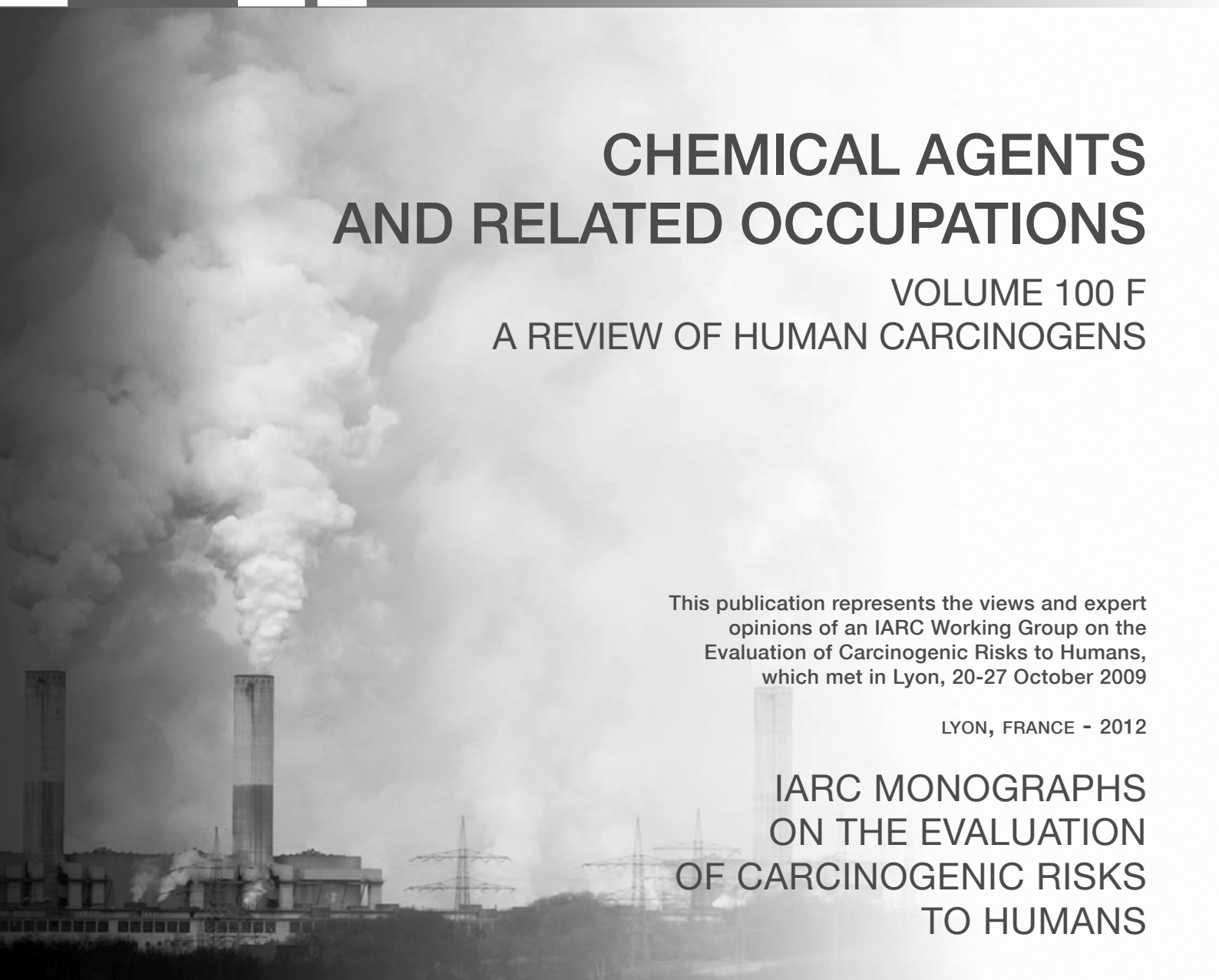
IARC MONOGRAPHS



CHEMICAL AGENTS AND RELATED OCCUPATIONS

VOLUME 100 F
A REVIEW OF HUMAN CARCINOGENS

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, 20-27 October 2009

LYON, FRANCE - 2012

IARC MONOGRAPHS ON THE EVALUATION OF CARCINOGENIC RISKS TO HUMANS

International Agency for Research on Cancer
World Health Organization

AFLATOXINS

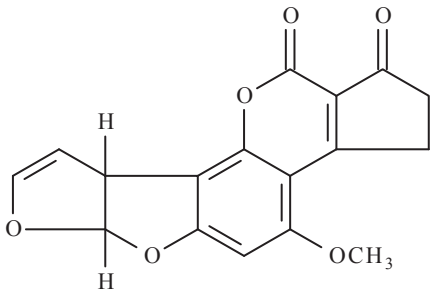
Aflatoxins were considered by previous IARC Working Groups in 1971, 1975, 1987, 1992 and 2002 (IARC, 1972, 1976, 1987, 1993 and 2002). Since that time new data have become available, which have been incorporated in this *Monograph*, and taken into consideration in the present evaluation.

1. Exposure Data

1.1 Identification of the agents

1.1.1 Aflatoxin B1

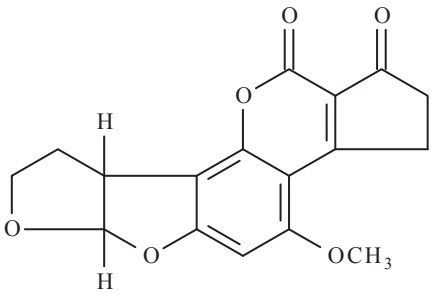
Chem. Abstr. Serv. Reg. No.: 1162-65-8
Chem. Abstr. Serv. Name:
(6a*R*,9a*S*)-2,3,6a,9a-Tetrahydro-4-methoxycyclopenta[*c*]furo-(3′,2′:4,5)furo[2,3-*h*][*l*]benzopyran-1,11-dione



$C_{17}H_{12}O_6$
Relative molecular mass: 312.3

1.1.2 Aflatoxin B2

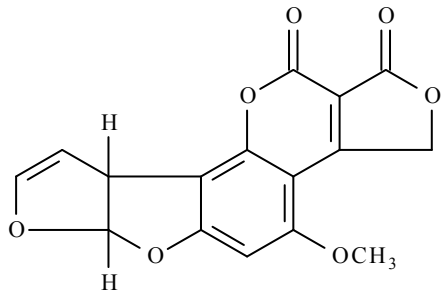
Chem. Abstr. Serv. Reg. No.: 7220-81-7
Chem. Abstr. Serv. Name:
(6a*R*,9a*S*)-2,3,6a,8,9,9a-Hexahydro-4-methoxycyclopenta[*c*]-furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione



$C_{17}H_{14}O_6$
Relative molecular mass: 314.3

1.1.3 Aflatoxin G1

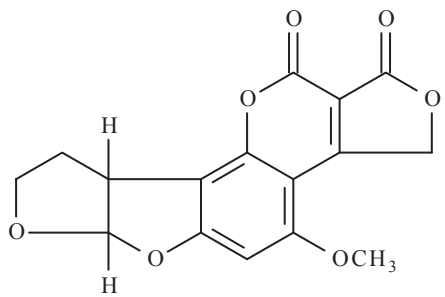
Chem. Abstr. Serv. Reg. No.: 1165-39-5
Chem. Abstr. Serv. Name: (7a*R*,10a*S*)-3,4,7a,10a-Tetrahydro-5-methoxy-1*H*,12*H*-furo-[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione



$C_{17}H_{12}O_7$
Relative molecular mass: 328.3

1.1.4 Aflatoxin G2

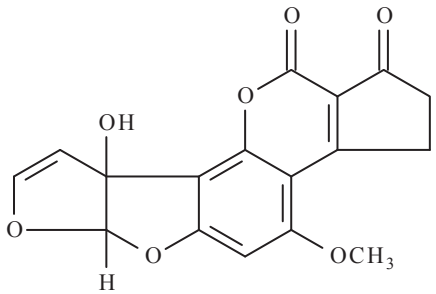
Chem. Abstr. Serv. Reg. No.: 7241-98-7
Chem. Abstr. Serv. Name: (7a*R*,10a*S*)-3,4,7a,9,10,10a-Hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione



$C_{17}H_{14}O_7$
Relative molecular mass: 330.3

1.1.5 Aflatoxin M1

Chem. Abstr. Serv. Reg. No.: 6795-23-9
Chem. Abstr. Serv. Name: (6a*R*,9a*R*)-2,3,6a,9a-Tetrahydro-9a-hydroxy-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione



$C_{17}H_{12}O_7$
Relative molecular mass: 328.3

Description: Aflatoxins form colourless to pale-yellow crystals. Intensely fluorescent in ultraviolet light, emitting blue (aflatoxins B1 and B2) or green (aflatoxin G1) and green-blue (aflatoxin G2) fluorescence, from which the designations B and G were derived, or blue-violet fluorescence (aflatoxin M1).

Solubility: Very slightly soluble in water (10–30 µg/mL); insoluble in non-polar solvents; freely soluble in moderately polar organic solvents (e.g. chloroform and methanol) and especially in dimethyl sulfoxide (IARC, 2002).

1.2 Sources and uses

Aflatoxins are produced primarily by the common fungus *Aspergillus flavus* and the closely related species *A. parasiticus*. These are well defined species: *A. flavus* produces only B aflatoxins and sometimes the mycotoxin cyclopiazonic acid (CPA), while *A. parasiticus* produces both B and G aflatoxins, but not CPA. Aflatoxin M1 is a metabolite of aflatoxin B1 that can occur in milk and milk products from animals

consuming feed contaminated with B aflatoxins (IARC, 2002).

Aspergillus species capable of producing aflatoxins include *A. flavus*, *A. parasiticus*, *A. nomius*, *A. pseudotamarii*, *A. bombycis*, *A. ochraceoroseus*, and *A. australis* (IARC, 2002). *A. flavus* and *A. parasiticus* are responsible for the largest proportion of aflatoxins found in foodstuffs throughout the world. Of the other species, only *A. australis*, which appears to be widespread in the southern hemisphere and is common in Australian peanut soils, may also be an important source of aflatoxins in some countries (IARC, 2002).

Because of the importance of aflatoxins, *A. flavus* has become the most widely reported foodborne fungus – even with the proviso that *A. parasiticus* is sometimes not differentiated from *A. flavus* in general mycological studies. *A. flavus* is especially abundant in the tropics. Levels of *A. flavus* in warm temperate climates such as in the USA and Australia are generally much lower, while the occurrence of *A. flavus* is uncommon in cool temperate climates, except in foods and feeds imported from tropical countries (IARC, 2002).

The major hosts of *A. flavus* among food and feed commodities are maize, peanuts, and cottonseed [Note: the terms maize and peanuts will be used throughout this Volume for corn and groundnuts, respectively]. In addition, various spices sometimes contain aflatoxins, while tree nuts are contaminated less frequently. Small amounts of aflatoxins may be found in a wide range of other foods (IARC, 2002).

It seems probable that although *A. parasiticus* occurs in the same geographical range as *A. flavus*, it is less widely distributed. In particular, it has been found only rarely in south-eastern Asia. The food-related hosts of *A. parasiticus* are similar to those of *A. flavus*, except that *A. parasiticus* is very uncommon in maize (IARC, 2002).

With maize, peanuts, and cottonseed, invasion of plants and developing seed or nut by *Aspergillus spp.* may occur before harvest, resulting in potentially high levels of aflatoxins in these commodities and the continuing difficulty to eliminate aflatoxins from these products. With other crops, prevention of the formation of aflatoxins relies mainly on avoidance of contamination after harvest by use of rapid drying and good storage practice (IARC, 2002).

Apart from natural formation, aflatoxins are produced only in small quantities for research purposes, by fermentation of *A. flavus* or *A. parasiticus* on solid substrates or media in the laboratory. Aflatoxins are extracted by solvents and purified by chromatography (IARC, 1993).

1.3 Human exposure

1.3.1 Exposure of the general population

Dietary intake is the primary non-occupational source of human exposure to aflatoxins. Intakes in the range of nanograms to micrograms per day occur mainly through consumption of maize and peanuts, which are dietary staples in some tropical countries (IARC, 2002).

Aflatoxins have been found in a variety of agricultural commodities, but the most pronounced contamination has been encountered in maize, peanuts, cottonseed, and tree nuts. An extensive review of the amounts of aflatoxins in commodities in North America, South America, Europe, Asia and Africa was included in *IARC Monograph* Volume 56 (IARC, 1993). More recent data were compiled in *IARC Monograph* Volume 82 (IARC, 2002).

Surveys of selected foods for the presence of aflatoxins in many countries have continued to detect some level of contamination; the amounts are highly variable, ranging from < 0.1 µg/kg to hundreds of µg/kg depending on source, food type, climate, storage conditions, and other factors (IARC, 2002). The fraction of samples with detectable levels of aflatoxin B1 or total aflatoxins (B1, B2, G1 and G2) can range from a few percent (e.g. 6.9% of imported peanuts

Table 1.1 Estimated numbers of workers exposed to aflatoxins in the European Union

Industry, occupational activity	
Education services	740
Research and scientific institutes	460
Food manufacturing	320
Water transport	200
Medical, dental, other health, veterinary services	100
Land transport	20
TOTAL	1840

From: CAREX (1999)

in Japan, 1999–2000; Okano *et al.*, 2003) to as much as 30% or more (e.g. maize in some parts of Latin America and Asia (IARC, 2002). Data on the occurrence of aflatoxin M1 in milk were summarized in the previous *IARC Monograph* (IARC, 1993).

From the point of view of dietary intake, aflatoxins in staple foods such as maize are almost all pervading. This contamination poses a far greater problem in the tropics than in temperate zones of the world. However, because of the movement of agricultural commodities around the globe, no region of the world is free from aflatoxins (IARC, 2002).

International exposure estimates on the intake of aflatoxins were summarized in *IARC Monograph* Volume 82 (IARC, 2002). These estimates include data from the 1995 compendium, Worldwide Regulations for Mycotoxins and the 1998 and 2001 reports of the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1998, 2001). The occurrence and assessment of aflatoxins in human biological fluids and tissues (e.g. cord blood, cord serum, and breast milk) were summarized in the previous *IARC Monograph* (IARC, 2002).

Several recent studies have addressed the early detection, prevention and control of aflatoxins in the food and feed chain around the world (Williams *et al.*, 2004; Kabak *et al.*, 2006; Magan, 2006; Strosnider *et al.*, 2006; Bryden, 2007; Kendra & Dyer, 2007; Magan & Aldred, 2007; Wagacha & Muthomi, 2008). These publications described pre- and post- harvest strategies (such as field management, use of biological and chemical agents, improved drying and storage conditions, irradiation, moisture control, biocompetitiveness and biotechnology (e.g. transgenic expression of maize-specific genes)) and early detection methods (such as molecular imprinted polymers, lateral-flow devices, and molecular-based technology).

1.3.2 Occupational exposure

Occupational exposure to aflatoxins can occur during processing and handling of contaminated grains, particularly animal feed. Airborne concentrations at the workplace are typically in the ng/m^3-range, but higher concentrations (up to µg/m^3) have been reported.

Estimates of the number of workers potentially exposed to aflatoxins in Europe have been developed by CAREX, an international information system on occupational exposures to known and suspected carcinogens collected in the period 1990–1993. This CAREX (CARcinogen EXposure) database provides selected exposure data and documented estimates of the number of exposed workers by country, carcinogen, and industry (Kauppinen *et al.*, 2000). Table 1.1 presents the results for aflatoxins in the European Union (CAREX, 1999).

Few studies have evaluated occupational exposures to aflatoxins (IARC, 2002).

Selim *et al.* (1998) collected dust samples from 28 farms in the United States during harvest and unloading, animal feeding, and bin cleaning. Aflatoxin concentrations ranged from 0.00004 to 4.8 μg/m^3. The lowest concentrations were detected during harvest and unloading, the highest during bin cleaning.

Brera *et al.* (2002) collected and analysed a total of 44 full-shift samples (26 personal samples, 18 ambient-air samples) to determine airborne concentrations of aflatoxins B1, B2, G1, and G2 in dust collected at three food-processing plants (cocoa, coffee, and spices) in Tuscany, Italy. Concentrations ranged from below the detection limit (< 0.002 ng/m^3), to 0.130 ng/m^3.

2. Cancer in Humans

2.1 Hepatocellular carcinoma

2.1.1 Previous evaluation

Aflatoxins were last evaluated in *IARC Monograph* Volume 82 (2002) and confirmed as a Group-1 agent. The weight of evidence for the classification of the aflatoxins as Group-1 carcinogens was driven by statistically significantly increased risks for hepatocellular carcinoma (HCC) in individuals exposed to aflatoxins, as measured by aflatoxin-specific biomarkers in cohort studies in Shanghai and Taiwan, China (Ross *et al.*, 1992; Qian *et al.*, 1994; Wang *et al.*, 1996). This effect was independent of exposure to hepatitis B virus (HBV); however, when HBV status was included in the analysis, a greater than multiplicative interaction between aflatoxin exposure and HBV infection was found.

2.1.2 Cohort studies

See Table 2.1 available at http://monographs.iarc.fr/ENG/Monographs/vol100F/100F-18-Table2.1.pdf

There has been no recent update of the cohort studied by Ross *et al.* (1992) and Qian *et al.* (1994). However, the cohort of Wang *et al.* (1996) has been extensively updated in three subsequent reports (Wu *et al.* 2007a, b, 2009). In these studies, the risk for HCC was significantly elevated for subjects with high concentrations of aflatoxin metabolites in the urine. Subjects who were seropositive for the hepatitis-B surface antigen (HBsAg) and had high aflatoxin exposure were at higher risk than those with high aflatoxin exposure only, or HBsAg-seropositivity only. There seemed to be no correlation with polycyclic aromatic hydrocarbon(PAH)-albumin-adduct formation (Wu *et al.* 2007a). The risk was elevated in those with urinary concentrations of the biomarker 8-oxodeoxyguanosine (8-oxodG) above the median, who were also HBsAg-positive (Wu *et al.*, 2007b). In one small cohort the risk for HCC from aflatoxin exposure was also elevated (Ming *et al.* 2002).

2.1.3 Case-series and case–control studies

(a) Aflatoxin-specific TP53 mutations

In recent years, epidemiological and experimental studies have linked exposures to aflatoxin with the formation of a specific mutation in codon 249 in the *TP53* tumour-suppressor gene, which has provided an important biological target for risk assessment. The identification of a strong mechanistic link between exposure to aflatoxin and mutation in *TP53* has triggered analyses of this codon-249 mutation in tumour tissues and blood samples in populations at high risk for HCC. In case-series of HCC patients in China, the prevalence of this mutation ranged from 36–54% (Jackson *et al.*, 2001, 2003; Stern *et al.*, 2001; Ming *et al.*, 2002). In the one case–control study in China, Huang *et al.* (2003) found an adjusted odds ratio of 22.1 (95%CI: 3.2–91.7) for the presence of a codon-249 *TP53* mutation among HCC cases compared with controls. In contrast, case-series in Africa found a much

lower prevalence of this type of mutation in some populations, ranging from 1% in one study in Egypt to 35% in The Gambia, West Africa.

(b) Metabolic polymorphisms and HCC risk from aflatoxin

See Table 2.2 available at http://monographs.iarc.fr/ENG/Monographs/vol100F/100F-18-Table2.2.pdf.

The availability of aflatoxin-specific biomarkers has enhanced the possibility to monitor individual exposure to this agent. In three case–control studies (two nested within cohorts) an analysis of a variety of genetic polymorphisms as probable modifiers of risk from aflatoxin, has been undertaken in regions of high HCC incidence (Sun *et al.*, 2001; McGlynn *et al.*, 2003; Kirk *et al.*, 2005). These polymorphisms are predicated on the hypothesis that enhanced detoxication or activation pathways of aflatoxin exposure will be a surrogate biomarker of exposure. All studies were limited because of small numbers of subjects in high-risk strata, but two studies were consistent in finding an increased risk for HCC among those with the *GSTM1*-null genotype, and in one of these studies the risk was elevated among those with the highest consumption of peanuts (an index of consumption of aflatoxin-contaminated food).

(c) Aflatoxin biomarkers of exposure

See Table 2.3 available at http://monographs.iarc.fr/ENG/Monographs/vol100F/100F-18-Table2.3.pdf.

Biomarkers of exposure to aflatoxin have been evaluated for association with risk for HCC in two case–control studies. The risk was significantly higher in those who were HBAsG-positive (Omer *et al.*, 2001, 2004; Liu *et al.*, 2008), in those who carried the *GSTM1*-null genotype (Omer *et al.*, 2001), and in those with oxidative stress (Liu *et al.*, 2008). In one study, it was determined that the attributable risk for the effects of exposure to aflatoxin and HBsAg-positivity was of the order of 80% (Omer *et al.*, 2004).

2.2 Synthesis

Geographically distinct cohort studies in Shanghai and Taiwan, China have independently found statistically significant effects of exposure to aflatoxin on the development of HCC. These results, buttressed by the information from several case-series and case–control studies also confirm that in the presence of HBV exposure, as judged by HBsAg status, there is a greater than multiplicative interaction between aflatoxin and HBV, increasing the risk for HCC. Further evidence of the role of aflatoxins in the development of HCC was gained from studies that demonstrated the ability of aflatoxins to induce a specific mutation in codon 249 of the *TP53* tumour-suppressor gene.

3. Cancer in Experimental Animals

3.1 Previous evaluations

Carcinogenicity studies in experimental animals, with administration of aflatoxin mixtures and aflatoxin B1, B2, M1, G1, or G2 to rats, mice, hamsters, salmon, trout, ducks, tree shrews, woodchucks and monkeys by several routes of exposure have been previously reviewed (IARC, 1993, 2002).

See Table 3.1.

The two previous IARC evaluations concluded that there was *sufficient evidence* for the carcinogenicity in experimental animals of naturally occurring mixtures of aflatoxins and of the individual aflatoxins B1, G1, and M1; there was *limited evidence* for aflatoxin B2, and *inadequate* evidence for aflatoxin G2. This *Monograph* reviews relevant carcinogenicity studies published since 2002.

Table 3.1 Carcinogenicity studies in experimental animals exposed to aflatoxins

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Rat, F344 (M) 59 wk Wogan *et al.* (1971)	0, 3, 750 µg (total dose) aflatoxin B2 5 d/wk, ip for 8 wk	Hepatocellular carcinomas: 0/10 and 3/9 at 57–59 wk	[NS]	Group size NR
Rat, F344 (M) 68 wk Wogan *et al.* (1971)	0, 700, 1400, 2000 µg (total dose) aflatoxin G1 by oral gavage 4 d/wk for 2.5 or 8 wk	Hepatocellular carcinomas: 0/10, 0/3, 3/5, 18/18 Kidney: adenocarcinomas: 4/26 dosed animals	[$P < 0.05$], two higher doses –	Group size NR
Rat, F344 (M) 100 wk Wogan & Paglialunga *et al.* (1974)	0 (control) or 25 µg aflatoxin M1 5 d/wk for 8 wk, oral gavage 12–29/group	Liver tumours: 0/12, 1/29	[NS]	Purity > 99%
Rat, F344 (M, F) 18 mo Frayssinet & Lafarge-Frayssinet (1990)	30% peanut-oil cake (control) or diet with 1000 ppb aflatoxin B1 and 170 ppb aflatoxin G1 19–20/group (M), 10–11/group (F)	Liver carcinomas (M): 0/20, 18/19 Liver carcinomas (F): 0/10, 5/11	[$P < 0.0001$] [$P < 0.05$]	
Rat, Wistar WAG (MF) 18 mo Frayssinet & Lafarge-Frayssinet (1990)	30% peanut oil cake (control) or diet with 1000 ppb aflatoxin B1 and 170 ppb aflatoxin G1 17–20/group (M), 10–11/group (F)	Liver carcinomas (M): 0/20, 17/17 Liver carcinomas (F): 0/10, 9/11	[$P < 0.0001$] [$P < 0.005$]	
Rat, F344 (M) 21 mo Hsieh *et al.* (1984)	0 (control), 5 or 50 µg/kg of diet aflatoxin M1 18–25/group	Benign and malignant liver tumours: 0/21, 0/25, 6/18*	*[$P < 0.01$]	* includes 2 hepatocellular carcinomas
Rat, MRC (M, F) 100–105 wk Butler *et al.* (1969)	0 (control), 20 or 60 µg aflatoxin G1/animal in the drinking-water, 5 d/wk/20 wk 11–15/group/sex	Benign and malignant liver tumours 0/15, 2/15, 9/11 (M) 0/15, 1/15, 12/15 (F)	[significant], high dose (M, F)	Liver tumours were mainly hepatocellular carcinomas
Rat, Wistar 64 wk Hao *et al.* (2009)	0 (control) or 100–200 µg aflatoxin B1/kg bw 1–3×/wk, ip 11–25/group	Hepatocellular carcinomas: control, 0/11; treated, 19/25	[$P < 0.0001$]	Sex NR
Trout (*S. gairdneri*) up to 16 mo Ayres *et al.* (1971)	0 (control), 4 ppb aflatoxin B1, 8 ppb aflatoxin B1, and 20 ppb aflatoxin G1 in diet 20–57/group	Liver hepatomas (12 mo): 0/20; 10/40; 40/57; 1/20 Liver hepatomas (16 mo): 0/40; 14/40; 32/40; 7/40	[$P < 0.05$], [$P < 0.0001$], [NS] [$P < 0.0001$],[$P < 0.0001$], [$P < 0.05$]	

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Trout (fry) up to 12 mo Bailey *et al.* (1994a)	Positive controls received 4 µg/kg aflatoxin B1 in diet for 12 mo. Positive controls received 20 µg/kg aflatoxin B1 in diet for 2 wk. Positive controls received aflatoxin M1 (80 or 800 µg/kg) for 2 wk. Positive controls received 64 µg/kg aflatoxin B1 for 2 wk Control and treated groups received a maximum of 8 µg/kg aflatoxin M1 for 2 wk (n = 110, total)	Liver: 34% (39/116) tumours at 12 mo Liver: 37% (68/186) tumours at 9 mo Liver: 5.7% (11/193) and 50% tumours, respectively at 9 mo Liver: 29% (80/278) tumours at 12 mo Liver: no tumours (0/110) at 12 mo in both groups	[significant] [significant] [significant] [significant] –	Study was designed to look at treatment of food source to reduce effect of aflatoxin contamination of feed. Liver-tumour data shown here are only for the 'positive controls' given aflatoxins in the diet. Liver tumours were predominantly hepatocellular carcinomas and mixed carcinomas (> 70%).
Trout (*O. mykiss*, Shasta strain) (fry) 9 mo Bailey *et al.* (1994b)	0, 4, 8, 16, 32, 64 ng aflatoxin B1 or aflatoxicol in diet for two wk 200 controls/group; 400 treated/group	Liver tumours: 0/192, 25/382, 98/387, 194/389, 287/389, 302/383 for aflatoxin B1. Aflatoxicol also caused liver tumours.	[significant]	Liver tumours were predominantly hepatocellular carcinomas and mixed carcinomas (> 80%).
Trout (*O. mykiss*, Shasta strain) (fry) 13 mo Bailey *et al.* (1994b)	0, 0.01, 0.025, 0.05, 0.1, 0.25, 0.5 µg/ml aflatoxin B1 or aflatoxicol solution exposure of embryo for 1 h and diet exposure at swimup for 13 mo 400 treated/group	Liver tumours: 1/349, 15/346, 59/348, 131/343, 191/343, 254/347, 252/313 for aflatoxin B1 Aflatoxicol also caused liver tumours	[significant]	Diet exposure unclear. Liver tumours were predominantly hepatocellular carcinomas and mixed carcinomas (> 70%).
Trout (fry) up to 12 mo Bailey *et al.* (1998)	4–64 µg/kg of aflatoxin B1, aflatoxicol, aflatoxin M1, aflatoxicol M1 in diet for 2 wk 120 treated/group	Liver tumour response: aflatoxin B1 (1.000); aflatoxicol (0.936); aflatoxin M1 (0.086); aflatoxicol M1 (0.041)		Tumour response is relative to aflatoxin B1, 1.000. Liver tumours were predominantly malignant (> 80%).
Trout (fry) 13 mo Tilton *et al.* (2005)	0 (control) or 0.5 µg/mL aflatoxin B1 in 0.01% ethanol for 30 min (exposure in tank), ≈400/group	Liver tumours: control 0/~400; treated, 20/~400 (30% hepatocellular carcinomas, 70% mixed carcinomas)	[significant]	Limited reporting of study

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Transgenic mouse TGF-β1 and wild type (C57Bl/6J x CBA) 12 mo Schnur *et al.* (1999)	Aflatoxin B1 (6 µg/kg bw) given as a single ip injection to wild type and transgenic mice 11 wild type, 12 transgenic (exposed) 9 wild type, 19 transgenic (controls)	Liver neoplasms: 0/9 wild type and 0/19 transgenic controls; 3/11 wild type and 3/12* exposed transgenic animals	*[$P < 0.05$]	Limited reporting of study. Transgenic mice overexpress TGF-β1. Liver tumours were mainly adenomas. Sex unspecified.
Transgenic mouse (+/–) FVB/N (wild type), p53 (+/–), HBVTg, and HBVTg-p53 (+/–) (M, F) 12–13 mo Cullen *et al.* (2009)	FVB/N; FVB/N + 1 mg/kg bw aflatoxin B1, single injection, ip; HBVTg, HBVTg + aflatoxin B1; p53 (+/–); p53 (+/–) + aflatoxin B1; HBVTg-p53 (+/–); HBVTg-p53 (+/–) + aflatoxin B1 15–30/group	Liver neoplasms (M): 0/19, 2/21, 0/32, 3/20, 1/30, 1/15, 0/29, 5/24* Liver neoplasms (F): 0/21, 0/20, 0/23, 0/19, 0/19, 0/17, 0/22, 2/29	*$P < 0.01$	Liver neoplasm only in groups exposed to aflatoxin B1. Aflatoxin B1 increased the incidence in HBVTg and p53 (+/–) mice.
Transgenic mouse with C3H/HeN background 11 mo Takahashi *et al.* (2002)	XPA+/–, +/–, –/– with 0.6 or 1.5 mg/kg bw aflatoxin B1 as single injection, ip 11–30/group	Liver carcinomas 0.6 mg/kg: 0%, 13%, 50%* 1.5 mg/kg: 6%, 6%, 38%*	*$P < 0.05$	Also significant for benign liver tumours and tumour multiplicity at 0.6 mg/kg. Also significant for liver tumour multiplicity at 1.5 mg/kg.
Transgenic mouse Hupki (human *TP53* knock-in) 18 mo Tong *et al.* (2006)	Wild type (129/Sv background); Hupki; Wild type + 6 µg aflatoxin B1, as single ip injection; Hupki + 6 µg aflatoxin B1, as single ip injection 21–46/group	Hepatocellular adenomas: 0/30, 0/46, 9/21, 6/34 Hepatocellular carcinomas: 0/30, 0/46, 4/21, 15/34	$P = 0.041$ in Hupki with aflatoxin B1 compared with wild type with aflatoxin B1 $P = 0.057$ in Hupki with aflatoxin B1 compared with wild type with aflatoxin B1	Sex NR
Mouse NIH 58–74 wk Huang *et al.* (2004)	Aflatoxin G1: 0 (control), 3 µg/kg bw or 30 µg/kg bw by gavage 3x/wk for 24 wk 10–14/group	Lung adenocarcinomas: 0% (0/11), 30% (3/10), and 43% (6/14)	High dose, $P = 0.02$	Sex NR
Tree shrew 160 wk Su, *et al.* (2004)	Dietary (milk) dose of 0 (control) or 200–400 µg aflatoxin B1/kg bw/d 20–29/group	Hepatocellular carcinomas: control, 0/20; aflatoxin B1-treated, 6/29	[$P < 0.05$]	Sex NR

Table 3.1 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Tree shrew (M, F) 160 wk Li *et al.* (1999)	Control, HBV+, aflatoxin B1-treated, HBV+/aflatoxin B1-treated Aflatoxin B1 (150 µg/kg bw/d) in feed for 105 wk 4–11/sex/group	Hepatocellular carcinomas in 67% (14/21) of males and females (combined) that were injected with HBV and fed aflatoxin B1. Aflatoxin alone resulted in 30% (3/10) hepatocellular carcinomas (male and female combined). No tumours in the two other groups	$P < 0.01$ (HBV and aflatoxin B1 group compared with aflatoxin B1 group).	Age NR
Tree shrew 150 wk Duan *et al.* (2005)	Dietary (milk) dose of 0 (control) or 150 µg/kg bw/d, 5 x/wk for 105 wk 13–48/group	Hepatocellular carcinomas: control, 0/13; aflatoxin B1, 35/48	[$P < 0.0001$]	Age and sex NR
Tree shrew (M, F) 90 wk Li *et al.* (2008)	Dietary (milk) dose of 0 (control) or 400 µg/kg bw/d 12–15/group	Hepatocellular carcinomas: control, 0/12; aflatoxin B1, 11/15	[$P < 0.0001$]	Age NR
Woodchuck (M, F) 25 wk Bannasch *et al.* (1995)	Control, WHV+, aflatoxin B1 (20–40 µg/kg bw in diet) and WHV+/aflatoxin B1 treated 6/group	Liver tumours: 0/9, 5/9, 0/5, 2/5		Animals were 10 mo of age

bw, body weight; d, day or days; HCC, hepatocellular carcinoma; ip, intraperitoneal; min, minute or minutes; mo, month or months; NR, not reported; NS, not significant; WHV, woodchuck hepatitis virus; wk, week or weeks; XPA, *Xeroderma pigmentosum* (a protein, involved in nucleotide excision-repair)

3.2 Aflatoxin B1

3.2.1 Transgenic mouse

An 11-month study was conducted with transgenic mice deficient in the XPA (*Xeroderma pigmentosum* A) protein. This protein recognizes various types of DNA damage, binds to the damaged DNA region and functions in the first step of the nucleotide excision-repair process. Treatment of these *XPA*$^{-/-}$ mice with a single dose of aflatoxin B1 given by intraperitoneal injection resulted in an increased incidence of liver carcinomas compared with the incidence in wild-type mice (Takahashi *et al.*, 2002). An 18-month study in Hupki (human *TP53* knock-in) transgenic mice that received a single dose of aflatoxin B1 by intraperitoneal injection, showed increased incidences of hepatocellular adenomas (P = 0.041) and carcinomas (P = 0.057) (Tong *et al.*, 2006). A 12–13 month study in FVB/N and *p53*$^{+/-}$ mice (with or without transgenic hepatitis-B virus expression) exposed to a single dose of aflatoxin B1 by intraperitoneal injection, showed liver tumours (hepatocellular adenomas and carcinomas combined) in *p53*$^{+/-}$ HBV-transgenic male mice (Cullen *et al.*, 2009).

These three studies in mice confirm earlier findings of Schnur *et al.* (1999) of an increased incidence of liver tumours (mainly adenomas) in TGF-β1-transgenic mice given aflatoxin B1.

3.2.2 Rat

An intraperitoneal study in Wistar rats confirmed that aflatoxin B1 is a liver carcinogen in this species (Hao *et al.*, 2009).

3.2.3 Tree shrew

A carcinogenicity study to detect alterations in the *p53* and *p21* genes in hepatocellular carcinomas in tree shrews infected with HBV showed an increased incidence of hepatocellular carcinomas in animals that had received aflatoxin by the oral route (Su *et al.*, 2004). This finding was confirmed by Duan *et al.* (2005) and Li *et al.* (2008) in similar studies.

3.2.4 Trout

A study by Tilton *et al.* (2005) confirmed that aflatoxin B1 is a liver carcinogen in trout. In this study, trout embryos were exposed for 30 minutes to water containing 50 ppb aflatoxin F1, and kept for a further 13 months.

3.3 Aflatoxin G1

3.3.1 Mouse

A 58–74-week study in NIH mice given aflatoxin G1 by gavage resulted in an increased incidence in lung adenocarcinomas (Huang *et al.*, 2004).

3.4 Synthesis

Table 3.1 lists the more recent studies described above and also summarizes several of the previously evaluated studies.

Results of additional carcinogenicity studies in animals reported since the previous IARC evaluations are consistent with the conclusions of previous Working Groups. Studies performed with trouts (whole-body exposure), in transgenic mouse models (by intraperitoneal injection), in mice (by gavage), and in tree shrews (via the diet) strengthen the original conclusions of *sufficient evidence* for carcinogenicity in experimental animals of aflatoxin B1 and G1. Aflatoxin B1 increases the incidence of liver cancer in rats, tree shrews, trouts, and transgenic mice. Aflatoxin G1 increases the incidence of liver cancer in rats.

4. Other Relevant Data

Experimental studies on aflatoxins have been reviewed in previous *IARC Monographs* (IARC, 1993, 2002). There is an extensive body of information related to the mechanism of aflatoxin-induced carcinogenicity, encompassing data on toxicokinetics, metabolism, genotoxicity, molecular biology, interactive effects with HBV, and human susceptibility factors. Aflatoxins are naturally occurring mycotoxins that are well documented hepatocarcinogens in humans (IARC, 1993, 2002; Gomaa *et al.*, 2008). At least 13 different types of aflatoxin are found naturally. Aflatoxin B1 is considered the most potent of the aflatoxins and is produced by *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxin B1 is genotoxic in prokaryotic and eukaryotic systems *in vitro*, including cultured human cells, and *in vivo* in humans and in a variety of animal species. Exposure to aflatoxin B1 induces adducts to DNA and albumin, gene mutations and chromosomal alterations including micronuclei and sister chromatid exchange, and mitotic recombination. Exposure to aflatoxin B1 is mechanistically associated with a specific AGG→AGT transversion mutation in codon 249 of the *TP53* gene in human hepatocellular carcinoma, providing mechanistic support for a causal link between exposure and disease (Gomaa *et al.*, 2008).

The key steps in the mechanism of carcinogenicity of aflatoxins involve metabolism to the reactive *exo*-epoxide, binding of the *exo*-epoxide to DNA resulting in formation of DNA adducts, and miscoding in replicating DNA, which leads to development of mutations with eventual progression to tumours. Biological interactions with HBV also play a role in the hepatic carcinogenicity of aflatoxins in humans (IARC, 2002).

4.1 Toxicokinetics

Rigorous quantitative comparisons of dietary intakes and the amount of aflatoxin metabolites in body fluids following absorption and distribution are lacking. As noted in previous *Monographs* (IARC, 1993, 2002), aflatoxin M1 concentrations in human urine and human breast milk have been correlated with dietary aflatoxin intake (Gan *et al.*, 1988; JECFA, 2001). Using aflatoxin-specific monoclonal antibody-based immunoaffinity chromatography, Wild *et al.* (1992) measured aflatoxin concentrations in cooked foods in a village in The Gambia. Estimated intakes of aflatoxins were less than those derived from the levels of aflatoxin–serum adducts and the concentrations in urine of the same individuals (Wild *et al.*, 1992). In humans, as in other species, the DNA binding and carcinogenicity of aflatoxin B1 result from its conversion to the 8,9-epoxide by cytochrome P450 (CYP) enzymes (Essigmann *et al.*, 1982; Guengerich, *et al.*, 1998). There is interindividual variation in the rate of activation of aflatoxins, including differences between children and adults. These differences may be relevant to the pharmacokinetics of aflatoxins, which in humans have still not been fully elucidated (Ramsdell & Eaton, 1990; Wild *et al.*, 1990).

Factors that explain differences in the response to aflatoxin between human individuals and animal species and strains include the proportion of aflatoxin metabolized to the 8,9-*exo*-epoxide (mainly by CYP enzymes) relative to other, much less toxic metabolites, and the prevalence of pathways that lead to the formation of non-toxic conjugates with reduced mutagenicity and cytotoxicity (Guengerich *et al.*, 1998).

After dermal application, aflatoxin B1 is absorbed via the skin in rats (Wei *et al.*, 1970). Aflatoxins are absorbed from the gut of sheep (Wilson *et al.*, 1985) and rats (Kumagai, 1989) and distributed via the blood, not by the lymphatic

system. In rats, absorption after intratracheal instillation is more rapid than after an oral dose, but the body distribution and excretion patterns are not different for these two routes of administration (Coulombe & Sharma, 1985). When a tracheally administered dose was first adsorbed onto dust, the binding of aflatoxin B1 to lung and tracheal DNA was increased and retention in the trachea was prolonged, compared with administration of microcrystalline aflatoxin B1 alone (Coulombe *et al.*, 1991). Aflatoxin is also rapidly absorbed after inhalation by the rat, resulting in the formation of hepatic DNA adducts (Zarba *et al.*, 1992). Aflatoxin B1 as weIl as aflatoxin M_1 are concentrated in the liver of rats 30 minutes after an intraperitoneal or oral dose of 7 mg/kg bw ^{14}C-aflatoxin B1; at 24 hours, both aflatoxins were detected only as traces (Wogan, 1969). In-vitro studies with bovine melanin have shown that unmetabolized aflatoxin B1 binds reversibly to this pigment (Larsson *et al.*, 1988).

More aflatoxin-B1 metabolites are usually excreted in rat faeces than in urine after intraperitoneal injection of [^{14}C]-ring-labelled aflatoxin B1 (Wogan, 1969). Intraperitoneal co-injection of [^{3}H]-glutathione and aflatoxin B1 (AFB1) in rats showed that the excretion of [^{3}H]-GSH–AFB1conjugates proceeds almost exclusively through the bile: 14% of the radioactivity was excreted as the conjugate by this route, and only traces were found in urine (Emerole, 1981). Degradation of aflatoxin B1–glutathione conjugate by enzymes of the mercapturic-acid pathway has been described in rat-kidney preparations *in vitro* (Moss *et al.*, 1985). The extent of urinary excretion of aflatoxin B1–mercapturate, together with the sulfate and glucuronide conjugates, correlates with species-sensitivity to aflatoxin B1 (Raj & Lotlikar, 1984).

In a more recent study, aflatoxin B1 (AFB1) was administered to rats by gavage for nine consecutive days at eight dose levels ranging from 50 pg/kg bw to 55 µg/kg bw (Scholl *et al.*, 2006). The dose–response relationship was linear-quadratic, with an upward curvature at higher doses. The adduct yield [(pg Lys-AFB1/mg albumin)/(µg AFB1/kg body wt)] increased sixfold, nonlinearly with the dose between the 0.05- and 55-µg AFB_1/kg bw groups, and showed the onset of saturation in the highest dose group, where the adduct yield was approximately 2%.

A recent study by Jubert *et al.* (2009) investigated aflatoxin-B1 pharmacokinetics in human volunteers by use of microdosing techniques and Accelerator Mass Spectrometry (AMS). The kinetics of low-dose aflatoxin B1 were investigated in three volunteers who received an oral dose of 30 ng [^{14}C]-labelled aflatoxin. AMS was used to measure the levels of aflatoxin equivalents in plasma and urine. Pharmacokinetic modelling of absorption and disposition showed that excretion was rapid, with 95% of the total urinary aflatoxin-B1 equivalents produced within the first 24 hours. Absorption of aflatoxin-B1 equivalents into the systemic circulation was also rapid, with peak concentrations being reached within approximately 1 hour. Changes in plasma concentrations of aflatoxin-B1 equivalents following intervention in each subject mirrored those seen in urine. The authors did not discriminate between free aflatoxin B1 and its various metabolites or conjugates. Based on total [^{14}C] equivalents, aflatoxin B1 was rapidly absorbed into plasma in all volunteers, with first-order kinetics.

4.2 Metabolism

The metabolism of aflatoxin B1 in humans and laboratory animals has been well characterized (Essigmann *et al.*, 1982; Eaton & Gallagher, 1994; McLean & Dutton, 1995; Gallagher *et al.*, 1996; Code *et al.*, 1997; Guengerich *et al.*, 1998; Ueng *et al.*, 1998; IARC, 2002). CYP1A2, 2B6, 3A4, 3A5, 3A7 and GSTM1 are enzymes that mediate aflatoxin metabolism in humans. The overall contribution of these enzymes to aflatoxin-B1 metabolism *in vivo* will depend not

only on their affinity but also on their expression level in human liver, where CYP3A4 is predominant. This enzyme mediates the formation of the *exo*-epoxide and aflatoxin Q_1, while CYP1A2 can generate some *exo*-epoxide but also a high proportion of *endo*-epoxide and aflatoxin M_1. *In vitro* evidence that both these enzymes are responsible for aflatoxin metabolism in humans has been substantiated by biomarker studies. Aflatoxins M_1 and Q_1, produced by CYP1A2 and 3A4, respectively, are present in the urine of individuals exposed to aflatoxin (Ross *et al.*, 1992; Qian *et al.*, 1994). In humans, as in other species, the DNA-binding and carcinogenicity of aflatoxin B1 result from its conversion to the aflatoxin B1 8,9-*exo*-epoxide by CYP3A4 (Essigmann *et al.*, 1982). This epoxide is highly reactive and is the main mediator of cellular injury (McLean & Dutton, 1995).

CYP3A5, in contrast to CYP3A4, metabolizes aflatoxin B1 mainly to the *exo*-8,9-epoxide but is about 100-fold less efficient in catalysing 3-hydroxylation of aflatoxin B1 to yield the aflatoxin Q_1 metabolite (Wang *et al.*, 1998). Hepatic CYP3A5 expression differs markedly between individuals. Factors that explain the variation in response to aflatoxin among human individuals, animal species and strains include the proportion of aflatoxin metabolized to the 8,9-*exo* and *endo*-epoxide relative to other, much less toxic metabolites and the prevalence of pathways forming non-toxic conjugates with reduced mutagenicity and cytotoxicity (Eaton & Gallagher, 1994; McLean & Dutton, 1995; Guengerich *et al.*, 1998).

The expression of enzymes involved in aflatoxin metabolism can be modulated with chemopreventive agents, resulting in inhibition of DNA-adduct formation and hepatocarcinogenesis, as has been demonstrated in rats. Oltipraz is a chemopreventive agent that increases glutathione conjugation and inhibits the activity of some cytochrome P450 enzymes (e.g. CYP1A2). Results from clinical trials with oltipraz in the People's Republic of China are consistent with experimental data in showing that, following dietary exposure to aflatoxins, modulation of the metabolism of aflatoxins can lead to reduced levels of DNA adducts (IARC, 2002; Kensler *et al.*, 2005).

There are marked interspecies differences in sensitivity to aflatoxin-induced carcinogenesis (Gorelick, 1990; Eaton & Gallagher, 1994; Eaton & Groopman, 1994). For example, the adult mouse is almost completely refractory to tumour formation except under conditions of partial hepatectomy, or as a result of liver injury through expression of transgenically induced hepatitis-B virus antigens. In contrast, the rat is extremely sensitive. A considerable part of this interspecies variation is understood in terms of differences in activation and detoxification activities of aflatoxin-metabolizing enzymes in the pathways described above (IARC, 2002). Microsomal preparations from mice show a higher specific activity for aflatoxin-B1 8,9-epoxide production than those from the rat (Ramsdell & Eaton, 1990). However, in the mouse, the resistance to aflatoxin carcinogenesis is largely, if not exclusively, explained by the constitutive hepatic expression of an α-class GST, mGSTA3–3, a detoxifying enzyme with a high affinity for aflatoxin B1 8,9-epoxide (Buetler & Eaton, 1992; Hayes *et al.*, 1992). In contrast, rats do not constitutively express a GST isoform with high epoxide-conjugating activity, but they do express an inducible α-class GST (rGSTA5–5) with high activity. The induction of this enzyme plays a major role in the resistance of rats to aflatoxin-B_1-induced hepatocarcinogenicity following treatment with enzyme inducers including oltipraz, ethoxyquin and butylated hydroxyanisole (Kensler *et al.*, 1986, 1987; Hayes *et al.*, 1991, 1994; Pulford & Hayes, 1996).

Current knowledge of the molecular mechanisms of aflatoxin-induced carcinogenesis contributes to the understanding of the nature of the biological interaction between hepatitis B virus (HBV) and aflatoxins in determining the

risk for hepatocellular carcinoma (IARC, 2002). In Asia and Africa, where the majority of cases are found, aflatoxins and hepatitis viruses (HBV and HCV) are important factors giving rise to extraordinarily high incidence rates (24.2–35.5/100000) of hepatocellular carcinoma. In these areas, HBV-induced chronic active hepatitis and cirrhosis constitute major risk factors for liver cancer.

Infection with HBV may increase aflatoxin metabolism. In HBV-infected children in The Gambia there was a higher level of aflatoxin–albumin adducts than in non-infected children, an observation consistent with altered aflatoxin metabolism (Allen *et al.*, 1992; Turner *et al.*, 2000). However, similar studies in adults did not show such differences (Groopman *et al.*, 1992; Wild *et al.*, 2000). Glutathione *S*-transferase activity is reduced in human liver in the presence of HBV infection (Zhou *et al.*, 1997). In HBV-transgenic mice, liver injury is associated with increased expression of cytochrome P450 enzymes (Kirby *et al.*, 1994).

4.3 Aflatoxin–albumin adducts

4.3.1 Aflatoxin–albumin adducts as biomarkers of exposure in children

Gong *et al.* (2003) conducted a cross-sectional study in Benin and Togo to investigate aflatoxin exposure in children around the time of weaning and correlated these data with food consumption, socioeconomic status, agro-ecological zone of residence, and anthropometric measures. Blood samples from 479 children (age, 9 months to 5 years) from 16 villages in four agro-ecological zones were assayed for aflatoxin–albumin adducts as a measure of recent (2–3 months) past exposure. Aflatoxin–albumin adducts were detected in 475/479 (99%) children (geometric mean 32.8 pg/mg, 95%CI: 25.3–42.5). Adduct levels varied markedly across agro-ecological zones, with mean values being approximately four times higher in the central than in the northern region. The aflatoxin–albumin adduct level increased with age up to three years, and was significantly (P = 0.0001) related to weaning status of the 1–3-year age group: weaned children had approximately twofold higher mean aflatoxin–albumin adduct levels (38 pg aflatoxin–lysine equivalents per mg of albumin [pg/mg]) than those receiving a mixture of breast milk and solid foods, after adjustment for age, sex, agro-ecological zone, and socioeconomic status. A higher intake of maize, but not peanuts, in the preceding week was correlated with higher aflatoxin–albumin adduct levels in the children. The prevalence of stunted growth (height for age Z-score, HAZ) and being underweight (weight for age Z-score, WAZ) were 33% and 29%, respectively, by World Health Organization criteria. Children in these two categories had 30–40% higher mean aflatoxin–albumin levels than the remainder of the children, and strong dose–response relationships were observed between aflatoxin–albumin levels and the extent of stunting and being underweight. Polychronaki *et al.* (2008) investigated aflatoxin exposure in Egyptian children (n = 50; age, 1–2.5 years) by assessing urinary aflatoxin metabolites (AFM1, AFB1, AFB2, AFG1, AFG2). Samples from Guinean children (n = 50; age, 2–4 years) were analysed in parallel, providing a comparison with a region of established, frequent exposure to aflatoxin. Overall, aflatoxins were less frequently present in Egyptian (38%) than in Guinean urine samples (86%) (P < 0.001). For AFM1, the geometric mean level in Guinea (16.3 pg/ml; 95%CI: 10.1–26.6 pg/ml) was six times higher (P < 0.001) than in Egypt (2.7 pg/ml; 95%CI: 2.5–2.8 pg/ml).

4.3.2 Aflatoxin–albumin adducts as biomarkers of exposure in intervention trials

The aflatoxin-biomarker studies in populations at high risk for HCC have stimulated the development of interventions to reduce exposure to aflatoxins. In the study by Turner *et al.* (2005), aflatoxin biomarkers were used to assess whether post-harvest measures to restrict aflatoxin contamination of peanut crops could reduce exposure in the lower Kindia region of Guinea. Farms from 20 villages were included, ten of which implemented a package of post-harvest measures to restrict aflatoxin contamination of the peanut crops; ten controls followed usual post-harvest practices. The concentrations of aflatoxin–albumin adducts from 600 people were measured immediately after harvest, and three and five months later, to monitor the effectiveness of the intervention. In control villages the mean aflatoxin–albumin concentration increased from 5.5 pg/mg (95%CI: 4.7–6.1) immediately after harvest to 18.7 pg/mg (17.0–20.6) five months later. By contrast, the mean aflatoxin–albumin concentration in intervention villages after five months of peanuts storage was similar to that immediately post-harvest (7.2 pg/mg [6.2–8.4] *vs* 8.0 pg/mg [7.0–9.2]). At five months, the mean adduct concentration in intervention villages was less than 50% of the values in control villages (8.0 *vs* 18.7 pg/mg; $P < 0.0001$). About a third of the people had non-detectable aflatoxin–albumin concentrations at harvest. At five months, five persons (2%) in the control villages had non-detectable adduct concentrations, compared with 47 (20%) of the subjects in the intervention group ($P < 0.0001$).

4.4 Aflatoxin–DNA adducts

Formation of DNA adducts through reaction with metabolically activated aflatoxin is well characterized. The primary site of adduct formation in DNA is the *N7* position of the guanine base (Guengerich *et al.*, 1998). Aflatoxin B1 is activated to its 8,9-*exo*-epoxide, which reacts with DNA to form the 8,9-dihydro-8-(*N7*-guanosinyl)-9-hydroxy aflatoxin B1 (AFB1–*N7*-Guo) adduct. This adduct represents more than 98% of the total adducts formed by the 8,9-*exo*-epoxide (Guengerich *et al.*, 1998).

The positively charged imidazole ring of the guanosine adduct promotes depurination and consequently, apurinic site formation. As a result, the purine-adduct aflatoxin-*N7*-guanine can be measured in the urine (see below). Under slightly alkaline conditions, the imidazole ring of AFB1–*N7*-Guo is opened and forms the more stable – not depurinating – ring-open aflatoxin B1–formamidopyrimidine adduct (Groopman *et al.*, 1981).

DNA and protein adducts of aflatoxin have been detected in many studies in human liver and in body fluids. Some studies related the level of adducts to polymorphisms in metabolizing enzymes, to investigate interindividual susceptibility to aflatoxin (IARC, 1993, 2002).

4.4.1 Aflatoxin–DNA adducts as biomarkers in intervention trials

Egner *et al.* (2001) reported on a clinical trial with chlorophyllin in Qidong County, People's Republic China. Chlorophyllin is a mixture of semisynthetic, water-soluble derivatives of chlorophyll that has been shown in animal models to be an effective inhibitor of aflatoxin-induced hepatocarcinogenesis by blocking the bioavailability of the carcinogen. A total of 180 adults from Qidong were randomly assigned to ingest 100 mg of chlorophyllin or a placebo three times a day for four months. The primary endpoint was modulation of levels of aflatoxin-N7-guanine adducts in urine samples collected three months into the intervention. Chlorophyllin consumption at each meal led to an overall 55% reduction ($P = 0.036$) in median urinary levels of this

aflatoxin biomarker compared with concentrations in the urine of those taking the placebo.

Kensler *et al.* (2005) described a randomized, placebo-controlled chemoprevention trial aimed at testing whether drinking hot-water infusions of three-day-old broccoli sprouts, containing defined concentrations of glucosinolates, could alter the disposition of aflatoxin. Two hundred healthy adults drank infusions containing either 400 µmol or < 3 µmol glucoraphanin (control value) nightly for two weeks. An inverse association was observed for excretion of dithiocarbamates and aflatoxin-DNA adducts ($P = 0.002$; $R = 0.31$) in individuals who consumed broccoli-sprout glucosinolates.

4.5 Mutagenicity

Aflatoxin B1 induces mutations in *Salmonella typhimurium* strains TA98 and TA100, and causes unscheduled DNA synthesis, chromosomal aberrations, sister chromatid exchange, micronucleus formation and cell transformation in various *in vivo* and *in vitro* mammalian systems. For its mutagenicity, aflatoxin B1 is strongly dependent on metabolic activation with a rat-liver S9 fraction: the mutagenicity in *Salmonella* tester strains TA98 and TA100 without S9 was approximately 1000 times lower than in the presence of S9 (IARC, 1993, 2002).

Aflatoxin B1 can induce mitotic recombination in addition to point mutations (IARC, 2002). This has been demonstrated in both yeast and mammalian cells. In human lymphoblastoid cells, aflatoxin B1 treatment resulted in mitotic recombination and loss of heterozygosity. A reversion assay demonstrated aflatoxin B1-induced intrachromosomal recombination in a mutant cell-line derived from V79 cells that harbour an inactivating tandem-duplication in the *Hprt* gene. Aflatoxin B1 also induced recombination in minisatellite sequences in yeast expressing recombinant human CYP1A2. Liver tumours in HBV-transgenic mice – accumulating hepatitis-B surface antigen in the endoplasmic reticulum of the hepatocytes – treated with aflatoxin B1 transplacentally contained rearrangements in minisatellite sequences after transplacental exposure to aflatoxin B1; no such alterations were seen in non-treated animals (Kaplanski *et al.*, 1997). These findings suggest that aflatoxin can induce genetic instability in addition to point mutations. Mitotic recombination and genetic instability may therefore be two mechanisms by which aflatoxin may contribute to genetic alterations, such as loss of heterozygosity, in hepatocollular carcinoma.

Efforts to correlate biomarkers of aflatoxin exposure (i.e. adduct levels) with mutation indiction have given mixed results. In human subjects from Qidong County, People's Republic of China, aflatoxin exposure was determined as high or low by measuring aflatoxin–albumin adduct levels in serum in comparison with the *HPRT* mutant frequency in lymphocytes. A higher *HPRT* mutant frequency was observed in subjects with high compared with low aflatoxin exposure (Wang *et al.*, 1999). In a study in The Gambia, chromosomal aberrations, micronuclei and sister chromatid exchange were studied in 35 adults, 32 of whom had measurable concentrations of aflatoxin–albumin adducts. There was no correlation within this group between the cytogenetic alterations and aflatoxin–albumin adducts in peripheral blood at the individual level. In a further study, blood samples of 29 individuals of the same group were tested for DNA damage in the single-cell gel electrophoresis (comet) assay, but no correlation was observed with aflatoxin–albumin adducts or *GSTM1* genotype (Anderson *et al.*, 1999).

4.6 Molecular lesions

It has been suggested that exposure to aflatoxin B1 can lead to hepatocellular carcinoma through induction of a specific mutation in codon 249 of the *TP53* tumour-suppressor gene (Gomaa

et al., 2008). Indeed, molecular analyses of human hepatocellular carcinomas have revealed a high prevalence of an AGG→AGT (Arg → Ser) transversion at codon 249 of the *TP53* tumour-suppressor gene (249ser mutation) in tumours from areas of the world where aflatoxin exposure was reported to be high (Montesano *et al.*, 1997). A large number of studies have been published on aflatoxin exposure and *TP53* mutations; two meta-analyses examined the relationship between aflatoxin exposure, HBV infection and *TP53* mutations in 20 (Lasky & Magder, 1997) and in 48 published studies (Stern *et al.*, 2001).

In geographical correlation studies, exposure to aflatoxin was associated with a specific G→T transversion in codon 249 of the *TP53* gene in human hepatocellular carcinoma. This alteration is consistent with the formation of the major aflatoxin B1–*N7*-guanine adduct and the observation that G→T mutations are predominant in cell culture and animal model systems. The high prevalence of the codon-249 mutation in human hepatocellular carcinoma, however, is only partly explained in experimental studies by sequence-specific binding and mutation induced by aflatoxin B1, or by an altered function of the p53 protein in studies of hepatocyte growth and transformation.

Preneoplastic lesions have been examined to define the time point in the natural history of hepatocellular carcinoma when the *TP53* mutation occurs. Hulla *et al.* (1993) examined six hyperplastic nodules from rat liver that had developed three weeks after intraperitoneal injection with aflatoxin B1 followed by partial hepatectomy. No mutations at the codon-249 equivalent were found. In other studies mice received intraperitoneal injections of aflatoxin B1 and were examined for tumours six to 14 months later (Tam *et al.*, 1999). Of the 71 lung tumours examined, 79% showed positive nuclear p53-staining. Analysis of microdissected tumour samples revealed mutations in different codons in exons 5, 6 and 7. Direct sequencing showed 26 mutations, which included nine G:C to A:T transitions, 11 A:T to G:C transitions and five transversions (two G:C to T:A, two T:A to A:T and one A:T to C:G). The high mutation frequency and heterogeneous staining pattern suggested that *TP53* mutations occur relatively late in aflatoxin-B1-induced mouse lung tumorigenesis.

Investigations have been conducted to establish which DNA adduct is the most likely precursor of the mutations induced by aflatoxin B1. In several experimental systems these mutations are certainly consistent with the main carcinogen-binding occurring at guanine in DNA, leading to G→T transversions (IARC, 1993, 2002). When a pS189 shuttle vector was modified by aflatoxin B1 and then replicated in human Ad293 cells, predominantly G→T transversions were detected (Trottier *et al.*, 1992). However, other types of mutation have also been observed with aflatoxin B1. For example, Levy *et al.* (1992) transfected an aflatoxin-B_1-modified shuttle vector into DNA repair-deficient (XP) or -proficient human (GM0637) fibroblasts, and examined mutations in the *SUP-F* marker gene. Higher mutation frequencies were observed in the DNA repair-deficient cells and the location of mutations was significantly affected by repair proficiency. The majority of mutations were at GC base pairs: 50–70% were G →T transversions, but G→C transversions and G→A transitions were also frequent. A polymerase stop-assay was used to examine the of aflatoxin-B_1-binding site within the shuttle vector: no strong correlation was found between initial binding sites and subsequent hotspots for mutation. This suggests that processing of adducts, e.g. during DNA replication and repair, can influence not only the overall mutation frequency but also the distribution of mutations within a gene.

A host-mediated assay was used to determine the pattern of mutagenesis induced by aflatoxin B1 in the *lacI* gene of *E. coli* bacteria recovered from rat liver. Most of the 281 forward mutations analysed were base substitutions at GC base

pairs; over half were GC→TA transversions, with other mutations evenly divided between GC→AT transitions and GC→CG transversions (Prieto-Alamo *et al.*, 1996).

In a human lymphoblastoid cell line (h1A2v2) expressing the human recombinant CYP1A1 enzyme, aflatoxin B1 (4 ng/mL; 25 hours) produced a hotspot GC→TA transversion mutation at base pair 209 in exon 3 of the *HPRT* gene in 10–17% of all mutants. This hotspot occurred at a GGGGGG sequence (target base underlined) (Cariello *et al.*, 1994).

Bailey *et al.* (1996) studied the induction of mutations resulting from two of the principal forms of DNA damage induced by aflatoxin B1, namely the AFB1–*N7*-Guo adduct and the ensuing apurinic sites, by site-directed mutagenesis. Single-stranded M13 bacteriophage DNA containing a unique AFB1–*N7*-Guo adduct or an apurinic site was used to transform *E. coli*. The predominant mutations with AFB1–*N7*-Guo were G→T transversions targeted to the site of the original adduct (approximately 74%), with lower frequencies of G→A transitions (13–18%) and G→C transversions (1–3%). Using *E. coli* strains differing in biochemical activity of the UmuDC- and MucAB proteins – involved in processing of apurinic sites by insertion of dAMP – the authors showed that the mutations observed with AFB1–*N7*-Guo were not predominantly a simple result of depurination of the initial adduct. A significant number of base substitutions were located at the base 5′ to the site of the original adduct, representing around 13% of the total mutations. This induction of mutation at the base adjacent to the original site of damage was not observed with apurinic sites as the mutagenic lesion. It was suggested that this reflects interference with DNA replication following the intercalation of aflatoxin-B1–8,9-epoxide (Gopalakrishnan *et al.*, 1990).

4.7 Synthesis

Several key steps in the development of hepatocellular carcinoma induced by exposure to aflatoxin are well accepted (Wild & Montesano, 2009), and provide strong evidence that the mechanism of action of this agent involves metabolic activation to a genotoxic metabolite, formation of DNA adducts, and modification of the *TP53* gene. The concurrent presence of hepatitis B virus increases the incidence of hepatic tumours in humans. Aflatoxin B1 is the most common and potent of the aflatoxins. It is metabolized predominantly in the liver to an AFB1–8,9-*exo*-epoxide, which forms a promutagenic AFB1-*N7*-guanine DNA adduct that results in G→T transversion mutations. In human hepatocellular cancers in areas where aflatoxin exposure is high, up to 50% of tumours have been shown to harbour a specific AGG→AGT point mutation in codon 249 of the *TP53* tumour-suppressor gene (codon 249Ser mutation) (Hussain *et al.*, 2007; Wild & Montesano, 2009).

5. Evaluation

There is *sufficient evidence* in humans for the carcinogenicity of aflatoxins. Aflatoxins cause cancer of the liver (hepatocellular carcinoma).

There is *sufficient evidence* in experimental animals for the carcinogenicity of naturally occurring mixtures of aflatoxins, and of aflatoxin B1, G1 and M1.

There is *limited evidence* in experimental animals for the carcinogenicity of aflatoxin B2.

There is *inadequate evidence* in experimental animals for the carcinogenicity of aflatoxin G2.

There is strong evidence that the carcinogenicity of aflatoxins operates by a genotoxic mechanism of action that involves metabolic activation to a genotoxic epoxide metabolite, formation of DNA adducts, and modification of the TP53 gene. In human hepatocellular

carcinoma from areas where exposure to aflatoxins is high, up to 50% of tumours have been shown to harbour a specific point mutation in the *TP53* tumour-suppressor gene.

Aflatoxins are *carcinogenic to humans (Group 1)*.

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