Cell Proliferation in the Liver and Thyroid of C57B1/10J Mice after Dietary Administration of Chlordane

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Chlordane is a polychlorinated hydrocarbon that causes liver enlargement and induces mixed-function oxidases similar to those induced by phenobarbitone in the mouse. We have assessed the hepatocarcinogenicity (after 2 years) and the time course (over 6 months) of liver and thyroid cell proliferation in C57B1/10J mice exposed to chlordane at 50 ppm in the diet, using the same batch of food for both carcinogenicity and cell proliferation studies. In the bioassay, 15/39 survivors had hepatocellular adenomas and a further 5/59 had carcinomas, compared with less than 5% incidence of primary hepatic tumors in concurrent controls. Among unscheduled deaths, 1/40 adenomas and 2/40 carcinomas were recorded. There were no macroscopically observed thyroid lesions. In the proliferation study, mice were killed on days 4, 5, 8, 15, 29, 99, and 190 after the start of dosing. Withdrawal groups were included from days 29 to 99 and from days 190 to 247. Replicating cells were labeled via bromodeoxyuridine delivered by osmotic minipump for 3 days before necropsy. In the thyroid, the peak labeling index (LI) was seen on day 5 (LI = 5.99 ± 2.90% versus 1.00 ± 20% in controls), while in the liver the peak was on day 8 (9.0 ± 1.6% versus 0.5 ± 0.4% in controls). Both organs had an elevated LI for the first month of dosing, but while the thyroid follicular LI was similar to control at 99 and 190 days, the liver LI was significantly elevated at all time points except in the withdrawal groups. However, there appeared to be a trend toward the control LI at longer time points, suggesting that elevated hepatocyte replication may not have occurred throughout the duration of the oncogenicity study.

Introduction

The kinetic response of hepatocytes to chronic insult by nongenotoxic hepatocarcinogens has been the subject of considerable recent interest. In several studies on the rodent liver, attention has been focused on the significance of observations of protracted proliferative activity in hepatocytes of rats exposed to inducers of cytochrome P450 type IVA1 of different carcinogenic potencies (1–3). Little information is currently available on the existence of such prolonged proliferative activity in the livers of mice of the C57B1 strain, which has a low background incidence of spontaneous liver tumors.

In the present study, the proliferative response of hepatocytes and thyroid follicular cells of the C57B1/10J mouse to chlordane was analyzed. The C57B1/10J mouse has a low incidence of hepatocellular and thyroid tumors and is used routinely for cancer bioassays within ICI. Chlordane was chosen as the test article for the studies reported here because of its known oncogenicity to the liver of both B6C3F1 and C57B1/6 mice (4–6) and because it is known to induce mixed-function oxidase enzymes similar to those induced by phenobarbitone (isozyme form II) (7,8). Thyroid follicular cells were analyzed for proliferative activity (as well as hepatocytes) to monitor any long-term response of the thyroid to the increased thyroid hormone metabolism, which would be expected to result from increased cytochrome P450 activity in the liver and increased liver size (9).

Two animal studies are reported in this paper. A

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chronic hepatocarcinogenicity assay was conducted with chlordane at 50 ppm in the diet of C57B1/10J mice for 2 years, and a parallel study of 6 months' duration with interim kills was conducted for analyzing liver histopathology and hepatocyte and thyroid follicular cell proliferation during preneoplastic stages. The same batch of food was used for both studies.

Materials and Methods

Hepatocarcinogenicity Assay

One hundred male C57B1/10J mice were given chlordane at 50 ppm in the diet up to 24 months. At necropsy, a thorough macroscopic examination was conducted, and liver samples were placed in neutral-buffered formalin for subsequent sectioning and histopathological analysis. The control incidence of hepatocellular tumors was derived from a concurrent bioassay within ICI.

Cell Proliferation Study

Groups of five male mice were killed on days 2, 3, 4, 5, 8, 15, 29, 99, and 190 after the start of dosing. Withdrawal groups were included from days 29 to 99 and 190 to 247 and control groups on days 4, 29, 99, and 190. Replicating cells were labeled by infusing 5-bromo-2'-deoxyuridine (BrdU; 15 mg/mL at 1 μL/hr via mini-osmotic pumps (model 1003D) implanted subcutaneously 3 days before necropsy on days 4, 5, 8, 15, 29, 190, and 247.

Samples were put in formalin from liver (left and right lateral and median lobes), thyroid, and duodenum (the latter as a control for BrdU immunostaining). Serial sections were cut from each block to permit hematoxylin and eosin (H&E) and anti-BrdU immunostaining. At least 1000 cells from each liver lobe and the thyroid were scored for labeled nuclei.

Results

Histopathology

Hepatocarcinogenicity Assay. The body weight data from the cell proliferation study show a reduction of 12% in chlordane-treated mice after 190 days. This indicates that the dose of 50 ppm represents an approximate maximum tolerated dose. No macroscopic abnormalities were noted in the thyroids at necropsy (these tissues were not examined histopathologically).

The liver histopathology findings are summarized in Table 1. At 2 years, the overall incidence of hepatocellular tumors was about 50%, with a higher incidence of adenomas than carcinomas. Among the unscheduled deaths, the first tumor was not observed until 21 months of dosing. The incidence of all types of hepatic tumor in 400 untreated male C57B1/10J mice was 2.0% after 2 years in a recent colony control study (data not shown).

| Tumor type            | Terminal kills | Incidental kills | Totals |
|-----------------------|----------------|-----------------|--------|
| Hepatocellular adenoma| 16/39          | 1/40            | 16/79  |
| Hepatocellular carcinoma| 5/39          | 2/40            | 7/79   |
| All tumors            | 20/39          | 3/40            | 23/79  |

The incidence of all types of hepatic tumors in 400 untreated male C57B1/10J mice was 2.5% after 2 years in a recent colony study (unpublished data).

Cell Proliferation

Thyroid. The data for thyroid follicular cell proliferation was summarized in Figure 2. The peak labeling index (LI) of thyroid cells was observed after 5 days of chlordane treatment (6.0 ± 2.9% in treated versus 1.0 ± 0.2% in controls). The LI was significantly greater in chlordane-treated mice than in control at all time points up to and including day 99 (p < 0.01), but there was no significant difference after 190 days. There was no observed elevation of LI among withdrawal animals. The distribution of replicating cells among individual follicles was also recorded during the study, but there was no evidence that any individual follicles contained an excessive number of stained nuclei (data not shown).

Liver. Qualitative examination of the slides revealed no clear zonal distribution of labeled hepatocytes in control or treated groups. The time course of hepatocyte proliferation is shown in Figure 3.

The peak hepatocyte LI was observed after 8 days of chlordane treatment (9.0 ± 1.6% in treated versus 0.5 ± 0.4% in controls). This decreased relatively quickly to 5.3 ± 1.1% by day 29, but thereafter the rate of decrease was slower. By day 190 (the end of dosing), the LI in treated animals was still 2-fold higher than in controls (1.7 ± 0.5% versus 0.8 ± 0.2%). The LI among withdrawal animals was compatible with concurrent controls in each case.
**Discussion**

These data show that chlordane is hepatocarcinogenic to male C57B1/10J mice at 50 ppm in the diet, but it does not obviously induce thyroid tumors. Chlordane induced extensive liver enlargement, which was due to both sustained hepatocyte hypertrophy as shown by histopathology, and to hyperplasia, as shown by immunocytochemical detection of S-phase hepatocyte nuclei. In the thyroid, follicular cell turnover was stimulated up to the 3-month time point, but this was not accompanied by any change in thyroid pathology. Among control mice, the rate of hepatocyte turnover ranged from 0.5% at 6 weeks of age to a maximum of 1.9% at 10 weeks. Subsequent observations were 1.0% at 19 weeks and 0.76% at 32 weeks. These results are comparable with data reported for the B6C3F1 mouse (10,11), suggesting that there is little difference in the rate of hepatocyte replication in B6C3F1 and C57B1/10J mice. A similar comparison is not available for the thyroid, where the LI in control C57B1/10J mice was 1.0% at 6 weeks of age and reduced to 0.2% by the 6-month time point.

**Figure 1.** Time course of change of liver/body weight ratios of chlordane-treated mice, expressed as percentage of control.

**Figure 2.** Time course of thyroid follicular cell S-phase labeling. A minimum of 1000 thyroid follicular cells were scored per section.
Table 2. Summary of histopathological findings in H&E sections of liver from C57B1/10J mice fed chlordane at 50 ppm in the diet for up to 6 months.

| Treatment | Days after start of dosing |
|-----------|---------------------------|
|           | 2  | 3  | 4  | 5  | 8  | 15 | 29 | 29+WD | 99 | 190 | 190+WD |
| Control   | (5)|    |    |    |    |    |    |       |    |     |       |
| Mitoses   | (5)|    |    |    |    |    |    |       |    |     |       |
| Hypertrophy |     |    |    |    |    |    |    |       |    |     |       |
| 50 ppm Chlordane | (5)| (5)| (10)| (10)| (10)| (5)| (5)| (4)| (5)| (4) |
| Mitoses   | 2  | 4  | 1  | 6  | 6  | 1  | 1  | 1    |    |     |       |
| Hypertrophy |     |    |    |    |    |    |    |       |    |     |       |
| Minimal   | 1  |    |    |    |    |    |    |       |    |     |       |
| Mild      |    |    | 2  | 10 |    |    |    |       |    |     |       |
| Moderate  |    |    |    |    |    | 5  | 5  | 5    |    |     |       |
| Severe    |    |    |    |    |    | 4  | 5  | 2    |    |     |       |

WD, withdrawal; see text for details.

Figure 3. (A) Time course of hepatocyte S-phase labeling. The results from the individual liver lobes have been amalgamated, so at least 4000 hepatocytes were scored from each animal. (B) Time course of hepatocyte labeling, shown as a line graph with a linear time scale.
It has been proposed that hepatocyte proliferation plays a pivotal role in the tumorigenicity of various nongenotoxicants that cause liver enlargement in rodents (1,3,12). Two phases of the proliferative response (“acute” and “sustained”) have been identified, and we discuss these in relation to our data with chlordane.

**Acute Proliferative Response**

With chlordane, the peak of hepatocyte LI was observed after 8 days of dosing and the initial wave of hepatocyte proliferation after the start of dosing lasted for 4 weeks. This is later than that reported in some other studies in mice with different compounds. Thus, Styles et al. (13) noted a maximum duration of the proliferative wave after methylclofenapate treatment of about 7 days, and Ward et al. (14) reported no elevated hepatocyte proliferation in mice given [3H] thymidine in 14 days of phenobarbixone treatment. It is, however, compatible with the results of Klaunig et al. (10) and Seglin et al. (15), who reported elevated hepatocyte proliferation in B6C3F1 mice treated with phenobarbixone or hexachlorocyclohexane (HCH; another polychlorinated hydrocarbon used as a pesticide) for 3, 7, and 14 days. Additionally, Lindroos et al. (16) have recently demonstrated elevated hepatocyte LI in rats treated with phenobarbixone or HCH for up to 10 days. The comparison with other reported data is difficult because factors such as different labeling procedures, age of the animals used, and different mouse strains need to be taken into account.

The time course of mouse hepatocyte proliferation with methyl clofenapate (which also has a long half-life) is inversely proportional to the dose (12), with a shorter response at higher doses. Although the dose of chlordane given in the present study is the effective maximum tolerated dose for an oncogenicity study, it would be of interest to determine the shape of the proliferative response at higher subacute doses.

**Sustained Proliferative Response**

Hepatocyte proliferation was elevated between days 29 and 190 with chlordane, although, in contrast to findings with the potent nongenotoxic hepatocarcinogens, methyl clofenapate and Wy 14,643 in the rat (1,2,17), the degree of elevation constantly decayed during this period. Chlordane also induced tumors with a much longer latency period than these other compounds, a finding that may relate to the (predicted) continued decay in cell turnover after 6 months.

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