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Liver Histopathologic Alterations in the Frog *Rana ridibunda* from a Small River of Northern Greece

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Abstract The various histopathologic alterations detected in the liver of the frog *Rana ridibunda* from a small river of Northern Greece were investigated. In the livers of the frogs collected from this river, there was an increase in the area occupied by melanomacrophages as well as an increase in their color intensity. Mild karyomegaly and polyploidy, together with solitary and focal accumulation of infiltrates (neutrophils and lymphocytes), was evident. Three different kinds of foci were detected basophilic focus, vacuolated focus, and a special type of focus with nodules surrounded by collagen. Some bile ducts were found to be filled with periodic acid schiff (PAS)-positive amorphous material mixed with cellular debris. In liver foci of dense connective tissue, with structures resembling bile ductuli, were detected. The latter finding supports the conclusion that this might be early holangiofibroma. Progressive fibrosis around bile ducts, which extended into the hepatic parenchyma, was obvious. Given that the river under study was previously characterized as being moderately to heavily polluted, it was concluded that a number of environmental factors, including heavy metals, might be responsible for these histopathologic alterations.

Introduction

It is well known that organisms are repeatedly exposed to low doses of a variety of chemical pollutants of environmental or occupational origin. The environmental contaminants from both natural factors and human activities have received considerable study. In many ecosystems, human-induced changes have overwhelmed the natural biogeochemical discharges of various pollutants, such as trace elements.

During the last few years, many amphibian populations have been decreasing dramatically, and extinction caused by man-made changes in the environment (Carey and Bryant 1995), has occurred in a few species. Frogs are more vulnerable than other vertebrates to environmental contaminants because frog eggs are not protected by semiimpervious shells and frog skin is water permeable (Dwellman and Trueb 1986). *Rana ridibunda* is a water frog widely distributed in Europe, especially very common in Balkans, with a wide distribution in Greece. Few researchers have worked on the relationship between the frog *R. ridibunda* and organochlorine pesticides (Loumbourdis 1998) or heavy metals (Sura et al. 2006; Loumbourdis and Wray 1998). The latter researchers studied the concentration of 14 heavy metals in the liver and whole body of this frog living in a small river of Northern Greece (Tafros 66), which is known to be polluted by effluents along its entire length.

Melanomacrophages (MMCs) are focal accumulations of pigmented macrophages in liver, spleen and, more rarely, kidney of fishes (Couillard and Hodson 1996) and also in liver of frogs (Loumbourdis and Vogiatzis 2002). MMCs may contain four types of brown to black pigments, namely, melanin, lipofuscin, ceroid, and hemosiderin/ferritin. MMC proliferation has been associated with several natural factors, such as normal aging, starvation, and infectious diseases (Wolke 1992). Increased density of MMCs has also been observed in experimentally treated fish (Couillard and Hodson 1996), amphibians (Loumbourdis and Vogiatzis 2002), and naturally polluted frogs (Fenoglio et al. 2005).

The ability of melanin to scavenge reactive oxygen species (*ROS*) (Rozanowska et al. 1999) suggests that

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melanin could protect pigment cells against the oxidative stress that may accompany the formation of ROS in these cells. Melanin has also been proposed as a novel biochemical mechanism involving the accumulation, stimulatory action, and eventually detoxification of metal ions (McGraw 2003).

One of the most significant cytochemical approaches to carcinogenesis research was the discovery of focal preneoplastic and early neoplastic lesions consisting of cells with characteristic altered phenotypes (Bannasch 1984). The identification of the altered cell populations in a number of tissues provided the basis for studies on different aspects of carcinogenesis. Evidence exists that a sequence of distinct histologic lesions leads from clear to basophilic cell foci and finally to tumors (Bannasch et al. 1989). Identification of foci with hematoxylin and eosin–stained sections is still regarded as the most reliable approach for the diagnosis and identification of preneoplastic liver lesions. Various types of possible preneoplastic foci have been described thoroughly (Boardman et al. 1997).

It is widely accepted that cholangiocellular tumors may develop from oval cells by way cholangiofibrotic lesions (Hsia et al. 1992). These cholangiofibrotic lesions, which are considered to be prestages of cholangiocellular tumors, exhibit a marked accumulation of mucous substances, which is typical of cholangiocellular but not hepatocellular tumors (Bannasch et al. 1991).

Hepatic fibrosis is regarded as a common response to chronic liver injury and is characterized by excessive deposition of extracellular matrix components (Friedman 1993). It has been shown that oxidative stress can stimulate fibroblast (Murrel et al. 1990) and hepatic stellate cell (HSC) proliferation (Lee et al. 1995) and collagen synthesis (Montosi et al. 1996; Fredman 1995) both *in vitro* and *in vivo.*

The aim of this study was (1) to assess the various histologic alterations observed in frogs collected from a small river, called Tafros 66, approximately 100 km southwest of from the city of Thessaloniki, an area earlier classified as moderately to heavily polluted by heavy metals (Loumbourdis and Wray 1998) and (2) to compare the data with those of frogs collected from the area of Pertouli, some 200 km southwest of Thessaloniki at an altitude of 1100 m.

Materials and Methods

Study Area

Fifteen adult *R. ridibunda* (8 female and 7 male frogs) were collected from the river Tafros 66, which flows through the Pella, Imathia, and Pieria provinces of Central Macedonia, Northern Greece. The river irrigates a broad area, cultivated mainly with fruit trees and to a lesser extent cotton and maize corn. Another 14 frogs (7 male and 7 female frogs) were collected from the Pertouli area.

Test Organisms

Up arrival at the laboratory, frogs were sexed and anaesthetized with MS222, individually weighed, and snout-to-vent length was measured. After anaesthetization, the belly was excised, and the liver was removed and weighed. Data concerning methods employed for quantitative and qualitative detection of heavy metals as well as concentrations of heavy metals in the liver of the frogs from the Tafros 66 area were previously presented (Loumbourdis and Wray 1998).

Tissue Preparations

Part of each liver of the above frogs was fixed in 10% neutral buffered formalin and then dehydrated, cleaned, and embedded in paraffin. The paraffin blocks were sectioned at 5 to 7 μm using a Leitz-Wetzlar 1400 microtome (Leitz, West Germany) and a series of sections were stained with H&E. Another series of sections underwent the same treatment, except that of staining, because the MMCs showed the same results in stained and unstained sections. However, the use of unstained sections was preferable. In terms of area and intensity of staining measurements, the results taken from unstained sections were more realistic because they were devoid of unnecessary additional staining.

For histochemical analysis, PAS stain for neutral polysaccharides (Pearse 1985) and Sirius red stain for collagen (Juncueira et al. 1979) were used. To avoid differing intensity of staining in different tissues, all sections to be stained by the same method were stained simultaneously.

In the unstained samples, the pigment cells retained a light to dark brown colour, altogether distinguishable from the background. Lehr's technique (1997), using Adobe Photoshop 7.0 (Adobe Systems), was applied to measure the area occupied by pigments, as well as their colour intensity on the unstained sections. These images were stored as jpeg files. The method has been thoroughly described elsewhere (Loumbourdis and Vogiatzis 2002).

The frogs collected from the Pertouli area were subjected exactly to the same treatment. They were used more or less as control animals, because the area is thought to be one of most unpolluted areas of Greece. All statistical comparisons were performed using Mann-Whitney nonparametric U test at a $p = 0.05$ level of significance.

Table 1 Mean values (± SE) for body length, hepatosomatic index, surface area and colour intensity of MMCs in the liver of adult frog *R. ridibunda* from Pertouli (controls) and Tafros 66. Numbers in parentheses, sample size

Population	Body length (mm)	HSI	Surface	Intensity
Control (14)	256 ± 4.03	3.02 ± 0.49	2.64 ± 0.18[a]	164.59 ± 6.14[b]
Tafros 66 (15)	248 ± 0.49	2.77 ± 0.16	7.75 ± 0.73[a]	122.8 ± 4.16[b]

[a,b] Statistically significant differences between groups

HSI: liver wt/body wt × 100; surface area: μm^2; Intensity: Arbitrary units

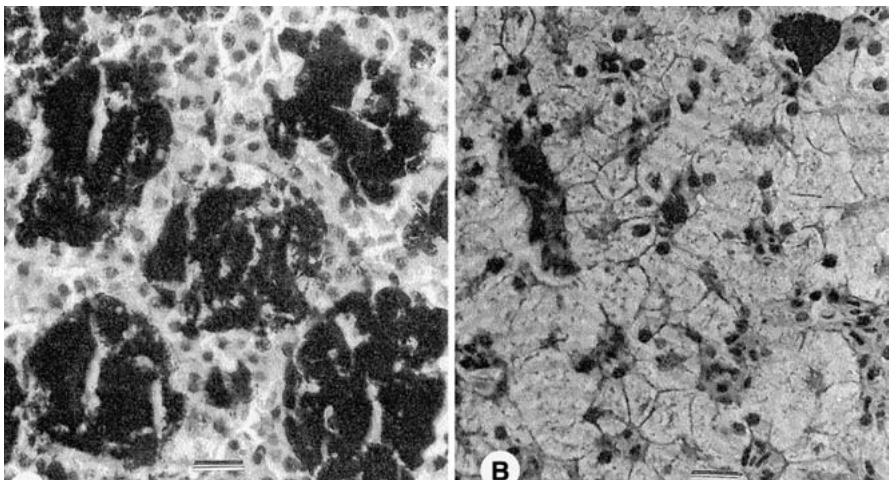

Fig. 1 Area occupied by MMCs in Tafros 66 (a) and control 66 (b) frogs. Note the enormous difference between the two samples. H&E stain. Bar = 25 μm

Results

Surface and Color Intensity

The surface covered by the MMCs appeared to be statistically significant compared with the control animals (Table 1). Color intensity of the pigments was stronger in the animals from Tafros 66 compared with control values (Table 1). There was a 25% decrease in the value of the mean color range of the MMCs compared with the respective value in the control group (Table 1). It should be noted, however, that the smaller the value of color intensity, the darker the sample, because in the range of 0 to 255, 0 corresponds to maximum darkness. Figs. 1a and 1b indicate two extreme differences in the area covered by MMCs and their staining intensity by comparing control frogs with frogs from Tafros 66.

Histopathologic Findings

Gross examination of frog livers from the Tafros 66 area did not reveal any macroscopic alterations in liver structure in any of the studied animals. Karyomegaly, as well as karyomegaly probably related to polyploidy, was evident (Fig. 2). Diffused infiltrates were evident either singly or in small groups. One interesting finding of this research was the discovery of a unique formation into some bile ducts. This formation consisted of a slightly eosinophilic amorphous mass, mixed with pycnotic dead cells, which filled

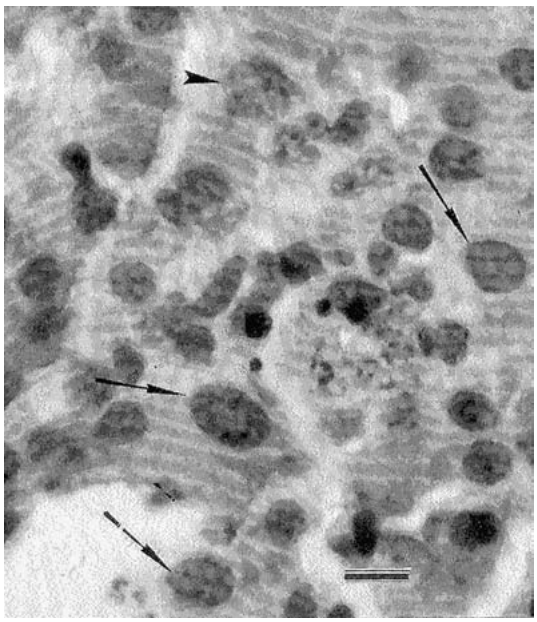

Fig. 2 A polyploid (arrowhead) and some karyomegalic (arrows) hepatocytes from a Tafros 66 frog. H&E stain. Bar represents 25 μm

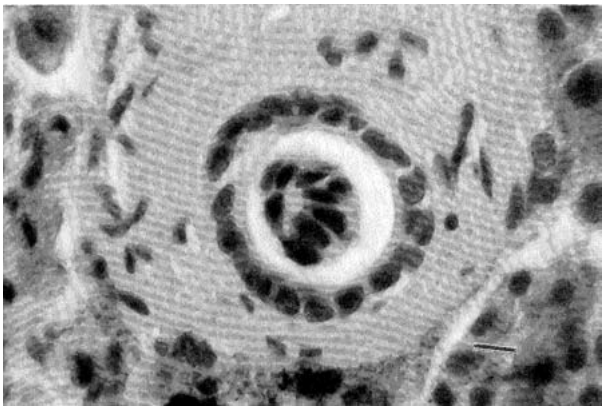

Fig. 3 A biliary duct from a Tafros 66 frog, filled with an amorphous eosinophilic mass mixed with pycnotic sloughed epithelial cells. H&E stain. Bar = 10 μm

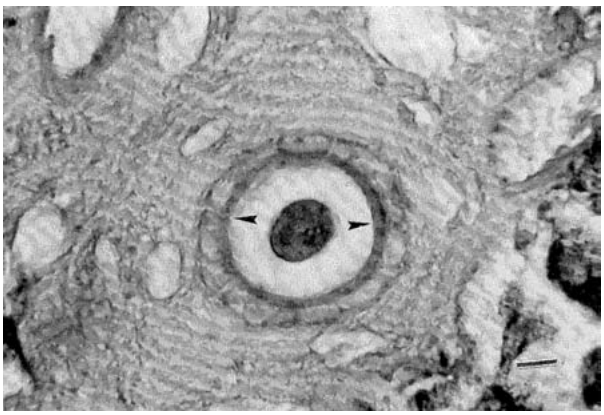

Fig. 4 The same area as in Fig. 3, from the same frog, stained with PAS. The amorphous mass was strongly PAS positive. Note that the glycocalyx of the bile duct (arrowheads) was also strongly PAS positive. PAS stain. Bar = 10 μm

the lumen of these bile ducts (Fig. 3). Staining by PAS method showed that the amorphous mass was strongly PAS positive (Fig. 4). A tangential section of such a duct (not shown) shown that such a mass may occupy the entire bile duct or at least a large part of it.

In one of the frogs having bile ducts filled with PAS-positive material, a densely fibrotic area, containing ducts surrounded mainly by epithelial cells, was detected. These cells had the classic appearance of bile duct cells (Fig. 5). In the same animal, an increase in the number and the lumen diameter of bile preductuli was also evident (Fig. 6).

An interesting finding in this study was the various types of foci observed in the animals. The first type of focus was characterized as classic basophilic focus. Its cells were small and strongly basophilic, and the limits of the focus were not strictly distinguishable from neighboring hepatocytes.

In the second type of focus, no regular hepatocytes, but only infiltrates, dead cells and large vacuoles resembling

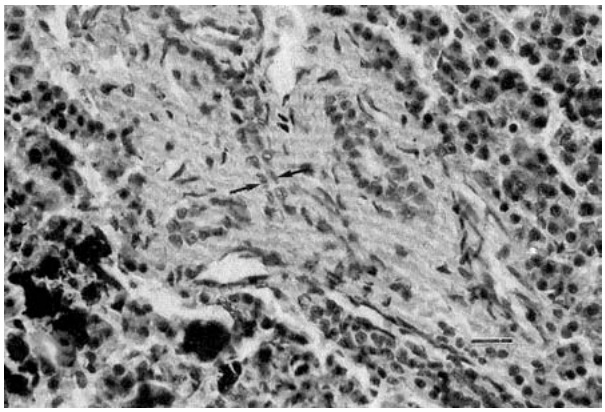

Fig. 5 A section from the liver of a frog from Tafros 66 area. A dense fibrotic area with duct-like structures lined with cuboidal epithelium was evident. The arrows point to two such ductal cells. H&E stain. The bar = 25 μm

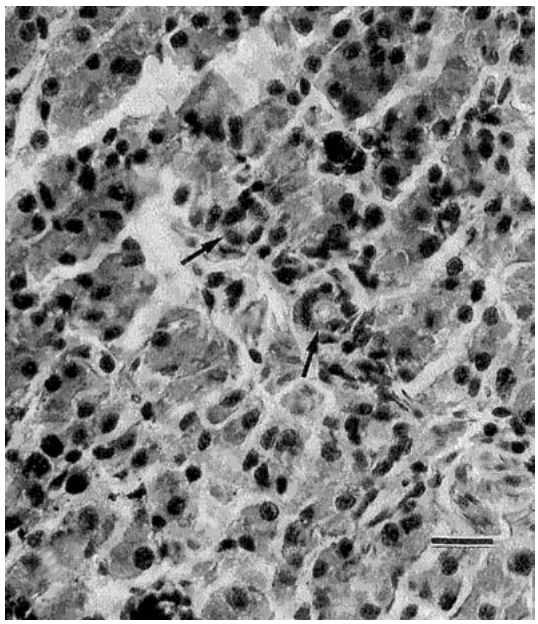

Fig. 6 Bile canaliculi (arrows) with enlarged lumen from the liver of a Tafros 66 frog. H&E stain. The bar = 25 μm

fat vacuoles, were observed. The focus was completely differentiated from adjacent hepatocytes (Fig. 7). This second focus was characterized as vacuolated focus.

The third focus was different from those of the previous types. The focus consisted of regular hepatocytes and large vacuoles. Sirius red stain showed that in the focus, there were distinguishable nodules surrounded by connective tissue (Fig. 8). In each section, three such similar foci were detected, all along the periphery (lower and higher) of the section.

In some studied animals, light fibrosis was observed. The fibrosis began in the regions around the large biliary

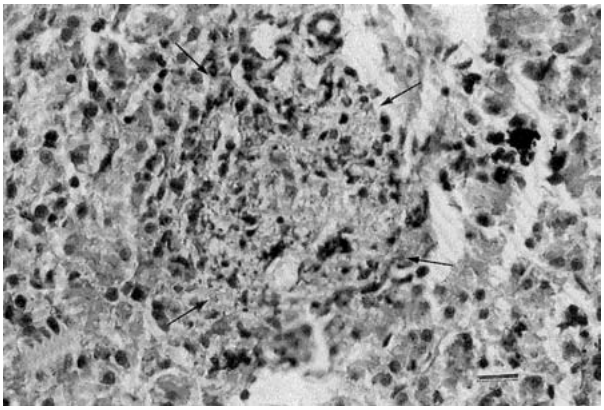

Fig. 7 A vacuolated focus (surrounded by arrows). Note the intense vacuolation, pycnotic cells, and infiltrates (mainly lymphocytes). H&E stain. Bar = 2 μm

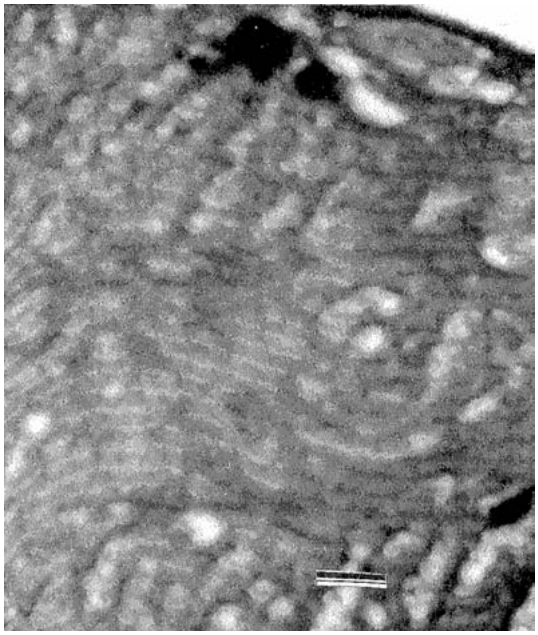

Fig. 8 A focus consisting of nodules surrounded by collagen fibers from a Tafros 66 frog. Sirius red stain. Bar = 25 μm

ducts, resulting in portal–portal linkage and, in certain regions, it extended to the adjacent parenchyma (Fig. 9).

In the frogs collected from Pertouli area, no such complicated histopathologic alterations were observed. Slight cytomegaly and some focal and mainly solitary infiltrates, all considered to be of spontaneous origin, were detected. Similarly, a mild vacuolation, most probably of spontaneous origin, was also detected.

Discussion

The area of the livers of Tafros 66 frogs occupied by the MMCs was roughly similar to that observed in animals

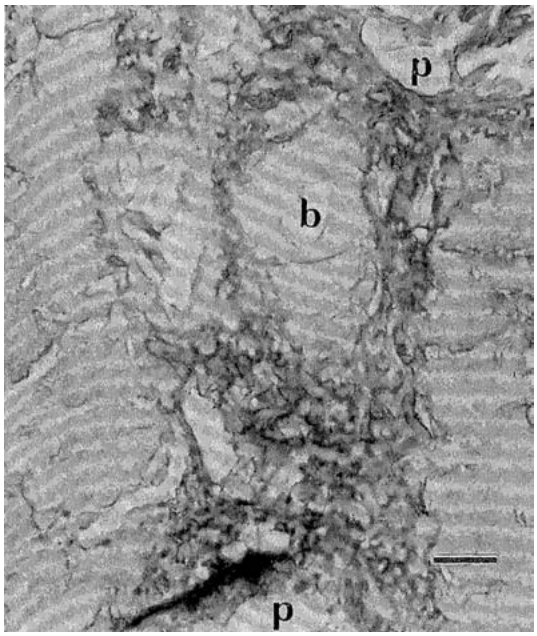

Fig. 9 Portal–portal linkage of collagenous fibers from a Tafros 66 frog. P-portal vein; b-bile duct. Sirius red stain. Bar = 10 μm

exposed to cadmium (Loumbourdis and Vogiatzis 2002). These values were also almost equal to those reported by Couillard and Hodson (1996) for Atlantic tomcod in regions of estuaries of rivers of Canada that accept sewage from paper mill effluents. The reasons for the increased surface of MMCs is not well known. Similarly, an increase in surface covered by MMCs was reported in amphibians exposed to low temperature (Barni et al. 1999), low oxygen (Frangioni et al. 2000), and heavy metals (Loumbourdis and Vogiatzis 2002; Fenoglio et al. 2005). Although in a previous study (Loumbourdis and Vogiatzis 2002), a relationship between cadmium and the surface covered by MMCs was found, MMCs can not at present be used as biomarkers of pollution by heavy metals because as noted previously, many other factors may be responsible for such an increase. More comprehensive and detailed studies should be performed to evaluate any relationship between heavy metals and MMCs as well as to establish firmly such a relationship.

In recent years, there has been increasing interest on the possible role of melanin as a scavenger of free radicals (Rozanowska et al. 1998) and other toxic substance. Such an increase in melanin content could be effective in protecting frogs from free radicals or other possible toxic substances.

Regarding sequestration of heavy metals, it should be noted that among the structural units of melanins are several negatively charged, carboxyl (COOH–) functional groups that serve as cation chelators (Riley 1997). These substituents allow melanin pigments to selectively bind

positively charged particles, such as transition metals, and sequester them in the pigmented cells. In this case, the metal-binding properties of melanin pigments should allow tracking of the distribution of hazardous heavy metals. Loumbourdis and Vogiatzis (2002), studying the relationship between MMCs and cadmium, discussed this possibility.

Regarding frogs collected from the Pertouli area, it should be noted that the surface of frog livers covered by MMCs was much smaller compared with frogs used as controls in an earlier study (Loumbourdis and Vogiatzis 2002). However, as was noted in that study, the frogs were collected from a ''relatively unpolluted'' area, which did not exclude same degree of pollution. In fact, the high concentration of copper detected in the livers of these frogs indicated that the area was to some extent polluted by heavy metals, however, the purpose of that study was comparative, *i.e.*, to comparison of unexposed versus exposed (to cadmium) frogs.

Hepatocellular alteration foci may represent progenitor lesions from which hepatocellular neoplasia may arise. These foci are composed of hepatocytes that have cytologic (clear, acidophilic, and basophilic cells) or histochemical (glycogen storage, iron-loading resistance) alterations. Their boundaries show little or no compression of adjacent parenchyma. Foci, such those observed in this study, are generally found in much greater numbers in the livers of rodents treated with carcinogens. In experimental studies of liver neoplasia using rats exposed to potent carcinogens, the initial change detected in liver tissue consisted of foci of such cellular alterations. These foci may eventually develop into hepatocellular neoplasms. Because of their consistent production by known hepatocarcinogens and their ultimate temporal relationship with neoplasia, these foci of cellular alterations are operationally regarded as preneoplastic lesions (Boardman et al. 1997).

Of particular interest is the strong PAS-positive amorphous mass filling some lumens of bile ducts and mixed with cellular debris from sloughed epithelial cells. The strong PAS reactivity showed that this amorphous mass consisted of polysaccharides. A similar condition has been reported in rats (Bannasch and Massner 1976), and they were referred to as cholangiofibroma. According to Hascheck and Rousseaux (1998), cholangiofibroma constitutes a benign liver tumor that has some characteristics in common with cholangiocarcinoma. The cholangiofibroma has an extensive collagenous connective tissue stroma surrounding atypical bile ducts. The duct-like structures are usually filled with necrotic cellular debris from sloughed epithelial cells and mucous substances that stain intensely with PAS. Indeed, the hypothesized cholangiofibroma of this study may represent a very early stage of cholangiofibroma compared with those described in rats exposed to known carcinogens (Hadjiolov and Bitch 1997; Lemmer et al. 2004).

The existence of an extensive fibrous area with duct-like structures in liver has been described by other researchers. Such a description, quite similar to that described in this study, is from Tan et al. (2002), who noted that these ductules proliferate along the fibrous septa, are lined by cuboidal cells, and usually have inconspicuous lumen. This description is quite similar to that in our study. The different cell types lining the lumen of these ducts may also show that these might be very early holangiofibromas and that the epithelial cells lining the lumen are in an early stage of differentiation from oval cells.

In this study, no special types of goblet cells in an intensive secretory state, similar to that observed in rats with extensive holangiofibroma, were detected, making it difficult to explain the origin of the mucus found in the biliary ducts. However, taking into consideration the fact that the glycocalyx of the cells in the biliary ducts containing mucus was intensely PAS positive, combined with the fact that the biliary ducts, which did not contain mucus, were PAS negative, one can hypothesize that the secretion arises from goblet cells or other types of mucous cells found in other sites of liver. Moreover, there is a strong possibility that this PAS-positive material may arise from excessive focal storage of glycogen (glycogenosis).

Baroni et al. (1998) provided insights on the role played by damaged hepatocytes in the stimulation of HSC proliferation and collagen synthesis. Hepatocytes were cultured by the above researchers in the presence of a prooxidant agent–released product, which stimulated HSC proliferation and collagen accumulation. They presumed that these *in vitro* observations mimiced, some profibrogenic situations *in vivo*, whereas HSCs are activated in the presence of minimal hepatocyte damage. Schiller et al. (1998) concluded that in guinea pigs, combined administration of aflatoxin plus copper resulted in a more-or-less pronounced fibrosis. Extensive fibrosis is presumed to be attributed to carcinogenesis by enhancing DNA hypomethylation, which is thought to be one possible mechanism of carcinogenesis because fibrosis may interfere with methyl delivery through blood flow (Schiller et al. 1998).

Allen et al. (2004) reviewed a number of studies in an attempt to identify specific pathologic end points that can be used individually and/or in concert with other findings to predict carcinogenicity. They proposed that hepatocellular hypertrophy, hepatocellular cytomegaly, and hepatocellular necrosis are the best predictors of liver cancer in mice. The investigators stated that in mice, the presence of a chemical showing a positive response in these three liver lesions has a very high likelihood of being a carcinogen in the chronic study. We described, to some extent, all of these factors as being present in this study. Karyomegaly was more

pronounced in the kidneys of frogs exposed to lead, just as described in an earlier study (Loumbourdis 2003).

All of the histopathologic alterations observed in this study indicated that the animals collected from Tafros 66 were under intense environmental stress. It is likely that these alterations may have been caused by factors presented in excess and for long times, thus leading to the various histopathologic alterations. In an earlier study (Loumbourdis and Wray 1998), we recorded in livers of the same type of frogs extremely high concentrations of copper (1041 μg/g), zinc (681 μg/g), and chromium (41 μg/g) as well as high concentrations of lead (9 μg/g). The corresponding values for the frogs collected from the Pertouli area were 24 μg/g for copper, 56 μg/g for zinc, 0.85 μg/g for chromium, and 0.9 μg/g for lead (all the previous values are in μg/g dry weight). Ebara et al. (2000) recorded high accumulations of copper as copper metallothionein in the area of hepatocellular carcinoma in rats, suggesting that copper may play a role in hepatocellular carcinoma. Chromium (VI) directly reacts with biologic material to produce ROS, which are able to cause DNA damage and gene mutation, leading thus to early carcinogenesis (Beyersmann 2002). It has been postulated that these two metals, together with some other metals such as cadmium, cobalt, and nickel, efficiently inhibit the repair of DNA damage. Therefore, these metals are assumed to enhance the extent of tumor initiation caused by other agents (Beyersmann 2002). Tafros 66, a small river, flows through three highly agricultural provinces that are extensively irrigated and sprayed pesticides and fungicides containing copper and zinc. This river also accepts the effluents of many medium-sized cities, towns, and villages, which contribute to the pollution of the river with urban runoff. It is known that urban runoff contributes significantly to pollution with chromium (Owe et al. 1982). Along the river there are also a number of light industries that discharge their effluents into the river. Many of these (unknown) pollutants, together with the heavy metals mentioned previously and found to be in excess, may also contribute to the histopathologic alterations observed in this study. Extensive ecologic, biochemical, and histologic studies are therefore required to determine the exact factor(s) controlling these alterations.

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