Reversibility and Irreversibility of Liver Tumors in Mice Induced by the $\alpha$ Isomer of 1,2,3,4,5,6-Hexachlorocyclohexane

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SUMMARY

The characteristics of liver tumors in mice induced by the $\alpha$ isomer of 1,2,3,4,5,6-hexachlorocyclohexane ($\alpha$-BHC), were studied with emphasis on their reversibility or irreversibility. Male 8-week-old DDY mice were fed basal diet supplemented with 500 ppm of $\alpha$-BHC for 16, 20, 24, and 36 weeks and then were fed basal diet without $\alpha$-BHC for 4, 8, 12, 16, 24, or 36 weeks. At various intervals, 13 to 20 mice were killed for light and electron microscopic observations. The incidences of liver tumors in mice induced by $\alpha$-BHC increased progressively on continuous administration of $\alpha$-BHC, but when its administration was discontinued some tumors disappeared. Histologically, after $\alpha$-BHC administration for 24 weeks, most tumors were nodular hyperplasias, and there were only a few well-differentiated hepatocellular carcinomas. However, 60 or 72 weeks after the beginning of the experiment, most of the liver tumors were hepatocellular carcinomas and there were only a few nodular hyperplasias. At a later stage, 60 or 72 weeks, the liver parenchymal tissue in nontumorous areas was essentially normal, but small foci were occasionally seen in nontumorous areas that were composed of remaining hyperplastic nodular cells, phagocytic cells, Kupffer cells, and leukocytes. These findings suggest that the reversible tumors were usually nodular hyperplasias whereas the irreversible tumors were hepatocellular carcinomas. After $\alpha$-BHC administration was stopped, many mesenchymal cells infiltrated the nodular hyperplastic lesions, and degenerated liver cells were found. These observations indicate that mesenchymal cell elements may be important in reversing the growth of liver tumors induced by $\alpha$-BHC.

INTRODUCTION

Increasing numbers of synthetic chemicals including pesticides and naturally occurring carcinogens have been identified in our environment. Many of the organochloride pesticides are highly stable and persist in the environment and some of them, such as aldrin, dieldrin (3, 5), and DDT (23–25), have been demonstrated to have carcinogenic activity when given p.o. to experimental animals, especially mice. Recently, the carcinogenic activity of the organochloride pesticide $\alpha$-BHC has also been observed in mice (11, 12, 16, 20) and rats (10).

The question of the nature of the liver tumors induced by chemical carcinogens in mice has frequently been raised (9, 19). One opinion is that liver tumors in mice do not have a neoplastic character because they show no signs of invasion or metastasis to distant organs and because their histological patterns do not resemble those of malignant tumors. That is, some of them do not resemble the lesions described as hepatocellular carcinomas in rats or man (18). With regard to this problem, Kyriazis et al. (14) reported that detection of a high rate of lung metastasis was possible only through detailed histological examination, not simply by macroscopic examination of whole lungs. The observations reported here provide some suggestions for histological criteria of cancer of tumors in mouse liver induced by chemicals.

MATERIALS AND METHODS

Three hundred forty-one male 8-week-old DDY mice (Awazu Animal Farm, Osaka, Japan), initially weighing about 18.6 g, were divided into 5 groups of 21 to 140 animals. They were fed basal diet (commercial stock diet; Oriental Yeast Co., Tokyo, Japan) supplemented with 500 ppm of $\alpha$-BHC (Tokyo Kasei Chemical Co., Tokyo, Japan) for different periods as shown in Table 1. Twenty-one mice were fed basal diet containing $\alpha$-BHC for 16 weeks and then sacrificed (Group 1). A total of 300 mice were fed basal diet containing $\alpha$-BHC for 20 (Group 2), 24 (Group 3), and 36 (Group 4) weeks. Then a total of 248 animals were killed or maintained on basal diet without $\alpha$-BHC for different periods of 4, 8, and 12 (Group 2); 4, 8, 12, 16, 24, and 36 (Group 3); or 12, 24, and 36 (Group 4) weeks. Twenty control mice (Group 5) were fed basal diet without $\alpha$-BHC for 72 weeks. Twelve to 20 mice were killed at these intervals. Mice that died during the experiment were excluded. The sample of $\alpha$-BHC used appeared to be over 99.0% pure by gas chromatography. Mixed diets were prepared once a week and kept in a cold room. Mice were housed individually in aluminum cages in an air-conditioned room under natural
lighting conditions and were given the experimental diets and tap water ad libitum.

Before examination animals were fasted for 18 hr. Then they were killed with ether and weighed. All mice were examined macroscopically for tumors. Then the liver of each animal was weighed, and pieces were taken for light and electron microscopic studies.

For histological examination, the tissues were fixed in 10% buffered neutral formalin solution. All tissues were stained with hematoxylin and eosin, and selected tissues were stained with Mallory’s, van Gieson’s, or periodic acid-Schiff stain.

For ultrastructural studies, samples of nonneoplastic or neoplastic areas of tissues from animals that had received α-BHC were fixed with glutaraldehyde or osmic acid solution. The fixed specimens were embedded in Epon resin by a routine method for electron microscopy. Ultrathin sections were stained with saturated uranyl acetate and then lead citrate and were examined in a Hitachi Model HU-11D electron microscope. Sections for light microscopy were stained with 0.1% toluidine blue in Veronal buffer, pH 5.0.

RESULTS

Changes of Liver Weight. The changes in liver weight as a percentage of the body weight in each group are summarized in Table 1. In general, the liver weight was significantly more in mice fed a diet containing α-BHC than in control animals. However, the liver weight decreased when the diet with α-BHC was replaced by diet without α-BHC. It remained low as long as this diet was continued, except at the very end of the experiment when it tended to increase again due to tumor development. Results were similar in the groups fed α-BHC diet for 16, 20, 24, and 36 weeks (Groups 1 to 4).

Gross Findings of the Livers. In the livers of mice fed a diet containing α-BHC continuously, multiple grayish- or yellowish-white liver tumors of up to 1.0 cm in diameter were seen after 16 weeks. The livers of mice fed diet containing α-BHC for 20, 24, and 36 weeks had an irregular surface with many large, grayish- or yellowish-white tumors of up to 2.0 cm in diameter.

The characteristic changes of the liver were noticed in Groups 2 to 4 when the α-BHC diet was discontinued; namely, 4, 8, 12, or 16 weeks after this diet was stopped, most mice had a smooth-surfaced liver and no large white or yellowish tumors. However, in a few mice the liver contained small, dark red nodules of up to 0.5 cm in diameter. Some dark red nodules were observed 4 weeks after the diet containing α-BHC was stopped, and their number first gradually increased until 8 or 12 weeks in place of yellowish-white nodules after the diet was stopped and then gradually decreased.

Later, 24 or 36 weeks after the diet containing α-BHC was stopped, the livers had multiple white or yellowish tumors of up to 1.5 cm in diameter. Areas of tumors frequently showed hemorrhagic reactions and nontumorous areas occasionally appeared to be cirrhotic.

Histological Findings in Tumorous and Nontumorous Areas. In the mice during continuous administration of α-BHC, the centrolobular liver cells were hypertrophied in nontumorous lesions after 16 weeks. Similar hypertrophic liver cells were seen after 20, 24, and 36 weeks (Fig. 1). However, 8 or 12 weeks after the diet containing α-BHC was stopped, liver cell hypertrophy in nontumorous areas had disappeared completely (Fig. 2).

The liver tumors induced by α-BHC were nodular hyperplasias or well-differentiated hepatocellular carcinomas. The areas of nodular hyperplasia in mice receiving α-BHC were usually sharply demarcated from the surrounding liver tissues and had a few sinusoids and blood vessels. The cells...
in these areas had irregular cytoplasm, but mitotic figures were rare (Fig. 4).

When the diet containing α-BHC was discontinued, dark red nodules usually appeared. These were not clearly demarcated from the surrounding liver tissues. These areas were composed of various kinds of cells, such as large phagocytic cells, Kupffer cells, lymphocytes, and polymorphonuclear leukocytes, and the nodular hyperplastic cells frequently showed nuclear irregularities (Figs. 5 and 6). In these areas, small foci of myeloid metaplasia were occasionally seen. A few typical hyperplastic nodules and some areas of well-differentiated hepatocellular carcinoma were also found.

At a later stage, 60 or 72 weeks in the experiment, almost all the tumors appeared to be well-differentiated hepatocellular carcinomas. Areas of tumor showed large trabecular patterns with distended sinusoidal spaces and mitotic figures (Fig. 8). Nuclear irregularities and hemorrhagic changes were seen (Fig. 7). In nontumorous lesions in this stage, hepatic cells and periportal regions were essentially normal, although small foci of phagocytic cells and histiocytes were found as the remains of the dark red nodules. Areas of typical nodular hyperplasia were very rare.

No metastatic foci were found by careful examination of serially sectioned specimen of lungs, kidneys, and regional lymph nodes.

**Ultrastructural Findings in Tumorous or Nontumorous Areas.** In areas of nodular hyperplasia, the cytoplasm of the cells was elongated, the mitochondria were irregularly shaped, and the amount of smooth endoplasmic reticulum was greatly increased. The nuclei were usually smooth, and inclusions were frequently seen. Cytoplasmic areas occasionally contained concentrated lamellar bodies of various size.

In areas of well-differentiated hepatocellular carcinoma, the nuclei were irregular in shape, inclusions were frequently seen, and the nucleoplasm contained loose chromatin material and had low electron density. The nuclei were enlarged and the nucleolonemata were deformed. Glycogen granules were not seen, but the number of free ribosomes on cytoplasmic areas was increased. Cells of nonnodular areas had an increased amount of smooth endoplasmic reticulum, but nuclear irregularities were rare.

Areas of dark red nodules had a very peculiar appearance. These nodules were occasionally composed of different kinds of cells, such as phagocytic cells, monocytes, Kupffer cells, and remaining nodular hyperplastic cells. Many lysosomes were noticed in the cytoplasm (Fig. 9).

The cells in remaining areas of nodular hyperplasia had irregularly shaped mitochondria and a decreased amount of smooth endoplasmic reticulum. Glycogen granules were observed in the cytoplasm of some cells in dark red nodules. The nuclei were irregular in shape, and chromatin associated with the nucleolus was increased. Many degenerated cells were seen in hyperplastic nodules (Fig. 10). Some cells in dark red nodular areas had increased numbers of glycogen granules in their cytoplasm. Smooth endoplasmic reticulum and elongated mitochondria were seen in remaining areas of hyperplasia in mice fed first an α-BHC diet and then a diet without α-BHC for 12 weeks.

At a later stage of the experiment, after the diet containing α-BHC was stopped, areas of hepatocellular carcinoma contained irregularly shaped cells with irregular or oval nuclei. Their nucleoplasm contained loose chromatin material, their nucleoli were enlarged, and their nucleolonemata were deformed.

**Characteristic of α-BHC-induced Liver Tumors in Mice.** The incidences of liver tumors in mice induced by α-BHC are shown graphically in Chart 1. The incidence increased progressively on continuous administration of α-BHC up to 36 weeks, but when its administration was discontinued some tumors disappeared. Thus tumors induced by α-BHC may show either reversible or irreversible growth. After α-BHC administration for 24 weeks (Group 3), most tumors were nodular hyperplasias, and there were only a few well-differentiated hepatocellular carcinomas. However, 60 or 72 weeks from the beginning of the experiment (Groups 3 and 4), most of the liver tumors were hepatocellular carcinomas and there were only a few nodular hyperplasias. At this stage, the nontumorous liver parenchymal tissue was essentially normal, but small foci were occasionally seen in nontumorous areas that were composed of remaining hyperplastic nodular cells, phagocytic cells, Kupffer cells, and leukocytes. By electron microscopy, nodular hyperplastic cells could still be seen in the areas of dark red nodules containing mixed cells in the stage that most foci of nodular hyperplasia disappeared.

**DISCUSSION**

It has been found that liver tumors in mice induced by chemicals such as α-BHC continue to show dynamic morphologic, histological, and electron microscopic changes throughout the observation period. Therefore, in studies on chemical carcinogenesis neoplastic changes must be carefully judged by histological and electron microscopic observations, as well as by macroscopic examinations.

Tumor cells may arise as a result of both somatic mutation and abnormal differentiation of target cells induced by proximate carcinogens (17).

In carcinogenesis, various changes of the cells induced by carcinogens have been regarded as preneoplastic changes (7, 8). There is much evidence that nodular hyperplasias may be 1 site of origin of hepatocellular carcinomas.
These preneoplastic lesions of the liver have been analyzed biochemically, histologically, histochemically, biologically, and by electron microscopy, and the reversibility or irreversibility of preneoplastic changes has been discussed extensively (2, 6, 7, 13, 18, 22, 27). The present work showed clearly that the preneoplastic change of nodular hyperplasia of the liver in mice induced by α-BHC may be either reversible or irreversible.

Several investigators have reported that epitheliomesenchymal interactions influence the response of the epithelium in carcinogenesis (1, 4, 15). The present results show that tumor growth or progression is intimately related to the interactions of mesenchymal elements and liver parenchymal cells. Because after α-BHC administration was discontinued, many mesenchymal cells infiltrated the nodular hyperplastic lesions of hepatocyte proliferation that were encased in a very thin fibroconnective tissue compressing surrounding liver parenchyma (7). Also degenerated liver cells were in the collections of mesenchymal cells. These observations indicate that mesenchymal cell elements may play an important role in reversing the growth of liver tumors induced by α-BHC.

However, by electron microscopy, hyperplastic liver cells with irregularly shaped mitochondria had decreased smooth endoplasmic reticulum and an irregularly oval nucleus with several indentations, could still be seen in dark red nodules. Thus it is possible that these remaining cells in the dark red nodules showed cancer or irreversibility at a relatively early stage of the experiment (21).

The apparent regression or progression of the liver tumor nodule may be influenced by both intrinsic and extrinsic factors (17, 26). Intrinsic factors include the immunological state of the host, aging, hormonal effects, and species susceptibility, which were all found to influence tumor growth or progression. Extrinsic factors include the nutritional state of the host, carcinogenic or noncarcinogenic chemicals, viruses, irradiation, and pathological changes of the organs. Hepatocarcinogenesis was found to be inhibited by a high lipid diet. It was also observed that carcinogenic or noncarcinogenic chemicals, such as 3-methylcholanthrene, 3,4-benzpyrene, and α-naphthylisothiocyanate had either inhibitory or promoting effects on liver carcinogenesis in experimental animals. Viral and chemical interactions on various mammary tumors in mice and rats have also been reported by many investigators.

Therefore, various intrinsic and extrinsic factors may influence the reversibility or irreversibility of preneoplastic lesions. Further analysis of these factors may provide an effective method for inhibiting tumor growth.

REFERENCES

Fig. 1. Symmetrical cell hypertrophy in the liver of a mouse treated with α-BHC for 16 weeks. H & E, × 100.

Fig. 2. Nontumorous area of the liver in a mouse fed α-BHC diet for 24 weeks and then a diet without α-BHC for 12 weeks. H & E, × 100.

Fig. 3. Nodular hyperplasia induced by α-BHC diet for 24 weeks. Note compression of surrounding liver parenchymal tissue. H & E, × 100.

Fig. 4. Well-differentiated hepatocellular carcinoma in a mouse treated with α-BHC for 36 weeks. H & E, × 100.

Fig. 5. Area of a dark red nodule in a mouse 8 weeks after discontinuation of α-BHC diet. Many mesenchymal cell elements, macrophages, and lymphocytes are seen infiltrating the hyperplastic nodular area. H & E, × 100.

Fig. 6. Higher magnification of a dark red nodule, showing nuclear irregularities of liver cells and macrophages or giant cells. H & E, × 200.

Fig. 7. Typical hepatocellular carcinoma in a mouse fed α-BHC diet for 24 weeks and then basal diet for 36 weeks. Dilation of sinusoidal spaces and an increased number of nuclei in cell cords are seen with hemorrhagic necrosis. H & E, × 100.

Fig. 8. Higher magnification of typical hepatocellular carcinoma. Tumor cells and nuclei are irregular in size and shape. H & E, × 200.

Fig. 9. Liver of a mouse fed α-BHC diet for 24 weeks and then basal diet for 4 weeks. Mesenchymal cells (MC) and phagocytic cells (PC) are seen. Many lysosomes are seen in the cytoplasm of macrophages. Degenerated liver cells (DC) are also seen. × 5000.

Fig. 10. Cells in a remaining area of nodular hyperplasia (NHC) in a mouse fed α-BHC diet for 24 weeks. The amount of smooth endoplasmic reticulum is decreased. Some degenerated cells are seen. × 5000.
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