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Pathological Mechanisms of Hepatic Tumour Formation in Rats Exposed Chronically to Dietary Hexachlorobenzene

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The chronic dietary administration of hexachlorobenzene (HCB) to rats for a year or more results in the formation of liver tumours described as hepatocellular carcinomas, hepatomas or haemangiomas. The hepatotoxicity of HCB, which is greatest in hamsters and rats, gives rise to peliosis and necrosis with haemosiderosis. This pattern of hepatotoxicity indicates vascular damage, which through haemosiderosis could increase not only the toxic effect of HCB to hepatocytes but also its tumourogenic potential. The present study confirmed vascular damage by the identification of widespread fibrin deposits in the livers of rats chronically exposed to HCB, using an antibody to rat fibrin. Based on our study we suggest that the formation of hepatomas and haemangiomas with elements of peliosis (cystic blood-filled cavities) could be explained by the compensatory hyperplastic responses to hepatocellular necrosis and by the simultaneous loss of hepatocellular cords. The accumulation of iron in the liver would strongly potentiate the development of hepatic tumours, as has been found in HCB and polychlorinated biphenyl-treated mice with iron overload. The implications of this non-genotoxic mechanism of hepatoma formation for the assessment of human health risk are discussed.

INTRODUCTION

The induction of liver tumours in rodents chronically administered a diet containing the fungicide hexachlorobenzene (HCB) has been well documented. Thus, hamsters developed hepatomas and haemangioendotheliomas,[1] mice hepatocellular carcinomas[2] and rats hepatomas[3] after feeding HCB for up to 90 weeks. More recently, hepatic iron over-load has been shown to accelerate the development of hepatocellular nodules and carcinomas in mice exposed to dietary HCB[4,5] and polychlorinated biphenyls (PCBs).[6] The experimental evidence of iron involvement in the formation of liver tumours may be related to the observed high incidence of hepatocellular carcinoma in humans with haemochromatosis.[7] We also examined in rats the influence of increased hepatic iron levels on the induction of liver tumours by HCB.[8] The tumours that resulted were morphologically similar to the hepatomas or haemangiomas seen previously in rats exposed to HCB.[9,10] As the development of haemangiomas could involve a different series of events from that occurring in the development of hepatocellular carcinoma,[9] we have examined the role of vascular injury in rats exposed to HCB, which is known to produce haemosiderosis.[10] The use of an antifibrin antibody allowed us to examine whether haemosiderosis was associated with vascular injury and localized extravasation of blood. If this did occur, the development of this type of haemangiomatous liver tumour could be a form of adaptive response to liver injury, where the promotion and progression induced by cell proliferation are the most important rate-limiting steps in tumour formation.

MATERIALS AND METHODS

Chemicals

Organic analytical grade hexachlorobenzene (HCB) with no detectable 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or related compounds was obtained from BDH, Poole, UK. Imferon (50 mg Fe ml^{-1}) was obtained from Fisons Ltd., Loughborough, UK.

Dietary treatment of rats with hexachlorobenzene (HCB)

All rats were from experiments reported previously.[3,8,9] AGUS and F344 female rats 7–10 weeks of age were fed a powdered 41BM diet (Christopher Hill Group Ltd., Poole, UK) mixed with arachis oil (2%). The HCB was administered as 0.01 or 0.02% of the diet for up to 90 weeks. Rats were fed *ad libitum* in a negative pressure isolator kept at 21°C in a 12-h light/12-h dark cycle. Animals were killed with carbon dioxide, the livers removed and immersion fixed in 10% neutral buffered formalin. Representative blocks of each liver lobe of each rat were embedded in paraffin wax and 5-μm sections were stained with haematoxylin and eosin (H&E) and Perls' stain for iron. Duplicate sections were immunostained with the guinea-pig anti-rat fibrin antibody.[11]

A shorter term exposure of female F344 rats to HCB was also conducted in which a single subcutaneous injection of Imferon (1 ml 100 g^{-1} body wt) was given

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to animals before commencement of HCB feeding. Rats given Imferon only were used as a control group for the possible induction of liver tumours by Imferon, and another group of rats was fed the control 41BM diet. After 65 weeks all rats were sacrificed and the livers treated as described above.

Immunohistochemical staining for rat fibrin

The immunoperoxidase technique was used to stain for rat fibrin in dewaxed 5-μm formalin-fixed liver sections as described previously.[11] The antiserum used was a guinea-pig anti-rat fibrin antiserum and was used at a dilution of 1:40 in an indirect immunoperoxidase technique. The second antibody was a peroxidase conjugated rabbit anti-guinea-pig antiserum used at a dilution of 1:100.

RESULTS

In the two 90-week studies, 10 out of 13 AGUS rats[3] had hyperplastic nodules in their livers and all eight F344 rats[9] also had nodules (Fig. 1). In both groups there was abundant haemosiderin in hepatocytes (Fig. 2) as well as the more usual haemosiderin deposits in sinusodial cells. There was also a high incidence of fibrin throughout the liver, either in the central veins (Fig. 3) or in the sinusoids (see Table 1). In nearly every case where there was sinusoidal telangiectasis there was also intrasinusoidal fibrin in the dilated sinusoidal areas, demonstrated by immunostaining (Fig. 4), although this was not so readily seen in the H&E sections. Three of the long-term dosed rats also had evidence of peliosis hepatis in the hyperplastic nodules (Fig. 5) and hepatocellular necrosis.

With rats exposed to HCB for a shorter period (65 weeks) less than half had nodules (three out of eight) with HCB alone, whereas all rats pretreated with a single subcutaneous injection of iron/dextran complex (Imferon)[8] showed nodules. This accords with previous studies showing that Imferon accelerates the development of nodules and carcinomas in mice treated with HCB.[4] Moreover hepatocellular necrosis and fibrin deposition in areas of sinusoidal dilation were also more marked and the development of sinusoidal telangiectasis was accelerated (for summary, see Table 1). The rats treated for 65 weeks with only HCB had significant intravascular and intrasinusoidal fibrin where there were nodules and areas of telangiectasis,but there was no evidence of iron accumulation in hepatocytes at this early stage.

Control rats in the above studies did not develop hepatic tumours and liver sections did not contain stainable iron other than in occasional macrophages, which is a normal feature of ageing rodent livers. No significant fibrin deposits were found in the blood vessels or sinusoids of control rats by immunostaining with antifibrin antibody.

DISCUSSION

Treatment with dimethylnitrosamine (DMN) has yielded liver tumours described as haemangioendothelial sarcomas[12] in hamsters, haemangioendotheliomas[13,14] and hepatomas[15] in adult mice and 'malignant primary hepatic tumours', often blood filled and with thrombi present[16] in rats. Acute DMN toxicity in the rat liver has also been shown to give rise to sinusoidal fibrin deposits.[17] The formation of liver haemangioendotheliomas in hamsters was seen as a simple proliferation of reticuloendothelial cells developing into a tumour.[12] The characteristic pattern of haemangioendothelial sarcoma formation in mice developed from haemorrhagic necrosis of hepatic cells followed in sequence by the development of peliosis-like cystic lesions succeeded hyperplasia of the endothelial cells of the cyst wall. However, proliferation of the vascular endothelial cells in hepatic nodules often does not give rise to a multilayered vascular endothelium covering the hyperplastic hepatic cords, indicative of haemangioendothelioma. Indeed, the demonstration that 'blood cysts' induced in mice by repeated injections of urethane could be transplanted was a particularly important confirmation of their neoplastic character.[18] This transplantation experiment was performed because it was felt by the authors that the macroscopic appearance, histological structure and clinical evolution were not enough to determine their pathological significance. This was due in part to the vascular areas of the tumours being lined by only a single layer of flattened elongated endothelial cells.

The consistent demonstration of significant amounts of intravascular fibrin in the central veins and sinusoids of livers of rats exposed to a chronic dietary intake of HCB (see Table 1) is evidence of toxicity to the vasculature that is occurring on a continuous basis. This is supported by evidence of iron accumulation in the livers of rodents treated with HCB noted previously[10] (and in our present studies) and supports the explanation that such iron accumulation may be due partly to repeated damage to the vasculature, resulting in extravasation of blood. The finding of iron in hepatocytes, in particular, is strong evidence of severe chronic intrahepatic bleeding, as occurs with chronic endotoxin damage to the liver.[19] There was also a consistent finding of intrasinusoidal fibrin deposits in the areas of widened hepatic sinusoids (also called sinusoidal telangiectasis), which led to blood lakes or peliosis hepatis, due to loss of liver cords. The formation of nodules with telangiectasis and associated fibrin deposits increased with age, suggesting that the vascular injury caused by HCB was age-related as has been found with disseminated intravascular coagulation in ageing rats.[11] Old rats have been shown experimentally to hypercoagulate in response to vascular endothelial damage, especially after repeated episodes, which constitute the Shwartzman reaction,[20] increasing the likelihood of such fibrin deposition with age. Evidence of vascular endothelial damage in areas of telangiectasis would also explain the eventual loss of the hepatic cords necessary for peliosis to develop. The vascular endothelial damage would expose hepatocytes to toxic material (HCB or metabolites) carried

Figure 1. Hepatic nodule found in an F344 rat after 90 weeks of dietary exposure to HCB. (H&E, ×31.)

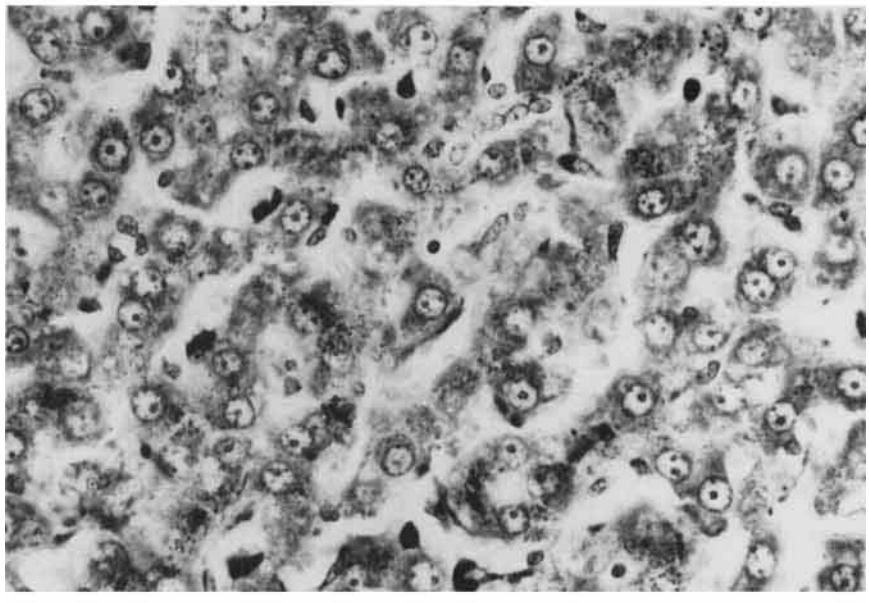

Figure 2. Accumulation of stainable haemosiderin granules in hepatocytes and sinusoidal cells of an F344 rat liver after 90 weeks of dietary HCB exposure. (Perl's reaction for iron, ×620.)

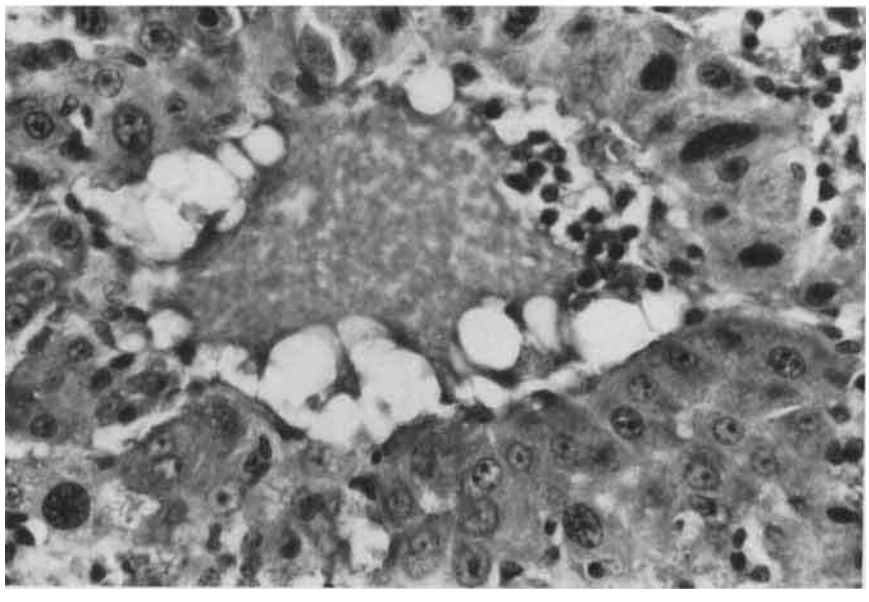

Figure 3. Immunostaining of F344 rat liver for fibrin present in the central veins. Note the inflammatory cells attached to the intravascular fibrin deposit. (Immunoperoxidase stain, ×620.)

Table 1. Summary of pathological changes in the livers of rats exposed to dietary HCB for various periods of time

Time on HCB diet	No. of rats with nodules/ hyperplasia	No. with intravascular fibrin	No. with intrasinusoidal fibrin	No. with telangiectasis	No. with Perls' stained iron deposits in hepatocytes
65 weeks	3/8	3/8	3/8	3/8	0/8
65 weeks + Fe	8/8	8/8	8/8	8/8	8/8
90 weeks (1980 study AGUS rats)	10/13	11/13	12/13	9/13	13/13
90 weeks (1985 study F344 rats)	8/8	6/8	7/8	8/8	8/8

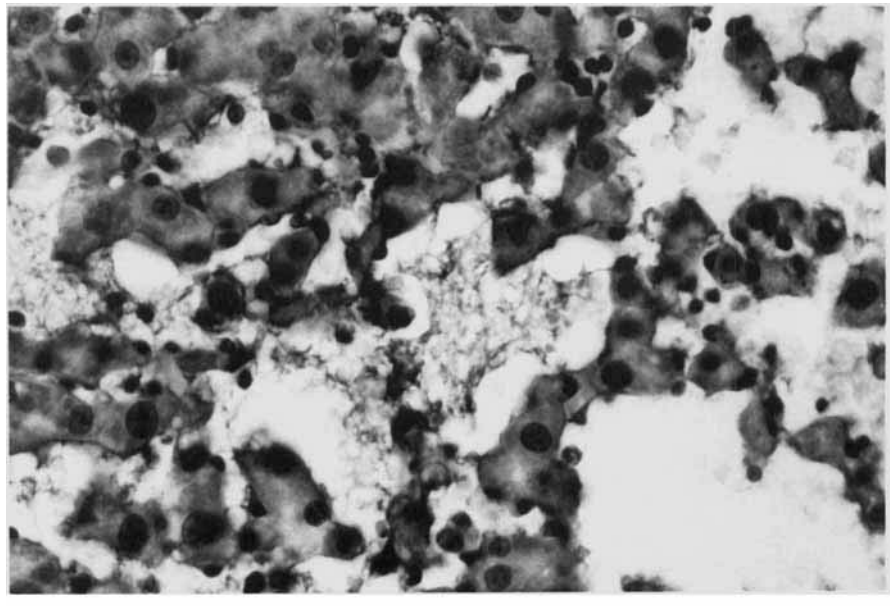

Figure 4. Area of liver of rat fed HCB for 90 weeks with dilated sinusoids and fibrin deposits within the sinusoids and attached to the cells lining the sinusoids. (Immunoperoxidase stain ×620.)

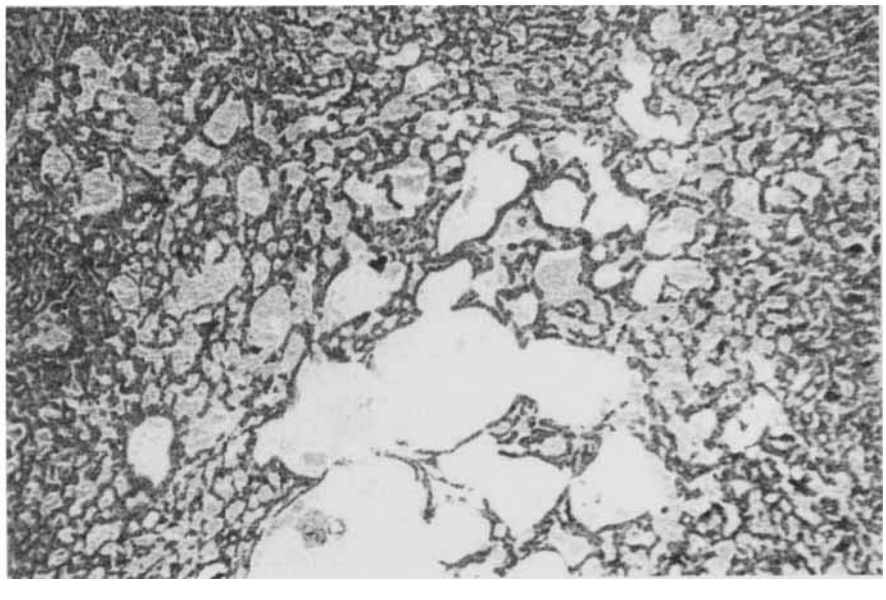

Figure 5. Sinusoidal telangiectasis and liver cord atrophy in an F344 rat fed HCB for 90 weeks. (H&E, ×155.)

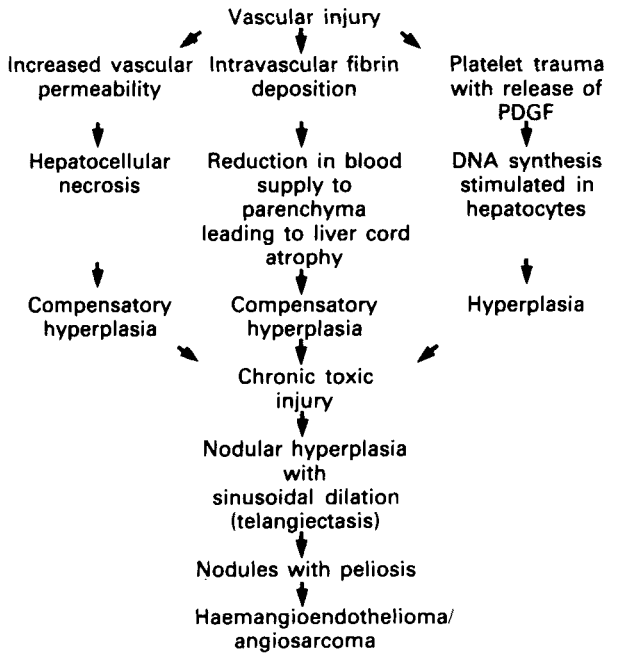

Figure 6. Pathways to liver tumour formation in rats exposed to HCB, involving vascular injury as a primary lesion in the liver. PDGF = platelet-derived growth factor.

through sinusoids, as indicated by necrosis in the hepatocellular cords in areas of telangiectasis. Thus we have two of the necessary conditions that can contribute to compensatory hyperplasia in the liver, reduction in blood supply[21,22] with fibrin deposition[23] and loss of parenchymal cell mass. Haemangiomas have been associated in man with localized nodular proliferation of the liver.[24] The detailed examination of 36 cases of focal nodular hyperplasia[25] and 64 autopsy cases of nodular regenerative hyperplasia in man[26] led to the conclusion that vascular malformation and secondary non-specific tissue adaption to the heterogeneous distribution of blood flow in the liver was the cause of nodule formation and 'cirrhosis telangiectasis hepatis'. If vascular damage due to HCB was chronic, necrosis, with possible alterations in blood supply, occurring in areas with fibrin deposits and compensatory hyperplasia would also be continuously in operation, favouring the formation of hyperplastic nodules. Fibrin deposition and damage to sinusoidal vascular endothelium would also favour localized atrophy of the liver due to increased toxic necrosis. This in turn would cause atrophy of the liver cords, leading at first to telangiectasis and then peliosis hepatis after a prolonged period of exposure to the toxin. The accumulation of iron in hepatocytes, as well as other liver cells, is also likely to favour liver growth and to potentiate the initiation of hepatoma, as has been found experimentally with both HCB- and PCB-treated mice with iron overload.[4–6]. Hexachlorobenzene has also been found to alter iron metabolism in the liver and this could also increase iron transport into hepatocytes, resulting in an increase in haemosiderin in the long term.

The idea of alternating hyperplasia and atrophy leading to the development of hepatoma and haemangioma is summarized in Fig. 6. The concept of formation of multiple hyperplastic nodules with areas of sinusoidal telangiectasis or peliosis by this mechanism can be envisaged as an adaptive response to a toxic phenomenon occurring by an epigenetic mechanism involving prolonged tissue injury,[27] rather than a deregulation of cell growth that occurs in the development of hepatocellular carcinomas due to a genotoxic mechanism. The maintenance of a normal lobular architecture with portal triads within the hyperplastic nodules was further evidence that the development of these tumours was essentially benign rather than malignant, although this does not imply that they do not have malignant potential.

How can nodules that have no degree of peliosis, only sinusoidal dilation with portal triads still evident, be interpreted? Are they putative premalignant lesions that will progress to peliotic nodules due to repeated haemorrhaging and atrophy, or are they hepatomas that may progress to carcinomas as proposed in Fig. 6. Furthermore, will they eventually progress past this stage to haemangioendothelioma or angiosarcoma if the chronic stimulation of the pathological process is maintained. This is particularly important in the chronic rodent toxicology studies that are used for the risk assessment of cancer in humans.[28] A compound tested in two species of rodents but that only causes hepatocellular carcinoma in one will be viewed as less of a risk in terms of human exposure than one that caused hepatocellular carcinomas in both. It was noticeable in previous studies, where mice were exposed to HCB in chronic dietary studies, that the tumours that developed were hepatocellular carcinomas and that there was no evidence of toxicity to the liver vasculature and no evidence of telangiectasis or peliosis present in the tumours examined. For the specific case of HCB there is no problem in risk assessment because the rats that develop hepatomas or haemangiomas also develop hepatocellular carcinomas, so the question of carcinogenicity is not an issue. Where problems of interpretation may arise are in cases where only hepatoma or haemangiomas are found at the end of a study.

The relative interpretation of the significance of these types of lesions is probably best considered in terms of dose reponse for exposure. The relative rate at which a nodule with sinusoidal dilation will progress to a hepatoma with peliosis and then haemangioendothelioma (if hepatocellular carcinoma does not supervene) will be determined by the relative amount of chronic haemorrhaging necrosis, which is dose-related. The possibility that vascular damage and hypercoagulation will occur in old rats towards the end of a 2-year carcinogenicity study is particularly important, as it has been demonstrated that there is a considerable increase in the hypercoagulating response of rats to endotoxin at 2 years of age,[20] which could rapidly accelerate the formation of haemangiomas or hepatoma in the liver.

Non-genotoxic compounds to which humans are exposed only in very small, non-cumulative amounts, with no significant hepatotoxicity at that exposure level, or compounds to which the liver is not chronically exposed, could be considered as not a significant carcinogenic risk to humans, despite the occurrence of haemangiomas in rodent studies.

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