Evidence of a Threshold Dose for Promotion of Hepatocarcinogenesis by Hexachlorobenzene in a Rat Medium-Term Liver Bioassay

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Abstract: The potential of hexachlorobenzene (HCB) to promote the development of glutathione S-transferase placental form (GST-P) positive foci in the male F344/DuCrj (F344) rat liver was evaluated with 11 different dietary doses, ranging from 0.002 to 75 ppm, using a medium-term liver bioassay system. No mortalities caused by HCB treatment were encountered, and no clinical changes were apparent. The numbers and areas of GST-P positive foci in the 0.002 ppm to 40 ppm groups were comparable with the control values, but those in the 75 ppm HCB group were slightly, albeit not significantly, increased. The present results indicate that HCB exerts weak promotion potential for rat liver carcinogenesis, but only at a dietary level of 75 ppm (5.27 mg/kg/day), thus providing evidence for the existence of a threshold. (J Toxicol Pathol 2005; 18: 7–11)

Key words: hexachlorobenzene, hepatocarcinogenesis, medium-term bioassay system, promotion

Introduction

Hexachlorobenzene (HCB), a widespread environmental contaminant¹², has been used as a fungicide. The production and applications of HCB have decreased since the 1970s due to bans and restrictions of its use in many countries, but it still occurs as a by-product in the production of a number of chlorinated solvents and other industrial chemicals. Due to its chemical stability, persistence, and long-range transport, HCB can be found throughout the environment and is detectable in human milk, blood, and adipose tissue⁴⁻⁵.

In the 1950s, accidental exposure to high doses of HCB in Turkey resulted in toxic effects in more than 3,000 people⁶, most of whom were children. Many suffered from porphyria turcica⁷, characterized by hepatic porphyria and cutaneous skin lesions caused by disturbed porphyrin metabolism. HCB is accumulated in body fat and discharged, perhaps concentrated, in maternal milk. Infants born from mothers exposed to HCB developed Pembe Yara, characterized by high mortality, diarrhea, fever, hepatomegaly, and skin lesions in the absence of porphyria. Immunotoxic effects were also reported. Human exposure to unusually high levels of HCB has also been associated with soft tissue sarcoma and brain and thyroid cancers⁸.

In experimental animals, administration of HCB, a non-genotoxic carcinogen, has been shown to induce hepatocellular tumors and haemangioendotheliomas in the liver, and adenomas of the thyroid, bile ducts and kidney⁹¹⁰. A two-generation carcinogenicity study of HCB was carried out in rats with dietary concentrations of 0, 0.32, 1.6, 8.0 and 40.0 ppm, and it showed a significant increase in the incidence of neoplastic liver nodules in F1 females exposed to 40.0 ppm, but not in F1 males exposed to any dose¹¹. The promotional potential of HCB on rat liver carcinogenesis was also investigated using a medium-term liver bioassay system with 5 different dietary doses¹². The data revealed that HCB significantly increased both the number and area of putative preneoplastic liver lesions. These were identified as glutathione S-transferase placental form (GST-P) positive foci at exposures of 75 and 150 ppm, but only the number of GST-P positive foci was significantly elevated at levels of 3 and 15 ppm. Based on these results, it was assumed that 3 or 15 ppm would be the lowest level at which the promotional potential on liver tumor development would be evident.

HCB is still of concern as an environmental contaminant, and precise evaluation of its hepatocarcinogenic potency is very important. The Ito
medium-term liver bioassay\textsuperscript{13-15} has been recognized as a reliable tool for detecting hepatocarcinogenicity or hepatopromotional potential of chemicals in a relatively short period and uses GST-P positive foci as end-point lesions\textsuperscript{14,16,17}. In the present experiment, we re-evaluated the promotion of rat liver carcinogenesis with 11 different dietary doses of HCB, ranging from 0.002 to 75.0 ppm, using our medium-term rat liver bioassay.

**Materials and Methods**

**Animals**

Male F344/DuCrj rats were obtained at 5 weeks of age from Charles River Japan (Atsugi, Kanagawa, Japan). They were housed two or three to a plastic cage with hard wood chips for bedding, and fed a powdered diet MF (Oriental Yeast, Co., Ltd., Tokyo, Japan) and water ad libitum. The animals were kept in an environmentally controlled room maintained at a temperature of 22 ± 3°C, a relative humidity of 55 ± 10% and on a 12-h light/dark cycle. They were used in this study after a one-week acclimation period.

**Chemicals**

Diethylnitrosamine (DEN) was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan), and HCB was obtained from Aldrich Chemical Company Inc. (USA).

**Experimental design**

The experimental design is shown in Fig. 1. A total of 216 rats were divided into 12 groups (18 rats/group). All were given a single intraperitoneal (i.p.) injection of DEN (200 mg/kg body weight) dissolved in saline to initiate hepatocarcinogenesis. After 2 weeks, rats in group 1 received basal diet, while those in groups 2 to 12 were given the same diet containing HCB at 0.002, 0.01, 0.04, 0.15, 0.6, 1.6, 3.0, 8.0, 15.0, 40.0 or 75.0 ppm for the experimental duration of 6 weeks. The stability, concentrations and homogeneity of the test material in the diet were also confirmed at the first preparation. The content of the test material in the diets were analyzed by gas chromatography-mass spectrometry. On analysis of the homogeneity, analytical values from samples taken from three points in the blender (0.002, 0.6 and 75 ppm) were within average value ± 10%. On analysis of the content of HCB in the prepared diet (0.6, 3.0, 15 and 75 ppm), analytical values of prepared diet (0.6, 3.0, 15 and 75 ppm) were within nominal value ± 12%. The contents for dietary levels of 0.002 and 0.04 ppm in the preparation were about nominal value ± 25%. The stability of the HCB in the diets (0.002 ppm) was confirmed to be stable in the diet for 4 weeks at room temperature. The test material diet admixture was prepared by mixing the required amount of the test material with powdered basal diet in a blender for 30 min, and it was used within 4 weeks. All rats were subjected to two-thirds partial hepatectomy at week 3. Throughout the experiment, the animals had free access to food and water, and body weights were measured once a week. Food and water consumption were also recorded once a week. Surviving rats in each group were sacrificed for examination at week 8. At necropsy, livers were excised and 5-mm thick slices were cut with a razor blade and fixed in 10% buffered formalin for immunohistochemical staining of GST-P positive foci.

All experimental procedures were performed in accordance with the in-house guidelines for the Care and Use of Laboratory Animals at DIMS Institute of Medical Science.

**Immunohistochemical staining and measurement of GST-P positive foci**

The avidin-biotin-peroxidase complex (ABC) method was used to stain GST-P positive foci. After deparaffinization, liver sections were treated sequentially with normal goat serum, anti-rabbit GST-P antibody (MBL Co., Ltd., Nagoya, Japan; 1:2000), biotin-labeled goat anti-rabbit IgG (1:200) for 1 hour and ABC. The sites of peroxidase binding were visualized by the diaminobenzidine method and the nuclei were counter-stained with hematoxylin. The numbers and the areas of GST-P positive foci >0.2 mm in diameter and the total areas of the liver sections examined were measured using a color video image processor (IPAP, Sumika Technoservice Corp., Osaka, Japan).

**Statistical analysis**

The quantitative data expressed as mean ± standard deviation (SD) values were analyzed statistically (Stat Light 1998, Yukms Corp) for significance of differences for each parameter. The significance of differences between control (group 1) and treated groups (groups 2 to 12) in numerical data obtained for body weights, organ weights and quantitative values for GST-P positive hepatocytic foci was assessed using Bartlett’s test at P<0.05. If homogeneous, the data were analyzed with the one-sided Dunnett’s multiple parametric comparison test at P<0.05; and if not they were analyzed with one-sided Steel’s non-parametric comparative
No statistical analysis was performed for general conditions, food consumption and water consumption data.

Results

Neither mortality nor clinical changes related to the test material treatment were apparent in any of the groups. Dietary administration of HCB did not affect the growth curves of rats (Fig. 2).

No alteration in daily food and water consumption was observed in any group (data not shown). The average HCB intakes calculated from the nominal dietary levels, body weights and food consumption data are summarized in Table 1. The daily intake of HCB was about 5.27 mg/kg/day in rats fed 75 ppm HCB.

The final body and liver weights are summarized in Table 1. Final body weights were not significantly different between control group and HCB-treated groups. The absolute liver weights were significantly increased in groups receiving more than 15 ppm, and relative liver weights were significantly increased in groups given more than 8 ppm.

Data for the numbers and the areas of GST-P positive foci in the liver are summarized in Table 2. The numbers and the areas of GST-P positive foci per cm² of the liver in the rats given HCB after DEN were not significantly altered from the values of animals treated with DEN alone (group 1). However, both numbers and the areas of GST-P positive foci in the liver in the 75 ppm group (5.58 ± 2.04 (P=0.09), 0.52 ± 0.27 (P=0.09)) showed a tendency to increase as compared to the control group (4.07 ± 1.56, 0.33 ± 0.16).

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**Table 1.** Final Body Weight, Liver Weight, Food Consumption and HCB Intake Data

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dose (ppm)</th>
<th>Final Body weight (g)</th>
<th>Liver weight</th>
<th>Food consumption</th>
<th>HCB intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absolute (g)</td>
<td>Relative (g/100 g body weight)</td>
<td>(g/animal/day)</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>249 ± 10</td>
<td>5.9 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>14.3</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>252 ± 13</td>
<td>6.0 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>14.6</td>
</tr>
<tr>
<td>3</td>
<td>0.01</td>
<td>251 ± 12</td>
<td>6.1 ± 0.5</td>
<td>2.4 ± 0.2</td>
<td>14.6</td>
</tr>
<tr>
<td>4</td>
<td>0.04</td>
<td>250 ± 12</td>
<td>6.0 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>14.6</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>246 ± 12</td>
<td>6.0 ± 0.3</td>
<td>2.4 ± 0.1</td>
<td>14.4</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>253 ± 10</td>
<td>6.2 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>14.3</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>253 ± 14</td>
<td>6.1 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>14.3</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>255 ± 11</td>
<td>6.2 ± 0.4</td>
<td>2.4 ± 0.1</td>
<td>14.4</td>
</tr>
<tr>
<td>9</td>
<td>8.0</td>
<td>248 ± 13</td>
<td>6.1 ± 0.4</td>
<td>2.5 ± 0.1*</td>
<td>14.2</td>
</tr>
<tr>
<td>10</td>
<td>15.0</td>
<td>251 ± 9</td>
<td>6.3 ± 0.4*</td>
<td>2.5 ± 0.1**</td>
<td>14.3</td>
</tr>
<tr>
<td>11</td>
<td>40.0</td>
<td>253 ± 9</td>
<td>6.6 ± 0.3**</td>
<td>2.6 ± 0.1**</td>
<td>14.6</td>
</tr>
<tr>
<td>12</td>
<td>75.0</td>
<td>256 ± 12</td>
<td>7.6 ± 0.5**</td>
<td>3.0 ± 0.2**</td>
<td>14.5</td>
</tr>
</tbody>
</table>

*: Significantly different from group 1 at P<0.05 (Steel’s test).
**: Significantly different from group 1 at P<0.01 (Steel’s test).

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Fig. 2. Animal growth curves
Table 2. Numbers and Areas of GST-P-Positive Foci in the Livers of Rats

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Dose (ppm)</th>
<th>No. of rats examined</th>
<th>GST-P-positive foci Number (No./cm²)</th>
<th>Area (mm²/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>18</td>
<td>4.07 ± 1.56¹</td>
<td>0.33 ± 0.16</td>
</tr>
<tr>
<td>2</td>
<td>0.002</td>
<td>18</td>
<td>3.72 ± 1.16</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>3</td>
<td>0.01</td>
<td>18</td>
<td>4.37 ± 2.24</td>
<td>0.57 ± 0.64</td>
</tr>
<tr>
<td>4</td>
<td>0.04</td>
<td>18</td>
<td>4.26 ± 2.39</td>
<td>0.34 ± 0.27</td>
</tr>
<tr>
<td>5</td>
<td>0.15</td>
<td>16</td>
<td>4.15 ± 2.11</td>
<td>0.36 ± 0.25</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>18</td>
<td>4.02 ± 1.85</td>
<td>0.41 ± 0.29</td>
</tr>
<tr>
<td>7</td>
<td>1.6</td>
<td>17</td>
<td>3.97 ± 1.69</td>
<td>0.35 ± 0.20</td>
</tr>
<tr>
<td>8</td>
<td>3.0</td>
<td>18</td>
<td>4.41 ± 1.66</td>
<td>0.40 ± 0.26</td>
</tr>
<tr>
<td>9</td>
<td>8.0</td>
<td>18</td>
<td>4.81 ± 2.82</td>
<td>0.43 ± 0.31</td>
</tr>
<tr>
<td>10</td>
<td>15.0</td>
<td>18</td>
<td>3.67 ± 1.39</td>
<td>0.31 ± 0.16</td>
</tr>
<tr>
<td>11</td>
<td>40.0</td>
<td>18</td>
<td>4.86 ± 2.01</td>
<td>0.37 ± 0.19</td>
</tr>
<tr>
<td>12</td>
<td>75.0</td>
<td>18</td>
<td>5.58 ± 2.04</td>
<td>0.52 ± 0.27</td>
</tr>
</tbody>
</table>

¹: Mean ± SD.

Discussion

In the present study we employed the Ito medium-term rat liver bioassay to assess the lowest level for the promoting effects of HCB on rat liver carcinogenesis. This system has been established on the basis of extensive scientific research over a period of more than 20 years and assessment of 313 chemicals has revealed that the assay system is highly effective for detecting hepatocarcinogens, bridging the gap between traditional long-term carcinogenicity tests and short-term screening assays. The efficacy of this system for detection of carcinogenicity is established, particularly for agents targeting the liver, as extensively discussed at a special meeting of the International Agency for Research on Cancer in 1999. The most recent edition of “Casarett and Doull’s “TOXICOLOGY” introduces this medium-term protocol as an alternative bioassay. Furthermore, this bioassay was recommended as an acceptable alternative to the long-term rodent carcinogenicity test at the Fourth International Conference on Harmonization. Since the required time is relatively short and the quantitative measurement of GST-P positive foci is very sensitive, it is relatively easy to conduct studies with many different doses of chemical to assess dose-dependence of carcinogenic potency. In particular, a Japanese research group has been approaching this very important issue using this assay system.

A HCB dose response study was carried out using the medium-term liver bioassay and it was found that among 4 doses of 3, 15, 75 and 150 ppm in diet, the upper two doses of HCB significantly increased the number and area of GST-P positive foci over the control values, whereas the doses of 3 or 15 ppm had borderline effects, because only the numbers of GST-P positive foci were increased. In another report, no promotional effect of HCB was evident at a dose of 50 ppm in this medium-term liver bioassay. However, in that report a wide range of doses were not investigated, therefore we decided that these studies were not sufficient for evaluating the threshold dose of HCB. With liver tumor formation as an end-point parameter, 40 ppm of HCB induced tumors in females but not males in a two-generation study. Under these circumstances, re-evaluation of the probable threshold level of HCB with regard to promotion of carcinogenesis is an important issue, because of its significance as an environmental contaminant. Therefore, in the present study, using a medium-term liver bioassay, the promotional effect of HCB was evaluated with numerous dose levels (12 levels, including a control level) to determine the no-effect level. The stability, concentrations and homogeneity of HCB in the diet were also determined to confirm the experimental reliability. The numbers and areas of GST-P positive foci in the 0.002 to 40 ppm groups were comparable to the control values, and only at the level of 75 ppm was a marginal increase in both parameters observed. Thus, from the present data, the dietary level of 75 ppm (5.27 mg/kg/day) should be considered as a borderline dose exerting promotion effects on liver lesion development in the rat.

The lower susceptibility of males than females to liver tumors induced by HCB was evident in several studies. However, it was demonstrated that larger liver tumors developed in males than females after a long promotion period following DEN-initiation, so the use of only males in the present medium-term liver bioassay, can be considered as validated.

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References


