4. Other Data Relevant for Evaluation of Carcinogenicity and its Mechanisms

4.1 Pathology of infection

4.1.1 *Humans*

(*a*) *Opisthorchis viverrini*

Tansurat (1971) described the detailed pathological features of infection with *O. viverrini* on the basis of 70 autopsied cases in Thailand. In early infections, there was no epithelial hyperplasia or fibrous proliferation. In chronic infections, there was proliferation of epithelial cells with formation of glandular acini, similar to the adenomatous changes in clonorchiasis, and there were varying degrees of periductal fibrosis. Enlargement of the liver is observed in most cases of opisthorchiasis, especially in cases of massive infection. The weight of the liver in massive infections is more than double the normal (3000–3500 g); the maximal weight recorded was 4000 g.

The major microscopic changes (Riganti *et al.*, 1989) are confined to the large and medium-sized bile ducts where the flukes are harboured. The cellular infiltrates consist of lymphocytes, monocytes, eosinophils and some plasma cells. Dilatation of the bile ducts, hyperplasia, desquamation and proliferation of the bile-duct lining cells, glandular formation and fibrosis of the periductal connective tissue of the walls are the commonest features. The gross and microscopic characteristics of human opisthorchiasis in 22 adults and seven children were similar, and the pathological changes were well established within 7–15 years after *O. viverrini* infection; however, dilatation of the gall-bladder, chronic cholecystitis and carcinoma were found only in adults.

In chronic and heavy infections, various degrees of cellular infiltration are caused by superimposed bacterial infection. This may result in suppurative cholangitis, and the infection may extend into the parenchyma of the liver tissue, causing cholangiohepatitis with abscess formation. Of 70 cases of advanced opisthorchiasis seen at autopsy, 10 showed multiple abscesses in the liver. The abscesses varied in diameter, from 5 to 10 mm; some ruptured into the right pleural cavity, and in some infections the lower lobe of the right lung was involved (Prijyanonda & Tandhanand, 1961).

In heavy infections with *Opisthorchis*, adult parasites are always discovered in the gall-bladder, the common bile duct and the pancreatic duct (Pungpak *et al.*, 1985, 1987). As in the large and medium-sized bile ducts, the parasites give rise to chronic cholecystitis. When there is superimposed bacterial infection, empyema of the gall-bladder may result. No stone formation was seen, however, either in the bile ducts or in the gall-bladder in one series of 70 cases at autopsy (Tansurat, 1971) or in another series of 154 cases (Koompirochana *et al.*, 1978). This finding is in contrast to that seen for clonorchiasis, in which cholelithiasis is one of the most serious complications (Rim, 1986). A number of biliary tract abnormalities associated with moderate to heavy *O. viverrini* infection were demonstrated by ultrasonography (Elkins *et al.*, 1990; Mairiang *et al.*, 1992). According to Mairiang *et al.* (1993), abnormal findings seen at ultrasonography improved dramatically after treatment with praziquantel.

(*b*) *Opisthorchis felineus*

Hepatic lesions produced by *O. felineus* are similar to those caused by *O. viverrini*. In the course of their development, they initiate inflammatory and proliferative changes of the biliary epithelium, which continue after the worms have matured and are accompanied by fibrosis of the distal biliary ducts. If the infection is intensified by continued exposure, the pathological process may extend to the bile ducts and gall-bladder and result in cirrhosis. The degree of pathogenicity and clinical involvement depends largely on the number of parasites and the duration of infection. Usually, small numbers of worms do not cause serious damage and do not give rise to clinical signs. In the Russian Federation, many apparently healthy people have been found to be infected; however, their worm burden was light, with an average of no more than 200 eggs/g faeces (Bronshtein *et al.*, 1991). When several hundred or thousand worms are present, severe damage to the liver and pancreas can occur (Rim, 1982a).

Hyperplasia of the epithelium of the larger bile ducts with cholangitis is much commoner. Advanced hepatic cirrhosis is rare. Occasionally, carcinoma of the bile ducts or of the pancreas, with metastases into the epigastric lymph nodes, is responsible for death (Faust *et al.*, 1970).

(*c*) *Clonorchis sinensis*

Most of the information on the pathological manifestations of *C. sinensis* comes from Hou's (1955) study of 500 autopsy cases. The liver appears grossly normal in light infections, but in heavy infections there is localized dilatation of the slightly thickened peripheral bile ducts (which can be seen on the surface beneath Glisson's capsule as pale-blue or greenish-blue blobs) and some atrophy of the parenchymal cells. The dilatation of bile ducts

is invariably caused by obstruction of the common bile duct by a stone, a tumour or inflammatory stricture resulting from cholangitis. Under these circumstances, nearly all the medium-sized bile ducts are dilated and filled with clear or turbid bile, with or without worms.

The major microscopic findings in the early stage of clonorchiasis are periductal oedema and acute inflammatory cellular responses in the bile duct walls. The bile ducts show not only desquamation but also marked hyperplasia of epithelial cells. Subsequently, marked goblet-cell metaplasia of ductal epithelial cells is seen, and remarkable adenomatous hyperplasia appears in the mucosa. Periductal connective tissue is increased around the biliary passages and the portal tract. In the chronic stage of infection, the ductal tissue is gradually replaced by fibrous tissue (sometimes described as cholangiofibrosis), which causes thickening of the bile duct wall (Hou, 1955).

The microscopic changes vary with the intensity and duration of infection and the coexistence of bacterial infections. Without secondary bacterial infection, the genuine histological changes are usually represented by a characteristic adenomatous formation, periductal fibrosis and heavy eosinophilic infiltration. With secondary bacterial infection, however, biliary obstruction is common and is due to adenomatous proliferation, calculi and cholangitis (Hou, 1955).

Extrahepatic involvement is relatively common in *C. sinensis* infection. Hou and Pang (1964) reported that 19/300 clonorchiasis patients had pancreatic involvement; Chan and Teoh (1967) found *C. sinensis* in 24 of 64 cases seen at autopsy. Adult fluke invasion of the pancreatic ducts occurs most frequently in heavy infections, but the pathological changes are usually less extensive than those in the intrahepatic bile ducts. The flukes reside in the main pancreatic duct and its tributary ducts. The changes are similar to those seen in the hepatic lesions: namely, adenomatous hyperplasia of ductal epithelium and, sometimes, squamous metaplasia (Chen *et al.*, 1994).

One of the most characteristic complications of clonorchiasis is formation of calculi in the intrahepatic biliary passages. It is sometimes accompanied by suppurative cholangitis, cholecystitis and biliary abscesses or so-called cholangiohepatitis and, ultimately, cholangiocarcinoma (Rim, 1986). The occurrence of calculi in clonorchiasis is due to bile stagnation caused by mechanical obstruction and the presence of worms and ova, which become nuclei for hepatolithiasis. Intra- and extrahepatic bile-duct calculi are composed almost entirely of bilirubin carbonate. According to Chen *et al.* (1994), the formation of pigmented stones in clonorchiasis can be attributed to changes in the concentrations of bilirubin, cholesterol, phospholipids and bile acids and the activity of bacterial glucuronidase in bile stagnation caused by mechanical obstruction. An increase in bacterial glucuronidase activity following *Escherichia coli* infection and glycoprotein in the bile favours the formation of pigmented stones (Guo *et al.*, 1990).

With goblet-cell metaplasia of the bile-duct epithelium, the bile has a high content of mucin, which combines with the presence of the helminth and its ova in the bile duct to cause cholestasis and to furnish a favourable environment for secondary bacterial infection. The most frequent infection is with *E. coli*, which induces ascending cholangitis from the intestine. Chou *et al.* (1976) studied mucin from 17 cases of clonorchiasis-associated

cholangiocarcinoma seen at autopsy. Histochemically, the mucins were qualitatively similar to those secreted by normal and *C. sinensis*-infected bile ducts, but the concentration of carboxymucins was reduced and sulfomucins were absent or present in only trace amounts in the neoplastic epithelium. Sulfomucins were abundant, however, in the hyperplastic epithelium of patients with clonorchiasis. The authors concluded that sulfomucins are valuable in differentiating hyperplastic bile ducts from cholangiocarcinoma.

Acute suppurative cholangitis may be caused by blockage of extrahepatic biliary ducts by masses of dead worms. Gallstones and the results of inflammation by bacterial infection often cause recurrent pyogenic cholangitis (Hou, 1955; Ong, 1962; Teoh, 1963). In a study of 525 *Clonorchis*-infected patients, only three had egg-induced lesions: an eosinophilic granuloma in the gall-bladder, a giant-cell reaction in the liver and pulmonary embolism (Sun, 1984). Periductal egg granulomas are rarely found (Sun, 1980).

Morphological studies by many investigators in Hong Kong and the Republic of Korea (Hou, 1956; Chou & Gibson, 1970; Kim *et al.*, 1974) indicate that carcinomas usually arise in association with pre-existing epithelial changes, which vary from hyperplasia to dysplasia and adenomatous formation in the secondary intrahepatic bile ducts.

Human cholangiocarcinoma can be divided into two macroscopic types according to the site of involvement, peripheral (intrahepatic) and hilar (extrahepatic). The peripheral type has multicentric growth as seen most frequently in *Clonorchis*-related neoplasms in patients, all of whom had histories of recurrent pyogenic cholangitis (Parkin *et al.*, 1993).

Of 38 subjects from Hong Kong chronically infected with *C. sinensis*, only one patient with cholangiocarcinoma had cirrhosis, whereas all but one patient with hepatocellular carcinoma had cirrhosis (Purtilo, 1976).

4.1.2 *Experimental systems*

(*a*) *Opisthorchis viverrini*

The pathological changes seen in the livers of cats, rabbits, guinea-pigs, hamsters and albino rats, which are considered to be suitable hosts, are grossly similar to those seen in man. After metacercariae are fed to animals, they grow into adult worms in the liver within about 30 days. The size of the worms found differs with species and is dependent on their size (Wykoff, 1958). Most studies of carcinogenesis have been conducted in Syrian hamsters, as the other species do not develop cholangiocarcinoma.

Bhamarapravati *et al.* (1978) described the histopathological response of Syrian hamsters to *O. viverrini* infection. The early changes consisted of an acute inflammatory reaction involving the second-order bile ducts and partial flattening of the epithelial cells, especially those in contact with the flukes. The main finding was foci of varying size consisting of liver cells that had undergone haemorrhagic and coagulation necrosis. Some multinucleated, foreign body-type giant cells were seen at the edge of the necrotic areas, but flukes were not found in these foci. The inflammatory reaction in the early stage of infection was predominantly eosinophilic infiltration of the portal areas, with some neutrophils and mononuclear cells. The dilated ducts showed hyperplasia and an atypical epithelial lining, which was piled up in places. An increase in the number of goblet cells was also evident. As the flukes developed into adults, they induced hyperplasia and adenomatous formations of

the bile-duct epithelium. There was also a granulomatous response to adult flukes and eggs. Resolution of the granulomas around eggs led to periductal and portal scarring and fibrosis. The major findings were two types of granuloma—one in response to the dying adult flukes and the other to the eggs. Dead or dying worms lying in bile ducts were surrounded by a granulomatous mass which consisted of eosinophilic, homogeneous, foamy material and various numbers of neutrophils, eosinophils and foamy macrophages. Granulomatous masses in the lumina of the ducts were usually connected to granulomatous masses in the periductal tissue through ulcerated areas of the mucosa. Numerous epithelioid granulomas containing eggs were seen in the periductal areas, occasionally extending into the lumen through the mucosal ulcers to connect with other granulomatous masses. The centres of the granulomas consisted of homogeneous eosinophilic precipitates and necrotic cellular debris. The shells of the eggs in some of the granulomas had been ingested by multinucleated giant cells, and in some granulomas the eggs were calcified.

(*b*) *Opisthorchis felineus*

The presence of *O. felineus* causes irritation of the intrahepatic bile ducts and pancreatic ducts, leading to a catarrhal inflammation and desquamation of the epithelium (Soulsby, 1965). As seen in *O. viverrini* and *C. sinensis* infections, extensive hyperplasia of the biliary system, papillomatous and adenomatous changes in bile ducts, cystic dilatation, necrosis and secondary atrophy of the hepatic cells, and extensive fibrosis occur in experimental animals (Rim, 1982b).

Formation of granulomas in the walls of bile ducts around *O. felineus* eggs was observed at days 20–25 of experiments in Syrian hamsters (Zubov & Mukanov, 1976).

(*c*) *Clonorchis sinensis*

Many laboratory animals are sensitive to *C. sinensis*. Rabbits and guinea-pigs are the most susceptible; rats, Syrian hamsters and dogs are relatively susceptible; and mice are the least susceptible of these species. The degree of pathological change depends on both the intensity and the duration of infection. The major pathological findings in the livers of animals with clonorchiasis are in the biliary system, which the helminths inhabit. The most characteristic pathological change in infection is diffuse adenomatous tissue formation in the secondary bile ducts. Desquamation, hyperplasia of lining epithelial cells, regeneration and adenomatous hyperplasia are seen (Hou, 1965b; Kim *et al.*, 1974).

Microscopically, periductal and ductal aggregations of inflammatory cell infiltrates are usually profound in the acute stage and consist of lymphocytes, plasma cells, histiocytes and fibrosis. Hyperplasia of epithelial cells is frequent (Rim, 1982b). Small eosinophilic abscesses and focal liver cell necrosis may be present, but the hepatic lobular structure remains intact (Chen *et al.*, 1994).

Cha *et al.* (1991) noted in rats infected repeatedly with *C. sinensis* that a heavy eosinophilic infiltration appeared around the bile duct after two to four weeks. The cells were then replaced by massive mononuclear cells, which often formed lymphoid follicles. In similarly infected mice, the epithelial cells of the bile duct were changed to secretory cells, which secreted hyalinized materials into the lumen of the bile duct. Inflammatory cells infiltrated the adjacent hepatic parenchyma and formed microabscesses.

Clonorchis infection induces severe hyperplasia of epithelial cells and metaplasia of mucopolysaccharide producing cells in the biliary epithelium (Lee, S.H. *et al.*, 1978; Song *et al.*, 1989; Hong *et al.*, 1990). In a study of the proliferative activity of bile-duct epithelial cells in clonorchiasis by immunostaining bromodeoxyuridine incorporated into the DNA of cells in the S phase of division (Risio *et al.*, 1988), the greatest rate was found mainly in cells located at the base of the mucosal layer (Hong *et al.*, 1993). The authors suggested that mucosal epithelial cells of bile ducts infected with *C. sinensis* become hyperplastic mainly by direct and local stimulation by the worms.

Hepatic changes in rabbits in the early stage (first two weeks) of infection were reversible after treatment with praziquantel; however, some of the biliary epithelial changes that occurred in the chronic stage (12 weeks) of infection were irreversible (Lee *et al.*, 1989).

In guinea-pigs infected with *C. sinensis*, the biliary epithelium had an increased prevalence of mucin granules, cytoplasmic projection into the lumen, decreased numbers of microvilli and obstruction of the bile canaliculi. Blurring or irregularity of intercellular lateral interdigitation was observed in most of the biliary epithelium. Hepatocytes showed dilatation of endoplasmic reticulum and destruction of cristae in some mitochondria (Lee, Y.S. *et al.*, 1978).

4.1.3 *Comparison of humans and experimental animals*

The general pathological features of clonorchiasis and opisthorchiasis are similar in human cases and experimental animals, including the Syrian hamster, which is the most commonly used species in carcinogenicity studies. The changes involve predominantly the intrahepatic bile ducts and pancreatic ducts. The initial changes, such as desquamation of the bile-duct lining cells, are followed by hyperplasia of the cells lining the intrahepatic bile ducts and are identical in humans and in the acute stage of experimental infections. In chronic infections in humans and experimental animals, adenomatous hyperplasia of the bile ducts, heavy eosinophilic infiltration and periductal fibrosis occur. Secondary bacterial infections, especially ascending infection with *E. coli*, result in multiple hepatic abscesses and cholangiohepatitis in livers infected by both *Opisthorchis* and *Clonorchis*.

4.2 Other observations relevant to the interpretation of carcinogenicity and mechanisms of carcinogenesis

4.2.1 *Humans*

Inflammatory responses in host tissues challenged by infections or inflammatory agents have been postulated to play a role in the development of cancers which arise in infected organisms (for reviews, see Gentile & Gentile, 1994; Ohshima & Bartsch, 1994). Reactive oxygen species and nitrates, nitrites and various nitrosating agents are produced to kill invading microorganisms and helminths. Polymorphonuclear leukocytes play a prominent role in the production of these host defence agents (for reviews, see Preussmann & Eisenbrand, 1984; Shepard *et al.*, 1987). The radicals have been shown to induce genetic damage in normal host tissues adjacent to the site of inflammation, producing DNA strand breaks, mutations and chromosomal aberrations (Weitzman & Stossel, 1981; Birnboim,

1982). While no data on humans are available to verify these observations, increased levels of urinary nitrates and salivary nitrites are found in *O. viverrini*-infected individuals.

Srianujata *et al.* (1984) reported significantly higher concentrations of nitrate (2–2.8 times) and nitrite (2–5.6 times) in the saliva of inhabitants in a high-risk area for cholangiocarcinoma in North-east Thailand than in subjects in Bangkok (low-risk area). Nitrate levels in urine were also significantly higher (1.5–3 times) in the subjects from the high-risk areas. Srianujata *et al.* (1987) also reported higher levels of nitrite (1.8 times) and *N*-nitrosoproline (2.6 times) in the urine of subjects infected with *O. viverrini* than in uninfected subjects from the same area of North-east Thailand. Haswell-Elkins *et al.* (1994b) confirmed these observations in a study in North-east Thailand in which diet and smoking were controlled for; they also demonstrated decreased concentrations of nitrates and nitrites in these subjects after treatment with praziquantel. Srivatanakul *et al.* (1991c), in a study in which diet and smoking were not controlled for, reported that subjects living in high-risk areas for fluke infection who had antibodies to *O. viverrini* had a 10-fold greater potential for endogenous nitrosation, measured on the basis of urinary levels of *N*-nitrosoproline after proline ingestion, than individuals who did not have antibodies.

Cholangiocarcinomas from *O. viverrini*-infected patients differed from those in uninfected patients with respect to point mutations in the c-Ki-*ras* proto-oncogene: mutations were found at codon 12 of this gene in five of nine individuals in Japan who had cholangiocarcinoma but no concomitant fluke infection, but not in six patients from Thailand who harboured both cholangiocarcinoma and fluke infection (Tsuda *et al.*, 1992). Similar results were reported by Kiba *et al.* (1993), who found, however, that a mutation at the *p53* tumour suppressor gene was similar in the two sets of cholangiocarcinoma patients, all but one being GC→AT transitions in a highly conserved GpG site.

4.2.2 *Experimental systems*

In male Syrian hamsters and jirds (*Meriones unguiculatus*), 220 days after experimental infection with *O. viverrini*, marked proliferation of smooth endoplasmic reticulum was observed in hepatocytes, and lobed and enlarged nuclei and mitochondria were seen which showed significant pathological degeneration, up to lysis. There was also accumulation of intermediate filaments in adjacent bile-duct epithelia and in the epithelium of the gall-bladder (Adam *et al.*, 1993). Depressed lymphoproliferative response to phytohaemagglutinin stimulation has also been described in Syrian hamsters infected with *O. viverrini*, suggesting an immunodepressive effect (Wongratanacheewin *et al.*, 1987).

The role of *O. viverrini* in enhancing host response to chemical carcinogens (particularly nitrosamines) has been well documented in Syrian hamster models (see section 3.2). A significant increase in the proportion of water-soluble aflatoxin B_1 metabolites was found in hamsters infected with liver flukes over that measured in uninfected animals (Makarananda *et al.*, 1991), suggesting increased expression of enzymes that metabolize aflatoxin B_1. A cytochrome P450 isozyme(s) (CYP2A) has been identified in the livers of hamsters infected with *O. viverrini*, the activity of which increased nonuniformly in male but not female animals, the highest levels of activity occurring in hepatocytes immediately adjacent to areas of inflammation. This increase occurred in spite of a decrease in the total hepatic P450 content. The enzyme was shown to contribute up to 50–60% of the metabolism

of hepatic aflatoxin B_1 and *N*-nitrosodiethylamine in infected males and 20–40% in infected females (Kirby *et al.*, 1994).

Immunohistochemical analysis of aflatoxin B_1–DNA adducts in parasitized animals indicated that the greatest numbers of adducts occurred in the regions of highest CYP2A activity. Studies with a related liver fluke, *Fasciola hepatica*, also showed enhanced cytochrome P450-related activation of aflatoxin B_1 into a mutagen by liver extracts from fluke-infected mice over that with extracts prepared from livers of uninfected animals (Gentile & DeRuiter, 1981).

Nitrosamine and nitrate biosynthesis mediated by nitric oxide synthase was found to be increased in *O. viverrini*-infected Syrian hamsters, and nitric oxide synthase activity in liver cytosol was twice as high in infected as in untreated hamsters The enzyme was located in macrophages and eosinophils which accumulated at the site of the infection (Ohshima *et al.*, 1994).