Responsiveness of Rat Nonparenchymal Liver Cells and Spleen Cells to the Decomposition of Erythrocytes Exposed to Di-*n*-butyl Phthalate and Its Metabolite

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We have reported that di-*n*-butyl phthalate (DBP) caused the depletion of circulating iron, characterized by the release of iron from both haemoglobin (Hb) and transferrin (Tf). The present study investigated whether the erythrocytes from DBP-treated rats were destroyed by nonparenchymal liver cells (NPC, including Kupffer cells) or spleen cells (SC). In the *in vivo* study, there were observed depletions of Hb in the blood and of iron in the hepatic Tf fraction, as well as an accumulation of iron in the hepatic hemosiderin (Hs) and splenic Tf fractions. In the *in vitro* study, mono-butyl phthalate (MBP), a metabolite of DBP, caused a depletion of iron in the plasma Tf, although a direct release of iron from Tf was not detectable. When erythrocytes from DBP-treated rats and erythrocytes preincubated with MBP both were incubated with NPC, respectively, the Hb was decomposed and the iron also accumulated in the cell debris. However, when the two kinds of erythrocytes were incubated with SC, respectively, no decomposition of Hb was observed at low and medium doses, but the highest dose induced an accumulation of iron to Tf. Therefore, the NPC may contribute in part to the decomposition of DBP- or MBP-affected erythrocytes.

Key words phthalate; iron release; transferrin; haemoglobin; hemosiderin; nonparenchymal liver cell

It is well known that phthalates cause testicular damage.²⁻⁷⁾ Mechanisms of the damage were responsible for producing the Sertoli cell damage histologically²⁻¹³⁾ and biochemically.^{4-7,14-17)} Fukuoka *et al.*^{5,6,18,19)} and Zhou *et al.*⁷⁾ proposed a possible mechanism, the loss of circulating iron, which could account for the Sertoli cell damage based on three phenomena: 1) di-*n*-butyl phthalate (DBP) and its metabolite (mono-butyl phthalate (MBP)) induced the release of iron from haemoglobin (Hb) during Heinz body formation in erythrocytes, 2) the release of iron from transferrin (Tf) was accelerated by the acidity of MBP and 3) the iron released was accumulated into the hemosiderin (Hs)-fraction of the liver. Therefore, it is necessary to explain the loss of circulating iron from the interactions of the three phenomena.

Naughton *et al.*²⁰⁾ have reported that senescent erythrocytes are selectively recognized and removed from the circulation by macrophages located primarily in the spleen and liver. Therefore, we investigated a loss of circulating iron from the effects of nonparenchymal liver cells (NPC) or spleen cells (SC) on DBP- or MBP-affected erythrocytes and Tf, respectively.

MATERIALS AND METHODS

Chemicals DBP (purity 99.9%) and assay kits for iron level were purchased from Wako Pure Chemical Ind. Ltd., (Tokyo).

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.²¹⁾

Rat Tf was purchased from Sigma. Horseradish peroxidase-goat anti-rabbit IgG (H+L) was purchased from Zymed Laboratory, Inc. (California). Rabbit-anti rat Tf antiserum was purchased from Cappel (England).

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Other reagents were obtained from commercial sources.

Animals and Dosing Adult male Wistar strain rats (Imamichi rats, Nippon Bio-supp. Center, Tokyo, weight range, 200—220 g) were treated with neat DBP as a single oral dose (mentioned below) after housing for one week in this study. Control rats received 0.9% saline (1.0 ml).

General Procedure Nine rats received neat DBP (8.6 mmol/kg body weight), the dose of which caused testicular atrophy within one week⁵⁾ and severe sloughing of mature germ cells at 6 h after the administration.⁶⁾ Six rats from each of the control and treated groups were killed at 3 h and the three remaining treated rats were sacrificed at 6 h to demonstrate the sloughing of germ cells.

Blood was collected from the abdominal aorta of the control and DBP-treated rats, and erythrocytes were isolated from the plasma by centrifugation at 1000 × *g* for 10 min, washed three times with saline (5 vol.), and subjected to biochemical examination, respectively. After the examination, the erythrocytes from the control rats were combined to use in the *in vitro* studies. Plasma for the *in vitro* studies was collected from the six control rats and diluted to 50% with 66 mM sodium phosphate (pH 7.4).

Urinary samples were collected at 3 and 6 h after administration and used to measure iron concentrations.

The right testes from the 3 h- and 6 h-treated rats were subjected to biochemical examination and the left ones from the 6 h-treated rats were subjected to histological examination.

A portion of the liver was fixed in 10% neutral buffered formalin and embedded in paraffin. After removal of paraffin, sections of the liver were stained with 2% potassium ferricyanide–1% HCl and 2% potassium ferrocyanide–1% HCl, respectively. A portion of the remaining liver and spleen were used to isolate ferritin (Ft) and Hs fractions as described below.

The liver and spleen for *in vitro* studies were obtained from another two control rats and were used for the preparation of non-parenchymal liver cells and spleen cells, respectively.

MBP was used in concentrations ranging from 0.1 to 6.8 μmol in the initial incubation-medium, since in the *in vivo* studies, MBP levels were 1.8 $\mu\text{mol}/\text{ml}$ in the plasma and 90 nmol/ml in the 50%-erythrocyte-suspension at 3 h after oral dosing of DBP (8.6 mmol/kg).¹⁸⁾

Hematological Examination of Erythrocytes The number of erythrocytes and mean corpuscular volume (MCV) were measured using a Sysmex M-2000 equipped with Sysmex DA-1000 (Toa Iyodensi, Ltd., Japan).

Separation of Ft, Hs, and Tf Fractions in the Liver and Spleen Fractionations of Hs and Ft were carried out according to the methods of Vidnes and Helgeland,²²⁾ and Setsuda,²³⁾ respectively from both the liver and spleen. The liver was homogenized with distilled water (10 vol.). The homogenate was filtered and centrifuged at $600 \times g$ at 4°C for 15 min to give a sedimentary Hs fraction and supernatant (Tf and Hb) fraction. The sediment was washed with water (3 ml) and the washed water was combined with the supernatants. After centrifugation at $10000 \times g$ at 4°C for 20 min, a portion of the supernatant was heated at 75°C for 10 min, followed by centrifugation at $5000 \times g$ for 15 min to give a soluble fraction as Ft fraction.²²⁾

The spleen was homogenized with 0.15 M NaCl (4 vol.). The homogenate was filtered and centrifuged at $5000 \times g$ at 4°C for 30 min to give a sedimentary Hs fraction and supernatant (Tf and Hb) fraction. The sediment was washed with 0.15 M NaCl (1 ml) and the washed solution was combined with the supernatants. A portion of the supernatant was heated at 75°C for 10 min and centrifuged at $5000 \times g$ at 4°C for 15 min to give a solution. The solution was adjusted to pH 4.8, centrifuged, and ammonium sulfate was added to reach 50%. The mixture was then allowed to stand at 2–10°C for 18 h, and centrifuged at $2500 \times g$ at 4°C for 15 min to give a sedimentary fraction. The fraction was dissolved in 0.15 M NaCl (1 ml) as Ft fraction.²³⁾

The sedimentary Hs fraction was subjected to Hs-iron measurement. The supernatant was also subjected to the measurement of Hb and Tf.

Measurement of Iron and Hb Levels of non-heme iron and Hb were determined using an assay kit, Fe-mono Kainos (by the yellow-green complex formation method with 2-nitroso-5-(*N*-propyl-*N*-sulfo-propylamino)phenol in the presence of a reducing agent, Kainos) and Hemoglobin B Test Wako (by sodium lauryl sulfate method, Wako Pure Chemicals), respectively.

Total iron binding capacity (TIBC) was determined by combining two assay kits, Fe-Kainos (the iron sequestered by Tf after the addition of ferrous iron, Kainos) and Fe-mono Kainos (Kainos). Each value represents an average of the duplicates.

Total iron in the blood, organs and fraction samples was digested completely with concentrated nitric acid (1.0 ml; specific gravity 1.38 available for metal ions) at 110°C for 2 d and was then analyzed using a Hitachi atomic absorption spectrometer (Model 170-50) at 248.3 nm.

Enzyme Immunoassay of Tf Tf was measured in duplicate samples by an enzyme immunoassay, according to the method of Fukuoka *et al.*⁶⁾ The samples used were supernatants at $105000 \times g$ of the separated cell fraction mentioned above. Twenty μg of Tf was solubilized in 10 ml of phosphate buffer containing saline (PBS; 134 mM NaCl in 20 mM phosphate buffer, pH 7.3). Flat-bottomed polyvinyl microtitre plates (Nunc, Denmark) were coated with Tf in PBS by adding 100 ng in 50 μl buffer to each well. The plates were incubated at 20°C under cover for 2 h and washed with 0.1% Tween 20 in PBS (PBS-T). After four wash cycles, the plates were treated with samples or standard protein (Tf) preincubated with anti-serum (1:20000) (rabbit anti-rat Tf) in PBS. After incubation at 20°C for 2 h and four wash cycles with PBS-T, 50 μl of a solution of horseradish peroxidase labelled goat-anti-rabbit IgG diluted 1:1000 in PBS-T and the plates were incubated at 20°C for 2 h. Four wash cycles followed. Fifty μl of substrate solution (10 μl of 30% H_2O_2 and 10 mg-tablet of *ortho*-phenylenediamine-HCl in 25 ml of citrate-phosphate buffer (2.4 mM citric acid and 51 mM Na_2HPO_4 , pH 5.0)) was added to each plate. The plates were then incubated at 20°C for 30 min in darkness. The substrate solution was made immediately before use. The reaction was terminated after exactly 30 min by adding 50 μl of 1.5 M H_2SO_4 to each well. The absorbance was measured at 492 nm in a Titertek Multiscan (Flow Laboratories).

Incubation of Rat Plasma with MBP The diluted plasma (1 ml) was added in a solution of MBP in 5 mM sodium phosphate-buffer (pH 8.0) and the concentrations of MBP were adjusted at 0, 0.1, 0.3, 0.7, 1.4, 3.4 and 6.8 μmol in the total mixture (1.5 ml). The mixture was incubated at 37°C for 2 h. A portion of the incubation mixture was used to measure TIBC and Tf-iron levels. The rest was passed through a disposable filter, Sartorius SM MW 20000, by centrifugation at $1000 \times g$ for 10 min. The filtrate was measured for levels in iron-containing proteins with a molecular weight less than 20000.

Preparation of NPC and SC NPC were isolated from rats by *in situ* perfusion of the liver with collagenase. The cells were separated into two fractions, parenchymal hepatocytes and non-parenchymal cells, by differential centrifugation according to the method of Shimaoka *et al.*²⁴⁾

SC were isolated from the spleen of control rats according to the modified method of Mishell-Dutton²⁵⁾. The spleen was minced in Hank's solution (12 ml), allowed to stand for 30 min at 25°C, and then separated into the cell debris and a supernatant cell fraction. The supernatant cell fraction was used for incubation studies.

Effects of NPC on the Erythrocytes Isolated from Rats Administered DBP A suspension (60 μl) of the erythrocytes ($1.3\text{--}1.7 \times 10^5$ cell/ μl) from control rats was incubated with NPC (2.5×10^5 cells) in L-15 medium (1 ml) at 37°C for 120 min. The mixture was centrifuged at $1000 \times g$ for 10 min to give pellets and supernatant fractions. The supernatant fraction was subjected to the measurement of iron levels, and the pellets were lysed with 5 mM phosphate buffer (pH 8.0) (1 ml) to measure Hb levels.

Effect of NPC and/or Plasma on Erythrocytes Treated with MBP A suspension (20 μ l) of the erythrocytes ($9.0 \times 10^6/\mu$ l) from control rats was incubated with MBP (0.1, 0.2, 0.7, 1.4, 3.4 and 6.8 μ mol) in a solution of 66 mM sodium phosphate–5.5 mM glucose (500 μ l, pH 7.4) and 50% rat plasma in 66 mM sodium phosphate buffer (pH 7.4, 500 μ l) under conditions which formed Heinz bodies.¹⁸⁾ After incubation for 5 min, NPC (500 μ l, 1.5×10^6 cells/ml in L-15 medium) were added into the above mixture and incubated at 37 °C for 2 h (in the case without NPC, 500 μ l of L-15 medium was added and incubated).

The incubation mixture was centrifuged at $1000 \times g$ for 10 min to give sedimental and supernatant fractions. The supernatants were measured for iron concentration. The sediment was lysed with distilled water (500 μ l) and gave a lysed solution and ghost-pellets. The lysed solution was used to measure the levels of Hb, free iron and low molecular protein-iron filtrated through Centriscarto SM13249 (Molecular weight, 20000, Sartorius, Japan). The ghost-cell debris was used to measure iron levels by an atomic absorption spectrometer.

Effect of SC on Erythrocytes Treated with DBP or MBP *In Vivo* Study: Erythrocytes were isolated from rats administered DBP and from the control rats as mentioned above. Twenty μ l of the washed erythrocytes (6.0 – 7.0×10^4 cells/ μ l) was incubated with a suspension (500 μ l) of SC (7.0 – 7.5×10^6 cells/ml) at 37 °C for 2 h.

In Vitro Study: An erythrocyte-suspension (20 μ l) from the control rats was incubated with a solution (pH 7.3) of MBP (0, 0.1, 0.2, 0.7, 1.4, 3.4 or 6.8 μ mol) in Hanks' solution (1000 μ l) with/without 50% plasma (500 μ l) at 37 °C for 5 min. To the mixture was the above suspension (500 μ l, pH 7.3) of SC added and the mixture was incubated at 37 °C for 2 h.

The above incubation mixture was centrifuged at $1000 \times g$ for 10 min to give sedimentary and supernatant fractions. A portion of the supernatant was used to measure iron and Tf-iron levels. The rest was passed through a disposable filter, Sartorius SM MW 20000, by centrifugation at $1000 \times g$ for 10 min. The filtrate was measured for iron levels in both free-iron and iron-containing protein with a molecular weight of less than 20000. The sediment was lysed with distilled water (500 μ l) and gave a lysed solution and ghost-cell debris. The lysed solution was used to measure levels of Hb.

RESULTS

Histopathological Examinations A histopathological examination was carried out, since it was necessary to know whether the histopathological results were in accord with the previous data^{4,5)} before this study was performed. The testes obtained from rats at 6 h postadministration showed sloughing of the germ cells in the DBP-treated rats, so it was then proved to be of use for a mechanical investigation of testicular damage.

Kupffer cells and Hs in the liver were not stained with two reagents which detected Fe(II) and Fe(III), respectively. However, protein-mass in the hepatic Hs fraction from DBP-treated rats and NPC-debris from DBP

(MBP)-affected erythrocytes were stained with the reagent for Fe(II) but not Fe(III). A stained blue colour was eluted from the protein mass into the reagent solution in course of time. This suggests that Hb might be decomposed or denaturated to ferrous proteins and that the proteins might not be fixed or might be washed out during the washing procedure on the liver-slide glass.

Hematological Examination Erythrocytes from DBP-treated rats showed no changes in mean corpuscular volume (MCV) when compared with that of the controls: 60.6 ± 0.9 fl for the control and 58.2 ± 1.5 for erythrocytes from DBP-treated animals.

Biochemical Examination Urinary iron levels were 0.03 ± 0.01 μ g and 1.49 ± 0.13 μ g for 3 h and 6 h-control rats, respectively, and 0.26 ± 0.06 μ g and 2.69 ± 0.19 μ g in the 3 h and 6 h-urine of DBP-treated rats, respectively. The excretion levels of iron were significantly higher ($p < 0.01$) in the treated rats than in the controls.

Table 1 shows rat body and organ weights. Of the organs, the liver, spleen and testes showed a tendency to decrease in weight, but it was not significant. The ratio of testes to body weight showed a significant decrease, in accord with the previous data.^{6,18)}

Table 2 shows concentrations of iron and Hb in the blood, liver, spleen or right testis. Iron depletion was observed in the blood and testis of the treated rats. Iron levels in hepatic fractions showed significant increases in

Table 1. Weights of Body, Liver, Spleen and Testis at 3 h after Oral Administration of DBP

Organ weight	Control	Treated
Body	217.0 ± 2.9	216.0 ± 4.2
Liver	11.5 ± 0.7	10.0 ± 0.4
Liver/body $\times 10^{-3}$	52.9 ± 1.6	$46.3 \pm 2.9^*$
Spleen $\times 10^{-1}$	6.0 ± 0.3	5.9 ± 0.5
Spleen/body $\times 10^{-4}$	25.7 ± 1.5	25.6 ± 2.1
Right testis $\times 10^{-1}$	9.1 ± 0.1	8.5 ± 0.4
Left testis $\times 10^{-1}$	9.2 ± 0.1	8.5 ± 0.5
Testes/body $\times 10^{-4}$	85.9 ± 2.0	$78.2 \pm 1.3^*$

Data are expressed as g and are means $\pm$ S.D. ($n=6$); not significant except for asterisks. * Significant for $p < 0.05$, compared with the respective controls; Dunnett-type mean rank test.³⁸⁾

Table 2. Levels of Iron and Hb after Oral Administration of DBP

Blood and organs	Control	Treated
Total iron (μ g/ml blood or g organ)		
Blood	624.1 ± 3.9	$528.0 \pm 5.8^{**}$
Liver ^{a)} Hs fraction	6.3 ± 0.2	$7.5 \pm 0.1^*$
Tf fraction ^{b)}	10.8 ± 0.7	$7.9 \pm 0.6^*$
Ft fraction	0.6 ± 0.02	0.7 ± 0.08
Spleen ^{a)} Hs fraction	3.5 ± 0.4	3.3 ± 0.3
Tf fraction ^{b)}	13.9 ± 2.2	$18.7 \pm 3.6^*$
Ft fraction	41.4 ± 4.1	33.5 ± 3.1
Right testis	26.0 ± 1.3	$20.4 \pm 0.5^*$
Hb		
Blood (mg/ml)	186.5 ± 6.2	$145.3 \pm 6.0^*$
Spleen (mg/ml)	49.6 ± 5.6	$18.6 \pm 3.5^{**}$

Values are means $\pm$ S.D. ($n=6$); not significant except for asterisks: * significant for $p < 0.05$; ** significant for $p < 0.01$, compared with the respective controls; Dunnett-type mean rank test.³⁸⁾ a) The fractionation was carried out according to the Materials and Methods. b) Tf levels in the fractions were 8.4 ± 0.2 mg for the control liver and $5.7 \pm 0.4^*$ mg for the treated liver, and 160.9 ± 18.9 μ g for the control spleen and 173.0 ± 12.7 μ g for the treated spleen.

Table 3. Levels of Tf (TIBC) and Tf-Iron in the Plasma at 3 h after Oral Administration of DBP or 2 h Incubation with MBP

Plasma	Control	DBT-treated
<i>In vivo</i>		
Tf (TIBC) $\mu\text{g/dl}$	542.9 $\pm$ 16.5	636.2 $\pm$ 35.6*
Tf-iron $\mu\text{g/dl}$	294.3 $\pm$ 68.3	142.2 $\pm$ 10.6*
Saturation %	52.1 $\pm$ 11.8	23.7 $\pm$ 2.9*
<i>In vitro</i>		
MBP	0 μmol	6.8 μmol
TIBC $\mu\text{g/dl}$	206.3 $\pm$ 8.8	209.9 $\pm$ 14.7
Tf-iron $\mu\text{g/dl}$	110.8 $\pm$ 0.1	105.0 $\pm$ 5.8
Saturation %	63.8 $\pm$ 13.1	62.4 $\pm$ 14.3

Values are means $\pm$ S.D. ($n=6$). *In vitro* experiment changes in TIBC and Tf-iron levels were not observed at doses of 0.7–3.4 μmol MBP in the medium when compared with the respective control (MBP 0 μmol). An iron-containing compound with a molecular weight of less than 20000 was not detectable in the medium. * Significant for $p < 0.05$, compared with the respective controls; Dunnett-type mean rank test.³⁸⁾

the Hs fraction and a decreasing tendency in the other fractions. Tf levels (5.7 $\pm$ 0.4 mg) in the Tf fraction of the treated rats showed significant decreases ($p < 0.05$) when compared with those (8.4 $\pm$ 0.2 mg) of the controls. In the spleen, total iron levels showed a significant increase in the Tf fraction, although its Tf-levels did not change (160.9 $\pm$ 18.9 μg for control; 173.0 $\pm$ 12.7 μg for the treated rats).

Hb levels showed significant decreases in the blood and spleen. Hb levels in the liver were eliminated in the table because its levels varied by bleeding upon liver removal.

Table 3 shows plasma Tf (TIBC) and Tf-iron levels in the *in vivo* and *in vitro* studies. In *in vivo* studies, the TIBC in DBP-treated rat was higher, and its Tf-iron levels and the saturation ratio of Tf with iron were significantly lower, when compared with those of the controls, respectively. In *in vitro* studies, MBP, a metabolite of DBP, did not affect the levels in TIBC and Tf-iron up to the dose of 6.8 μmol .

Table 4 shows the effects of NPC and SC on the erythrocytes (RBC) from control and DBP-treated rats. As for the effects of NPC, the Hb ratio of post- to pre-incubation showed a significant decrease in RBC from the DBP-treated rats when compared with those in the control cells. Iron levels in the medium and the cell debris were higher in the RBC of DBP-treated rats than in those of the controls. This suggests that iron was released from the Hb of the DBP-affected erythrocytes and a portion of the iron was accumulated in the cell debris.

As for the effect of SC, a significant increase in both Hb levels and the Hb ratio was observed in the DBP-affected erythrocytes, without a change in the release of iron from Hb, when compared with those of the control. This suggests that the DBP-affected erythrocytes might resist being destroyed by SC or that iron might be released from components of the SC.

Table 5 shows the effects of the plasma Tf, NPC and SC on Hb and iron levels in erythrocytes exposed to MBP.

On incubation of the erythrocytes with MBP and plasma, the plasma Tf-iron levels increased at 3.4 μmol of MBP, and a decrease in Hb levels was observed at 6.8 μmol without changes in iron levels in the ghost. These

Table 4. Effect of NPC or SC on Erythrocytes (RBC) from the Control and DBP-Treated Rats

Cells	Control	DBP-treated
<i>NPC</i>		
Hb pg/RBC		
before incubation	25.5 $\pm$ 1.0	21.5 $\pm$ 0.8*
after 2 h incubation	23.7 $\pm$ 0.2	18.6 $\pm$ 0.7*
Hb ratio of the post- to the pre-incubation (%)	92.5 $\pm$ 0.7	86.5 $\pm$ 0.8*
Iron (ng/ml) in the medium	51.5 $\pm$ 2.5	89.4 $\pm$ 0.1**
Cell-debris ($\times 10^{-1}$ mg)	20.4 $\pm$ 0.3	24.2 $\pm$ 0.6*
Iron in the debris ($\times 10^{-1}$ μg)	55.6 $\pm$ 0.4	66.7 $\pm$ 0.2*
<i>SC</i>		
Hb pg/RBC		
after 2 h incubation	11.7 $\pm$ 0.5	13.7 $\pm$ 0.2*
Hb ratio of the post- to the pre-incubation (%)	49.3 $\pm$ 1.6	67.4 $\pm$ 2.5*
Iron (ng/ml) in the medium	252.4 $\pm$ 37.3	253.5 $\pm$ 36.7

Values are means $\pm$ S.D. ($n=6$). * Significant for $p < 0.05$; ** significant for $p < 0.01$, compared with the respective controls; Dunnett-type mean rank test.³⁸⁾

suggest that Tf might catch iron released from the Hb of the MBP-affected erythrocytes.

When NPC existed in the incubation medium, Hb levels decreased and iron levels in the cell debris increased at 6.8 μmol of MBP, significantly, but Tf-iron levels in the medium did not change. The results suggest that the MBP-affected erythrocytes might be destroyed by NPC and the released iron might be accumulated into the debris of NPC and/or the ghost.

When NPC was replaced with SC, the Hb levels were not affected by up to 6.8 μmol of MBP. However, there was observed an increase in Tf-iron and decreases in free iron and/or low molecular protein-iron at the range of 0.7–6.8 μmol . Yet, when plasma did not exist in the medium, the Hb levels descended significantly at 6.8 μmol and the iron increased ascendingly in the medium at the ranges from 0.7 to 6.8 μmol . These results suggest the possibility that MBP might induce the release of iron from components of SC, and that the released iron may be captured to plasma-Tf.

DISCUSSION

Mechanisms of iron depletion have been investigated partially from the metabolism of Hb and iron in the liver, spleen and bone marrow^{18,19)}: DBP was metabolized to MBP, and MBP might then accelerate the dissociation of Hb and Tf and the accumulation of the iron in the liver and spleen, along with the depletion of Hb and iron in bone marrow. It was necessary to determine which would be depleted first from circulating iron, Tf or Hb, by MBP in liver and spleen cells. In the *in vivo* studies, since there was observed a loss of Tf-iron in the liver, accompanied by the enhancement of iron as Hs-iron (Table 2), the loss might be attributed to the depletion of iron in the plasma Tf (Table 3). The *in vitro* studies showed the possibility that iron was released from MBP-affected erythrocytes (Table 5) but not from the plasma Tf (Table 3), and that the released iron was caught by Tf, not the ghost (Table

Table 5. Effects of MBP on the Erythrocytes (RBC) in the Plasma, NPC and SC

MBP in medium	0 μmol^a	0.7	1.4	3.4	6.8
2 h incubation with rat plasma					
Hb pg/RBC	22.6 $\pm$ 2.4	19.6 $\pm$ 3.8	22.6 $\pm$ 1.0	20.6 $\pm$ 1.0	19.2 $\pm$ 0.9*
Tf-iron in the medium ($\mu\text{g/dl}$)	84.0 $\pm$ 2.2	86.2 $\pm$ 1.8	91.3 $\pm$ 4.3	96.5 $\pm$ 2.4*	99.8 $\pm$ 3.1*
Iron in the ghost ($\mu\text{g/dl}$)	28.3 $\pm$ 1.3	24.0 $\pm$ 2.0	25.5 $\pm$ 2.0	29.8 $\pm$ 3.7	28.9 $\pm$ 1.1
2 h incubation with NPC and plasma					
Hb pg/RBC	20.4 $\pm$ 0.8	20.5 $\pm$ 1.0	19.3 $\pm$ 1.1	17.9 $\pm$ 1.2	16.6 $\pm$ 0.8*
Tf-iron in the medium ($\mu\text{g/dl}$)	83.7 $\pm$ 1.8	82.5 $\pm$ 2.5	83.5 $\pm$ 2.5	82.6 $\pm$ 3.2	81.3 $\pm$ 3.7
Iron in the cell debris (ng/ml)	3.8 $\pm$ 0.4	4.2 $\pm$ 1.0	4.5 $\pm$ 0.9	5.3 $\pm$ 1.2	9.8 $\pm$ 1.2*
2 h incubation with SC and plasma					
Hb pg/RBC	17.7 $\pm$ 0.9	19.0 $\pm$ 0.8	18.8 $\pm$ 1.0	19.4 $\pm$ 0.9	17.4 $\pm$ 0.7
Tf-iron in the medium ($\mu\text{g/dl}$)	72.2 $\pm$ 0.7	80.4 $\pm$ 3.1**	80.4 $\pm$ 2.2**	80.5 $\pm$ 3.2**	80.7 $\pm$ 1.7**
LMP-iron ^b in the medium (ng/ml)	22.6 $\pm$ 1.8	12.5 $\pm$ 1.1**	12.2 $\pm$ 1.0**	12.2 $\pm$ 1.7**	11.0 $\pm$ 0.5**
2 h incubation with SC					
Hb pg/RBC	23.8 $\pm$ 1.2	23.2 $\pm$ 1.5	23.2 $\pm$ 1.4	20.6 $\pm$ 1.6	19.1 $\pm$ 1.2*
Iron released into medium (ng/ml)	16.2 $\pm$ 0.3	32.8 $\pm$ 4.9*	38.3 $\pm$ 7.1**	52.5 $\pm$ 5.2**	53.9 $\pm$ 4.3**

* Significant for $p < 0.05$; ** significant for $p < 0.01$, compared with the respective controls; Dunnett-type mean rank test.³⁸⁾ Values are means $\pm$ S.D. ($n = 6$). a) When erythrocytes were incubated with MBP at doses of 0.1, 0.2 and 0.4 μmol , the results showed no significant difference from those of the respective controls (0 μmol). The data at 0.1–0.4 μmol were omitted from this table. b) LMP-iron represents a mixture of free iron and iron-containing proteins with molecular weights less than 20000.

5). Therefore, it was conceivable that the erythrocytes might be destroyed by the liver Kupffer cells and that the iron might be released from the Hb and accumulate in the hepatic Hs fraction, resulting in part in iron-depletion in the blood (Table 2).

The accumulation of iron in the Hs fraction (Table 2) in the *in vivo* studies was in accord with the results obtained by the *in vitro* studies, in which NPC seemed to destroy the erythrocytes from DBP-treated rats and the iron was captured in the cell debris of NPC (Table 4). Similarly, when erythrocytes were preincubated with MBP and then incubated with NPC, iron released from the Hb was accumulated in the cell debris of NPC, not to Tf (Table 5). When NPC did not exist in the incubation medium, the iron was captured to Tf (not in the ghost) (Table 5). This suggested that the released iron might be oxidized in order to be captured to Tf. The released iron existed as Fe^{2+} -proteins in both the cell-debris and Hs fraction (see the Results). Furthermore, it has been shown that the Fe^{2+} ion is not caught or binds weakly to Tf.^{26–29)}

As for SC, it seemingly destroyed the erythrocytes from the control rats much more than those from DBP-treated animals, accompanied by capture of the released iron to Tf (Table 2). The iron of senescent erythrocytes in the spleen was utilized in the bone marrow to reticulocytes for the biosynthesis of Hb³⁰⁾ via Tf-iron. However, *in vitro* studies suggested that the enhancement of Tf-iron might not contribute to the decomposition of the Hb. MBP might release iron from a component of SC (Table 5). When erythrocytes were exposed to MBP at the range of 0.3 to 3.4 μmol , the Hb was not decomposed by incubation with SC, irrespective of the existence of plasma. Nevertheless, increases in iron were observed in the medium and in the Tf when added the plasma. Although further study remains to determine whether MBP releases iron from a component of SC directly or indirectly by macrophages, it is conceivable that the DBP- or MBP-affected erythrocytes might be destroyed mainly by NPC rather than SC.

Normal erythrocytes undergo autooxidation to a small extent (about 1% of the total Hb) with the methemoglobin (MetHb) formed converted back to functional Hb by

MetHb reductase.³¹⁾ The activity of the reductase showed a tendency to decrease in the DBP- or MBP-affected erythrocytes, accompanied by significant decreases in glutathion (GSH) levels and in GSH reductase activity.¹⁸⁾ It is known that autooxidation represents the initial reaction that is followed by reactions destructive not only to Hb but also to other components of the erythrocytes³²⁾ during hemichrome formation, protein denaturation and Heinz body formation (including cell aging).³¹⁾ DBP or MBP might induce abnormal Hb by autooxidation due to the acidic microenvironment of MBP.¹⁸⁾ The rate of autooxidation significantly increases by a decrease in the pH value of the medium probably due to the protonation of the distal histidine of Hb.³³⁾ Considering that a Heinz body-like formation was observed with a higher incidence in both the arterial erythrocytes from DBP-treated rats¹⁸⁾ than the venous ones (unpublished data, not significant changes to the respective controls) and in the MBP-affected erythrocytes on air-bubbling (but not observed on non-bubbling),¹⁸⁾ it is conceivable that DBP or MBP might accelerate the autooxidation of Hb for progressive iron release accompanied by the depletion of GSH.

Since it was observed in this study that Hb was destroyed to insoluble Fe^{2+} -proteins in the NPC (liver Kupffer cells) and then a portion of the released iron was finally excreted to the urine, the decomposition of Hb might contribute to the depletion of circulating iron by combination with the previous results¹⁸⁾ in which MBP might induce iron-deficient erythrocytes in the blood.

It has also been shown that DEHP depleted Hb in mice and rats^{34,35)} and that the autooxidation was induced by other compounds which induced testicular atrophy, such as nitrobenzene³⁶⁾ and phenacetin.³⁷⁾

In conclusion, this study proposes a possible mechanisms causing the depletion of circulating iron. Erythrocytes exposed to DBP and MBP might be decomposed in the NPC (liver Kupffer cells), being predominant over SC, and the released iron might be accumulated in the NPC-debris as insoluble Fe^{2+} -proteins.

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