Acquired Resistance to Bromobenzene Hepatotoxicity by Repeated Treatment of Rats with Bromobenzene

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Abstract: It has often been observed that chemical-induced initial insult is no longer detected in examinations after additional consecutive treatments, suggesting that the target organs acquire resistance to chemical toxicity. In this study, we examined whether acquired resistance to toxicity would be observed after repeated treatments of a toxic dose of bromobenzene (BB). In Experiment 1, F344 male rats were intraperitoneally given BB at a dosage of 150 mg/kg/day. Based on the serum AST level 20 h after the first treatment, the rats were divided into 2 groups, Groups 2 and 3, which consisted of rats with elevated AST and rats without remarkable changes in AST activity, respectively. Subsequently, the rats were subjected to the same BB treatment regimen for 4 or 9 consecutive days. The control group (Group 1) was administered with the vehicle. The AST activity of Group 2 showed no remarkable changes from Day 3, indicating an acquired resistance to BB hepatotoxicity. Measurements of drug-metabolizing enzymes in Group 2 demonstrated a reduction in CYP contents and activities, and a strong induction of GST, which contributed to the resistance. The aminotransferase activities in Group 3, however, showed no changes throughout the dosing periods. In Experiment 2, the rats with the same BB treatment regimen as Experiment 1 were administered intraperitoneally with a single dosage of BB of 300 mg/kg. Although a higher dosing of BB caused hepatic injury in all three groups, the degree of injury in the two groups with BB treatment was much slighter than that in the control (vehicle + BB). These results indicate that not only Group 2 but also Group 3 acquired resistance to BB hepatotoxicity after repeated treatments. The exposure levels of BB in the two groups were lower than those of the control. Thus, changes in the drug-metabolizing reaction related to the metabolism and detoxification of BB contributed to the resistance to BB hepatotoxicity.


Key words: resistance, bromobenzene, hepatotoxicity, drug-metabolizing enzymes, toxicokinetics, rat

Introduction

It is sometimes observed that chemical-induced initial insult is no longer detected in examinations at the end of repeated treatments, even though further treatments are continuously applied after the initial insult. This suggests that a toxic dose to target organs in the early stages of a dosing period eventually turns into a non-toxic dose as a result of repeated treatments, indicating that the animals and/or the target organs acquire a kind of resistance to the chemical’s toxicity.

A similar phenomenon, so-called autoprotection, is a well-known model in which one chemical modifies its own toxicity. In this instance, pretreatment of animals with a small dose of a chemical results in protection against a subsequently administered lethal dose of the same compound, and consequently the animals do not die even from a lethal dose²⁻⁴. Stimulated cell division by treatment with a sublethal dose of CCl₄ or thioacetamide exhibited less sensitivity to a lethal dose of the same compound due to the mechanism of autoprotection⁵⁻⁶. In addition, there have been several reports on decreased hepatotoxicity induced by acetaminophen in concomitant treatments with other compounds such as butylated hydroxyanisole⁷ or pregnenolone-16α-carbonitrile⁸⁻⁹, and by CCl₄ with SKF-525A¹⁰⁻¹³. Few reports, however, have demonstrated decreased toxicity after repeated treatment with a chemical at the same dose level.

Bromobenzene (BB), a typical hepatotoxicant, is an industrial solvent that causes centrilobular necrosis of the liver in rats and mice¹⁴⁻¹⁶. As a possible mechanism causing hepatotoxicity, epoxides, the metabolic intermediates of BB, which are formed by a phase I reaction like that of the cytochrome P450 (CYP) mixed function oxidase system, are considered to contribute to BB-induced hepatic necrosis. Most of the epoxides are converted to glutathione conjugates and dihydrodiols via glutathione S-transferase and epoxide
hydrolase, respectively, and are then finally detoxified\textsuperscript{17-20}. Pretreatment with phenobarbital (PB), a well-known hepatic drug-metabolizing enzyme inducer, enhances BB hepatotoxicity. This augmented hepatotoxicity is considered to arise from excessive 3,4-epoxide, generated by increases in covalent binding to cellular macromolecules due to the PB pretreatment, causing consequent liver necrosis\textsuperscript{21}. However, whether repeated treatment of BB results in resistance to BB-induced hepatotoxicity has not yet been examined.

These findings led us to examine whether or not rats treated with repeated toxic doses of BB would acquire resistance to BB-induced hepatotoxicity, and whether changes in phase I and phase II enzymes are related to resistance. The dose of 150 mg/kg BB used in this study was chosen because 1 mmol/kg (corresponding to 157 mg/kg) or more significantly increased serum aminotransferase activities\textsuperscript{15}.

**Materials and Methods**

**Animals**

Male Fischer-344 rats at 6 weeks of age (Charles River Japan Inc.) were transferred to a test room (barrier system) and acclimatized to the environmental conditions for 8 days. Then dosing was commenced at 7 weeks of age. The animal room was maintained at 22.3 to 25.9°C with 46.2 to 65.2% relative humidity, with an illumination time of 12 hours (7:00 to 19:00)/day and ventilation of 10 to 15 air changes per hour. The range of animal body weights at the start of dosing was between 135 and 150 g. The animals were housed individually in bracket cages (Automatic self-washing type: Nippon Cage Co., Ltd.), fed ad libitum with a radiosterilized (30 kGy, \textsuperscript{60}Co-\gamma-ray) pellet diet (NMF: Oriental Yeast Industries, Ltd.) and tap water through an automatic water supply apparatus.

**Experimental designs (Fig. 1)**

Experiment 1: Blood samples were obtained from the tail veins of 45 rats prior to the start of treatment (Day 0), and the serum aminotransferase activities (AST and ALT) of the rats were measured with a portable autoanalyzer (FDC 5500V: FUJIFILM Medical Co., Ltd.).

Thirty rats were intraperitoneally given BB (Wako Pure Chemical Industries, Ltd.) in 15% corn oil suspension (Day 0). The dosing volume was set at 0.5 ml per 100 g body weight. Twenty hours after the first dosing (Day 1), blood samples were collected and serum aminotransferase (AST and ALT) activities were measured in the same manner as described above. Based on the AST level, the rats were divided into 2 groups: Groups 2 and 3 each consisted of 15 rats with more than 100 U/L and less than 100 U/L of AST activity but no ALT activity, respectively. Furthermore, each group was subdivided into 3 subgroups in such a way as to minimize deviation of the mean values among the subgroups (5 rats/group, Table 1). The rats of one subgroup from Groups 2 and 3 were autopsied immediately after grouping and the other subgroups of Groups 2 and 3 were sequentially administered the same BB treatment regimen for 3 or 8 consecutive days, in total 4- or 9-day treatments, respectively, before being autopsied on the day after the last treatment. The control group, Group 1, consisting of 5 rats per subgroup for each termination (Days 1, 4 and 9), received the vehicle.

To monitor changes in serum aminotransferase activities during the dosing period, the activities in the rats were measured on Days 0, 1, 2, 3, 4, 5, 7, 8 and 9. On Days 1 (immediately after grouping), 4 and 9, the rats were euthanized by exanguination via the abdominal aorta under anesthesia with ether, and were autopsied. The livers were removed and weighed individually. The relative liver

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
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<tbody>
<tr>
<td>Group 1\textsuperscript{a}</td>
<td>15</td>
<td>71.3 ± 13.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>15</td>
<td>218.0 ± 133.9</td>
</tr>
<tr>
<td>Group 3</td>
<td>15</td>
<td>82.5 ± 11.5</td>
</tr>
</tbody>
</table>

The rats were divided based on the AST level 20 h after the first administration of BB (150 mg/kg); Groups 2 and 3 consisted of rats with elevated AST (more than 100 U/L) and rats with no remarkable changes in AST values (less than 100 U/L), respectively. Group 1 (a) as the control consisted of rats treated with the vehicle only (corn oil). Values are the mean ± S.D.
weight to body weight was individually calculated based on the final body weight. The livers were fixed in 10% neutral buffered formalin solution. After fixation, the histopathological specimens were prepared according to standard procedures and were stained with hematoxylin-eosin.

Experiment 2: The rats were divided into Groups II (Group 2 same as Exp. 1) and III (Group 3 same as Exp. 1) in the same manner as in Experiment 1, and were given 150 mg/kg of BB in 15% corn oil suspension for 7 consecutive days in total. The control rats (Group I) were administered the same volume of vehicle for the same dosing periods as Groups II and III. Serum aminotransferase (AST and ALT) activities were measured on Days 0 (before the start of dosing), 1, 2, 3, 4, 5 and 7 (24 h after administration of 150 mg/kg BB on Day 6). On Day 7, all rats were administered once intraperitoneally with BB at a dosage of 300 mg/kg. Twenty-four hours (Day 8) after administration of 300 mg/kg BB, all animals were anesthetized with ether, and blood was sampled from the abdominal aorta for the measurement of serum aminotransferase (AST and ALT) activities with an auto analyzer (Toshiba Medical System Co., Ltd.). They were then autopsied. The livers were removed and fixed in 10% neutral buffered formalin solution. After fixation, histopathological specimens were prepared according to standard procedures and were stained with hematoxylin-eosin.

Determination of drug-metabolizing enzymes in Experiment 1

On Days 4 and 9, liver cytosolic and microsomal fractions from the rats were prepared according to the method of Omura and Sato. The P450 content was also determined using the method of Omura and Sato. 7-Alkoxycoumarin O-dealkylase (ACD) activities, i.e., 7-methoxy-, 7-ethoxy- and 7-propoxycoumarin O-dealkylase (MCD, ECD and PCD, respectively) activities, were determined by the method of Matsubara et al. UDP-Glucuronosyltransferase activity to p-nitrophenol as a substrate (UDP-GT activity) was measured by the method of Bock et al.

UDPGlucuronosyltransferase activity in p-nitrophenol as a substrate (UDP-GT activity) was measured by the method of Bock et al. Glutathione S-transferase activities using 1-chloro-2, 4-dinitrobenzene or 1, 2-dichloro-4-nitrobenzene as a substrate (GST-D and GST-C activities, respectively) were measured by the method of Habig et al. Glutathione (GSH) content was measured using an GSH Assay kit (BIOXYTECH GSH-400; OXIS Health Products, Inc.).

Statistical analysis

The mean value and standard deviation for each subgroup were calculated for serum aminotransferase activities, absolute and relative liver weights, values of measured drug-metabolizing enzymes and TK parameters. The liver weights, drug-metabolizing enzyme and AUC values in Experiment 1, and serum aminotransferase activities and AUC values in Experiment 2 were analyzed by the Dunnett test (p<0.01 and p<0.05).

Results

Experiment 1

At grouping on Day 1, the mean values of AST and ALT in Group 2, which comprised rats with more than 100 U/L of AST activity individually, were approximately threefold to fourfold higher than Group 1. The activities in Group 2 declined to the control levels on Day 3, even though treatment with the same dose of BB was continued after the
1st dosing. Further treatment until Day 9 induced no remarkable changes in the values, which were similar to those of Group 1 until the end of the treatment. On the other hand, Group 3, which consisted of rats with less than 100 U/L of AST activity in the grouping and with the same level of activities as before starting the treatment, showed no apparent changes throughout the dosing periods (Fig. 2).

There were no significant differences in body weights between Group 1 and Group 2 or 3 on Day 9. In the measurement of the liver weights on Days 1, 4 and 9, absolute liver weights of Groups 2 and 3 were higher than those of Group 1 on Days 4 and 9. The relative weights in both Groups 2 and 3 were also higher than Group 1 on Day 1 and thereafter (Fig. 3).

Although there were no macroscopic abnormalities in the liver, mild degeneration of the hepatocytes was found microscopically in four of the five rats of Group 2 autopsied on Day 1, and severe degeneration and mild necrosis of the hepatocytes were observed in the other rat (Fig. 4). No remarkable changes in the liver were observed in any of the five rats of Group 3. Mild centrilobular hypertrophy of the

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Fig. 2. Time-course of serum aminotransferase activities in rats treated repeatedly with BB (150 mg/kg/day) in Experiment 1. Rats with elevated AST levels (Group 2) and without remarkable change (Group 3) from the first BB dosing (150 mg/kg) were subsequently subjected to the same BB treatment. Control (Group 1) was administered the vehicle. Blood sampling was conducted from the tail veins of the rats. AST and ALT levels were measured prior to each administration. The number of animals tested was 15, 10 and 5 rats on Days 0 and 1, Days 2 to 4 and 5 to 9, respectively. Data represent the mean ± S.D. of each animal number.

![Graph of AST and ALT activities](image)

*Significant or **highly significant change in comparison to Group 1 (p<0.05, p<0.01, respectively).

Table 2. Body Weights at Each Termination in Experiment 1

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Body weights (g)</th>
<th>Day 1</th>
<th>Day 4</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>5</td>
<td>136.2 ± 10.3</td>
<td>144.3 ± 9.3</td>
<td>151.6 ± 9.2</td>
</tr>
<tr>
<td>Group 2</td>
<td>5</td>
<td>133.4 ± 8.3</td>
<td>148.0 ± 11.5</td>
<td>148.0 ± 12.2</td>
</tr>
<tr>
<td>Group 3</td>
<td>5</td>
<td>122.6* ± 8.0</td>
<td>148.0 ± 7.3</td>
<td>136.6 ± 11.3</td>
</tr>
</tbody>
</table>

Final body weights of rats at termination were measured prior to anesthesia with ether. Group 1 as the control consisted of rats treated with the vehicle only (corn oil). Values are the means ± SD. *Significant change in comparison to Group 1 as the control (p<0.05).
hepatocytes was found in all the rats of Group 2 and three rats of Group 3 on Day 4. On Day 9, mild centrilobular hypertrophy of the hepatocytes was also found in all the rats of Group 3. The changes in Group 2 on Day 9 were comparable with those on Day 4. Neither degeneration nor necrosis of hepatocytes was found in any group on Days 4 or 9.

On Day 4, the P450 content significantly decreased in both Groups 2 and 3 compared with Group 1. In addition, ACD activities, as well as MCD, ECD and PCD activities,
significantly decreased in Group 2. ECD and PCD activities also decreased in Group 3. There were no notable differences between Groups 2 and 3 on Day 4. On Day 9, no significant changes in the P450 content of either Group 2 or 3 were observed compared with Group 1. For ACD activities, only PCD activity was significantly reduced in both Groups 2 and 3 (Fig. 5). Western blot analysis revealed that both CYP3A and CYP4A proteins, which cross-react with each antibody, apparently decreased in Group 2 on Day 4; CYP4A protein returned to the control level on Day 9. CYP3A protein on Day 9 tended to increase compared with that on Day 4, while the intensity of the bands in Group 2 was still weaker than that of Group 1 on Day 9 (Fig. 7). There were no remarkable changes of CYP2C6, 2C11 or 2E1 in any group during the dosing periods. On the other hand, UDP-GT activity and GST activities were augmented approximately 4- and 1.5-fold, respectively, in both Groups 2 and 3 and remained at similar levels on Day 9. GSH content in Groups 2 and 3 was also approximately 2-fold higher than in Group 1 on Days 4 and 9 (Fig. 6).
The maximum plasma concentration of BB following the first administration in both groups was approximately 20 \( \mu \text{g/ml} \). The concentration declined and was not detected at 24 h after the treatment. Exposure levels as AUC values in Groups 2 and 3 were 124 and 91 \( \mu \text{g·hr/ml} \), respectively. Measurement of the plasma concentration of BB conducted on Days 4 and 8 demonstrated that the kinetic profiles were mostly similar to those on Day 0. Plasma concentrations followed the same pattern on both days. Although the AUC values in both groups showed a tendency to increase on Day 4, the values on Day 8 were equivalent to those on Day 0. There were no significant differences in the AUC values between both groups (Fig. 8).

**Experiment 2**

Rats were divided into two groups, Groups II and III, as in Experiment 1. Groups II and III were then treated with BB, 150 mg/kg, for 7 consecutive days. The AST and ALT activities of the rats in all groups underwent the same changes as in Experiment 1. After that, the rats including Group I (the vehicle control rats) were given a single 300 mg/kg dosage of BB. The AST and ALT values dramatically increased in all the rats. However, the AST values of Groups II and III were approximately 20- and 60-fold lower than Group I, respectively (Fig. 9). Additionally, the ALT values of Groups II and III were 15- and 50-fold lower than Group I, respectively (Fig. 9). Centrilobular degeneration and/or necrosis of the hepatocytes were also microscopically observed in all rats. Microscopic examination revealed that mild to moderate necrosis of the hepatocytes in all the rats of Group I was observed, though mild necrosis was found in 3 of 5 rats in Group II and there was no necrosis in Group III. Thus, the order of the degree of the change was as follows: Group I >> Group II > Group III (Fig. 10).

The plasma concentration of BB was measured at 1, 2, 4, 8 and 24 h after the single administration of 300 mg/kg BB. The maximum concentration in Groups I and Group III were 34 \( \mu \text{g/ml} \) and 31 \( \mu \text{g/ml} \), respectively, though the concentration (21 \( \mu \text{g/ml} \)) in Group II was slightly lower than that of the other groups. The AUC values in Group I and Group III were 320 and 260 \( \mu \text{g·hr/ml} \), respectively, whereas a lower AUC value (200 \( \mu \text{g·hr/ml} \)) was observed in Group II. In comparison with the AUC value of Group I, that of Group II was significantly lower, while that of Group III showed no significant difference (Fig. 11).
In Experiment 1, we examined whether or not repeated dosing with a chemical would reduce the chemical-induced initial insult caused by the chemical itself, using the hepatotoxicant BB.

Serum aminotransferase activities, as indicators of hepatic injury, increased approximately 3-fold after a single administration of 150 mg/kg BB (Group 2). Degeneration and/or necrosis of hepatocytes was found microscopically in Group 2. The aminotransferase activities declined to normal levels as did those of the vehicle control (Group 1) on Day 3 and thereafter, even though further BB treatments were administered to the injured rats. Increased liver weight and hypertrophy of the hepatocytes were observed from Day 4, while neither the degeneration nor the necrosis of the hepatocytes observed on Day 1 was found after that. Thus, at least Group 2 was considered to acquire resistance to the BB hepatotoxicity resulting from repeated treatments. On the other hand, no remarkable changes without transient elevation of aminotransferase activities were observed microscopically in Group 3. Further treatment to Group 3 caused no apparent changes, and the values followed the same levels as Group 1 throughout the dosing periods. Liver weights significantly increased to the same extent as those on Days 4 and 9, corresponding to hypertrophy of the liver weights.

Discussion

In Experiment 1, we examined whether or not repeated dosing with a chemical would reduce the chemical-induced initial insult caused by the chemical itself, using the hepatotoxicant BB. Serum aminotransferase activities, as indicators of hepatic injury, increased approximately 3-fold after a single administration of 150 mg/kg BB (Group 2). Degeneration and/or necrosis of hepatocytes was found microscopically in Group 2. The aminotransferase activities declined to normal levels as did those of the vehicle control (Group 1) on Day 3 and thereafter, even though further BB treatments were administered to the injured rats. Increased liver weight and hypertrophy of the hepatocytes were observed from Day 4, while neither the degeneration nor the necrosis of the hepatocytes observed on Day 1 was found after that. Thus, at least Group 2 was considered to acquire resistance to the BB hepatotoxicity resulting from repeated treatments. On the other hand, no remarkable changes without transient elevation of aminotransferase activities were observed microscopically in Group 3. Further treatment to Group 3 caused no apparent changes, and the values followed the same levels as Group 1 throughout the dosing periods. Liver weights significantly increased to the same extent as those on Days 4 and 9, corresponding to hypertrophy of the liver weights.
hepatocytes. In the measurement of drug-metabolizing enzymes, both the P450 content and ACD activities in Groups 2 and 3 significantly decreased on Day 4. GST (GST-C and GST-D) activities and GSH content related to drug conjugation increased 1.5- and 2-fold in both groups, respectively. On Day 9, however, there were no significant changes in the P450 content, and among ACD activities, only PCD activity declined. The phase II conjugation reaction on Day 9 significantly increased to the same extent as that on Day 4. Therefore, the changes in the drug-metabolizing enzymes related to the metabolism and detoxification of BB were responsible for the resistance to the BB hepatotoxicity in Experiment 1. In particular, the induced phase II reaction was considered to be significantly involved, rather than the reduced phase I reaction, due to the lack of reduction in P450 content in Group 2 on Day 9.

Two possible factors may be involved in the acquired resistance observed in this study. The intermediate metabolite (3,4-oxide) might decrease as a result of the decreased activities of phase I metabolism and consequently less of the metabolite was detoxified effectively by the enhanced conjugation activities. Strubelt et al. reported that decreased CYP contributed to an impaired sensitivity to APAP-induced hepatotoxicity resulting from a 3-day APAP treatment at 1000 mg/kg. Shayiq et al. also demonstrated that a lethal dose of APAP following an 8-day dosing at sublethal doses provided resistance, in which the down-regulation of CYP to metabolize APAP to NAPQI and increased GSH were involved. On the other hand, it was unclear whether without elevated aminotransferase activities Group 3 would develop resistance to hepatotoxicity as in Experiment 1, even though Group 3 was also considered to indicate acquired resistance, since it showed the same profiles of drug-metabolizing enzymes as in Group 2 as a result of repeated treatments.

For further confirmation of the resistance in Experiment 1, rats receiving the same regimen as Experiment 1 were given twice the dosage of BB (300 mg/kg). According to the results, serum aminotransferase activities increased in all rats with BB, whereas the extent in Groups II (same as Group 2 in Exp. 1) and III (same as Group 3 in Exp. 1) was significantly lower than that in the control (Group 1). Cirrhotic degeneration and/or necrosis of hepatocytes were also microscopically observed in all rats. Severe hepatocellular necrosis, in particular, was found in all the control rats. The degree of hepatic injury was in the following order: Group 1 >> Group II > Group III. Hence, it was obvious that Group III also acquired resistance with repeated BB treatments, as did Group 2/II. However, the differences in the AUC levels between the BB-treated groups and the control failed to take into account the different degree of hepatic injury. The livers of rats with resistance were exposed to the same levels of BB as the control, but the changes in the drug-metabolizing enzymes as described above are thought to function to effectively detoxify. Moreover, there were differences in the degree of hepatic damage between Groups II and III, indicating a distinguishable difference in the grade of the acquired resistance. These findings, however, may suggest that the other enzymes not measured in this study could also be involved in the resistance.

Western blot analysis in Experiment 1 indicated that BB treatment impaired CYP3A and CYP4A proteins. On Day 9, CYP4A recovered to normal levels, though increased CYP3A still was lower than Group 1. Although BB hepatotoxicity has been shown to be enhanced under conditions where drug-metabolizing enzymes have been induced by phenobarbital, CYP isoenzymes related to the impaired hepatotoxicity of BB remain obscure. The present results demonstrate that CYP3A and/or CYP4A might predominantly be involved in BB metabolism if decreased production of BB metabolites contributes to the resistance as described above.

It was concluded that the degree of severity of the BB-induced initial insult declined since the whole body and/or the liver acquired resistance to BB hepatotoxicity after repeated treatments. The changes in the hepatic drug-metabolizing enzymes, in particular, induced phase II conjugation, resulting in the metabolism of BB, and the AUC levels of BB contributed to the resistance. However, other factors may also have contributed to the resistance, since the present findings failed to fully explain the mechanism of the acquired resistance.

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References