Studies on Lead-Binding Protein and Interaction Between Lead and Selenium in the Human Erythrocytes

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Abstract: To clarify the function of Pb-binding protein and the interaction between lead and selenium in human erythrocytes, the following experiments were performed. (a) blood from a man who has not been exposed to lead occupationally, (b) blood from lead-exposed workers and (c) blood from control subjects working in the same plant were used. (a) was incubated with Pb and/or Se. Hemolysates of rinsed erythrocytes were applied onto a Bio-gel A 1.5 m column and eluted by 40 mM Tris-HCl buffer (pH 7.0). As the results, (1) Pb in erythrocytes was found in ALAD fraction. When Pb was added *in vitro* at 1.25 μ M of final concentration, Pb content of ALAD fraction increased and the other Pb-containing fraction appeared at the position of a smaller molecular size than that of Hb fraction. When Pb dose added was increased to 2.5 or 10 μ M, Pb contents in the newly appeared fraction was larger than that in ALAD fraction. The affinity of Pb with the protein in the new fraction was weak, and Pb was released from the protein by ultrafiltration. (2) There were main and sub-peaks containing Pb in erythrocytes from Pb-workers; the former was ALAD fraction and the others were smaller molecules than ALAD. The peak of Pb observed with the blood from a worker who has worked at the same plant for more than 20 years (chronic exposure) corresponded with the new peak which appeared *in vitro* Pb exposure. (3) As to the interaction between Pb and Se, there was no effect of Se added *in vitro* on the position of Pb-containing fraction and on Pb amounts in the chromatographic profile. When Se was incubated with blood, however, coexistence of Pb made Se concentration in erythrocytes high compared with the case of Se alone.

Key words: Lead, Selenium, 5-Aminolevulinic acid dehydratase (ALAD)

Introduction

Lead (Pb) is one of the commonest elements that contaminate the environments. Pb is considered to be an element which has some toxic effects on men and animals, and has no physiological function in organisms. Selenium (Se) is known as one of the essential elements. In the case of Se deficiency, therefore, Se deficient diseases such as Keshan disease appears¹⁾. Se exerts a harmful effect when

it is loaded in excessive dose²⁾. Se belongs to the elements which are regarded as useful only in a very narrow range.

On the other hand, it is known that Se interacts with heavy metals such as mercury, cadmium, arsenic, tin, copper and so on³⁾. There have been reports on the interaction between Pb and Se in the organism⁴⁻¹⁹⁾. Most of them have showed that Se has some protective effects on Pb toxicity⁵⁻¹⁹⁾, or that Pb has some antagonistic effects on Se toxicity^{4, 10, 11)}. It has also been reported that Pb and Se are synergistic^{11, 12)}.

Erythrocytes are important binding sites and retain large portion of Pb in blood, but there have not been enough

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reported studies on the interaction between Pb and Se in erythrocytes. In our previous study on lead-exposed workers²⁰⁾, we determined concentrations of 23 elements in plasma and erythrocytes from 70 male workers, and found that Se concentrations in erythrocytes significantly increased according to the increasing of lead concentrations. There was positive correlation between Pb and Se concentrations in erythrocytes. We considered, therefore, that there may be the existence of Se and Pb binding protein(s) in erythrocytes. We tried this experimental study to investigate the Pb-binding protein and the interaction between Pb and Se in erythrocytes.

Materials and Methods

When we planned this experiment using human blood samples, we obtained informed consent from the donors.

I. Experiments on *in vitro* exposure to lead

Heparinized venous blood was obtained from a male donor with no history of occupational exposure to lead. A sample of 2 ml of whole blood was incubated with 0.5 ml of 5% glucose solution (a), and 0.5 ml of 5% glucose solution containing 6.25 μM lead acetate $[(\text{CH}_3\text{COO})_2\text{Pb}\cdot 3\text{H}_2\text{O}]$ (1.25 μM of final Pb concentration) (b), 12.5 μM lead acetate (2.5 μM of final Pb concentration) (c), and 50 μM lead acetate (10 μM of final Pb concentration) (d). After 60 min incubation at 37 °C, the samples were left at 4 °C for 20 h. Then they were centrifuged at 3,000 rpm for 15 min, and the packed erythrocytes were rinsed 5 times with 5% glucose solution. Each erythrocyte sample was supplemented by 40 mM Tris-HCl buffer (pH 7.0) till the original hematocrit value was restored, and the erythrocyte samples were stored at -80 °C for at least 1 h. After thawing, all the lysate was centrifuged at 24,000 g at 4 °C for 30 min. A sample of 1380 μl of the erythrocyte supernatant (ES) was applied onto a Bio-gel A 1.5 m column (1.6 $\times$ 90 cm), which was equilibrated with 40 mM Tris-HCl buffer (pH 7.0), and eluted with the same buffer. Eluates from the column were collected in 5.6 ml/tube.

II. Experiments on *in vitro* exposure to lead and/or selenium

Heparinized venous blood was obtained from the same donor as noted above. Two ml of whole blood was incubated with 0.5 ml of 0.5% glucose solution (a), and 0.5 ml of 0.5% glucose solution containing 50 μM lead acetate (b), 50 μM sodium selenite (Na_2SeO_3) (c), and 50 μM sodium selenite and 50 μM lead acetate (d). Incubation conditions and the following procedures were the same as in the above experiment.

III. Experiments on blood of lead-exposed workers

Heparinized venous blood samples were obtained from (a) a male worker as a control subject, (b) a male worker of subacute occupational exposure to Pb and (c) a male worker of chronic occupational exposure to Pb. These three workers have been working at the same steel wire plant. The job of Pb-treating in this plant means working near the furnace containing melting Pb. The worker (a) was not included in a Pb-treating job and whose Pb concentration in blood was 6.4 $\mu\text{g}/\text{dl}$. The worker (b) has been working at the same plant for 3 years, and whose Pb concentrations in whole blood at the periodical medical checks were 24.8 and 22.4 $\mu\text{g}/\text{dl}$ in the first 2 years, then elevated to 51.4 $\mu\text{g}/\text{dl}$ at the last year. The worker (c) has been working at the same plant for more than 20 years and whose Pb concentration in whole blood was 39.9 $\mu\text{g}/\text{dl}$ at the last medical check and was in the range from 43.3 to 51.8 $\mu\text{g}/\text{dl}$ in past 3 years. The workers (b) and (c) were on the same Pb-treating job in last one year.

The blood samples from the subjects were separated for erythrocytes. Each erythrocyte sample was rinsed with 5% glucose solution 5 times then was supplemented by 40 mM Tris-HCl buffer (pH 7.0) till the original hematocrit value was restored. The following procedure was same with above two experiments.

Determination of protein, 5-aminolevulinic acid dehydratase (ALAD) activity, Pb and Se concentrations

Protein concentration in each eluate fraction was determined from both the absorbance at 280 nm and the colorimetric method of Bradford²¹⁾. ALAD activity in whole blood or erythrocytes was assayed by the method of Chiba *et al.*²⁰⁾. When ALAD activity in the eluate was assayed, 100 μl of the substrate solution containing 25 mM 5-aminolevulinic acid, 50 mM dithiothreitol and 1 mM zinc sulfate in 0.25 M sodium phosphate buffer (pH 6.8) was mixed with 500 μl of eluate and incubated at 37 °C for 60 min. Pb concentrations in ES and in the eluate were determined with flameless atomic absorption spectrophotometer (Hitachi Z-8100) after the addition of one tenth volume of 10% HNO_3 to the samples. Se concentrations were determined for ES and eluate in Experiments II and III with the fluorometer (Japan Spectroscopic Co. Ltd, FP-770) according to the modified Watkinson's procedure²²⁾.

Estimation of molecular weight and ultrafiltration

The relation between the eluting positions and molecular

sizes was ascertained by applying marker proteins on the same column. Ferriitin (MW 450,000), catarase (MW 240,000), bovine albumin (67,000), chymotrypsinogen (MW 25,000) and myoglobin (MW 17,000) were used. Fractions containing Pb were pooled in each experiment and applied to ultrafiltration (Diaflo ultrafilter YM-30, Amicon Inc.) for concentrating and further gel-filtration.

Results

I. Experiments on *in vitro* exposure to lead

Figure 1 shows the chromatographic profiles of blood incubated with different concentrations of Pb. In each profile there were two clear protein peaks. The main one, which appeared at the fraction No. 21 to 26, corresponded to the peak of hemoglobin (Hb); the minor one, which eluted in void volume and was bigger molecular size, was the peak of some unknown protein(s). There were two peaks of ALAD activities; i.e. a higher one eluted before the Hb peak and a lower one at the same position of the small protein peak at the void. The main ALAD peak at fraction No. 17–19 corresponded to 240~260 KD which agreed with the molecular weight of ALAD already estimated²³.

It was found that Pb in blood of the subject without occupational exposure to lead existed in ALAD fraction (Fig. 1a). When Pb was added in the incubation mixture, Pb existing in ALAD fraction was markedly increased and a new Pb peak (fraction No. 27) was eluted after Hb fraction. These 2 peaks were almost even in size when 1.25 μM Pb was added. When Pb dose was increased to 2.5 or 10 μM Pb, the distribution ratio of Pb was larger in the newly appeared peak than in the ALAD fraction.

II. Experiments on *in vitro* exposure to lead and selenium

Figure 2 shows the chromatographic profiles of incubation with glucose solution (Fig. 2a), with 10 μM Pb (Fig. 2b), with 10 μM Se (Fig. 2c), and with both 10 μM of Pb and Se (Fig. 2d). The profiles of the protein and ALAD activity were similar to the results shown in Fig. 1. As to Pb, there were 3 peaks in both Fig. 2-b and Fig. 2-d, in which the incubation mixture contained 10 μM Pb. The highest and latest Pb peak (fraction No. 27–29) was located after the Hb peak; the second peak (fraction No. 17–19) corresponded with the ALAD peak; and the lowest peak (fraction No. 10–11) was located at the void position. For the incubation mixtures without addition of Pb shown in Fig. 2a and Fig. 2c, only small amounts of Pb were detected, mainly in the ALAD fraction.

Se was found mainly in the Hb fraction as shown in each profile of Fig. 2. When Se was added solely in the incubation mixture, Se in Hb fraction was markedly increased (Fig. 2c). When Se was added together with Pb, Se formed a shoulder at fraction No. 28 after the main peak at Hb fraction (Fig. 2d). It should be noted that the total Se amounts in the eluate shown in Fig. 2d were larger than that shown in Fig. 2c, which was incubated with 10 μM Se alone. The Se concentration in the ES which was applied onto column of the former one was 549 ng/ml, and that of the latter one was 246 ng/ml.

Fig. 1. Chromatographic profiles of protein, ALAD activity, and Pb in the human erythrocytes incubated with 5% glucose solution containing Pb acetate. Blood was incubated with (a) 0, (b) 1.25, (c) 2.5, and (d) 10 μM Pb of final concentrations. ----: protein, —: ALAD activity, —: Pb concentration.

III. Experiments on blood from lead-exposed workers

Pb and Se concentrations in each ES used in experiment III are shown in Table 1. The chromatographic profiles of two lead-exposed workers and a non-lead exposed worker (control) are shown in Fig. 3. The distribution patterns of protein were similar to those of the *in vitro* experiments except that there was no detectable peak at the void position. An ALAD peak was eluted before the Hb peak. The highest

Pb peak corresponded to the ALAD peak. In Fig. 3a, there are a few minor Pb peaks other than the ALAD fractions. In Fig. 3b, there was a second Pb peak between the ALAD and Hb fractions. In Fig. 3c, the second Pb peak was eluted after Hb fraction, which was similar to the position which appeared in the *in vitro* exposure (Figs. 1b–1d, 2b, and 2d). The main peak of Se was found at the Hb fraction.

To attempt further gel-filtration of the secondary appeared

Table 1. Pb and Se concentrations in the erythrocyte supernatant (ES) of workers

worker	Pb (ng/ml)	Se (ng/ml)
control	44.3	78
chronic exposure to Pb	164	87
sub-acute exposure to Pb	277	97

Fig. 2. Chromatographic profiles of protein, ALAD activity, Pb, and Se in the human erythrocytes incubated with 5% glucose solution containing Pb acetate and/or sodium selenite. Blood was incubated (a) without Pb and Se, (b) 10 μ M Pb, (c) 10 μ M Se, and (d) 10 μ M Pb and 10 μ M Se of their final concentrations. ----: protein, —: ALAD activity, —: Pb concentration, ----: Se concentration.

Fig. 3. Chromatographic profiles of protein, ALAD activity, Pb, and Se in the erythrocytes of lead-exposed workers (a) control, (b) subacute exposure, (c) chronic exposure, ----: protein, —: ALAD activity, —: Pb concentration, ----: Se concentration.

peak of Pb which was estimated to be 30~50 kD from the eluting position, the fractions from the *in vitro* exposure and those from lead-workers' blood were individually pooled and concentrated by ultrafiltration. From 70 to 80% of Pb came into filtrates indicating that the molecular weight of Pb-containing molecule was lower than 30 kD.

Discussion

Lead is a universal element which human beings ingest from the environment, foods, and drinking water. Lead is commonly found in the blood of humans. In blood, more than 95% of Pb is found in erythrocytes, and this distribution ratio in erythrocytes is emphasized according to the increasing body burden of lead²⁰. On the other hand, it is well known that ALAD is in erythrocytes and that it is the most sensitive biological indicator of Pb. ALAD is the second enzyme of heme biosynthesis and its molecular weight has been estimated to be 250 kD²³. From the eluting position of ALAD in the results of the present experiments, its molecular weight was estimated to be 240~260 kD which agreed with the other results reported²⁴.

In the human adults living in urban areas in Japan, Pb concentrations in the blood are roughly assessed to be about 5 µg/dl blood, and the largest amounts of Pb may be found in ALAD fraction (Figs. 3a and 1a). When Pb-loading is increased in people such as Pb exposed workers, Pb is taken into ALAD fraction and inhibits the activity of ALAD. The inhibition of ALAD activity by Pb was not observed in Figs. 3b and 3c, because SH reagent and zinc were added to the incubation mixture for the assay of eluate. When Pb is more increased, Pb appears in the other fraction in addition to ALAD fraction (Figs. 3b and 3c). The newly appeared peak seen in the chronic exposed workers (Fig. 3c) was also seen in the *in vitro* exposure to Pb (Fig. 1b~1d). In the *in vitro* study, the minimum Pb exposed dose, 1.25 µM added to incubation mixture, was calculated to be 26 µg Pb/dl blood. From the results of ultrafiltration of the secondary appeared peak of Pb, it is considered that Pb in this fraction bound to a protein or other molecules with a weak affinity and easily dissociate from these molecules. In the case of subacute exposure, the eluting position of newly appeared Pb peak shifted to an earlier numbers of fractions compared with the chronic exposed worker. It is not clear at present, if the subpeak in the subacute exposure appeared there temporarily or not.

A small Pb peak along with ALA-D activity at void volume was found in Figs. 1 and 2, but not in Fig. 3. In the

experiments shown in Figs. 1 and 2, the blood samples used were from the same man. It is not considered that ALAD might form dimer or any particular molecule during sample preparation. It is considered that a part of ALAD might combine with a somewhat large molecule such as globin during long-time incubation, and then it might be found at the void position. Contrary to these samples, those in Fig. 3 were prepared without such incubation process.

As to the interaction between Pb and Se, we tried *in vitro* addition of equimolar amounts of Pb and Se. Se concentration in ES incubated with Se and Pb was much higher (549 ng/ml) than that in ES incubated with Se alone (246 ng/ml) suggesting that Pb may enhance the attaching of Se to proteins in erythrocytes, or help Se to retain in erythrocytes. As shown in Figs. 2b and 2d, Se had no remarkable effect on Pb eluting pattern, while eluting pattern of Se was changed by simultaneous addition of Pb. When Se only was incubated with blood *in vitro*, the eluting position of Se was coincide with that of hemoglobin. When both Se and Pb were incubated with blood *in vitro*, main peak of Se also appeared at the position of hemoglobin, in addition, Se concentrations increased in the wide range from void to hemoglobin and shoulder peak appeared at fraction number 27~28 (Fig. 2d). The position of this shoulder coincide with Pb peak suggesting the binding of Se with Pb-binding protein. This fact did not contradict the results from blood of Pb-workers, as shown in Fig. 3. This fact supports the finding that erythrocyte Se concentrations increased according to the increase in Pb concentrations as described in the introduction and we surveyed in 70 lead-workers²⁰. The mechanism will be studied in the near future.

References

- 1) Chen X, Yang G, Chen J, Chen X, Wen Z, Ge K (1980) Studies on the relations of selenium and Keshan disease, *Biol Trace Elem Res* **2**, 91-107.
- 2) US Department of Health and Human Services (1984) Epidemiologic notes and reports —Selenium intoxication—. New York, *Morb Mort Weekly Rep* **33**, 157-8.
- 3) Himeno S (1996) Selenium. In: Health and elements —their basic knowledge—. eds. by Chiba M, Suzuki KT, 72-79, Nanzando, Tokyo.
- 4) Chiba M, Fujimoto N, Oyamada N, Kikuchi M (1985) Interaction between selenium and tin, selenium and lead, and their effects on ALAD activity in blood. *Biol Trace Elem Res* **8**, 263-82.

- 5) Uminska R (1990) Selenium in human environment. *Rocz Panstw Zakl Hig* **41**, 25–34.
- 6) Chowdhury BA, Chandra RK (1987) Biological and health implications of toxic heavy metal and essential trace element interactions. *Prog Food Nutr Sci* **11**, 55–113.
- 7) Beckman L, Nordenson I (1986) Interaction between some common genotoxic agents. *Hum Hered* **36**, 397–401.
- 8) Flora SJ, Singh S, Tandon SK (1983) Role of selenium in protection against lead intoxication. *Acta. Pharmacol Toxicol Copenh* **53**, 28–32.
- 9) Goldman M, Dillon RD (1982) Interaction of selenium and lead on several aspects of thyroid function in Peking ducklings. *Res Commun Chem Pathol Pharmacol* **37**, 487–90.
- 10) Schrauzer GN (1987) Effects of selenium antagonists on cancer susceptibility: new aspects of chronic heavy metal toxicity. *Sangyo Ika Daigaku Zasshi* **9** (suppl.), 208–15.
- 11) Donaldson WE, McGowan C (1989) Lead toxicity in chickens. Interaction with toxic dietary levels of selenium. *Biol Trace Elem Res* **20**, 127–33.
- 12) Khan MZ, Szarek J, Krasnodebska Depta A, Koncicki A (1993) Effects of concurrent administration of lead and selenium on some haematological and biochemical parameters of broiler chickens. *Acta Vet Hung* **41**, 123–37.
- 13) Gerhardsson L, Brune D, Nordberg GF, Wester PO (1988) Multielemental assay of tissues of deceased smelter workers and controls. *Sci Total Environ* **74**, 97–110.
- 14) Khan MZ, Szarek J, Markiewicz K (1993) Effects of oral administration of toxic levels of lead and selenium upon concentration of different elements in the livers of broiler chicks. *Zentralbl Veterinarmed A* **40**, 652–64.
- 15) Neathery MW, Miller WJ, Gentry RP, Crowe CT, Alfaro E, Fielding AS, Pugh DG, Blackmon DM (1987) Influence of high dietary lead on selenium metabolism in dairy calves. *J Dairy Sci* **70**, 645–52.
- 16) Gustafson A, Schutz A, Andersson P, Skerfving S (1987) Small effect on plasma selenium level by occupational lead exposure. *Sci Total Environ* **66**, 39–43.
- 17) Gustafson A, Hedner P, Schutz A, Skerfving S (1989) Occupational lead exposure and pituitary function. *Int Arch Occup Environ Health* **61**, 277–81.
- 18) Xu B, Chia SE, Ong CN (1994) Concentrations of cadmium, lead, selenium, and zinc in human blood and seminal plasma. *Biol Trace Elem Res* **40**, 49–57.
- 19) Tandon SK, Dhawan M, Kumar A, Flora SJ (1992) Influence of selenium supplementation during chelation of lead in rats. *Indian J Physiol Pharmacol* **36**, 201–4.
- 20) Chiba M, Shinohara A, Matsushita K, Watanabe H, Inaba Y (1996) Indices of lead-exposure in blood and urine of lead-exposed workers and concentrations of major and trace elements and activities of SOD, GSH-Px and catalase in their blood. *Tohoku J Exp Med* **178**, 49–62.
- 21) Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–54.
- 22) Watkinson JH (1966) Fluorometric determination of selenium in biological materials with 2,3-diaminonaphtalene. *Anal Chem* **38**, 92–7.
- 23) Anderson PM, Desnik RJ (1979) Purification and properties of δ -aminolevulinic acid dehydratase from human erythrocytes. *J Biol Chem* **254**, 6924–30.
- 24) Sakai T, et al (1982) Relationship between distribution of lead in erythrocytes *in vivo* and *in vitro* and inhibition of ALA-D. *Br J Ind Med* **39**, 382–7.