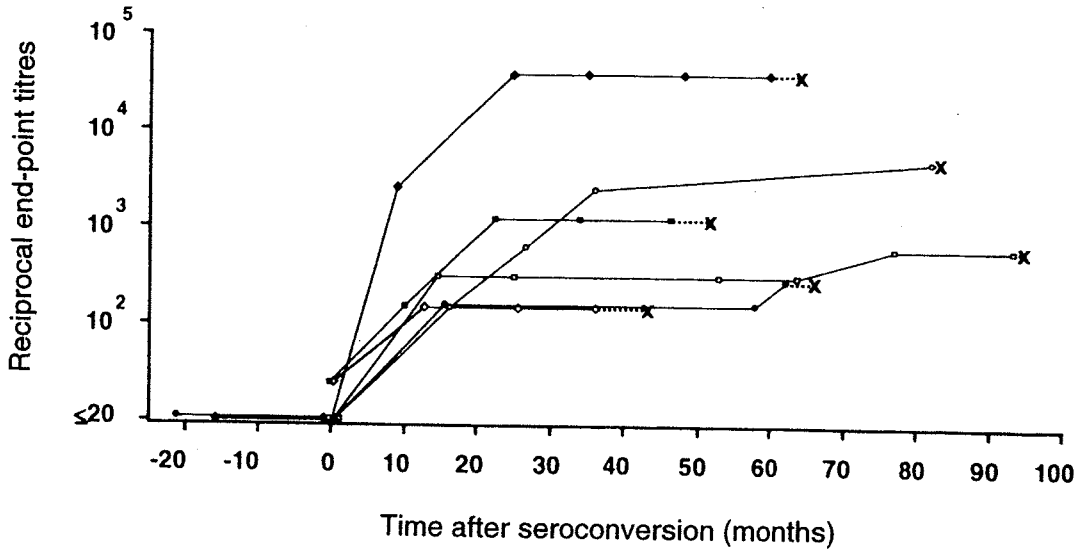
From Gao *et al.* (1996a,b)

Date of seroconversion was estimated to be the mid-point between last negative and first positive serological test. For comparison, seropositivity for KSHV/HHV8 by immunoassay for latent nuclear antigen (dashed line) is plotted against seropositivity by BCP-1 immunofluorescence (solid line). Fifty percent of the Kaposi's sarcoma patients were seropositive 46 months before onset of the disease by BCP-1 immunofluorescence assay. Error bars are standard errors of the mean calculated from a binomial distribution.

Under the electron microscope, the cells are large, with lobulated nuclei containing marginated heterochromatin and prominent rope-like nucleolonemas. The cytoplasm is moderate in amount and exhibits short, blunt, surface projections. KSHV/HHV8 particles are not identified in the cytoplasm, but nuclear particles measuring 110 nm have been observed (Renne *et al.*, 1996a; Said *et al.*, 1996a,b).

Primary effusion lymphoma cells have indeterminate (null) immunophenotypes, lacking expression of any lineage-associated B- or T-lymphocyte antigens (Table 7), but usually express the common leukocyte antigen CD45. A B-cell lineage is indicated by the presence of clonal immunoglobulin gene rearrangement (Knowles *et al.*, 1989; Cesarman *et al.*, 1995b; Komanduri *et al.*, 1996). The B-cell derivation is also supported by the monoclonal nature of primary effusion lymphoma, as demonstrated by a consistent rearrangement of the immunoglobulin genes and by expression of monotypic κ or λ mRNA in the cell cytoplasm (Nador *et al.*, 1996). Primary effusion lymphoma cells usually express activation markers such as CD30, CD38, CD71 and epithelial membrane antigen.

Figure 6. Immunoglobulin-γ end-point titres for six AIDS patients with Kaposi's sarcoma from whom three or more samples were drawn after seroconversion (immunofluorescence titre > 1:160 and a fourfold or greater rise in end-point titre)



From Gao *et al.* (1996a)

Titres remained elevated for 36–93 months after seroconversion, until onset of Kaposi's sarcoma (X), consistent with a prolonged antibody response after primary infection

These cells consistently lack the molecular defects commonly associated with neoplasia of mature B cells, including activation of the proto-oncogenes *c-myc*, *bcl-2*, *bcl-6*, *N-ras* and *K-ras* or mutations of *p53* (Cesarman *et al.*, 1995a; Carbone *et al.*, 1996a; Nador *et al.*, 1996). Cytogenetic studies have shown complex, hyperdiploid karyotypes. Alterations of the chromosomal region 1q21–q23 have been reported, which are also present in other EBV-positive AIDS-related lymphomas (Ansari *et al.*, 1996).

The levels of IL-6 and IL-10, which are involved in B-cell proliferation and differentiation, are both markedly elevated (340–16 000-fold higher than in normal human plasma) in primary effusion lymphoma. Expression of both IL-6 and IL-6 receptor transcripts in some cells suggests a paracrine mechanism for continued B-cell proliferation (Komanduri *et al.*, 1996).

In patients with AIDS, primary effusion lymphoma is a fulminant lymphoproliferation, and the median survival time is less than six months (Komanduri *et al.*, 1996; Nador *et al.*, 1996); however, a more indolent course has been documented in immunocompetent patients (Strauchen *et al.*, 1996).

Table 7. Immunophenotypic types of primary effusion lymphoma cells

CD45 (leukocyte common antigen)	+
TdT	–
Activation markers	
CD30	+
CD38	+
CD71	+
HLA-DR	+
Epithelial membrane antigen	+
T-Cell markers	
CD2	–
CD3	–
CD4	–
CD5	–
CD7	–
CD8	–
B-Cell markers	
CD19	–
CD20	–
CD22	–
CD23	–
Other markers	
CD10	–
CD14	–
CD15 (Reed-Sternberg antigen)	–

From Cesarman *et al.* (1995a,b), Ansari *et al.* (1996) and Nador *et al.* (1996)

2.2.1.2 *Descriptive epidemiology*

Very little is known about the distribution and epidemiological characteristics of primary effusion lymphoma. Because it is rare, its incidence remains to be established. Although primary effusion lymphoma was first described among AIDS patients (Knowles *et al.*, 1988), in whom it occurs mainly at an advanced stage of the disease (Komanduri *et al.*, 1996), it has also been reported in HIV-negative individuals (Nador *et al.*, 1995). In addition, like Kaposi's sarcoma, with which it is closely linked, it is seen primarily in homosexual men and seldom in other groups at risk for HIV infection (Jaffe, 1996; Nador *et al.*, 1996).

Primary effusion lymphoma is distinct from another body cavity-based lymphoma, pyothorax-related B-cell lymphoma. These large B-cell lymphomas occur in patients with long-standing pyothorax resulting from artificial pneumothorax for the treatment of pulmonary tuberculosis or tuberculous pleuritis (Iuchi *et al.*, 1987, 1989). This tumour has been identified most often in Japan, with more than 50 cases in the literature, in comparison with a single series of three cases reported from a western country, France

(Martin *et al.*, 1994). The geographical distribution of pyothorax-related B-cell lymphoma may be due to the fact that artificial pneumothorax is used more frequently as a treatment modality in Japan. In common with primary effusion lymphoma, the tumour cells in pyothorax-related B-cell lymphoma nearly always contain EBV (14 of 14 in a study by Cesarman *et al.*, 1996b); however, in pyothorax-related B-cell lymphoma, pleural mass lesions are seen, *c-myc* rearrangements are present and KSHV/HHV8 is absent (Cesarman *et al.*, 1996b).

2.2.1.3 *Case reports and case series*

Table 8 summarizes 30 case reports of primary effusion lymphoma reported in the literature. Four other cases were associated with *c-myc* gene rearrangements and thus molecularly resembled Burkitt-type lymphomas (Nador *et al.*, 1996). Since these cases also had cytomorphological features similar to those of Burkitt's or Burkitt-like lymphomas, and two of the four also involved systemic lymphoma, Nador *et al.* (1996), who originally reported these cases as primary effusion lymphomas, argued that they should be classified as Burkitt-type lymphomas, despite their body cavity involvement. [The Working Group concluded that these cases could not be considered primary effusion lymphomas.] Effusions from the 30 patients all contained KSHV/HHV8 and in 26 of these the PCR product was confirmed by Southern blot hybridization. Twenty-five of the described cases occurred in HIV-infected homosexual men and three in uninfected elderly men who did not belong to any established HIV risk group. Two cases of primary effusion lymphomas have been described in HIV-negative women (Said *et al.*, 1996b).

The median $CD4^+$ count in the HIV-infected persons was 65, indicating that they were severely immunosuppressed at the time of diagnosis of primary effusion lymphoma (Table 8). Of the patients reported to be infected with HIV, 10/25 had previously or at the same time received a diagnosis of Kaposi's sarcoma. Similarly, two of five uninfected primary effusion lymphoma patients had Kaposi's sarcoma (Nador *et al.*, 1995, 1996; Said *et al.*, 1996b; Strauchen *et al.*, 1996).

Co-infection with EBV is common in primary effusion lymphomas; EBV monoclonality has been established in most cases (Komanduri *et al.*, 1996; Cesarman *et al.*, 1995a; Nador *et al.*, 1996). It is therefore of interest that several cell lines derived from these lymphomas, with genetic and immunological markers similar to those of the original lymphomas, were latently infected with KSHV/HHV8 but not EBV (Arvanitakis *et al.*, 1996; Renne *et al.*, 1996a; Said *et al.*, 1996a; Gao *et al.*, 1996b). KSHV/HHV8 has not been consistently detected in other lymphomas (see Table 9).

2.2.2 *Castleman's disease*

2.2.2.1 *Pathology and clinical presentation*

Castleman's disease, also referred to as angiofollicular or giant lymph node hyperplasia, is a rare, usually polyclonal, non-neoplastic disorder of unknown etiology (Castleman *et al.*, 1956). Two distinct histopathological variants with different clinical characteristics have been described: the hyaline vascular type and the plasma-cell type. The more common hyaline form presents primarily as a solitary mass, most frequently in

Table 8. Demographic, clinical and virological characteristics of patients with primary effusion lymphoma

Age (years)/ sex	HIV status	HIV risk factor	KS	$CD4^+$ count (cells/μl)	Location of effusion	Other sites of disease	KSHV/ HHV8	EBV		Reference
								Type	Clonality	
46/M	+	HS	–	561	Abdominal	None	+	2	Clonal	Chadburn *et al.* (1993); Cesarman *et al.* (1995a)
31/M	+	HS	–	NR	Pleural	None	+	1	Clonal	Knowles *et al.* (1989); Cesarman *et al.* (1995a)
40/M	+	HS	+	NR	Pleural	Submandibular gland, lymph nodes	+	1	Clonal	Knowles *et al.* (1989); Cesarman *et al.* (1995a)
35/M	+	HS	–	NR	Abdominal	None	+	1	Clonal	Walts *et al.* (1990); Cesarman *et al.* (1995a)
38/M	+	HS	–	NR	Pericardial	None	+	2	Clonal	Cesarman *et al.* (1995a)
58/M	+	HS	–	NR	Pleural	None	+	1	Clonal	Cesarman *et al.* (1995a)
37/M	+	HS	+	109	Pericardial	None	+	1	ND	Nador *et al.* (1996)
42/M	+	HS	–	NR	Pleural	Oesophageal lymph node, lung	+	2	ND	Nador *et al.* (1996)
53/M	+	HS	–	84	Abdominal	None	+	2	Clonal	Ansari *et al.* (1996)
43/M	+	HS	–	34	Pleural	None	+	2	Clonal	Ansari *et al.* (1996)
44/M	+	HS	+	25	Pleural	None	+	2	Clonal	Ansari *et al.* (1996)
44/M	+	HS	+	33	Pleural	None	+	1	Clonal	Ansari *et al.* (1996)

Table 8 (contd)

Age (years)/ sex	HIV status	HIV risk factor	KS	$CD4^+$ count (cells/μl)	Location of effusion	Other sites of disease	KSHV/ HHV8	EBV		Reference
								Type	Clonality	
54/M	+	HS	+	130	Pleural	None	+	1	Clonal	Ansari *et al.* (1996)
42/M	+	HS	+	NR	Pleural	None	+	1	Clonal	Gessain *et al.* (1997)
31/M	+	HS	–	NR	Peritoneal, pleural	Small intestine	+	EBER +	ND	Gessain *et al.* (1997)
35/M	+	HS, IVDU	–	58	Abdominal, pleural	None	+[a]	–		Komanduri *et al.* (1996)
40/M	+	HS	–	65	Abdominal	None	+	1	Polyclonal	Komanduri *et al.* (1996)
32/M	+	HS	–	91	Pleural	None	+	2	Clonal	Komanduri *et al.* (1996)
42/M	+	HS	–	181	Abdominal	Left atrial mass	+	1	Polyclonal	Komanduri *et al.* (1996)
31/M	+	HS	+	34	Pericardial	None	+	–		Komanduri *et al.* (1996)
32/M	+	HS	+	65	Pleural	None	+	–		Komanduri *et al.* (1996)
47/M	+	HS, IVDU	–	20	Abdominal, pleural	None	+[a]	1	Clonal	Komanduri *et al.* (1996)
40/M	+	HS	+	190	Abdominal	ND	+	–		Komanduri *et al.* (1996)
30/M	+	HS	–	NR	Pericardial	None	+[a]	1	ND	Walts *et al.* (1990); Cesarman *et al.* (1995a)
32/M	+	HS	+	NR	Pleural	None	+[a]	1	ND	Walts *et al.* (1990); Cesarman *et al.* (1995a)

Table 8 (contd)

Age (years)/ sex	HIV status	HIV risk factor	KS	$CD4^+$ count (cells/μl)	Location of effusion	Other sites of disease	KSHV/ HHV8	EBV		Reference
								Type	Clonality	
85/M	–	–	–	288	Pleural	None	+	–		Nador *et al.* (1995, 1996)
78/M	–	–	–	NR	Abdominal	None	+	1	Clonal	Nador *et al.* (1996)
94/M	–	–	+	60	Pleural	Peritoneaum Pericardium	+	–		Strauchen *et al.* (1996)
85/F	–	–	+	NR	Pleural	None	+	–		Said *et al.* (1996b)
46/F	–	–	–	NR	Silicone breast implant	None	+	–		Said *et al.* (1996b)

M, male; F, female; NR, not reported; ND, not determined; HS, homosexual man; IVDU, intravenous drug user; EBER, Epstein-Barr virus-encoded RNA

[a] Only analysis by polymerase chain reaction

Table 9. Presence of HHV8 in lymphoid neoplasias

Histology	Positive/tested
Cases unrelated to AIDS	
Acute lymphoblastic leukaemia	0/44
Chronic lymphocytic leukaemia	0/61
Prolymphocytic leukaemia	0/10
Lymphoplasmacytoid lymphoma	0/3
Mantle-cell lymphoma	0/14
Follicular lymphoma	0/60
Monocytoid lymphoma	0/3
MALT lymphoma	0/16
Hairy cell leukaemia	0/18
Multiple myeloma and plasmacytoma[a]	0/28
Diffuse large-cell lymphoma	0/65
Small non-cleaved (including Burkitt's) lymphoma	0/57
Cutaneous T-cell lymphoma	0/9
Peripheral T-cell lymphoma	0/20
Anaplastic large-cell lymphoma	0/17
Lymphoblastic lymphoma	0/4
Adult T-cell leukaemia/lymphoma	0/13
Post-transplant lymphoproliferation	0/23
Hodgkin's disease	0/49
Primary effusion lymphoma	8/8
AIDS-related lymphomas	
Small non-cleaved lymphoma	0/42
Diffuse large-cell lymphoma	0/39
Anaplastic large-cell lymphoma	0/5
Peripheral T-cell lymphoma	0/1
Hodgkin's disease	0/14
Primary effusion lymphoma	34/35

From Chang *et al.* (1994), Pastore *et al.* (1995), Cesarman *et al.* (1995a), Karcher & Alkan (1995), Nador *et al.* (1995), Ansari *et al.* (1996), Arvanitakis *et al.* (1996), Carbone *et al.* (1996a,b), Gaidano *et al.* (1996b, 1997), Luppi *et al.* (1996b), Nador *et al.* (1996), Otsuki *et al.* (1996), Said *et al.* (1996a), Strauchen *et al.* (1996), Gessain *et al.* (1997)

MALT, mucosa-associated lymphoid tissue

[a] See also section 2.2.3 (multiple myeloma)

the mediastinum or retroperitoneum, is asymptomatic and is usually curable surgically. The rare plasma-cell type is typically characterized by generalized lymphadenopathy, immunological abnormalities and type B symptoms.

The systemic variety, also designated multicentric Castleman's disease, is primarily of the plasma-cell type, but the hyaline type has occasionally been reported in a multicentric clinical appearance (Herrada & Cabanillas, 1995; Shahidi *et al.*, 1995). Associated clinical findings are necessary to make the diagnosis of multicentric Castleman's disease, since the pathological features in lymph nodes can be nonspecific (Peterson & Frizzera, 1993; Shahidi *et al.*, 1995). EBV was reported to be present in 9 of 16 cases of localized and multicentric Castleman's disease (Barozzi *et al.*, 1996). Multicentric Castleman's disease has an aggressive clinical course with a poor prognosis, and such patients are at increased risk for Kaposi's sarcoma and lymphomas (Peterson & Frizzera, 1993). It has been suggested that some of the immunological changes observed in HIV-negative patients with multicentric Castleman's disease are similar to those in HIV-infected individuals (Lane *et al.*, 1985; Vuillier *et al.*, 1988; Birx *et al.*, 1990; Boyd & James, 1992; Ishiyama *et al.*, 1996).

2.2.2.2 *Descriptive epidemiology*

About 70% of patients with all forms of Castleman's disease are under 30 years of age, and men are affected more often than women. Patients with multicentric Castleman's disease often tend to be in their fifties or sixties and to have increased risks for non-Hodgkin's lymphoma and Kaposi's sarcoma (Peterson & Frizzera, 1993).

2.2.2.3 *Case reports and case series*

Few reports have addressed the presence of KSHV/HHV8 in Castleman's disease (Table 10). Soulier *et al.* (1995) detected KSHV/HHV8 in all of 14 HIV-positive lesions from French patients with multicentric Castleman's disease, comprising six plasma-cell type, seven mixed and one hyaline vascular type. Seven of the patients also had Kaposi's sarcoma in the same tissue sample, and an additional two at another site; 64% had Kaposi's sarcoma both in the same tissue and elsewhere. Of 17 HIV-negative multicentric Castleman's disease lesions, seven (three plasma cell, two mixed, two hyaline type) contained KSHV/HHV8. Kaposi's sarcoma was diagnosed in one of these subjects. Whereas the vast majority of cases among HIV-positive patients were found to contain the virus by Southern blot, only two of the seven cases in HIV-negative patients found to be positive by PCR were positive by Southern blot. To evaluate the significance of the positivity rate in the HIV-negative patients, reactive lymph nodes from 34 HIV-seronegative control patients were analysed; only one KSHV/HHV8-positive case was found.

Dupin *et al.* (1995b) reported the finding of KSHV/HHV8 in PMBC from two HIV-infected men with Castleman's disease, one of whom had Kaposi's sarcoma. [The authors did not specify whether these cases were multicentric.] Tirelli *et al.* (1996) found KSHV/HHV8 in lesions from an HIV-positive woman with multicentric Castleman's disease whose husband was diagnosed with Kaposi's sarcoma.

Gessain *et al.* (1996) detected KSHV/HHV8 by PCR in cryopreserved lymph node biopsy samples from one of three HIV-negative patients with multicentric Castleman's disease but in none of three with localized disease. Of the HIV-positive subjects, three of four homosexual men with multicentric Castleman's disease had KSHV/HHV8 in their

Table 10. Detection of KSHV/HHV8 in patients with Castleman's disease

Reference (country)	HIV-negative (KSHV/ HHV8 positive/total)			HIV-positive (KSHV/ HHV8 positive/total)			Method	Comments
	HV	PL	Mixed	HV	PL	Mixed		
Soulier *et al.* (1995) (France)	2/3	3/9	2/5	1/1	6/6	7/7	PCR, Southern blot	All MCD HIV-positive: 9/14 with KS HIV-negative: 1/17 with KS
Corbellino *et al.* (1996d) (Italy)	0/2	4/4					PCR, Southern blot	None with KS or lymphoma
Barozzi *et al.* (1996) (Italy)	1/11		0/5				PCR	Mixed: all MCD HV: all localized Paraffin-embedded biopsies
Gessain *et al.* (1996) (France)	0/1	1/5			3/4		PCR	HIV-positive: 3 HHV8-positive had KS in other organs HIV-negative: none had KS

HV, hyaline vascular type; PL, plasma-cell type; mixed, both HV and PL; MCD, multicentric Castleman's disease

lymph nodes. Kaposi's sarcoma was diagnosed in three HIV-positive and KSHV/HHV8-positive men but not in the KSHV/HHV8-positive but HIV-negative woman. Semi-quantitative PCR showed a high KSHV/HHV8 viral load in the lesions of patients with and without HIV infection.

In a group of Italian HIV-negative patients, Corbellino *et al.* (1996d) found high levels of KSHV/HHV8 by PCR and Southern blot hybridization in biopsy samples from all of four cases of plasma-cell type Castleman's disease but in none of two cases of the hyaline vascular type. [The authors did not specify whether these cases were multicentric.] Neither Kaposi's sarcoma nor lymphoma was diagnosed in any of the patients. None of 20 lymph node biopsy samples from 15 HIV-infected drug abusers with persistent lymphadenopathy or from five HIV-negative patients with reactive lymphadenitis contained KSHV/HHV8. Two of the four cases of plasma-cell Castleman's disease were EBV-positive by PCR.

In archival formalin-fixed, paraffin wax-embedded biopsy material from HIV-negative Italian patients with Castleman's disease, Barozzi *et al.* (1996) found KSHV/HHV8 in one of 11 patients with the localized hyaline vascular type and in none of five patients with multicentric disease. PBMC and saliva were positive for KSHV/HHV8-specific sequences in the KSHV/HHV8-positive patients, whereas serum, faeces and urine were negative.

2.2.3 *Multiple myeloma*

Rettig *et al.* (1997) demonstrated the presence of KSHV/HHV8 DNA by PCR and in-situ hybridization in the cultured bone-marrow dendritic cells of 15 patients with multiple myeloma but not in the myeloma cells (plasma cells). The authors also demonstrated by RT-PCR the expression of v-*IL-6* in three of three cultured myeloma bone-marrow dendritic cells, suggesting that KSHV/HHV8–v-IL-6 contributes to the mechanism whereby bone-marrow dendritic cells infected with KSHV/HHV8 promote myeloma growth. KSHV/HHV8 was not detected by PCR in 28 DNA samples from myeloma specimens (Cesarman *et al.*, 1995a; Pastore *et al.*, 1995; Gessain *et al.*, 1997) or bone-marrow samples (Rettig *et al.*, 1997) in previous studies; this was attributed by the authors to dilution of the sample with uninfected cells.

2.2.4 *Other lymphoproliferative disorders*

With the exception of primary effusion lymphoma, most large series of lymphoid malignancies, including a variety of immunophenotypic categories of B- and T-cell tumours, have not been shown to contain KSHV/HHV8 (Chang *et al.*, 1994; Cesarman *et al.*, 1995a; Pastore *et al.*, 1995; Luppi *et al.*, 1996b; Gessain *et al.*, 1997; see Table 9). Bigoni *et al.* (1996), however, found that with nested and semiquantitative PCR 7–9% of PBMC from non-Hodgkin's lymphoma patients and patients with Hodgkin's disease contained KSHV/HHV8.

2.3 Other tumours

In studies of angiosarcoma in HIV-negative individuals, Gyulai *et al.* (1996a,b) reported one KSHV/HHV8-positive case. McDonagh *et al.* (1996) found KSHV/HHV8 by PCR in seven cases in the United States; one case was also tested by Southern blotting. These findings were not confirmed by other investigators (Chang *et al.*, 1994; Boshoff *et al.*, 1995a,b; Dictor *et al.*, 1996; Jin *et al.*, 1996a,b).

Rady *et al.* (1995) reported the widespread presence of KSHV/HHV8 by PCR in various skin tumours from four immunosuppressed patients, but in studies in Austria, Germany, Sweden and the United Kingdom this association could not be confirmed (Adams *et al.*, 1995; Boshoff *et al.*, 1996; Dictor *et al.*, 1996; Uthman *et al.*, 1996).

3. Studies of Cancer in Animal Models

No animal model for KSHV/HHV8 has so far been reported; however, several closely related rhadinoviruses, e.g. HVS, *Herpesvirus ateles* (HVA) and murid herpesvirus 4 (MHV68), cause lymphoid malignancies or polyclonal proliferation. In terms of its tropism for B-cells, MHV68 is the most closely similar to KSHV/HHV8; however, angiogenic proliferation is not seen with these animal viruses. Whether any of them will provide in-vivo models for KSHV/HHV8 is unknown. Bovine herpesvirus 4 (BHV-4), also a gamma-2 herpesvirus, has not been associated with specific lymphoid disease but is included in this monograph.

Small DNA fragments of two new primate rhadinoviruses, retroperitoneal fibromatosis herpesvirus of *Macaca nemestrina* (RFHVMn) and retroperitoneal fibromatosis herpesvirus of *Macaca mulatta* (RFHVMm), have recently been obtained from two macaque species in one colony (Rose *et al.*, 1997) and these may be closely related to KSHV/HHV8. The animals are also infected with D-type simian retrovirus type 2 and develop retroperitoneal and subcutaneous fibrosis with progressive fibrovascular proliferation, reminiscent of Kaposi's sarcoma lesions (Stromberg *et al.*, 1984; Bryant *et al.*, 1986).

3.1 *Herpesvirus saimiri* (saimiriine herpesvirus 2)

3.1.1 *Description*

HVS is a non-human primate rhadinovirus; it was first isolated in 1968 from the kidneys of squirrel monkeys (*Saimiri sciureus*), New World primates common to South America (Meléndez *et al.*, 1968). The virus is indigenous to squirrel monkeys and is transmitted horizontally from mother to infant in saliva. There is no evidence that HVS can induce tumours in squirrel monkeys (Jung & Desrosiers, 1994); however, when newborn squirrel monkeys were removed from their mothers immediately after birth and bred in captivity, some also being immunosuppressed with ciclosporin, later inoculation of HVS provoked viraemia and the development of antibody but no illness (Fleckenstein

& Desrosiers, 1982). There are no published reports of the development of tumours in squirrel monkeys in the wild after exposure to HVS.

Squirrel monkeys originating from discrete geographical zones were all found to be infected with HVS. The virus can be recovered routinely from PBMC and other tissues and organs of squirrel monkeys either naturally or experimentally infected with HVS. There is no evidence of seasonal variation in HVS transmission; however, under crowded conditions more virus is shed from the throat (Deinhardt *et al.*, 1974; Fleckenstein & Desrosiers, 1982; Jung & Desrosiers, 1994). Horizontal transmission of HVS by oropharyngeal secretions from a squirrel monkey to an owl monkey housed in the same isolation unit occurred experimentally (Barahona *et al.*, 1975).

HVS, like other gammaherpesviruses, remains latent in lymphocytes and can cause lymphomas, leukaemias and lymphoproliferative disorders in other species of New World primates — tamarins and marmosets (*Saguinus* and *Callithrix* species), owl monkeys (*Aotus trivirgatus*) and spider monkeys (*Ateles* species) — and in rabbits (*Oryctolagus cuniculus*) (Deinhardt *et al.*, 1974; Fleckenstein & Desrosiers, 1982; Jung & Desrosiers, 1994).

3.1.2 *Host range, virus isolation and virus multiplication*

HVS was first isolated by chance when it was observed that cultured kidney cells derived from a healthy squirrel monkey degenerated spontaneously, with cytopathic effects reminiscent of herpesvirus-infected cultures (Meléndez *et al.*, 1968). Specifically, hexagonal enveloped virions were found of approximately 140 nm, with capsids of 101–108 nm and cores of 50–60 nm (Deinhardt *et al.*, 1974). HVS can replicate in various monkey and human cell cultures. Higher infectivity titres were obtained in kidney cells from owl monkeys ($\geq 10^{6.2}$/mL) than in those from African green or rhesus monkeys. This experiment also showed that the infection with HVS is highly productive, contrary to most other gammaherpesviral infections *in vitro* (Ablashi *et al.*, 1972).

The first evidence that HVS could infect human fibroblast cells was provided by Ablashi *et al.* (1971a), who showed that infected cells produce infectious virus; however, the infectivity titres obtained were lower than those in owl monkey kidney cells. Simmer *et al.* (1991) showed that HVS persists in T-lymphoblastoid cells of various primate species, including human T cells. The human B-cell line (Raji) containing episomal EBV DNA could be persistently infected with HVS. A human pancreatic carcinoma cell line of epithelial origin could also be infected with HVS, and the cells contained both episomal and linear HVS DNA and produced HVS. Oie *et al.* (1973) reported that the MEST cell line, which is a spontaneously transformed cell line of rhesus monkey embryo origin, showed cytopathic effects after infection with HVS and, after two subpassages, provided a continuous source of HVS. HVS has tropism for T lymphocytes and can immortalize $CD8^+$ and $CD4^+$ cells (Biesinger *et al.*, 1992).

Dahlberg *et al.* (1988) showed that replication of HVS in human cells is semipermissive because of a block in the synthesis of certain late proteins. The studies were conducted with human fibroblasts (HEp-2), other epithelial cells (KHOS) and human T cells and the use of polyclonal and monoclonal antibodies to HVS (Dahlberg *et al.*, 1985).

3.1.3 *Host response: antibody detection*

Klein *et al.* (1973) identified the early and late antigens in HVS-infected Vero cells (an African green monkey continuous cell line) using sera obtained from squirrel monkeys (*Saimiri sciureus*) and HVS-infected white-lipped tamarins (*Saguinus fuscicollis*) and owl monkeys (*Aotus trivirgatus*). Two strikingly different patterns of staining of early antigen were observed, both limited to the nucleus (one trabecular and the other punctate), very similar to EBV EA(R) and EA(D). HVS-infected owl monkeys and white-lipped tamarins behaved quite differently from squirrel monkeys in their early antigen responses, and took two to three times longer to develop antibodies. The early antigen titres lagged behind those of late antigen. Antibodies appeared later in owl monkeys than in white-lipped tamarins; in squirrel monkeys, the virus could be isolated at the same time or somewhat later than the appearance of antibody. In tamarins, HVS was isolated before or at the time of appearance of antibody, whereas in owl monkeys, the virus was usually isolated one to two weeks before antibody was detected.

3.1.4 *Human exposure*

Of 150 animal caretakers who were bitten several times while handling squirrel monkeys or HVS-infected owl monkeys or marmosets, 11 (7.3%) were seropositive (Ablashi *et al.*, 1988). Four of 100 control adult sera (4%) also contained antibody to HVS as tested by immunofluorescence assay. The antibody titre to late antigen in these sera was between 1:10 and 1:80, and intense nuclear fluorescence reactivity was observed in cells in most of the sera. When these sera were analysed by radioimmunoprecipitation, two from the exposed persons were exceptionally reactive and the others weakly reactive. The sera precipitated mainly HVS major capsid protein (160 kDa) but also recognized several other proteins. Unexpectedly, follow-up sera from the caretakers several months later showed no antibodies. Whether these results were due to antibody cross-reactivity with KSHV/HHV8 or another primate rhadinovirus to which these individuals might have been exposed is unclear. Several attempts to isolate HVS from PBMC of the caretakers were unsuccessful.

3.1.5 *Molecular aspects*

HVS has a linear double-stranded DNA genome of about 155 kb with a central unique light region of 112 kb (36% G + C) flanked by variable numbers of 1.4-kb tandem repeats of heavy DNA (72% G + C) (Bankier *et al.*, 1985; Cameron *et al.*, 1989; Albrecht *et al.*, 1992; Jung & Desrosiers, 1994). Earlier studies showed that DNA sequences at the left terminus of light DNA are required for in-vitro immortalization and for the oncogenic phenotypes (Desrosiers *et al.*, 1986). Most HVS genes have ORF sequences that are similar to those of the KSHV/HHV8 and EBV genomes, and most of these genes are arranged in collinear order. Nevertheless, HVS and EBV differ with respect to transformation and latent stage replication (Kung & Medveczky, 1996).

HVS strains have been classified into subgroups A, B and C on the basis of molecular analysis, biological properties and oncogenic and transforming potential. Mutational analysis demonstrates that most of the left ORF of strain 11 (subgroup A) is required to

immortalize common marmoset T lymphocytes, but not for replication of the virus. An ORF designated *STP-C488* (subgroup C) was more potent in transforming Rat-1 cells (Jung *et al.*, 1991). DNA sequencing of the left end of the light strand revealed that HVS contains a gene coding for dihydrofolate reductase (DHFR) with high sequence homology to human *DHFR*. The HVS *DHFR* gene differs from mammalian and avian genes in that it lacks introns, suggesting that it may have been acquired through a process involving reverse transcriptase. The *DHFR* gene is also not required for viral multiplication or for in-vitro transformation (Fleckenstein & Desrosiers, 1982).

Murthy *et al.* (1986) studied RNA derived from 0.0–6.7 map units (7.4 kb) of HVS light DNA by northern blot hybridization and nuclease protection analysis. Although several poly(A)-containing RNAs were found in this region in permissively infected monolayer cells *in vitro*, these RNAs could not be detected in lymphoblastoid tumour cell lines. Instead, transformed T cells expressed four small RNAs of approximately 73, 105, 110 and 135 nucleotides derived from this region; these RNAs were not detected during the course of lytic infection of monolayer cells. HVS and EBV also differ with regard to other genes relevant to transformation and latency. In HVS, there are no *EBNA-1* or *oriP*-like sequences in the region of the genome that corresponds to these sites in EBV (see monograph on Epstein-Barr virus; Fleckenstein & Desrosiers, 1982; Jung & Desrosiers, 1994; Kung & Medveczky, 1996). Instead, HVS and other rhadinoviruses appear to have acquired from the host cells a block of genes at the left side of their genomes that is not found in lymphocryptoviruses. Many other viral genes of HVS have been identified which code for cytokines (Yao *et al.*, 1995), as has also been observed in KSHV/HHV8.

3.1.6 *Oncogenicity in non-human primates, rabbits and transgenic mice*

As indicated previously, HVS strains can be divided into A, B and C subgroups. HVS of subgroups A and C are highly oncogenic for a wide spectrum of New World primates, while HVS strains of subgroup B have limited tumour-inducing capacity. In rabbits, only subgroup C has oncogenic potential (Medveczky *et al.*, 1989).

HVS induces malignant lymphoma of the reticulum-cell type in cotton-topped tamarins (*Saguinus oedipus*) and white-lipped tamarins (*Saguinus fuscicollis*) (Meléndez *et al.*, 1969; Deinhardt *et al.*, 1974). Lymphocytic leukaemia was induced in owl monkeys (*Aotus trivirgatus*) inoculated with prototype strain of HVS (295C; Meléndez *et al.*, 1971). Lymphoproliferative disease was induced in cinnamon ringtail monkeys (*Cebus albifrons*) and African green monkeys (*Cercopithecus aethiops*; Meléndez *et al.*, 1972a).

Fleckenstein *et al.* (1978a) induced malignant lymphomas in 3/14 cotton-topped tamarins inoculated with isolated HVS DNA.

Five owl monkeys experimentally inoculated with HVS developed moderately well-differentiated malignant lymphomas with lymphogenous leukaemia within 57–178 days. HVS could be isolated from cells of the spleen, kidney, lung, tumour mass (lymph node), thymus and liver cultured from these organs. HVS could also be isolated from PBMC. In general, the leukocyte counts rose significantly above the baseline levels. Bone-marrow

samples showed eosinophilic hyperplasia and progressive infiltration by lymphocytes. Terminally, the bone marrow was intensely hypercellular, with 70% cells of the lymphogenous series (Ablashi *et al.*, 1971b). The tumour-bearing owl monkeys developed antibodies to HVS early and late antigens (Klein *et al.*, 1973) and membrane antigens (Prevost *et al.*, 1976).

Heat-inactivated HVS strain 295C induced malignant lymphoma in two owl monkeys, suggesting that HVS DNA, even though it may have been fragmented, can induce tumours (Ablashi *et al.*, 1973).

The common marmoset (*Callithrix jacchus*) did not develop tumours after inoculation with HVS, even though the virus was consistently present in the PBMC.

Daniel *et al.* (1974) induced lymphoid tumours in New Zealand white rabbits (*Oryctolagus cuniculus*) by intravenous inoculation of HVS, although not all of the inoculated animals developed tumours. Similar results were reported in an inbred strain III/J (Ablashi *et al.*, 1980). The clinical and pathological findings in these animals were nasal discharge, respiratory disorders leading to death, lymphocyte infiltrates in the nares, enlarged lymph nodes and generalized peripheral lymphoadenopathy. The animals developed antibody to HVS, and the virus could be isolated from lymph nodes, spleen and PBMC. All infected animals showed depressed cell-mediated immune response.

Kretschmer *et al.* (1996) showed that a previously identified ORF (*StpA*) which is necessary for oncogenicity in monkeys induces pleomorphic T-cell lymphomas in transgenic mice expressing StpA in a variety of organs.

3.1.7 *Transformation of mammalian cells* in vitro

Subgroup A and C strains of HVS efficiently transform marmoset PBMC to permanent autonomous growth *in vitro*, whereas cells transformed by subgroup B viruses are IL-2-dependent (Desrosiers *et al.*, 1986; Szomolanyi *et al.*, 1987).

Ablashi *et al.* (1985) established a T-cell line from the spleen of an HVS-infected New Zealand male rabbit which developed a well-differentiated lymphoma. This cell line was IL-2-dependent and was highly oncogenic in rabbits, since it produced well-differentiated lymphomas, and the animals died of lung complications shortly afterwards. The cells (7710 cell line) contained multiple copies of non-integrated circular HVS genome. As in other HVS-transformed non-producer cell lines, a large segment of light DNA was missing from the persistent circular viral DNA present in the 7710 cells.

Medveczky *et al.* (1993) showed that a collagen-like ORF-1 protein is expressed in permanent tumour-derived rabbit T cells and in transformed primate T cells infected *in vitro* with HVS group C strain 484-77. Antibody to ORF-1 protein was also found in rabbits bearing tumours, suggesting that this protein is expressed *in vivo*.

Pacheco-Castro *et al.* (1996) showed that HVS immortalized $\alpha\beta$ and $\gamma\delta$ human T-lineage cells derived from $CD34^+$ intrathymic precursors *in vitro*, and the $\gamma\delta$ lineage was IL-2-dependent.

3.2 *Herpesvirus ateles* (ateline herpesvirus 2)

3.2.1 *Description*

HVA is also a non-human primate rhadinovirus. Strain 810 was first isolated from a degenerating kidney-cell culture from a spider monkey (*Ateles geoffroyii*; Meléndez *et al.*, 1972b). Later, strain 73 of HVA was isolated from circulating lymphocytes of a spider monkey (*A. paniscus*) by co-cultivation with owl monkey kidney cells (Falk *et al.*, 1974). Immunological, biological and molecular analyses indicate that HVA is closely related to HVS but with homology to EBV. HVA is common in species of spider monkeys native to South America and does not cause disease in the host. HVA does not cross-react with other primate herpesviruses. The virus is latent in spider monkeys and can be isolated early from tissue and PBMC (Deinhardt *et al.*, 1974; Fleckenstein & Desrosiers, 1982; Jung & Desrosiers, 1994).

3.2.2 *Host range, cytopathogenicity and viral multiplication*

Although HVA is a T-lymphotropic herpesvirus, it replicates efficiently in monolayer cell cultures of fibroblasts and epithelial cells of animal origin (Deinhardt *et al.*, 1974; Jung & Desrosiers, 1994). HVA in monolayer cultures induces cytopathic effects, with discrete, enlarged, round multinucleated giant cells. Owl monkey kidney cells are an excellent source of high-titre infectious virus. The host range of cells that can be infected with HVA is more distinct than that of HVS: HVA infects primary cultures of African green monkey, owl monkey, marmoset and squirrel monkey kidney cells. The cytopathic effects are slower (more than five days), and HVA titres are 2–3 logs lower than those of HVS. Rabbit kidney cells could not be infected by strain 73 but were infected by strain 810, which also infected cells of hamster heart origin. Lymphocytes from owl monkeys, marmosets and humans could be infected and transformed (Deinhardt *et al.*, 1974; Falk *et al.*, 1974; Ablashi *et al.*, 1976).

Luetzeler *et al.* (1979), using HVA-73, studied the ultrastructural morphogenesis of HVA in owl monkey kidney cells. The replicative cycle paralleled, in general, that of HVS, two morphologically distinct inclusion bodies appearing in the nuclei early in infection.

3.2.3 *Molecular analysis*

Little is known about the molecular biology of HVA. The structural organization of its genome is similar to that of HVS. There has been no sub-classification of its strains. Interestingly, 35% of the light strands of the DNA of HVA and HVS anneal with each other, but with considerable mismatching of base sequences. There is 10% homology between the heavy regions of HVS and HVA, with at least 13% divergence of their base pairs (Fleckenstein *et al.*, 1978b).

3.2.4 *Oncogenicity in non-human primates*

Three cotton-topped tamarins and two owl monkeys were inoculated intramuscularly with HVA strain 810. Two of the tamarins died 28 days after inoculation, and one was

killed when moribund at 40 days; all animals had malignant lymphomas, and generalized enlargement of the lymph nodes and splenomegaly were seen grossly. The two owl monkeys were killed 42 days after inoculation in moribund condition. No significant lesions were seen in one, while in the other focal interstitial collections of lymphoblasts, reticulum cells and eosinophils were present in the kidneys and lung and there was lymphocytic hyperplasia in most lymph nodes (Meléndez *et al.*, 1972b).

Hunt *et al.* (1972) inoculated HVA strain 810 into 12 cotton-topped tamarins. All animals died within about one month with malignant lymphomas of the lymphoblastic type. Leukaemia developed in 11 of the animals. One of four control tamarins in contact with the infected animals also developed lymphoma and leukaemia, indicating horizontal transmission.

Laufs and Meléndez (1973) inoculated partially purified HVA DNA or lymphoid cells derived from tumorous lymph nodes taken from experimentally infected cotton-topped tamarins immediately after death into owl monkeys (*Aotus trivirgatus*) and marmosets (*Callithrix jacchus*), two other New World monkey species, and African green monkeys (*Cercopithecus aethiops*). The two owl monkeys died after 20 and 28 days, respectively, of unknown causes. The two common marmosets died 36 and 104 days after inoculation with generalized malignant lymphoma, which resembled the disease observed in cotton-topped tamarins. The two adult African green monkeys inoculated with tumour cells or with partially purified HVA DNA survived, however, but the virus could not be isolated from their PBMC, indicating that this species of Old World primate is not susceptible to HVA infection.

Three viral isolates of HVA induced fatal malignant lymphomas in six cotton-topped tamarins and two white-lipped tamarins, while one isolate tested in squirrel monkeys did not cause overt disease (Falk *et al.*, 1974).

Two common marmosets infected with HVA strain 73 developed lymphomas of a variety of cell types within 27 days after inoculation. Some of the lymphoma cells were giant cells resembling the Sternberg-Reed cells observed in human Hodgkin's disease (Ablashi *et al.*, 1978).

3.3 Bovine herpesvirus 4 (Movar herpesvirus)

BHV-4 is one of four known bovine herpesviruses. It was first isolated by Bartha *et al.* (1966) under the name 'Movar' strain and has a worldwide distribution. It is the only known bovine gammaherpesvirus and is most probably a rhadinovirus, since it is similar to HVS (Bublot *et al.*, 1992). It has been isolated in a variety of clinical conditions as well as from healthy cattle (reviewed by Thiry *et al.*, 1989, 1990, 1992a,b). The group BHV-4 includes a large number of antigenically related isolates that are distinct from other bovine herpesviruses (Potgieter & Maré, 1974; Staczek, 1990). The role of BHV-4 as the etiological agent of a distinct disease entity is still questionable, although its role in the etiology of some diseases of the eye and respiratory and genital tracts has been suggested (Thiry *et al.*, 1989, 1990).

3.3.1 *Classification*

BHV-4 was initially known as bovine cytomegalovirus and was classified as a beta-herpesvirus because only its biological characteristics were taken into account (Storz *et al.*, 1984). Bublot *et al.* (1992) used molecular data to show that BHV-4 belongs to the Gammaherpesvirinae. Careful examination of the BHV-4 genome showed its close relationship to other gammaherpesviruses such as HVS, which allowed classification of BHV-4 into the rhadinovirus group (Lomonte *et al.*, 1996).

3.3.2 *Description*

The morphology of BHV-4 is typical of that of a herpesvirus. The nucleocapsid is icosahedral, with a dense core within the capsid which is made up of a regular arrangement of short tubular capsomeres (Todd & Storz, 1983). The diameter of the naked nucleocapsid is about 90–100 nm, while enveloped virions have a diameter ranging from 115 to 150 nm (Smith *et al.*, 1972; Munz *et al.*, 1974).

The BHV-4 virion contains double-stranded DNA of approximately 145 kb (Todd & Storz, 1983; Ehlers *et al.*, 1985). The genomic structure of BHV-4, typical of group B herpesviruses (Roizman, 1982), is similar to that of HVS, i.e. it has a unique coding sequence (light DNA) of approximately 110 kb flanked by a (G + C)-rich tandem repeat region of 2.65 kb (in BHV-4 VT strain) called polyrepetitive DNA (Ehlers *et al.*, 1985; Bublot *et al.*, 1990). RNA derived from the heavy strand of DNA has not been detected in infected cells (Chang & van Santen, 1992).

The overall genomic organization of BHV-4 VT strain was determined by sequencing 33 segments of the coding region (light strand) of its genome (Bublot *et al.*, 1992). Twenty-seven sequences showed homology to proteins present in either the three herpesvirus subfamilies or in only the two gammaherpesviruses, EBV and HVS. In the former case, the homology scores were always higher in comparison with EBV and HVS proteins than with the same proteins in alpha- or betaherpesviruses. Twenty-three of the sequenced regions of BHV-4 had homologous counterparts in both HVS and EBV genes. Nineteen of these regions belong to the five blocks of genes that are conserved among gamma- and alphaherpesviruses and/or betaherpesviruses. Five BHV-4 sequences were homologous to genes present only in the gammaherpesvirus genomes. The BHV-4 amino-acid sequences were more closely related to those of gammaherpesviruses than to those of alpha- or betaherpesviruses. Furthermore, the homology of most of these sequences was closer to the homologous products of HVS than to the equivalent proteins of EBV. Therefore, on the basis of the overall conservation of the sequences, BHV-4 is more closely related to HVS than to EBV. Six of the 33 BHV-4 sequences were homologous to neither HVS nor EBV genes nor to any other herpesvirus genes. All of these six sequences were located outside the conserved gene blocks. Genomic regions that were found to vary in size between BHV-4 isolates or strains (Bublot *et al.*, 1990, 1991a; Thiry *et al.*, 1992b) were also located outside these blocks (Bublot *et al.*, 1992).

The presence of five blocks of conserved genes was demonstrated in the BHV-4 genome, as in EBV and HVS. The length of the BHV-4 conserved gene blocks was estimated and found to be more closely related to that of HVS than that of EBV. Only

HVS block 3 was longer than those of BHV-4 and EBV (Lomonte *et al.*, 1996). This difference in size was due to the presence of a repeated region in the HVS gene 48 which causes an expansion of the acidic C-terminal domain of the protein (Albrecht *et al.*, 1992). The space between the BHV-4 gene blocks was also more like that of HVS than EBV, particularly between blocks 3 and 4 and blocks 4 and 5. Finally, as in the HVS genome, no large internal repeats were found between blocks 4 and 5 as is the case in the EBV genome.

No genes were found in region C, located between the second and the third conserved blocks. This region was less than 100 nucleotides long in the HVS and BHV-4 genomes (Lomonte *et al.*, 1996), whereas in the EBV genome this region contains the *BKRF1* gene coding for the EBNA-1 protein (Baer *et al.*, 1984) which is expressed in EBV-immortalized B lymphocytes (Middleton *et al.*, 1991).

A gene coding for viral fas-associated death domain protein interleukin-1β converting enzyme (FLICE)-inhibiting proteins, which prevent apoptosis, has been identified in BHV-4 DNA (Thome *et al.*, 1997).

Glycoprotein gB is a heterodimer and is a major component of the BHV-4 virion, unlike gBs of EBV (gp110) and murine gammaherpesvirus 68 (Lomonte *et al.*, 1997). This glycoprotein corresponds to the gp10/17 described by Dubuisson *et al.* (1989a). BHV-4 gB arises from a 142-kDa precursor which undergoes cleavage to give rise to two covalently linked glycopolypeptides of 128 and 56 kDa, corresponding to the previously identified gp10 and gp17, respectively. The resulting heterodimer has an apparent molecular mass of 210 kDa (Lomonte *et al.*, 1997).

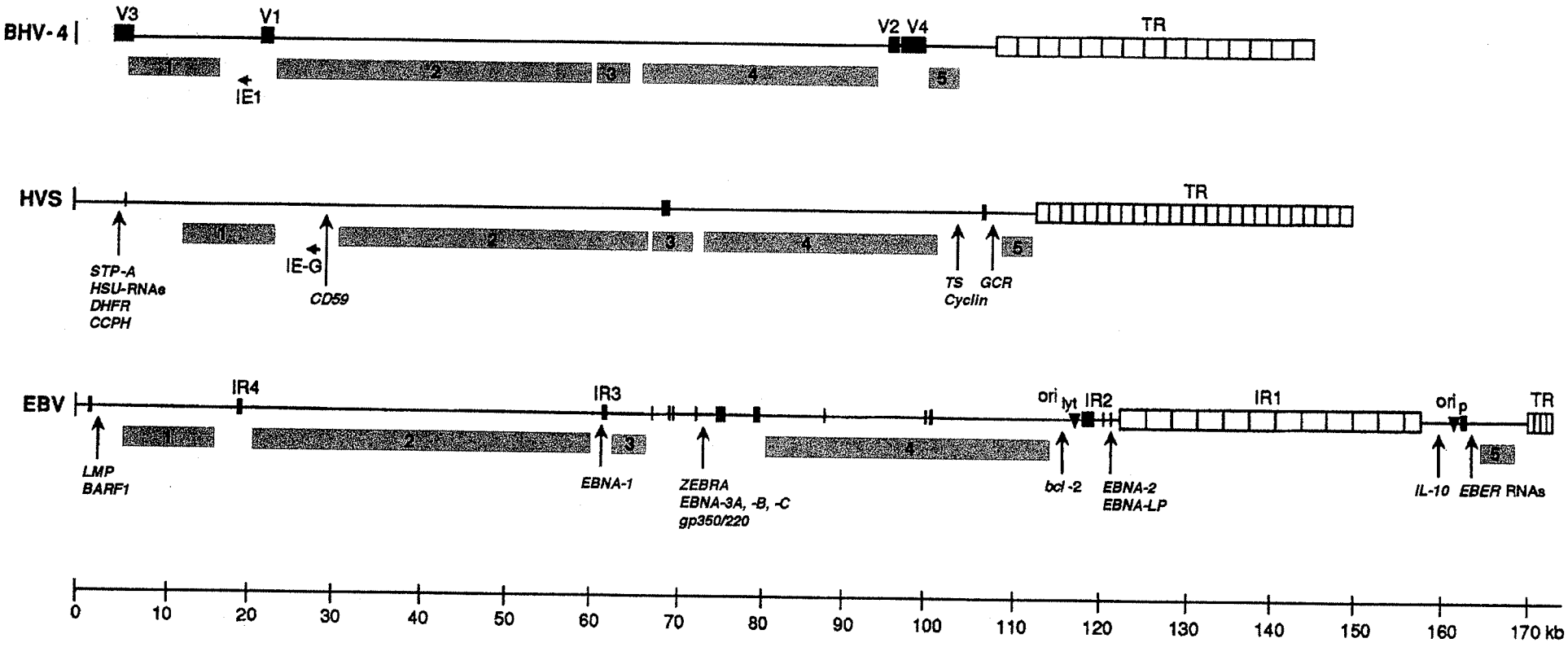
In summary, both genomic and protein analysis show the close homology of BHV-4 to HVS rather than to EBV (see Figure 7); therefore a possible relationship to KSHV/-HHV8 exists.

3.3.3 *Host range*

BHV-4 replicates in a variety of primary and established bovine cell cultures: primary kidney, testicle, lung, skin, spleen and thyroid, Madin Darby bovine kidney, Georgia bovine kidney, embryonic bovine kidney, embryonic bovine trachea, bovine bone marrow (BBM and FB4BM) and calf thymic lymphosarcoma cells (Bartha *et al.*, 1966; Luther *et al.*, 1971; Smith *et al.*, 1972; Parks & Kendrick, 1973; Rweyemamu & Loretu, 1973; Sass *et al.*, 1974; Theodoridis, 1978; Thiry *et al.*, 1981; Storz *et al.*, 1984; Theodoridis, 1985). BHV-4 has also been shown to replicate in cells of other animal species, including sheep, goats, pigs, dogs, rabbits, mink, horses, turkeys, geese, ferrets and potoroo (Luther *et al.*, 1971; Rweyemamu & Loretu, 1973; Kit *et al.*, 1986; Peterson & Goyal, 1988). Although the host range of BHV-4 *in vitro* is very broad, the virus does not replicate in cells of human (HeLa, Hep-2) or mouse (A31B77) origin or in chicken embryo fibroblasts (Luther *et al.*, 1971; Rweyemamu & Loretu, 1973). As in many other herpesviruses, the first receptor of BHV-4 is a heparin-like moiety on the cell surface, and its replication is cell cycle-dependent (Vanderplasschen *et al.*, 1993, 1995).

Of the ruminants, American bison *(Bison bison)* (Todd & Storz, 1983), African buffalo *(Syncerus caffer)* (Rossiter *et al.*, 1989), sheep (Van Opdenbosch *et al.*, 1986)

Figure 7. Order and spacing of gene blocks on the mature, linear genome of BHV-4 (V test strain), *Herpesvirus saimiri* (HVS strain; Albrecht *et al.*, 1992) and Epstein-Barr virus (EBV, B95-8 strain; Baer *et al.*, 1984)



Adapted from Bublot *et al.* (1992)

The five gene blocks that are conserved in gammaherpesviruses are indicated by the large shaded rectangles. Genes conserved in beta- and alphaherpesviruses are located in blocks 1, 2 and 4; blocks 3 and 5 contain only gammaherpesvirus-specific genes. The horizontal arrows indicate the positions of the major immediate-early gene of BHV-4 (*IE1*; van Santen, 1991) and HVS (*IE-G*). The vertical arrows indicate important genes located outside of the gene blocks and which are not conserved between EBV and HVS; the most commonly investigated genes are named: *STP-A*, saimiri transformation-associated protein; *HSU*-RNAs, *Herpesvirus saimiri* U-RNAs; *DHFR*, dihydrofolate reductase; *CCPH*, complement control protein homologue; *CD59*, cluster designation 59 homologue; *TS*, thymidylate synthase; *Cyclin*, cyclin family member homologue; *GCR*, G-coupled receptor homologue; *LMP*, latent membrane protein; *EBNA-2*, *-3A*, *-B*, *-C*, *-LP*, EBV nuclear antigen 2, 3A, 3B, 3C, leader protein; *bcl-2*, *bcl-2* proto-oncogene homologue; *IL-10*, interleukin 10 homologue; EBER, EBV-encoded RNA. EBV origins of replication are indicated by triangles and ori_{lyt} (origin of replication for the lytic cycle) and ori_p (plasmid origin of replication); terminal repeats (TR) and large internal repeats (IR) are represented by open rectangles; small internal repeated sequences are indicated by vertical lines or black rectangles. V1, V2, V3 and V4 are genomic regions which vary in size between BHV-4 isolates (Thiry *et al.*, 1992b). The orientation of the EBV genome is inverted relative to the conventional orientation (Baer *et al.*, 1984).

and goats (Moreno-Lopez *et al.*, 1989) undergo natural or experimental infection with BHV-4. European wild ruminants do not have antibodies to BHV-4 (Thiry *et al.*, 1988). The ability of BHV-4 to infect non-bovine ruminant species is understandable, considering the close phylogenetic relationships between these species.

BHV-4 can also infect species phylogenetically distant from ruminants: it has been isolated from a cat suffering from urolithiasis (Fabricant *et al.*, 1971). The latter isolate, designated 'feline herpesvirus type 2' or 'feline cell-associated herpesvirus', was characterized by restriction analysis (Kit *et al.*, 1986). Even if its role in feline disease is uncertain, the virus was undoubtedly isolated from cats and it has been shown to infect cats (Kruger *et al.*, 1990). The virus was a typical BHV-4 strain. Attempts to infect cats with the Movar 33/63 strain by conventional intranasal and intravenous routes were unsuccessful (Thiry *et al.*, 1991), perhaps due to a lack of adaptation of the virus to the cat rather than to strain specificity. The finding that this virus is a BHV-4 strain led to the disappearance of 'feline herpesvirus type 2' from the International Nomenclature.

The discovery that *Herpesvirus aotus* type 2, isolated from owl monkeys (*Aotus trivirgatus*), is also a strain of BHV-4 extends the range of species that are susceptible to BHV-4 (Bublot *et al.*, 1991b). This virus has been fully characterized; however, the genomic and protein differences from the prototype BHV-4 strains are compatible with the variations detected among BHV-4 strains (Bublot *et al.*, 1991b; Dubuisson *et al.*, 1991a). The antigenic relationship between *Herpesvirus aotus* type 2 and BHV-4 was proven by indirect fluorescence antibody testing with a broad panel of monoclonal antibodies raised against three BHV-4 glycoproteins and three other proteins and rabbit polyclonal sera specific for the two viruses (Bublot *et al.*, 1991b). The protein profile shows some variations between the two: glycoprotein gp6/gp10/gp17 of *Herpesvirus aotus* type 2 has a molecular mass of 160/131/52 kDa, with an extra band of 60 kDa which is probably a duplicate of the 52-kDa component; gp11 has a molecular mass of 109 kDa, while that of gp8 does not vary from those of BHV-4 isolates (Dubuisson *et al.*, 1991a). As a consequence, *Herpesvirus aotus* type 2 disappeared from the International Nomenclature of Herpesviruses (see Introduction, Table 2).

3.3.4 *Natural transmission*

BHV-4 is isolated in bovine species from both the anterior respiratory and genital tracts. Only respiratory infection has been studied experimentally, while other routes of inoculation, i.e. intravenous, intradermal and intratesticular, have been tested in cattle (Osorio & Reed, 1983; Dubuisson *et al.*, 1987, 1989b). Direct and indirect transmission of BHV-4 by means of infected materials is suggested.

3.3.5 *Evidence that bovine herpesvirus 4 causes disease*

After primary infection of cattle, BHV-4 replicates in mucosal cells and infects mononuclear cells, provoking generalized infection (Osorio & Reed, 1983). Viraemia is not always detected, but the virus can be re-isolated from many organs, including brain and spinal cord (Castrucci *et al.*, 1987; Dubuisson *et al.*, 1989b). BHV-4 may also infect the fetus (Kendrick *et al.*, 1976). Thereafter, BHV-4 establishes a latent infection. The spleen

is the main site of latency in rabbits (Osorio *et al.*, 1982, 1985); in cattle, the spleen is also the site where viral DNA is most frequently detected (Lopez *et al.*, 1996; Egyed *et al.*, 1996). In rabbits and cattle, non-B non-T cells and, presumably, monocytes and splenic macrophages are the main cells in which latent infection with BHV-4 is found (Osorio *et al.*, 1985; Lopez *et al.*, 1996); however, this issue is not definitely resolved, because BHV-4 can also replicate in lymphocytes (Egyed *et al.*, 1996). Latent virus can be reactivated by dexamethasone treatment, and re-excretion is shown to occur by the isolation of BHV-4 from peripheral blood leukocytes and nasal swabs (Krogman & McAdaragh, 1982; Dubuisson *et al.*, 1989b).

After viral entry, BHV-4 proteins are expressed in a cascade fashion, as for any herpesvirus. Trancription of two immediate-early genes (*IE1* and *IE2*) is initiated in infected cells (van Santen, 1991; Chang & van Santen, 1992; van Santen, 1993). IE1 and IE2 proteins share homology with IE110 of HSV and EBV R *trans*-activator, respectively (van Santen, 1991, 1993). IE2 was shown to *trans*-activate early (*E*) gene promoters, e.g. thymidine kinase gene (Zhang & van Santen, 1995) and late (*L*) gene (Bermudez-Cruz *et al.*, 1997). Expression of the *L* gene leads to abundant synthesis of a 1.7-kb RNA unique to BHV-4 (Bermudez-Cruz *et al.*, 1997). The precursor of the gB complex is expressed during the early phase and does not require DNA replication. Glycoproteins gp1, gp8 and gp11 are late proteins (Dubuisson *et al.*, 1991b,c, 1992a,b). BHV-4 DNA replication, and consequently the expression of late proteins, are dependent on the S phase of the cell cycle (Vanderplasschen *et al.*, 1995).

The contribution of BHV-4 to disease is speculative. BHV-4 has been designated as a passenger virus when isolated from ethmoidal tumours in Indian cattle (Moreno-Lopez *et al.*, 1989). The virus infects mononuclear blood cells, and this peculiarity allows it to be distributed to the whole body. Therefore, in primary infection and during reactivation of the latent state, BHV-4 can be reisolated from virtually all bovine tissues and organs. It is hard to discriminate between isolation by chance and a direct role of the virus in the observed lesions. BHV-4 has never been identified as a potential cause of tumours either *in vitro* or in cattle or rabbits *in vivo*.

3.3.6 *Isolates*

Two reference strains were isolated from clinical cases of conjunctivitis and respiratory disease: the Movar 33/63 strain in Europe (Bartha *et al.*, 1966) and the DN 599 strain in the United States (Mohanty *et al.*, 1971). Other strains were isolated from cases of respiratory disease (Smith *et al.*, 1972; Evermann *et al.*, 1984). In Africa, several herpesviral strains were isolated from people with a syndrome characterized by epididymitis and vaginitis (Maré & van Resenburg, 1961; Theodoridis, 1978, 1985), which are biologically similar to BHV-4 but have not yet been typed as BHV-4. The two Belgian reference strains, namely V. Test and LVR140, came from cases of orchitis and post-partum metritis, respectively (Thiry *et al.*, 1981; Wellemans *et al.*, 1984). Other BHV-4 strains were isolated from cases of genital disease in Italy (Castrucci *et al.*, 1986) and the United States (Parks & Kendrick, 1973; Evermann *et al.*, 1984). Reed *et al.* (1979) and Wellemans and Van Opdenbosch (1989) isolated BHV-4 from aborted

fetuses, and Reed *et al.* (1977) isolated BHV-4 from cases of mammary pustular dermatitis and ulcers of the udder. A few herpesviral strains biologically similar to BHV-4 were isolated from ruminal tumours (Kaminjolo *et al.*, 1972), tongue lesions (Rweyemamu & Loretu, 1973; Luini *et al.*, 1985) and diarrhoeal faeces (Eugster, 1978/-1979).

BHV-4 was also isolated or serologically suspected in cases of malignant catarrhal fever (Storz, 1968; Todd & Storz, 1983), lumpy skin disease (Alexander *et al.*, 1957; Rweyemamu & Loretu, 1973; House *et al.*, 1990), vesicular stomatitis (Evermann & Henry, 1989) and a variety of neoplasms such as lymphosarcoma (Potgieter & Maré, 1974), ocular squamous-cell carcinoma (Anson *et al.*, 1982) and T-cell lymphoma (Toho *et al.*, 1985).

BHV-4 is regularly isolated from healthy cattle. It has been recognized in renal-cell cultures (Luther *et al.*, 1971; Belák & Pálfi, 1974), in trigeminal ganglia (Homan & Easterday, 1981) and in triturated liver, lung and spleen (Krogman & McAdaragh, 1982).

3.4 Murid herpesvirus 4

Murine gammaherpesvirus 68 (MHV-68) is a naturally occurring rodent pathogen. Intranasal or intravenous administration of MHV-68 results in acute productive infection in B lymphocytes (Sarawar *et al.*, 1997) and lymphoproliferation (Sunil-Chandra *et al.*, 1992a,b, 1994; Sarawar *et al.*, 1997). The virus remains latent in B cells after primary infection (Sunil-Chandra *et* al., 1992b). Sequence analysis of its genome shows that it is most probably a rhadinovirus, more closely related to HVS and KSHV/HHV8 than to EBV (Mackett *et al.*, 1997).

3.5 Retroperitoneal fibromatosis herpesviruses

PCR with consensus gammaherpesvirus primers was used to detect small DNA fragments of two new, closely related rhadinoviruses in captive *Macaca nemestrina* and *M. mulatta* in one colony (Rose *et al.*, 1997). These animals were also infected with D-type simian retrovirus type 2 and suffered from retroperitoneal and subcutaneous fibrosis with progressive fibrovascular proliferation. This condition has some similarities to Kaposi's sarcoma (Tsai *et al.*, 1990; Rose *et al.*, 1997).

Earlier experiments had shown that cell cultures could be established from these lesions, which induced self-limited, transient spindle-cell proliferation, accompanied by pronounced vascularization when inoculated into nude mice (Tsai *et al.*, 1990, 1995).

The short sequences of these retroperitoneal fibromatosis herpesviruses presently available are derived from the polymerase gene and are about 70% identical at the nucleotide level (83–84% at the protein level) to the corresponding KSHV/HHV8 region (Rose *et al.*, 1997). If confirmed by a more extensive sequence analysis, these viruses could represent the closest relatives of KSHV/HHV so far. It may therefore prove to be a useful model for studying some aspects of Kaposi's sarcoma; however, nothing is yet known about the relative contributions of these new rhadinoviruses and simian retrovirus type 2 to the pathogenesis of retroperitoneal fibromatosis.

4. Other Data Relevant to an Evaluation of Carcinogenesis and its Mechanisms

4.1 Kaposi's sarcoma

4.1.1 *Cell biology*

4.1.1.1 *Origin of spindle cells*

Spindle cells surrounding slit-like spaces are characteristic of advanced Kaposi's sarcoma lesions. Endothelial cells (either vascular or lymphatic endothelium), cells from venous lymphatic junctions, fibroblasts, smooth-muscle cells, dermal dendrocytes and macrophages have all been proposed as possible progenitors of Kaposi's sarcoma spindle cells (reviewed by Roth *et al.*, 1992; Stürzl *et al.*, 1992a; Browning *et al.*, 1994; Kaaya *et al.*, 1995). Like normal lymphatic endothelial cells, spindle cells stain with the monoclonal antibody EN-4, which detects both vascular and lymphatic endothelium, but lack reactivity with the monoclonal antibody Pal-E, which reacts with blood-vessel but not lymphatic endothelial cells (Rappersberger *et al.*, 1990). Other markers for blood-vessel endothelium, such as OKM-5 and anti-factor VIII-related antigen (von Willebrand factor), stain Kaposi's sarcoma endothelial or spindle cells, although varying results have been reported by different laboratories. Studies with *Ulex europaeus* agglutinin 1, another marker for endothelial cells, have also produced contradictory results (Nadji *et al.*, 1981; Modlin *et al.*, 1983; Little *et al.*, 1986; Rappersberger *et al.*, 1990; further references in Roth *et al.*, 1992). Ultrastructural examination failed to show the presence of Weibel-Palade bodies, the storage vesicles for von Willebrand factor and therefore a characteristic feature of vascular endothelium, in spindle cells from Kaposi's sarcoma lesions (Rappersberger *et al.*, 1990). Staining with the monoclonal antibody BMA 120, which reacts with an antigen on endothelial cells, lends support to an endothelial origin of Kaposi's sarcoma cells (Roth *et al.*, 1988). Spindle cells and endothelia lining vascular spaces in Kaposi's sarcoma lesions express leukocyte adhesion molecule 1 and thrombomodulin, which are markers of lymphokine-activated endothelial cells (Zhang *et al.*, 1994). This observation further supports the notion that Kaposi's sarcoma spindle cells are of endothelial origin and are activated by growth factors (see below).

The staining of spindle cells with antibodies to CD14, CD68 and factor XIIIa, observed by some laboratories, has been interpreted as reflecting a link between these spindle cells and cells of the monocyte/macrophage lineage, possibly dermal dendrocytes (Nickoloff & Griffiths, 1989; Rappersberger *et al.*, 1990). These cells are distinct from Langerhans cells (Nickoloff & Griffiths, 1989). The staining of cultured Kaposi's sarcoma spindle cells with an antibody to smooth-muscle α actin (Weich *et al.*, 1991) and similar histochemical findings have been interpreted to suggest a relationship with smooth-muscle cells or myofibroblasts (reviewed by Roth *et al.*, 1992). These discrepant results suggest either that cells of different lineages can adopt a spindle-like morphology or that these markers are common to different cells of mesenchymal origin and Kaposi's sarcoma spindle cells derived from pluripotent mesenchymal progenitor cells. Currently,

CD34 (as detected by monoclonal antibody QBEND 10) is considered the best marker for Kaposi's sarcoma spindle cells (Russell Jones *et al.*, 1995).

Cells expressing markers characteristic for vascular or lymphatic endothelium from Kaposi's sarcoma lesions have been cultured in a number of laboratories (Delli Bovi *et al.*, 1986; Nakamura *et al.*, 1988; Roth *et al.*, 1988; Siegal *et al.*, 1990; Corbeil *et al.*, 1991; Herndier *et al.*, 1994), but cultures expressing smooth-muscle α actin (Albini *et al.*, 1988; Wittek *et al.*, 1991) and mixed populations (Siegal *et al.*, 1990; further references in Roth *et al.*, 1992) have also been reported. The lineage of these cultured cells has been defined by staining for similar markers as in studies *in situ*, notably vimentin and cytokeratin (to discriminate mesenchymal and epithelial cells, respectively), endothelial markers such as von Willebrand factor, Pal-E, OKM-5, BMA 120 (specific for blood-vessel endothelium), EN-4 and *Ulex europaeus* agglutinin 1 lectin (which reacts with blood-vessel and lymphatic endothelium), CD14 and factor XIIIa (for the monocyte/macrophage lineage), smooth-muscle α actin (smooth muscle and myofibroblasts) and others (reviewed by Roth *et al.*, 1992; Stürzl *et al.*, 1992a; Kaaya *et al.*, 1995). Spindle-shaped cells showing moderate expression of endothelial antigens have been cultured from peripheral blood of Kaposi's sarcoma patients (Browning *et al.*, 1994).

4.1.1.2 *Vascular lesions induced by Kaposi's sarcoma cell cultures in nude mice*

The various cell cultures established from Kaposi's sarcoma lesions differ in their ability to induce angiogenic lesions in nude mice. [The Working Group noted that the similarity of some of these lesions to Kaposi's sarcoma is controversial.] A cell line expressing endothelial markers, established by Siegal *et al.* (1990) and studied by Herndier *et al.* (1994), induced Kaposi's sarcoma-like tumours of human origin in nude mice. This cell line expressed the endothelial markers factor VIII, EN-4 and *Ulex europeaus* agglutinin 1 lectin. In addition, it produced high levels of urokinase plasminogen activator and plasminogen activator inhibitor 1 (Herndier *et al.*, 1994). Interestingly, plasminogen activator has been shown to be involved in the development of endothelial tumours in mice transgenic for the polyoma middle-T protein (Montesano *et al.*, 1990). A second cell line capable of causing tumours of human origin in nude mice has also been described (Lunardi-Iskandar *et al.*, 1995). The development of these cell lines suggests that a subpopulation of cells in Kaposi's sarcoma lesions may have progressed to a malignant phenotype; however, they do not contain KSHV/HHV8 DNA, and their relationship to spindle cells containing this virus (see section 4.5.1) is unclear.

A few other Kaposi's sarcoma cell cultures, also of an endothelial phenotype, are angiogenic *in vivo* but induce the growth of 'Kaposi's sarcoma-like' vascular lesions of murine origin when inoculated into nude mice (Nakamura *et al.*, 1988; Salahuddin *et al.*, 1988). Spindle-shaped cells grown from the peripheral blood of Kaposi's sarcoma patients have also been reported to induce angiogenesis in nude mice (Browning *et al.*, 1994). Although these cultures were not examined for KSHV/HHV8, similar cultures established more recently did contain viral DNA (Sirianni *et al.*, 1997).

Most other cell cultures, established by several laboratories (Roth *et al.*, 1988), were not angiogenic in nude mice (Delli Bovi *et al.*, 1986; Albini *et al.*, 1988; Roth *et al.*, 1988; Wittek *et al.*, 1991); furthermore, KSHV/HHV8 is either not present or is rapidly lost upon serial passage from such cultures (Ambroziak *et al.*, 1995; Lebbé *et al.*, 1995).

4.1.1.3 *Growth factors involved in proliferation of spindle cells*

The role of growth factors in the development of Kaposi's sarcoma has been studied in several laboratories, with inconsistent findings, probably because of the use of different cell types. The role of growth factors in the development of Kaposi's sarcoma *in vivo* and their interaction with KSHV/HHV8 are still unknown.

(*a*) *Fibroblast growth factors*

Basic fibroblast growth factor (FGF) is secreted by Kaposi's sarcoma cultures expressing endothelial cell markers and may promote the growth of these cells *in vitro* (Ensoli *et al.*, 1989). Other groups working with Kaposi's sarcoma cultures of either endothelial phenotype (Corbeil *et al.*, 1991) or mixed fibroblastoid/endothelial appearance (Werner *et al.*, 1989) also found FGF-like activity in supernatants of these cultures which stimulated the growth of normal fibroblasts and endothelial cells.

Members of the FGF family, including basic FGF and endothelial cell growth factor, are known to stimulate the growth of normal endothelial cells, and cultured Kaposi's sarcoma cells with endothelial characteristics have been shown to induce transient neoangiogenesis in nude mice (Nakamura *et al.*, 1988). The FGF family of cytokines may thus play a crucial role in the development of Kaposi's sarcoma. Expression of basic FGF and FGF5 has been shown to occur in spindle cells of Kaposi's sarcoma by in-situ hybridization (Xerri *et al.*, 1991). Acidic FGF and FGF6 are also expressed in these lesions (Li *et al.*, 1993), but the technique employed in this study (RT-PCR) did not permit identification of the cell type(s) that secrete(s) these two members of the FGF family. The importance of basic FGF in the development of experimental Kaposi's sarcoma-like lesions is further supported by the report that basic FGF-specific antisense oligonucleotide can inhibit the angiogenic effect of cultured Kaposi's sarcoma cells in nude mice (Ensoli *et al.*, 1994a).

(*b*) *Platelet-derived growth factor*

Both normal endothelial cells (Ensoli *et al.*, 1989; Roth *et al.*, 1989) and short-term cultures of Kaposi's sarcoma cells with endothelial characteristics (Ensoli *et al.*, 1989) produce platelet-derived growth factor (PDGF). Cultures that produce this factor thus do not require exogenous PDGF to promote proliferation (Ensoli *et al.*, 1989; Corbeil *et al.*, 1991). Short-term cultures were also shown to express mRNA for the receptors for PDGF-A and PDGF-B (Roth *et al.*, 1989; Werner *et al.*, 1990). Kaposi's sarcoma spindle cells express mRNA for the PDGF-β receptor *in vivo*, whereas mRNAs for PDGF-A and PDGF-B were expressed on some tumour cells located in the vicinity of slit-like spaces (Stürzl *et al.*, 1992b). PDGFs may therefore play a role in the pathogenesis of Kaposi's sarcoma.

(*c*) *Interleukin-1*

IL-1 has also been reported to be secreted by cultured Kaposi's sarcoma cells of the endothelial phenotype (Ensoli *et al.*, 1989; Corbeil *et al.*, 1991) and to have a potent stimulatory effect on these cells (Nakamura *et al.*, 1988).

(*d*) *Interleukin-6*

Cultured Kaposi's sarcoma cells of an endothelial phenotype secrete and proliferate in response to IL-6 (Miles *et al.*, 1990; Corbeil *et al.*, 1991). The expression of receptors for IL-6 on cultured cells has also been reported (Miles *et al.*, 1990), and Kaposi's sarcoma cells expressed IL-6 mRNA *in vivo* (Gillitzer & Berger, 1991). The former observation is of particular interest in the context of the IL-6 homologue encoded by KSHV/HHV8 (v-IL-6; see section 1.1); however, the v-IL-6 homologue is expressed only rarely in KSHV/HHV8-infected Kaposi's sarcoma spindle cells (Moore *et al.*, 1996b), suggesting that it does not play a major role in the pathogenesis of this disease.

(*e*) *Tumour necrosis factor* α

Tumour necrosis factor (TNF) α has a potent stimulatory effect on some Kaposi's sarcoma cell cultures (Nakamura *et al.*, 1988), but whether it is produced by cultures with endothelial characteristics is controversial (Ensoli *et al.*, 1989; Corbeil *et al.*, 1991). TNFα has been reported to be expressed by Kaposi's sarcoma cells in small amounts *in vivo* but was mainly found in epidermal cells adjacent to the tumour (Gillitzer & Berger, 1991), compatible with a possible paracrine role of this factor.

(*f*) *Miscellaneous growth factors*

Secretion of granulocyte–monocyte colony-stimulating factor and transforming growth factor β by Kaposi's sarcoma cell cultures with endothelial characteristics, but not by normal endothelial cells, has been reported (Ensoli *et al.*, 1989). The latter also promotes the growth of cultured Kaposi's sarcoma cells. In lesions, mature transforming growth factor β1 is found mainly in macrophage-like cells and not in spindle cells (Williams *et al.*, 1995). Hepatocyte growth factor (scatter factor) also promotes the growth of cultured Kaposi's sarcoma cells and may thus play a role in the pathogenesis of this disease (Naidu *et al.*, 1994).

4.1.1.4 *Role of HIV-1 Tat in promoting Kaposi's sarcoma lesions*

AIDS-associated Kaposi's sarcoma is clinically more aggressive than classic or endemic Kaposi's sarcoma, suggesting that HIV is a cofactor in the progression of this tumour. Experimental evidence suggests that the Tat protein of HIV-1 (see IARC, 1996) can enhance the growth of cultured 'endothelial' Kaposi's sarcoma cells (Ensoli *et al.*, 1990); the effect of Tat on other cell cultures was inconsistent (Roth *et al.*, 1992). Several cytokines, including TNF, IL-1 and interferon γ, can render normal endothelial and smooth-muscle cells susceptible to the growth-promoting effect of Tat (Barillari *et al.*, 1992), possibly by increasing the expression of integrin receptors which interact with Tat (Barillari *et al.*, 1993; Ensoli *et al.*, 1994a). Injection of Tat into nude mice

(Ensoli *et al.*, 1994b) or immunocompetent C57Bl mice (after incorporation into Matrigel; Albini *et al.*, 1994) induces angiogenesis, and this effect is potentiated by basic FGF (Ensoli *et al.*, 1994a,b) and heparin (Albini *et al.*, 1994, 1996b). Tat- and heparin-induced neoangiogenesis can be inhibited by the matrix metalloproteinase inhibitor TIMP-2 (Albini *et al.*, 1994), and Tat and basic FGF synergize to increase the expression of collagenase IV in nude mice (Ensoli *et al.*, 1994b). In addition, one group reported the emergence of Kaposi's sarcoma-like lesions in mice transgenic for HIV-1 *tat* (Vogel *et al.*, 1988); however, other lines of transgenic mice carrying the complete HIV-1 genome failed to develop similar lesions (Leonard *et al.*, 1988). Transgenic mice carrying the early region of BK virus included in a long terminal repeat-*tat* construct also develop 'Kaposi's sarcoma-like' lesions, in addition to other malignancies (Coralini *et al.*, 1993), and extracellular Tat released by tumour cell lines derived from these animals protects them from apoptosis under conditions of serum starvation (Campioni *et al.*, 1995). Tat can be released from HIV-infected cells and can act on HIV-uninfected cells. The growth promoting effect of extracellular Tat on cultured Kaposi's sarcoma cells and endothelial cells (Ensoli *et al.*, 1990; Barillari *et al.*, 1992) suggests that infection of cells not directly involved in the Kaposi's sarcoma lesion may be sufficient to trigger the sequence of events leading to the development of this tumour. In keeping with this interpretation, in *tat*-transgenic mice which did develop Kaposi's sarcoma-like lesions, expression of *tat* was not found in spindle cells but in neighbouring keratinocytes (Vogel *et al.*, 1988).

HIV-1 Tat has been reported to be detectable by histochemical techniques in Kaposi's sarcoma lesions in AIDS patients, probably originating from a few HIV-1-infected mononuclear cells (Ensoli *et al.*, 1994b). Thus, the angiogenic properties of Tat, alone or in concert with other growth factors, has been documented in a variety of experimental systems; however, the molecular basis for its angiogenic properties is still controversial. Tat has been reported to bind to $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins via an RGD sequence element in a manner similar to, and replaceable by, their physiological ligands fibronectin and vitronectin (Barillari *et al.*, 1993; Ensoli *et al.*, 1994b). Baboons infected with HIV-2, however, also developed Kaposi's sarcoma-like lesions, although HIV-2 Tat lacks an RGD domain (Barnett *et al.*, 1994). Tat binds with high affinity to the Flk-1/KDR receptor for vascular endothelial cell growth factor (Albini *et al.*, 1996c), and this interaction promotes angiogenesis. A basic heparin binding-like domain in *tat*, rather than the RGD domain, is thought to be involved in this interaction (Albini *et al.*, 1996b).

4.1.1.5 *Clonality of Kaposi's sarcoma lesions*

Individual Kaposi's sarcoma nodules in female patients have been shown, by studying X-inactivation markers, to contain monoclonal or oligoclonal cell populations (Rabkin *et al.*, 1995), and different nodules from the same patient have the same monoclonal origin (Rabkin *et al.*, 1997). Southern blotting of Kaposi's sarcoma DNA with KSHV/-HHV8 terminal-repeat probes showed one or a few bands, which is consistent with a monoclonal or oligoclonal expansion of virus-infected cells. Whether different lesions from the same patient have the same KSHV/HHV8 clonality pattern has not yet been

addressed by terminal-repeat hybridization, and it is not known whether this assay is sufficient to determine monoclonality (Russo *et al.*, 1996). Short-term cultures of biopsy samples from Kaposi's sarcomas have been found to contain chromosomal rearrangements, but no consistent pattern has emerged (Delli Bovi *et al.*, 1986). Two tumorigenic cell lines derived from Kaposi's sarcoma lesions have been reported to have a 3(p14) translocation in common (Popescu *et al.*, 1996); however, as neither is infected with KSHV/HHV8, the relationship of these findings to Kaposi's sarcoma spindle cells latently infected with the virus (see section 4.1.4) is unclear.

4.1.2 *Role of KSHV/HHV8 in development of Kaposi's sarcoma*

The consistent detection of KSHV/HHV8 in Kaposi's sarcoma biopsy samples and the epidemiological data discussed in Section 1 strongly support a causative role of KSHV/HHV8 in the pathogenesis of Kaposi's sarcoma. Although its precise role is not yet understood, the presently available data are compatible with the notion that KSHV/-HHV8 exerts a direct transforming effect on endothelial cells, the likely precursors of Kaposi's sarcoma spindle cells.

KSHV/HHV8 establishes a persistent infection in most Kaposi's sarcoma spindle cells, which involves the expression of at least three viral genes. All spindle cells, as well as the atypical endothelial cells of early lesions, express abundant mRNA for *ORF K12*, which encodes a putative small hydrophobic protein of unknown function (Zhong *et al.*, 1996; Stürzl *et al.*, 1997; Staskus *et al.*, 1997). Kaposi's sarcoma spindle cells also express the high-molecular-mass latent nuclear antigen (LNA) encoded by *ORF 73*, as shown by immunohistochemistry with affinity-purifed antibodies to this protein (Rainbow *et al.*, 1997). Expression of mRNA for the *ORF 72*-encoded D-type cyclin homologue has been demonstrated by in-situ hybridization (Stürzl *et al.*, 1997); however, as the mRNA encoding *ORF 73* extends through the *ORF 72/v-cyclin* gene, this result requires confirmation by immunohistochemical studies with specific antibodies. The *ORF K13* gene is also contained within the mRNAs encoding *ORF 72/v-cyclin* and *ORF 73/LNA* (Rainbow *et al.*, 1997) and may therefore be expressed in Kaposi's sarcoma spindle cells, but no immunohistochemical studies on the protein it expresses have yet been reported (see section 4.4.5). The KSHV/HHV8 homologue to *IL-6* (*ORF K2*) is expressed during latency in KSHV/HHV8-infected haematopoietic cells but is not generally expressed in Kaposi's sarcoma lesions (Moore *et al.*, 1996b).

A subpopulation (approximately 10%) of Kaposi's sarcoma spindle cells also expresses a polyadenylated nuclear *T1.1* RNA (Staskus *et al.*, 1997), which is abundant in primary effusion lymphoma cell lines induced into lytic replication (Renne *et al.*, 1996a; Zhong *et al.*, 1996). Expression of *T1.1* may therefore be indicative of lytic replication within Kaposi's sarcoma lesions, suggesting that a subpopulation of spindle cells can produce KSHV/HHV8 virions. Intranuclear herpesvirus-like particles or intranuclear inclusions characteristic of herpesviruses can be found in Kaposi's sarcoma tissues, indicating that the tumour is a source of productive infection (Walter *et al.*, 1984). Expression of *ORF 74*, encoding a functional chemokine receptor (Arvanitakis *et al.*, 1997), can

be found by RT-PCR, but it is not clear whether this gene is expressed during the latent or the lytic viral expression programme (Cesarman *et al.*, 1996a).

As discussed in section 1.1.6, v-cyclin can phosphorylate Rb and histone H1 and therefore dysregulate the cell cycle, but it has not so far been shown to have transforming properties of its own. The functions of *ORF 73/LNA* and *K12* are unknown; however, the expression of a limited set of genes in persistently infected cells is a familiar pattern with other (both oncogenic and non-oncogenic) herpesviruses and suggests that the role of KSHV/HHV8 in the pathogenesis of Kaposi's sarcoma is likely to be a direct one.

4.2 Primary effusion lymphomas

Primary effusion lymphomas have a unique constellation of features that distinguishes them from all other known lymphoproliferations: they are predominantly confined to 'body cavities' as an effusion; cells with cytomorphological features bridging those of large-cell immunoblastic and anaplastic large-cell lymphoma suggest that they represent mature B cells; a nil phenotype, although some primary effusion lymphomas with B- and/or T-cell markers have been described; clonal immunoglobulin gene rearrangements, further implying a B-cell phenotype; uniform lack of *c-myc* rearrangements and the presence of KSHV/HHV8 with or without EBV (see section 2.2.1). In at least one primary effusion lymphoma cell line tested, KSHV/HHV8 is clonal by terminal-repeat analysis (Russo *et al.*, 1996).

Most cases of primary effusion lymphoma have been described in severely immunocomprised individuals with HIV infection, in keeping with the notion that lack of immunosurveillance favours primary effusion lymphoma cell proliferation. Why these lymphomas persist predominantly as effusions, without lymph node or other lymphoid tissue involvement, is unclear, although a lack of 'homing markers' has been suggested (Karcher & Alkan, 1995).

Multiple, complex chromosomal abnormalities have been described in cases of primary effusion lymphoma; however, apart from a consistent absence of *c-myc* rearrangement, none of the described abnormalities is present in all cases (Ansari *et al.*, 1996).

In view of the frequent co-infection with EBV and KSHV in the cells of such tumours, these two viruses may act together to induce neoplastic transformation and/or the peculiar phenotypic features of these lymphomas. The presence of activation markers (similar in EBV-positive and EBV-negative KSHV/HHV8-positive cases) suggests that cell activation is secondary to viral infection (Ansari *et al.*, 1996; Nador *et al.*, 1996). Of note, all KSHV/HHV8-positive primary effusion lymphomas lack *c-myc* rearrangements. The few cases of 'effusion-based lymphomas' described that have *c-myc* rearrangements do not have a nil surface antigen phenotype, display different cytomorphological features and do not contain KSHV/HHV8 (see Table 8).

4.3 Multicentric Castleman's disease

Multicentric Castleman's disease is a polyclonal lymphoproliferation characterized by prominent vascularity in lymphoid tissue and associated systemic symptoms. There is a

strong association between multicentric Castleman's disease and Kaposi's sarcoma in patients with AIDS (see section 2.2.2).

Immunoregulatory abnormalities probably contribute to these lymphoproliferations. It was hypothesized that a virus acts as a cofactor, perhaps as a stimulus for cytokine production (Peterson & Frizzera, 1993). The association between multicentric Castleman's disease and the presence of IL-6 is notable. Thus, IL-6 is present at high levels in biopsy samples from patients with this disease, and PBMC from patients with multicentric disease secrete high levels of IL-6 (Yoshizaki *et al.*, 1989; Burger *et al.*, 1994). IL-6 has prominent actions on cells of the immune system, including stimulation of immunoglobulin synthesis by activated B cells and differentiation of cytotoxic T cells (Kikutani *et al.*, 1985). It also commits myeloid progenitors to differentiate into granulocytes and macrophages. IL-6 is synthesized in response to a number of stimuli, which include viruses and other cytokines such as IL-1 and TNF (Kishimoto, 1989). IL-6 also acts as an auto- and paracrine growth factor for multiple myeloma cells (Kawano *et al.*, 1988). Retroviral-mediated transfer of IL-6 into haematopoietic cells of mice results in a syndrome resembling multicentric Castleman's disease (Brandt *et al.*, 1990a,b). These mice develop anaemia, polyclonal hypergammaglobulinaemia, splenomegaly and peripheral lymphadenopathy, further supporting the hypothesis that dysregulated synthesis of IL-6 has a causative role in multicentric Castleman's disease.

KSHV/HHV8 is nearly always present in HIV-positive individuals with multicentric Castleman's disease; in immunocompetent hosts, the virus is present in about 40% of cases (see Table 10). The presence of HHV6 in two of five cases of multicentric Castleman's disease and EBV in two of five cases has also been reported (Barozzi *et al.*, 1996), but the significance of this finding is unknown.

4.4 Viral genes with cellular growth promoting or oncogenic potential

As discussed in Section 1 (Table 1), the KSHV/HHV8 genome contains multiple non-conserved viral genes, some of which are strikingly similar to human genes involved in cellular growth control. The specific viral genes discussed in this section are those with sequence similarity to known human oncogenes and/or are expressed during viral latency.

4.4.1 Open reading frame K1

The first gene at the left end of the KSHV/HHV8 genome (Figure 2), *ORF K1*, encodes a putative 289-amino acid, type I transmembrane protein, featuring a signal peptide, an extracellular domain with multiple cysteine residues, a typical transmembrane anchor domain and a short intracellular domain containing several potential tyrosine phosphorylation motifs (Russo *et al.*, 1996; Neipel *et al.*, 1997a). No experimental data are available to indicate that the K1 protein is phosphorylated, and neither its function nor its expression pattern in Kaposi's sarcoma-associated tumours has been described. The same genomic region is also highly variable between different strains of *Herpesvirus saimiri* (Jung *et al.*, 1991; Jung & Desrosiers, 1991; Biesinger *et al.*, 1995).

A comparison of deposited genomic sequences indicates that it may also be highly variable (Russo *et al.*, 1996; Lagunoff & Ganem, 1997; Neipel *et al.*, 1997a).

4.4.2 *Growth factor homologues*

Three KSHV/HHV8 genes have sequence similarity to members of the C-C-chemokine family (Russo *et al.*, 1996; Neipel *et al.*, 1997a; Nicholas *et al.*, 1997a,b). Two of these, encoded by *ORF 6* and *4* (v-MIP-I and v-MIP-II) are expressed at low levels in latently infected primary effusion lymphoma cells, and that expression increases during the lytic cycle of viral replication (Moore *et al.*, 1996b; Nicholas *et al.*, 1997a,b). v-MIP-I can interact with the C-CR5 co-receptor to inhibit the entry of some primary HIV strains (Moore *et al.*, 1996b).

The viral IL-6 homologue (v-IL-6), encoded by *ORF K2*, is 25% identical at the amino-acid level to human IL-6. Several groups have identified this protein (Moore *et al.*, 1996b; Russo *et al.*, 1996; Neipel *et al.*, 1997b; Nicholas *et al.*, 1997a,b) and shown that it supports the growth of the IL-6-dependent mouse myeloma cell line, B9 (Moore *et al.*, 1996b; Nicholas *et al.*, 1997b). It is expressed in latently infected lymphoma cell lines and primary effusion lymphoma tissue, but not generally in Kaposi's sarcoma tissue. Only a few scattered $CD20^+$ B cells expressing v-IL-6 were found in one of eight Kaposi's sarcoma specimens examined by immunohistochemistry (Moore *et al.*, 1996b). v-IL-6 activates the same Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway in HepG2 hepatoma cells as human IL-6; however, human IL-6 and v-IL-6 differ in their cellular IL-6 receptor interaction. Whereas human IL-6 requires both IL-6Rα and gp130 receptor protein for signal activation, v-IL-6 requires only gp130 (Molden *et al.*, 1997). Murine B9 plasmacytoid cell proliferation in response to v-IL-6 is inhibited by antibody to murine IL-6Rα, suggesting possible differences between human and mouse systems (Nicholas *et al.*, 1997b).

4.4.3 bcl-2 *homologue*

ORF 16 of KSHV/HHV8 encodes a homologue of the cellular anti-apoptotic protein bcl-2, with which it shares 16% sequence homology (Russo *et al.*, 1996; Cheng *et al.*, 1997; Neipel *et al.*, 1997a; Nicholas *et al.*, 1997a; Sarid *et al.*, 1997). The *BH1* and *BH2* domains, which are conserved in the bcl-2 family of proteins, are also found in the KSHV/HHV8 homologue. Functional studies indicate that bcl-2 prevents Bax-mediated toxicity or apoptosis in yeast, Sindbis virus-infected cells and transfected fibroblasts (Cheng *et al.*, 1997; Sarid *et al.*, 1997). There is contradictory evidence for the ability of v-*bcl-2* to heterodimerize with human *Bcl-2* or *Bax*: Sarid *et al.* (1997) found evidence of v-*bcl-2*–human *Bcl-2* interactions in a two-hybrid yeast system, but Cheng *et al.* (1997) found no specific interaction between the KSHV/HHV8 protein and other bcl-2-like proteins by co-immunoprecipitation. [This contradiction could be due to differences in the sensitivities or specificities of the assays used or to differences in the recombinant *bcl-2* constructs.] Both studies demonstrate, however, that the v-*bcl-2* has functional anti-apoptotic activity.

v-bcl-2 Transcripts can be induced in primary effusion lymphoma cell lines by 12-*O*-tetradecanoylphorbol 13-acetate and can be detected at low levels in Kaposi's sarcoma lesions and primary effusion lymphoma cell lines (Cheng *et al.*, 1997; Sarid *et al.*, 1997), similar to the proposed function of the EBV *bcl-2* homologue *BHRF1* (Roizman, 1993). [The function of *v-bcl-2* of KSHV/HHV8 may be to prolong the survival of lytically infected cells.] Since lytic virus replication is thought to be incompatible with survival of the cell (Roizman, 1993), expression of *BHRF1* may not play a direct role in EBV-mediated transformation.

4.4.4 *Viral interferon regulatory factor*

ORF K9 encodes the homologue of v-IRF (Moore *et al.*, 1996b; Russo *et al.*, 1996). This gene has low but significant homology to the IRF family of proteins responsible for interferon signal transduction. Two members of this pathway, IRF-1 and IRF-2, are antagonistic to each other in their effector functions (Taniguchi *et al.*, 1995a). IRF-1 positively regulates interferon signaling by binding to specific enhancer elements, called interferon-stimulated response elements, in the promoter regions of interferon-inducible genes. Activated transcription of interferon-stimulated genes results in phenotypic changes characteristic for the interferon-induced antiviral state, which include (but are not limited to) induction of CDKI p21, which can lead to shut-down of the cell cycle through inhibition of Rb (Taniguchi *et al.*, 1995a), and up-regulation of the major histocompatibility complex antigen (Taniguchi *et al.*, 1995b). *IRF-2* has oncogenic activities in NIH 3T3 cells which can be reversed by *IRF-1* overexpression (Harada *et al.*, 1993). Like KSHV/HHV8-encoded cytokine homologues, *v-IRF* is expressed in latently infected primary effusion lymphoma cell lines, and its expression is markedly enhanced after lytic cycle induction (Moore *et al.*, 1996b).

4.4.5 *Viral proteins that inhibit fas-associated death domain protein interleukin-1β converting enzyme (FLICE)*

ORF K13 encodes a small protein similar to the 'death effector' domains found in *ORF 71*-encoded proteins of three other rhadinoviruses, HVS, equine herpesvirus 2 and BHV-4 (Thome *et al.*, 1997); however, there is no significant, overall sequence similarity between KSHV/HHV8 *K13* and the *ORF 71* of other rhadinoviruses (Russo *et al.*, 1996). The protein of equine herpesvirus 2 encoded by *ORF 71* and a similar protein of molluscipoxvirus have been shown to act as dominant negative inhibitors of apoptotic signals by interfering with the interaction of fas-associated death domain protein (FADD) and tumour necrosis factor receptor-associated death domain protein (TRADD) and the assembly of the interleukin-1β-converting enzyme (ICE)-like protease complex and are therefore known as v-FLIPs (Bertin *et al.*, 1997; Thome *et al.*, 1997). No information on the function of the v-FLIP of KSHV/HHV8 is available, but conservation of the critical sequence elements suggests that it may also be able to inhibit this apoptotic pathway. Although the HVS v-FLIP may be expressed primarily during the lytic cycle of replication and not in tightly latent HVS-transformed human T-cells, the KSHV/HHV8 v-FLIP may be expressed in Kaposi's sarcoma spindle cells and primary effusion lym-

phoma cells, as its reading frame is included in mRNAs that encode the neighbouring ORF 72 (v-cyclin) and ORF 73 (LNA) proteins which are expressed in both Kaposi's sarcoma spindle and primary effusion lymphoma cells (Rainbow *et al.*, 1997). No direct evidence for its expression in endothelial tumour (spindle) or B-lymphoma cells is available.

4.4.6 *Viral cyclin*

ORF 72 of KSHV/HHV8, like that of HVS, encodes a protein that has about 30% amino-acid identity with human cellular cyclin D2 (Cesarman *et al.*, 1996a; Russo *et al.*, 1996; Li *et al.*, 1997). Cellular D-typed cyclins are implicated in the pathogenesis of several human malignancies (Sherr, 1995, 1996). *v-Cyclin* contains a region of high sequence similarity to the cyclin box domain of cellular cyclins, responsible for interactions with cyclin-dependent kinases (Chang *et al.*, 1996a; Godden-Kent *et al.*, 1997; Li *et al.*, 1997). The KSHV/HHV8 v-cyclin associates predominantly with cyclin-dependent kinase 6 and more weakly with kinase 4 (Godden-Kent *et al.*, 1997; Li *et al.*, 1997), and the cyclin-dependent kinase 6–v-cyclin complex can phosphorylate the physiological target of D-type cyclins, the retinoblastoma protein Rb (Chang *et al.*, 1996a; Godden-Kent *et al.*, 1997; Li *et al.*, 1997). Two-dimensional electrophoresis indicates that KSHV/HHV8 v-cyclin induces phosphorylation of Rb at authentic sites (Chang *et al.*, 1996a). The activation of cyclin-dependent kinase 6 activity induced by the v-cyclins of both HVS and KSHV/HHV8 is much more pronounced than that by human cyclin D2: the two have a broader specificity and can also phosphorylate histone H1 (Godden-Kent *et al.*, 1997; Li *et al.*, 1997). The activity of KSHV/HHV8 v-cyclin *in vivo* was demonstrated in the osteosarcoma cell line SAOS-2, which has homozygous deletions of both *Rb* alleles. When wild-type *Rb* is transfected into SAOS-2 cells, they stop replicating and take on a senescent phenotype characterized by an enlarged cytoplasm. Co-transfection of KSHV/HHV8 *v-cyclin* with *Rb* prevents entry of SAOS-2 into senescence, and the cells continue to proliferate (Chang *et al.*, 1996b).

KSHV/HHV8 *v-cyclin* is expressed in latently infected primary effusion lymphoma cell lines, in uncultured primary effusion lymphomas and in Kaposi's sarcoma tissue (Cesarman *et al.*, 1996a; Godden-Kent *et al.*, 1997). It is expressed in persistently infected cells in concert with K13/v-*FLIP* and ORF 73 (Rainbow *et al.*, 1997).

4.4.7 *Latency-associated nuclear antigen*

ORF 73 of KSHV/HHV8 encodes a large nuclear protein which is identical (Rainbow *et al.*, 1997) to the previously described high-molecular-mass (224–236 kDa) LNA (Gao *et al.*, 1996b) and a component of the LANA, defined by immunofluorescence (Gao *et al.*, 1996a; Kedes *et al.*, 1996). Its size varies between different virus isolates, due to varying lengths of the *moi* repeat region within this ORF (Russo *et al.*, 1996; Rainbow *et al.*, 1997). The *moi* region is translated as a long acidic repeat. *ORF 73*/LNA also encodes a leucine zipper region in the carboxy terminus of the protein (Russo *et al.*, 1996). Its apparent molecular mass (by sodium dodecyl sulfate polyacrylamide gel electrophoresis) is much higher than that predicted from its amino-acid sequence, pro-

bably because of the highly charged nature of the *moi* repeat region and possibly also as a result of post-translational modification. Post-translational modification may also explain the doublet nature of LNA on western blots (Gao *et al.*, 1996b; Rainbow *et al.*, 1997).

The function of ORF 73/LNA is not yet known. It is a latent protein, expressed in latently infected primary effusion lymphoma cell lines and Kaposi's sarcoma spindle cells (Gao *et al.*, 1996a,b; Rainbow *et al.*, 1997) and associated with subnuclear domains (Gao *et al.*, 1996a,b; Kedes *et al.*, 1996; Rainbow *et al.*, 1997), the identity of which remains to be resolved. There is no evidence that ORF 73/LNA has transforming properties. [It is conceivable that, in analogy with *trans*-activating proteins of other herpesviruses, it is primarily involved in controlling transcription of other viral genes but could also affect the expression of cellular genes.]

4.4.8 *G Protein-coupled receptor homologue*

ORF 74 of both KSHV/HHV8 and HVS encodes a homologue of a G protein-coupled receptor with seven membrane-spanning domains (Cesarman *et al.*, 1996a; Arvanitakis *et al.*, 1997). This receptor has highest sequence homology to IL-8 chemokine receptors and is also related to the EBI-1 cellular protein induced by EBV infection (Birkenbach *et al.*, 1993). Studies of transient expression of viral G-protein-coupled receptor suggest that it is constitutively active and does not require ligand binding or is activated by a ligand commonly expressed into cell culture medium. The receptor is unusual in that it can bind chemokines belonging to both the C-X-C and C-C families. Transfection of the KSHV/HHV8 G protein-coupled receptor enhances proliferation of rat kidney fibroblasts (NRK-49F), suggesting that it may contribute to tumour-cell proliferation (Arvanitakis *et al.*, 1997). Its increased expression in primary effusion lymphoma cell lines treated with 12-*O*-tetradecanoylphorbol 13-acetate suggests, however, that it is expressed primarily during lytic replication. While its expression in Kaposi's sarcoma tissue has been documented by RT-PCR (Cesarman *et al.*, 1996b), it is not clear whether it is expressed in persistently infected Kaposi's sarcoma spindle cells or in the few cells in these lesions that are undergoing lytic viral replication.

4.5 Summary of potential roles of KSHV/HHV8 in tumorigenesis

4.5.1 *Kaposi's sarcoma*

KSHV/HHV8 establishes a persistent infection in most Kaposi's sarcoma spindle cells, characterized by a restricted pattern of gene expression (Zhong *et al.*, 1996). All spindle cells and the atypical endothelial cells of early Kaposi's sarcoma lesions express abundant mRNA for *ORF K12*, which encodes a small hydrophobic protein of unknown function (Zhong *et al.*, 1996; Stürtzl *et al.*, 1997; Staskus *et al.*, 1997). Kaposi's sarcoma spindle cells also express the high-molecular-mass LANA encoded by *ORF 73*, as shown by immunohistochemistry with affinity-purified antibodies to this protein. Expression of mRNA for the *ORF 72*-encoded D-type-cyclin homologue has been demonstrated, but as the mRNA encoded *ORF 73/LANA* extends through the *ORF 72/v-cyclin* gene, this result

should be confirmed by immunohistochemical studies with v-cyclin. The *K13/v-FLIP* gene is also contained within the mRNAs encoding *ORF 72/v-cyclin* and *ORF 73/LANA* (Rainbow *et al.*, 1997) and may therefore also be expressed in Kaposi's sarcoma spindle cells, but no immunohistochemical studies on this protein have been reported. The KSHV/HHV8 homologue of IL-6 (*ORF K2*) is expressed during latency in KSHV/-HHV8-infected haematopoietic cells but is not generally expressed in Kaposi's sarcoma lesions (Moore *et al.*, 1996b). Expression of *ORF 74*, which encodes a functional chemokine receptor (Arvanitakis *et al.*, 1997), can be found by RT-PCR (Cesarman *et al.*, 1996c). KSHV/HHV8 virions have been seen in a subpopulation of Kaposi's sarcoma spindle cells and infiltrating haematopoietic cells (Orenstein *et al.*, 1997).

These findings indicate that all or nearly all endothelial tumour (spindle) cells in Kaposi's sarcoma lesions are infected with KSHV/HHV8 (Boshoff *et al.*, 1995b). There is not yet enough evidence to conclude that the gene expression programme of KSHV/-HHV8 (i.e. lytic and latent gene expression programmes) is similar to that of other herpesviruses. A set of viral genes that is expressed in primary effusion lymphoma cells (*ORF K12*, *ORF K13*, *ORF 72*, *ORF 73*) is not up-regulated by chemical treatment and could therefore represent latent genes that are also expressed in Kaposi's sarcoma spindle cells. Since a minority of these cells appears to undergo full lytic replication, it is too early to conclude whether KSHV/HHV8 establishes a latent infection in most spindle cells; however, it is also unclear whether a latent expression pattern is required for the expansion of spindle cells. The presently available evidence is insufficient to conclude whether a limited lytic expression programme is compatible with virus-mediated cellular proliferation.

4.5.2 *Primary effusion lymphoma*

Ninety percent of the described cases of primary effusion lymphoma contain both KSHV/HHV8 and EBV (see Section 1), and the cells contain multiple episomal copies of KSHV/HHV8 (Cesarman *et al.*, 1995a,b; Nador *et al.*, 1996). KSHV/HHV8 gene expression in primary effusion lymphoma has been studied mainly in derived cell lines, and the results may not fully reflect the gene expression pattern *in vivo*. It is, however, largely similar to that in Kaposi's sarcoma lesions (Zhong *et al.*, 1996; see section 4.5.1). In addition, of the genes investigated, *v-bcl-2*, *v-MIP-I*, *v-MIP-II*, *v-IRF*, *v-IL-6* and *v-GCR* are expressed at low levels in primary effusion lymphoma cell lines, and their expression can be up-regulated by treatment with phorbol esters and/or sodium butyrate. The untranslated *T1.1* RNA (see Section 1) is strongly expressed in unstimulated primary effusion lymphoma cell lines but can be further up-regulated by chemical treatment. Unlike Kaposi's sarcoma, primary effusion lymphoma cells strongly express v-IL-6 protein, as shown by immunohistochemistry of fresh primary effusion lymphoma cells (Moore *et al.*, 1996b).

In cell lines concomitantly infected with EBV and KSHV/HHV8, the EBV is monoclonal and its expression is limited to EBNA-1 and (in one case) LMP-2 (Moore *et al.*, 1996a; Nador *et al.*, 1996).

Despite attempts by several groups, there is currently no evidence that KSHV/HHV8 can transform or immortalize lymphocytes *in vitro*.

Given the low level of expression of several KSHV/HHV8 genes shown to dysregulate the cell cycle and/or prevent apoptosis, it is at least conceivable, although unproven, that these viral genes play an important role in the development of primary effusion lymphoma.

4.5.3 *Multicentric Castleman's disease*

Unlike Kaposi's sarcoma and primary effusion lymphoma, multicentric Castleman's disease is not universally associated with KSHV/HHV8 infection, although infection with this virus is very common in HIV-associated cases (see Section 2). Multicentric Castleman's disease is a polyclonal disorder and, like Kaposi's sarcoma, characterized by prominent angiogenesis. No published data are available on KSHV/HHV8 gene expression in these lesions. Given the polyclonal nature of the disease and the inconsistent association with KSHV/HHV8, it is likely that multicentric Castleman's disease has a multifactorial etiology and that the role of KSHV/HHV8 is indirect, conceivably mediated by virus-encoded cytokines.

4.6 Antiviral agents

Specific antiviral therapy has been evaluated in only a few studies and a case series (Morfeldt & Torssander, 1994; Jones *et al.*, 1995; Glesby *et al.*, 1996; Mocroft *et al.*, 1996). All of the currently available antiherpesvirus drugs that have been evaluated belong to the class of DNA polymerase inhibitors, which are active against lytic but not latent herpesviral infection. Morfeldt and Torssander (1994) described clinical regression of AIDS-associated Kaposi's sarcoma lesions in three of five patients after administration of high doses of phosphonoformic acid (foscarnet), but no untreated control patients were available for comparison. Jones *et al.* (1995) examined follow-up data on 20 228 HIV-positive persons and AIDS patients enrolled in a study of adult diseases, of whom 1033 (5%) developed Kaposi's sarcoma. Proportional hazards analysis was used to demonstrate that the risk of patients receiving foscarnet for developing Kaposi's sarcoma after a median of 14 months' follow-up was 30% of that of patients who did not receive herpesviral therapy, after taking into account CD4 count, age, sex, race, route of exposure to HIV, other opportunistic illnesses and antiretroviral therapy ($p = 0.001$). Similar analysis for gancyclovir (odds ratio, 1.0; $p = 0.8$) and acyclovir (odds ratio, 1.4; $p < 0.001$) showed either no effect or an increased risk for disease with therapy. A similar analysis of 935 participants in a multicentre study of AIDS showed a non-significant preventive effect against Kaposi's sarcoma for forscarnet (RR, 0.40; 95% CI, 0.05–3.10; $p = 0.38$) and gancyclovir (RR, 0.56; 95% CI, 0.22–1.44; $p = 0.23$) but not for acyclovir (Glesby *et al.*, 1996). Mocroft *et al.* (1996) followed 3688 patients with HIV infection or AIDS for a median of 4.2 years, of whom 598 (16%) developed Kaposi's sarcoma. After adjustment for sex, route of exposure to HIV, age, antiretroviral treatment, prophylaxis for *Pneumocystis carinii* pneumonia, opportunistic infections and CD4 count, use of foscarnet (relative hazard, 0.38; 95% CI, 0.15–0.95; $p = 0.038$) and

gancyclovir (relative hazard, 0.39; 95% CI, 0.19–0.84; p = 0.015) but not acyclovir (relative hazard, 1.1; 95% CI, 0.88–1.4; p = 0.40) were associated with a decreased risk of developing Kaposi's sarcoma.

Foscarnet has some direct antiretroviral activity (Sandstrom *et al.*, 1985) and may therefore have activity against Kaposi's sarcoma, but other opportunistic tumours and infections (aside from susceptibility to herpesviral infections) are not known to respond to foscarnet therapy.

Kedes and Ganem (1997) demonstrated that gancyclovir, foscarnet and cidofovir at pharmacological concentrations inhibit virion induction *in vitro* in the primary effusion lymphoma cell line BCBL-1, whereas acyclovir does not. An anti-retroviral protease inhibitor and ritonavir analog, A 77003, did not act on KSHV/HHV8 replication.

5. Summary of Data Reported and Evaluation

5.1 Virus–host interactions

Kaposi's sarcoma herpesvirus/human herpesvirus 8 (KSHV/HHV8) is a gamma-2 herpesvirus (a rhadinovirus) with a 165-kb genome. Its closest relatives are *Herpesvirus saimiri* (HVS), a tumorigenic rhadinovirus of New World primates, and a group of recently identified rhadinoviruses in Old World monkeys. It contains blocks of conserved herpesvirus genes that encode mainly structural proteins. In addition, several genes similar in sequence to other viral and cellular oncogenes and growth controlling factors are present in the KSHV/HHV8 genome. These include homologues of interleukin 6, the antiapoptotic protein bcl-2, a D-type cyclin and a chemokine receptor, some of which are known to be functional.

KSHV/HHV8 has been found in B cells, macrophages and dendritic cells *in vivo*. It establishes a persistent infection in endothelial Kaposi's sarcoma spindle cells and in primary effusion lymphoma cells, which involves a disease-specific pattern of expression, with at least four (in Kaposi's sarcoma) or seven (in primary effusion lymphoma cells) viral genes. Lytic replication occurs in a subpopulation of infected spindle and haematopoietic cells.

KSHV/HHV8 DNA is readily detected in Kaposi's sarcoma lesions, primary effusion lymphoma cells and some lymphoid tissue from patients with multicentric Castleman's disease by Southern blotting or polymerase chain reaction (PCR). In contrast, only small amounts of viral DNA are generally present in non-neoplastic tissue from KSHV/HHV8-infected individuals, in particular in peripheral blood mononuclear cells and semen, requiring the use of sensitive PCR techniques for detection. Serological methods have been developed for the detection of antibodies to a latent nuclear protein and to defined and undefined structural antigens, including immunofluorescence assays, enzyme-linked immunosorbent assays and western blotting. Serological and PCR testing of peripheral blood mononuclear cells and semen shows that infection with KSHV/HHV8 is uncommon among the general populations of northern Europe and the United States, but more common in some Mediterranean countries and frequent in parts of Africa; however,

precise estimates of prevalence rates, especially in non-endemic areas, are still not available. There is some evidence that KSHV/HHV8 is sexually transmitted, but other routes of transmission are likely and probably account for a high prevalence in parts of southern Europe and Africa.

5.2 Human carcinogenicity

DNA analysis has consistently demonstrated the presence of KSHV/HHV8 at high (> 90%) rates in Kaposi's sarcoma lesions and at a generally low rate in neoplastic and non-neoplastic tissues from control patients. The load of viral DNA is higher in tissue from Kaposi's sarcomas than in unaffected tissues from the same patients. When mononuclear cells from Kaposi's sarcoma patients and controls were examined by PCR, KSHV/HHV8 was detected in significantly more cases (up to 50%) than controls. Despite differences in sensitivity, in specificity and in the antigens examined, all of the available serological studies are consistent in showing high rates of antibody-positivity in Kaposi's sarcoma patients and lower rates of seropositivity among various controls. Studies among HIV-1-positive and -negative populations at different risks for Kaposi's sarcoma indicate that seroprevalence is generally in accordance with the risk for developing the disease. The limited number of longitudinal analyses based on either detection of KSHV/HHV8 DNA by PCR or the presence of antibodies to KSHV/HHV8 suggest that KSHV/HHV8 infection precedes the development of Kaposi's sarcoma in the majority of cases.

Thus, the strength of association between infection with this virus and Kaposi's sarcoma is high, as measured by PCR, Southern blotting and serology, with odds ratio greater than 10 being found in most studies involving large numbers of cases and well-defined controls. This association is found in studies with various designs and for all epidemiological types of Kaposi's sarcoma.

Primary effusion lymphoma has been recognized as a new disease entity only since the identification of KSHV/HHV8. It has a characteristic morphology and cell surface phenotype, and all of the cases reported in the literature that showed these characteristics have been found to contain KSHV/HHV8 DNA, sometimes at high copy numbers. The vast majority of cases also contain clonal EBV. Owing to the rarity of this malignancy, no epidemiological studies are yet available.

Multicentric Castelman's disease is a rare and usually polyclonal lymphoproliferative disorder. In studies based on very few cases, KSHV/HHV8 has been found in a substantial proportion of HIV-positive patients with this disorder, and a high proportion of these patients also had Kaposi's sarcoma; a much smaller proportion of HIV-negative cases of multicentric Castleman's disease showed KSHV/HHV8 DNA.

KSHV/HHV8 has occasionally been reported to be present in other tumours, but the results are inconsistent. Whereas some of these discrepant results probably reflect the marked geographical differences in KSHV/HHV8 prevalence and the fact that the virus can be detected at several body sites and in samples from some KSHV/HHV8-infected but healthy individuals, other reports are more difficult to explain and remain controversial.

5.3 Animal models

KSHV/HHV8 has not yet been tested for tumorigenicity in experimental animals; however, studies of related viruses have proved informative.

Herpesvirus saimiri (HVS) and *Herpesvirus ateles* (HVA) do not induce disease in their natural hosts, the two New World monkeys, squirrel monkeys and spider monkeys, but they induce tumours and/or lymphoproliferation in a variety of heterologous non-human primates. The natural host populations become infected early in life, perhaps through horizontal transmission, and maintain the virus in latency throughout their lives. Both HVS and HVA are T-lymphotropic viruses and readily transform and immortalize human and simian T cells. As they are gamma-2 herpesviruses (rhadinoviruses), HVS and HVA are more closely related to KSHV/HHV8 than to EBV. Lymphoid cell lines derived either by transformation *in vitro* or from tumour tissues contain viral DNA, express viral proteins and release variable amounts of virus and viral genome copies per cell. HVS induces lymphoid tumours in New Zealand white rabbbits.

There is evidence of gamma-2 herpesviruses in Old World monkeys, which may play a role in retroperitoneal fibromatosis, a condition with some similarities to Kaposi's sarcoma.

Bovine herpesvirus type 4 is another gamma-2 herpesvirus which includes a large number of antigenically related isolates distinct from other bovine herpesviruses. This virus has not been established as the etiological agent of a distinct disease entity, but its role in the etiology of some diseases of the genital tract has been suggested. It has never been identified as a potential cause of tumours.

Murid herpesvirus 4 is a B-lymphotropic gamma-2 herpesvirus that causes B-cell proliferation in mice. As it is a gamma-2 herpesvirus, it could serve as a model for KSHV/HHV8 infection.

5.4 Molecular mechanisms of carcinogenesis

The role of KSHV/HHV8 in the pathogenesis of Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease is still poorly understood. The virus is present in the endothelial tumour (spindle) cells of Kaposi's sarcoma lesions and in primary effusion lymphoma cells. The latter are of monoclonal origin, and there is evidence to suggest that Kaposi's sarcoma lesions are also monoclonal. The viral homologue of D-type cyclins, which can disrupt cell cycle control, is expressed in both these tumour types, as are some other proteins of as yet unknown function. In the case of primary effusion lymphoma, several growth factors, a growth factor regulatory protein and a growth factor receptor are also expressed. Some KSHV/HHV8-infected Kaposi's sarcoma spindle cells undergo lytic replication. It is therefore at present unclear whether, as in EBV, a latent programme of gene expression is required for cellular transformation, with lytic infection of spindle cells representing an abortive pathway. Some viral genes whose expression can be upregulated during lytic infection (e.g. several growth factors and a growth factor regulatory protein) may contribute to virus-mediated expansion of Kaposi's sarcoma spindle cells. There is a striking correspondence between genes

encoded by KSHV/HHV8 and human genes involved in the control of cell growth, which are induced after EBV infection. This suggests that the two viruses may use different strategies to modify the same cellular regulatory and signalling pathways. Similar considerations apply to the role of KSHV/HHV8 in the pathogenesis of primary effusion lymphoma, the cells of which can also undergo lytic infection *in vitro*.

KSHV/HHV8 is not always found in multicentric Castleman's disease, especially in HIV-negative cases. There are no published data on which cell type in these lesions harbours KSHV/HHV8; however, KSHV/HHV8 probably plays an indirect role in this disorder, conceivably involving cytokines such as viral interleukin-6, since cellular interleukin-6 has been implicated in its pathogenesis.

5.5 Evaluation

There is compelling but as yet limited evidence for a role of KSHV/HHV8 in the causation of Kaposi's sarcoma.

KSHV/HHV8 *is probably carcinogenic to humans (Group 2A).*

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Abbreviations

AIDS	acquired immunodeficiency syndrome
BHV	bovine herpesvirus
CI	confidence interval
CIN	cervical intraepithelial neoplasia
CMV	cytomegalovirus
CTL	cytotoxic T lymphocyte
DHFR	dihydrofolate reductase
EA	early antigen
EA(D)	diffuse early antigen
EA(R)	restricted early antigen
EBER	EBV-encoded RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FGF	fibroblast growth factor
FLICE	fas-associated death domain protein interleukin-1β converting enzyme
FLIP	FLICE-inhibiting protein
GMT	geometric mean titre
gp	glycoprotein
HHV	human herpesvirus
HIV	human immunodeficiency virus
HRS	Hodgkin and Reed-Sternberg
HSV	herpes simplex virus
HTLV	human T-cell lymphotropic virus
HVA	*Herpesvirus ateles*
HVP	*Herpesvirus papio*
HVS	*Herpesvirus saimiri*
ICE	interleukin-1β-converting enzyme
IF	interferon
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
Iscom	immunostimulating complex

JAK/STAT	Janus kinase-signal transducer and activator of transcription
kb	kilobase
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA	latency-associated nuclear antigen
LMP	latent membrane protein
LNA	latent nuclear antigen
LP	leader protein
mAb	monoclonal antibody
MALT	mucosa-associated lymphoid tissue
MDV	Marek's disease virus
MHC	major histocompatibility complex
MHV	murid herpesvirus
MIP	macrophage inflammatory protein
NK	natural killer
ORF	open reading frame
oriP	origin of plasmid RNA replication
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RAG	recombinase gene
RBP	recombination binding protein
RR	relative risk
RT	reverse transcriptase
SCID	severe combined immunodeficiency
SMR	standardized mortality ratio
TNF	tumour necrosis factor
TPA	12-*O*-tetradecanoylphorbol 13-acetate
TRAF	tumour necrosis factor receptor-associated factor
v	viral
VCA	viral capsid antigen
VZV	varicella–zoster virus

SUPPLEMENTARY CORRIGENDA TO VOLUMES 1–69

Volume 68

p. 33, line 25, add 'least' between 'were' and 'likely'.

CUMULATIVE CROSS INDEX TO *IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS*

The volume, page and year of publication are given. References to corrigenda are given in parentheses.

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