Mechanism of Testicular Atrophy Induced by Di-n-butyl Phthalate in Rats. VI. A Possible Origin of Testicular Iron Depletion

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In previous studies we have described mechanisms of testicular atrophy whereby di-n-butyl phthalate (DBP) caused a sloughing of the germ cells, prior to the testicular atrophy; this sloughing might be attributed to iron depletion in the blood and the testicular interstitial cells. To determine whether the iron depletion is mediated by iron-release from hemoglobin (Hb), the effects of DBP upon erythrocytes have been studied. In the in vivo studies, it was observed that DBP induced glutathione (GSH) depletion, a decrease in GSH reductase activity and Heinz body formation in the red blood cells, and iron release from Hb. In the in vitro studies, in which mono-n-butyl phthalate (MBP), a metabolite of DBP, was incubated with erythrocytes, Heinz bodies and iron release from Hb were observed.

The present study proposes that a mechanism for the testicular atrophy induced by DBP might involve Heinz body formation, accompanied by iron release from Hb followed by depletion of iron in the blood and testes.

Keywords di-n-butyl phthalate; rat testicular atrophy; erythrocyte; iron release; hemoglobin; Heinz body

Phthalate-induced damage results in Sertoli cell vacuolation followed by shedding of morphologically normal germ cells into the tubular lumen.1–4 The mechanisms of this damage have been mainly investigated using two compounds, di-2-ethylhexyl phthalate (DEHP) and di-n-butyl phthalate (DBP). The results of in vivo5–6 and in vitro7,8 experiments suggest that mono-2-ethylhexyl phthalate (MEHP), a metabolite of DEHP, is responsible for producing Sertoli cell damage. In our previous in vivo experiments,5–6 DBP caused a sloughing of mature germ cells in the early stages of damage, before the Sertoli cell vacuolation, and testicular atrophy at the final stage. Moreover, the testicular level of mono-n-butyl phthalate (MBP), a metabolite of DBP, was highest at between 1 and 3 h following oral postadministration, accompanied with both decreases in iron level9 and succinate dehydrogenase (SDH) activity,7,8 and an increase in lactate dehydrogenase activity (LDH)3 in the testis before severe sloughing of germ cells. Recently, we proposed a possible mechanism3,9 by which iron depletion in the blood might be associated with iron transfer from transferrin to hemosiderin in the liver to induce testicular iron depletion followed by the sloughing of germ cells. However, there remains an alternative mechanism, i.e. blood iron depletion may be caused by iron release from hemoglobin (Hb) directly.

We were therefore prompted to investigate the effects of DBP or MBP on iron release from Hb which is involved in the formation of Heinz bodies in vivo and in vitro.

MATERIALS AND METHODS

Materials DBP (purity 99.9%) and enzyme assay kits were purchased from Wako Pure Chemical Ind. Ltd. (Tokyo). Other reagents were obtained from commercial sources.

MBP (purity 99.9%) was synthesized by heating an equivalent mixture of phthalic anhydride and n-butyl alcohol at 120°C for 5 h, according to the method of Brunel.10

Animals and Dosing As reported previously,5–6) adult male Wistar rats (Imamichi rats, Nippon Bio-supp. Center, Tokyo, weight range, 380–430 g) were used: Control rats received 0.9% saline (1.0 ml) and all treated rats received neat DBP as a single oral dose of 2.4 g (8.6 mmol)/kg. Three rats were sacrificed at 3 h from reasons mentioned previously.7–9

General Procedure After the rats were killed at 3 h, their organs were individually weighed within 1 min of removal. The left and right testes were subjected to histological and biochemical examinations, respectively. The histological examination was carried out according to the method of Fukunoka et al.5,6) Blood samples were collected from the abdominal aorta and subjected to biochemical examinations.

Preparation of Blood Fractions and Biological Assays Freshly drawn blood samples were collected in a 5.0 ml tube containing heparin sodium (50 μl, 1000 U/ml) and a portion of the samples was used to measure the number of red blood cells and iron levels. The remainder of the samples was allowed to stand at 4°C for 30 min. Erythrocytes were separated from the plasma by centrifugation at 700 × g for 10 min and then washed three times with saline (5 ml).

The erythrocytes (1 vol.) were mixed with saline (1 vol.) to give an erythrocyte suspension which was used to measure Hb, methemoglobin (MHB) and glutathione (GSH), and the enzyme activities of glucose-6-phosphate dehydrogenase (G6PDH), methemoglobin reductase (MHB-R) and glutathione reductase (GSH-R) as well as Heinz body formation.

Hematological Examination of Erythrocytes The number of erythrocytes, Hb content and hematocrit were measured using a Sysmex M-2000 equipped with Sysmex DA-1000 (Toa Iyodensi Ltd.).

Heinz Body Formation In Vivo Heinz Body Observation: The erythrocyte suspensions (50 μl, about 6 × 10⁸ cells) obtained from control and DBP-treated rats were

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used to measure the numbers of erythrocytes and Heinz bodies: Ten $\mu$l of erythrocyte suspension (50 $\mu$l) was used to measure the number of red blood cells and 4 $\mu$l of the residual suspension was stained with a solution of methyl violet (16 $\mu$l; methyl violet (0.5 g) in saline (100 ml)) and Heinz bodies were measured under the microscope.

**In Vitro Heinz Body Formation:** The formation was observed by incubating erythrocytes with MBP according to a slightly modified method in the case of phenylhydrazine. MBP (2.95 mg, 6.66 mm) was dissolved in 66 mm phosphate buffer (pH 7.6, 2 ml) containing 5.5 mm glucose and the erythrocyte suspension (50 $\mu$l, about $6 \times 10^8$ cells) from control rats was added to the MBP solution. As a control, the erythrocyte suspension (50 $\mu$l) was added to the above phosphate-glucose buffer (2 ml). Each mixture was incubated at 37°C for 2 h and a portion (50 $\mu$l) of the mixture was stained with a solution of methyl violet (200 $\mu$l). The residual mixture was centrifuged at 700 $\times$ g for 10 min to give a supernatant and cells. The solution was filtered through Centricon 30. While the precipitated cells were lysed with water (2 ml). The filtrate and lysed solution were used to measure iron and MBP.

**GSH Content** GSH levels in the erythrocytes were determined by the Ellman method. Ten $\mu$l of the erythrocyte suspension (60 $\mu$l) was used to measure the number of red blood cells and Hb levels. The remaining suspension (50 $\mu$l) was lysed with water (2 ml) and the solution (2 ml) was mixed with a solution of EDTA (3 ml, metaphosphoric acid (0.835 g), EDTA (0.1 g), NaCl (15 g) and water (50 ml)). This solution was allowed to stand for 10 min and then filtered. The filtrate (0.5 ml) was diluted with 0.3 M Na$_2$HPO$_4$ (2 ml) and the absorbance (A1) was measured at 412 nm. A solution of DTNB (0.25 ml, dithio-bis(2-nitrobenzoic acid) (10 mg) in 1% sodium citrate (50 ml)) was added to the above mixture and the absorbance ($A_2$) was measured.

Concentration of GSH = ($A_1 - A_2$) / 13600 x 5/2 x 2.75/0.5 x 10$^5$/cell-number

**Enzyme Activities of G6PDH, MHBp (NADH-Ferricyanide Reductase), GSHR and LDH** The enzyme activities were assayed using the corresponding assay kits: G-6-PDH-UV Test (Boehringer Mannheim) for G6PDH and LDH UV Test Wako for LDH. The activities of MHBp and GSHR were determined according to the methods by Takesue and Omura, and Beutler, respectively.

**Measurement of the Iron, MBP and Hb Content of Erythrocytes** In Vitro Studies: Iron and MBP were isolated from erythrocytes as described in the experimental section “in vitro Heinz body formation” and then measured.

**In Vivo Studies:** The erythrocyte suspension (1.5 ml) was lysed with 5 mm sodium phosphate buffer (20 ml, pH 7.9) and centrifuged at 700 x g to separate the supernatant cytosol and erythrocyte-membrane-ghosts. The ghosts were washed with the same buffer (15 ml x 3). The cytosol and wash solutions were combined. A part (10.0 ml) of the combined solution was passed through Centrprep 30 (Amicon, Grace company) by centrifugation at 1500 x g to give a filtrate. A part (2.5 ml) of this filtrate was freeze-dried, dissolved in water (1 ml) and the iron level determined using an iron assay kit, Fe-Mono Kainos (Kainos Lab). A part (50 ml) of the remaining filtrate was adjusted to pH 4.0 by addition of 10% HCl and extracted with chloroform (50 ml x 3), followed by re-adjustment of the pH to 3.0 with 10% HCl and then extracted with chloroform (50 ml x 3).

In addition, the ghosts were suspended in 10% HCl (10 ml) and extracted with chloroform (10 ml x 3). MBP levels in the three chloroform extracts were determined by measuring the peak with a retention time of 8 min on a JASCO HPLC series instrument; a standard solution of MBP (10, 20, 40 and 100 $\mu$m) was used to give a calibration curve under the following conditions: column: Puresil 5 $\mu$m C$_18$, 120 Å, 4.6 x 150 mm; solvent system: 50% aqueous methanol and flow rate 0.5 ml/min.

**Statistical Analysis** Statistical analysis was carried out as described by Gad and Weil.

**RESULTS**

Left tests, which were subjected to histological examination, exhibited mild sloughing characteristic of the initial stage of damage as found in germ cells in DBP-treated rats, as reported previously.

**Heinz Body Formation** The formation was observed both in vivo and in vitro experiments: In the in vivo study, Heinz bodies were observed in 11—15% erythrocytes (136.7 ± 18.8 per 1000 erythrocytes) from the blood of untreated rats and in about 0.05% erythrocytes (0.5 ± 0.3 per 1000 cells) from the blood of controls. In the in vitro study, when MBP and erythrocyte suspension were incubated in 66 mm phosphate-glucose buffer (pH 7.6) by bubbling for 1 min every 15 min at 37°C for 2 h, Heinz bodies were observed under the microscope, 16—32% (266.7 ± 73.2 per 1000 cells) and 1—3% (16.6 ± 9.4 per 1000 cells) for the MBP-treated erythrocytes and controls, respectively. In both studies, the higher incidence of Heinz body-erythrocytes in the treated rats was significant when compared with those in the controls, but in the incubation without bubbling, the difference (1—4%) in both groups was not significant.

**Levels of Iron, GSH, Hb, MHBp, and MBP, and Enzyme Activities in Erythrocytes** Table I shows the body and testis weights, and iron levels in the right testis. A decrease in iron levels was observed as reported previously.

Table II shows the hematological findings and number of erythrocytes along with body weights and blood iron levels. Levels of Hb, mean corpuscular volume (MCV)
Table II. Hematological Findings for Rat Erythrocytes 3 h after Administration of DBP

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
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<tbody>
<tr>
<td>Body weight mg/ml blood</td>
<td>390 ± 8.1</td>
<td>409.3 ± 14.6</td>
</tr>
<tr>
<td>Iron µg/ml blood</td>
<td>612.4 ± 4.0</td>
<td>528.0 ± 7.8**</td>
</tr>
<tr>
<td>Red blood cells (RBc) × 10^6/µl</td>
<td>10.27 ± 0.18</td>
<td>9.86 ± 0.16</td>
</tr>
<tr>
<td>Hemoglobin (Hb) g/dl</td>
<td>18.0 ± 0.3</td>
<td>16.9 ± 0.5*</td>
</tr>
<tr>
<td>Hematocrit (HCT) %</td>
<td>50.7 ± 1.0</td>
<td>47.7 ± 0.9</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>9.0 ± 0.2</td>
<td>55.7 ± 0.7*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>20.9 ± 0.1</td>
<td>20.6 ± 0.1</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>35.5 ± 0.2</td>
<td>37.2 ± 0.6</td>
</tr>
</tbody>
</table>

*All values are means ± S.D., n = 3. **Corresponding to iron levels (624.6 ± 10.4 and 586.4 ± 10.3 µg/ml) for the control and treated groups, respectively, from the equation: Fe = Hb content × 4 × MW of Fe × (MW of Hb)−1. Abbreviations: MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration. * and ** Significantly different from control at p < 0.05 and p < 0.01, respectively.

Table III. Levels of Hb, Mhb and GSH and Activity of Enzymes in the Isolated Erythrocytes 3 h after Administration of DBP

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb pg/cell</td>
<td>20.9 ± 0.4</td>
<td>18.6 ± 0.2*</td>
</tr>
<tr>
<td>MHB pg/cell</td>
<td>3.7 ± 1.6</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>GSH × 10−2 µmol/cell</td>
<td>1.96 ± 0.39</td>
<td>1.30 ± 0.10*</td>
</tr>
<tr>
<td>G6PDH × 10−7 IU/cell</td>
<td>5.25 ± 0.47</td>
<td>4.78 ± 0.58</td>
</tr>
<tr>
<td>GSHR × 10−4 IU/cell</td>
<td>38.4 ± 2.16</td>
<td>25.9 ± 3.72**</td>
</tr>
<tr>
<td>LDH × 10−3 IU/cell</td>
<td>4.97 ± 0.68</td>
<td>4.82 ± 0.39</td>
</tr>
<tr>
<td>MHR × 10−3 IU/cell</td>
<td>0.98 ± 0.02</td>
<td>0.82 ± 0.09</td>
</tr>
</tbody>
</table>

*All values are means ± S.D., n = 3. * and ** Significantly different from control at p < 0.05 and p < 0.01, respectively.

Table IV. Levels of MBP and Released Iron in the Isolated Erythrocytes of DBP-Treated Rats

<table>
<thead>
<tr>
<th>MBP concentration (× 10−3 µmol/ml ES)</th>
<th>Iron levels in the cytosol of erythrocytes (× 10−5 µg/ml ES)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control rats</td>
<td>56.9 ± 6.6</td>
</tr>
<tr>
<td>DBP treated rats</td>
<td>361.6 ± 16.4*</td>
</tr>
<tr>
<td>Membrane-ghost</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cytosol at pH 7.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>at pH 4.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>at pH 3.0</td>
<td>96.7 ± 4.7</td>
</tr>
</tbody>
</table>

*All values are means ± S.D., n = 3 and n.d. = not detected. MBP was extracted from the erythrocyte membrane and the cytosol at various pH values. **Iron: non-heme iron. Levels of the iron released from Hb into the cytosol are calculated from the averaged differences between the treated rats and controls: 361.6 ± 56.9 ± 364.7 × 10−3 µg/ml ES, corresponding to 877.4 µg Hb/ml ES, ES, erythrocyte-suspension used. * Significantly different from the control at p < 0.05.

and iron were significantly reduced by DBP-treatment.

Table III shows levels of Hb, MHB and GSH, and the enzyme activities in the erythrocytes. The activity of GSHR was significantly reduced. Other enzyme activities tended to decrease in the erythrocytes from the DBP-treated rats but this was not significant. Hb and GSH levels were significantly reduced from the erythrocytes from the treated group. MHB levels tended to fall but this reduction was not significant.

Table IV shows levels of MBP and iron in the membrane-ghosts and cytosol of the erythrocytes from DBP-treated rats (Table III). MBP was extracted at pH 3.0 but not at pH 4.0 which allowed free MBP to be extracted. The presence of MBP was observed in the cytosol but not in the membrane when extraction was performed at pH 3.0. Cytosolic levels of non-heme iron in the erythrocytes from the DBP-treated rats were higher than those in controls. This suggests that MBP might induce iron release from Hb directly and/or by decomposition of Hb.

Table V shows the changes in iron and Hb levels in the erythrocytes under the conditions of the in vitro formation of Heinz bodies induced by MBP. The results show increased iron levels in the incubation medium and decreased Hb levels in the cytosol. These changes suggest that MBP crossed the erythrocyte membrane and released iron from Hb in the cytosol into the incubation-medium.

**DISCUSSION**

We reported in previous papers that the testicular transferrin (Tf) enhancement induced by DBP might be attributed to iron deficiency in the blood (including the testicular interstitial tissue) and this iron depletion might produce a decrease in SDH activity in the Sertoli cells, causing sloughing of the germ cells. In addition, we proposed a mechanism by which Hb and iron depletion in the blood might become involved in iron release from Tf-derived iron and/or Hb and in the accumulation of the released-iron as both liver-hemosiderin and spleen-ferritin. However, since serum Tf levels were not affected by DBP, it is conceivable that DBP (MBP) may denature or decompose Hb directly after crossing the erythrocyte membrane and the MBP-treated erythrocytes may reduce the binding affinity of Hb for oxygen, causing anoxia. Therefore, mechanisms for this iron depletion need to be investigated with respect to the behavior of iron in the erythrocytes.

Heinz bodies are aggregates of denatured Hb, termed hemicochrome, which are commonly seen by phase-contrast microscopy as dark spots, free in the cytoplasm or attached to the cytoplasmic surface of the erythrocyte membrane. Heinz bodies are believed to occur by either natural processes (including cell aging and disease) or drug induced formation. MHB in the red cell is converted first to a
reversible hemichrome, then to an irreversible one and, finally, into an aggregate, the Heinz body. 17, 18 Waugh et al. 19 have proposed that Heinz bodies occur naturally in three distinct ways: Firstly, Heinz bodies are believed to contribute to the structural instability of Hb, causing its denaturation, precipitation on the membrane and ultimately hemolytic anemia. 18 Secondly, Heinz bodies are seen in cells containing normal Hb under conditions of oxidant stress, suffering from a diminished capacity to maintain intracellular reducing power, e.g., in G6PDH and GSH peroxidase deficiency diseases 20–22 and in cells containing unstable Hb. 18 Thirdly, Heinz bodies are frequently observed in aged cells. 23, 24 In our study (Table III), GSH concentration and GSHR activity were significantly lower in the erythrocytes from DBP-treated rats than those from controls. Activities of G6PDH and MHBR showed a tendency to fall in the treated erythrocytes (Table III). Therefore, the results in Tables III–V suggest the possibility that DBP (MBP) might induce formation of Heinz bodies both/or by depletion of reducing power, mainly GSH and GSHR, and/or by producing an acidic microenvironment due to MBP. 9 Oxy Hb was oxidized to MHB dramatically at low pH 25 and GSH oxidized Hb to MHB in the presence of oxygen. 26, 27

As pointed out by Preisach et al. 17 if Heinz body formation was induced by a similar mechanism irrespective of natural or drug-induced formation, drug-affect erythrocytes may be selectively recognized and removed from the circulation by macrophages located primarily in the spleen and liver as well as in the case of senescent erythrocytes. 28–31 Recently, it was shown that phenylhydrazine, which induces Heinz bodies, can cross-link red cell band 3 protein (senescent antigen), resulting in the binding of autologous immunoglobulin G (IgG). 29 Recognition of this complex by macrophages triggers rapid erythropagocytosis in the spleen and possibly also the liver. 29 Therefore, this erythropagocytosis may reasonably explain the previous results 9 that DBP (MBP) induced iron accumulation in both hepatic hemosiderin and splenic ferritin along with Hb depletion in the liver and spleen.

In conclusion, this study is the first to report that iron depletion might be associated with Heinz body formation. The possible mechanisms for testicular atrophy induced by DBP are 1) that its early stage might involve the formation of Heinz bodies and then the iron in the Heinz body-erythrocytes might be transferred to hemosiderin in the liver 9 and to ferritin in the spleen, 9 subsequently causing iron depletion in the blood 5–9 2) that the iron depletion might induce testicular iron depletion 5–8 and affect the iron supply to the iron-sulfur subunit of SDH in the Sertoli cells, 8 resulting in a disruption 6, 7 of the energy transfer system between Sertoli and germ cells, and 3) that finally this disruption might cause sloughing of the germ cells which might then disappear from the testicular tubule, 5, 6 resulting in testicular atrophy. 5, 6

Further work is in progress on the direct effects of MBP on oxyHb and MHB with respect to hemichrome formation.

REFERENCES