Abstract: Biomarkers of exposure, effect, and susceptibility are reviewed in relation to lead exposure. Of the biomarkers of lead exposure, blood lead (Pb-B), mainly red cell lead, is a representative of soft tissue lead, and most widely used as measures of body burden and absorbed (internal) doses of lead. Urine lead (Pb-U) as well as plasma lead (Pb-P) increases exponentially with increasing Pb-B under a steady-state situation and is a reflection of recent exposure. The amount of lead in plasma and urine (MPb-P and MPb-U) after administration of a chelating agent (e.g. CaEDTA) can be useful for biomarkers of internal exposure of lead, reflecting the mobilizable pool of lead which consists of mainly blood and soft tissue lead with only a small fraction derived from bones. The critical effects in bone marrow arise mainly from the interaction of lead with some enzymatic process responsible for heme synthesis. The effects can be used for the biomarkers of effects. They are the inhibition of delta-aminolevulinic acid dehydratase (ALAD) and the variation in some metabolite concentrations (e.g. delta-aminolevulinic acid in urine (ALA-U), blood (ALA-B) or plasma (ALA-P), coproporphyrin in urine (CP), zinc protoporphyrin (ZP) in blood). The activities of pyrimidine nucleotidase (P5′N) and nicotinamide adenine dinucleotide synthetase (NADS) in blood are also decreased in lead exposure, and nucleotide contents in blood is altered in lead exposure. These effects of lead on human can be also useful biomarkers of effect. The differences in levels of heme precursors between two types of ALAD genotypes might be attributable to those in the affinity of different ALAD isozymes to lead. ALAD1 homozygotes have higher levels of ZP and ALA in comparison with ALAD2 carriers at the high lead exposure, suggesting that ALAD1 homozygotes might be more susceptible for disturbance in heme biosynthesis by lead than ALAD2 carriers.

Key words: Lead, Blood lead, Plasma lead, Biomarkers, Delta-aminolevulinic acid, Delta-aminolevulinic acid dehydratase, Zinc protoporphyrin, Pyrimidine 5′-nucleotidase, Genetic polymorphism
The measurements or techniques in BM can be used for the diagnosis and also risk assessment of the toxic agents in the field of environmental health, although BM should not be confused with diagnosis. The term, biological markers or biomarkers are general term for specific measurements reflecting an interaction between a biological system and an environmental agent, which may be chemical, physical, or biological\(^2\). Biomarkers are mainly referred to the measurements used in the diagnosis or risk assessment in the field of environmental health. In 1987, National Research Council (NRC)\(^3\) developed the concept of biomarkers, describing a continuum of events from exposure to the development of disease. Biomarkers are generally classified into three groups: biomarkers of exposure, effect, and susceptibility\(^3\). Recently the term is also used for the measures of BM in the field of industrial health or occupational medicine. The biomarkers of exposure and effect correspond to the monitoring of exposure and effect, respectively. Some of confounding factors in BM are related to the biomarkers of susceptibility.

Figure 1 shows biomarkers and disease progression with exposure to toxic chemicals. Chemicals entering the body produce internal doses as consequence of their disposition and metabolism. An exogenous substance or its metabolites or the product of an interaction between a xenobiotic agent and some target molecules or cells can be used for biomarkers of exposure. Biologically activated chemicals can bind functioning macromolecules such as enzymes, receptors, and DNAs, resulting in early effects of target tissues or organs. The early effect in biochemical process is usually reversible, and occurs within a short time after exposure (subclinical effect). It can be used for the biomarkers of effect, and may precede the altered structure or function of tissues, which may be irreversible and lead to clinical disease. The toxicokinetics and toxicodynamics of chemicals entering are affected by various individual factors, which are used as the biomarkers of susceptibility. They may be also defined as the indicators or measures of inherent or acquired abilities of individuals to respond to the specific xenobiotic exposure.

**Biological monitoring of lead workers**

Recently, typical symptoms of lead poisoning have not been observed, because of the improvement of industrial and environmental health, especially in highly industrialized countries. However, epidemiological studies have indicated that subclinical effects of lead occur at a low blood lead concentration (Pb-B). The American Conference of Governmental Industrial Hygienists (ACGIH) Guide to occupational exposure value-1993 described that Pb-B should be controlled to levels of 20 \(\mu g/dl\) or below\(^4\), because females, children, and fetuses are more sensitive to lead exposure than male adults\(^5\). Women of child bearing potential, whose Pb-B exceeds 10 \(\mu g/dl\), are at risk of delivering a child with a Pb-B over the current Center of Disease Control (CDC) guideline of 10 \(\mu g/dl\). CDC concluded that the tolerance value for reproductive-age women should be 10 \(\mu g/dl\) of Pb-B\(^8\). On the other hands, highly exposure to lead is still observed in some industries in developing countries and also in small industries of secondary smelting of lead even in industrialized countries.

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**Fig. 1.** Biomarkers of exposure, effects, and susceptibility in workers exposed to chemical substances
In the low level lead exposure, highly accurate determination of Pb-B remains to be developed, using an advanced analytical instrument such as Inductively coupled plasma mass spectrometry (ICP-MS), as well as graphite furnace atomic absorption spectrometry (GFAAS). It is also important to find useful biological markers for evaluation of subclinical effects of lead at low Pb-B levels such as 10–20 µg/dl. Usual diagnostic determinations of clinical laboratory such as hematological test like Hb and Hct are not useful for the biological monitoring at low level exposure. So we should develop a new technique for BM of lead exposure, which are mainly reviewed here.

**Biomarkers for Lead Exposure**

**Lead metabolism and exposure markers**

Inorganic lead is a cumulative toxin absorbed mainly by lungs and gastrointestinal tract; percutaneous absorption is minimal in humans, with exception of organic lead. Inhalation is the dominant pathway for lead exposure of workers in industries and 40–50% of inhaled lead may be transferred to bloodstream. In adult humans approximately 10% of the dietary lead is absorbed. Fig. 2 shows dynamic equilibrium of lead in human body. In blood, more than 98% of the lead are found in blood cells\(^9\), 26) . Blood lead (Pb-B), mainly red cell lead, is a representative of soft tissue lead, and most widely used as measures of body burden and absorbed (internal) doses of lead. The relationship between Pb-B and the concentration of lead in exposure sources is curvilinear\(^10–12\) . The half-life for lead in blood and other soft tissue is about 28–36 days\(^13\) .

Plasma lead (Pb-P) has an important role in lead metabolism, where it is the active center of the body lead pool, although the concentration is very low. Pb-P is equilibrium with the extra-cellular pool and is directly involved in all the movements of lead among the different biological compartments\(^14\) . Thus, lead in plasma circulates in the body, affects the body lead burden and causes the toxicity of lead in some soft tissues, such as bone marrow, kidney, brain and so on. The levels of Pb-P are sharply elevated with a sudden intake or acute exposure to lead and rapidly diminished by time elapse from it\(^9\), indicating that Pb-P is an index for very recent exposure\(^15\) . The half life is very short, maybe less than 1 hour.

Some fractions of absorbed lead are excreted into urine. The amount of urine lead (Pb-U) depends not only on the exposure conditions but also on the extent of body burden and kidney function. Pb-U as well as Pb-P increases exponentially with increasing Pb-B under a steady-state situation and is mainly a reflection of recent exposure\(^15\) . The amount of lead excreted in urine after administration (MPb-U) of a chelating agent (e.g CaEDTA) reflects the mobilizable pool of lead which consists of mainly blood and soft tissue lead with only a small fraction derived from bones\(^17\) . Recently authors reported that Pb-P after injection of chelating agent (MPb-P) can be used for evaluation of amounts of mobilized lead\(^19\) . A x-ray fluorescence technique has been developed for measuring lead concentration in bone, but the use of them are limited in some countries alone.

**Pb-B, P-U, Pb-P**

Blood lead concentrations are currently regarded as the most reliable index of exposure to lead. Over 95% of the blood lead is bound to the erythrocytes and seems to be in dynamic equilibrium with plasma lead\(^14, 30\) . Plasma lead is more diffusible than erythrocyte lead and more important for evaluating the toxic effect of lead because the diffusible form exerts an influence on lead concentrations of other compartments and produces critical effect in the various organs\(^14, 30, 54\) . Erythrocytes rather behave as a depository for lead\(^17\) , which might produce some disturbances in erythrocyte metabolism such as Na, K-ATPase, delta-aminolevulinic acid dehydratase (ALAD) and nicotinamide adenine dinucleotide synthetase (NADS) activity\(^18, 19, 21, 22\) .

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**Fig. 2. Dynamic interchange of body lead pool and biomarkers of exposure.**

Major compartments indicate in circles, whose area is not proportional to the size of the compartments. Approximate percentages in body burden and half-lives are shown in the circles. Biomarkers of exposure are indicated in italics. BC: Blood red cells, St: Soft tissue, TB: Trabecular bone, CB: Compact or cortical bone, Hr: Hours, Mo: Month, Yr: Years.
Ong and Lee\(^{19}\) have reported that more than 85% of the \(^{203}\)Pb incorporated into erythrocytes \textit{in vitro} is associated with the cytoplasmic components and the rest is bound to the stromal membrane. They suggested that the membrane-bound lead might affect the activity of Na, K-ATPase. Several workers\(^{23–25}\) have indicated that most of the labeled lead incorporated into erythrocytes \textit{in vitro} is found in hemoglobin fractions. By contrast, Sakai et al.\(^{20, 66}\) reported that half the lead in erythrocyte hemolysate from a lead-exposed worker was found in protein fractions with a high molecular weight, including ALAD, which had highest affinity for lead among erythrocyte components in vivo and in vitro.

Atomic absorption spectroscopy (AAS) is the method most widely used for lead in biological materials. Flame methods require the pretreatment of samples mineralized by wet or dry ash techniques, which can be applicable for all kinds of biological material, such as not only blood and urine, but also hair and nail. Currently, GFAAS are the most widely used techniques for Pb-B and Pb-U. Pb-P can be also determined by GFAAS after solvent extraction of plasma lead chelated with ammonium pyrrolidinedithiocarbamate (APDC)\(^9\). Since Pb-P levels are very low, maybe less than 1/100 of Pb-B, the procedure include so many steps and chemicals, which might cause the contamination of samples with exogenous lead.

ICP-MS is recently introduced for the determination of lead in biological materials\(^{16, 26–29, 98}\). Pb-P as well as Pb-B and Pb-U could be simply determined by ICP-MS\(^{16}\). For the analysis of lead in plasma, whole blood, and urine, samples are prepared by diluting 5, 50, and 80-fold, with nitric acid solution, respectively. One of the advantages for ICP-MS determination is of a wide range of linearity in the calibration curves as compared with AAS, where dynamic range is very narrow. Another advantage for ICP-MS determination is of simple procedure and also high sensitivity, which enables us to determine very low levels of Pb-P, without complicated and time consuming pretreatment.

Using ICP-MS, Schütz et al.\(^{26}\) has reported the ratio of Pb-P/Pb-B increased with increasing Pb-B, in other words, there is a logarithmic relationship between Pb-B and Pb-P. Based on the correlation equation by Sakai et al.\(^{16}\), Pb-P level corresponding to Pb-B levels of 400 \(\mu\)g/l is calculated to be about 1.9 \(\mu\)g/l. The Pb-P level is close to that in the report by Schütz et al.\(^{26}\), from whose data it is calculated to be about 2.2 \(\mu\)g/l.

**MPb-U, MPb-P**

The chelatable lead excreted in urine (MPb-U) is considered to be an excellent measure of potentially toxic fraction of the body lead burden\(^{51}\), and usually determined in urine collected for 24 h after calcium disodium ethylenediamine tetraacetic acid (CaEDTA) administration\(^{51}\). Recently, Sakai et al.\(^{20}\) have reported that Pb-P concentration at 2 h after the start of CaEDTA injection (MPb-P) is well correlated with the amount of lead excreted in urine for 24 h thereafter, and is a useful measure for the chelatable lead.

Thus, MPb-P as well as MPb-U are very important indices of lead as exposure, distribution, and health risk. However, the concentration of lead in plasma or serum is not routinely measured, so far. One of the main reasons is the very low concentration of Pb-P, which is difficult to determine by AAS. In the AAS techniques, it needs trouble some procedures of chelation and extraction described above\(^9\), which might result in contamination of samples with exogenous lead. Recently, a highly sensitive instrument, ICP-MS, can be used for the measurement of lead in plasma or in serum\(^{16, 26, 98}\) as mentioned above.

**Biomarkers for Lead Effect**

**Lead effect on target organ**

The organs or tissues where the first biological effects can be observed with increasing amounts of chemicals are termed “critical organs or tissues”. The critical effects (subclinical effect) appear when the concentration reaches a critical level in critical organs or tissues. In lead exposure bone marrow, central and peripheral nervous system, kidney and digestive system are critical organs. Critical concentrations of lead in bone marrow can be detected by measuring the degree of disturbances in the heme biosynthetic pathway and nucleotide metabolisms. The decreased enzyme activity or the amounts of altered intermediates in the biochemical pathways and also other adverse effects in critical organs can be used for the biomarkers of effects.

**Dose-effect and does-response relationships**

The relationships between the amounts of dose and the degree of effect produced by the dose are denoted as dose-effect relationship and are an essential requirement for biological effect monitoring. The evaluation of degree of exposure or its effect is based on the dose-effect relationship. The dose-response relationship may be also useful for the evaluation of effect in a group level, because not all the subjects react in the same degree at an identical dose. Response is expressed by the parentage of subjects in the group who respond the dose and show a specific values of effect.
Lead effect on heme and nucleotide metabolisms

The critical effects in bone marrow arise mainly from the interaction of lead with some enzymatic process responsible for heme synthesis (Fig. 3). These interactions can be sensitively determined by the inhibition of delta-aminolevulinic acid dehydratase (ALAD) and the variation in some metabolite concentrations (e.g. delta-aminolevulinic acid in urine (ALA-U), blood (ALA-B) or plasma (ALA-P), coproporphyrin in urine (CP), zinc protoporphyrin (ZP) in blood). Analyses of porphyrins in biological materials have been developed along with the monitoring and diagnosis of lead effect on human during this century. The activities of pyrimidine nucleotidase (P5’N) and NADS activity in blood are also decreased in lead exposure, and nucleotide contents in blood is altered in lead exposure. These effects of lead on human can be also useful biomarkers of effect.

1) ALAD

ALAD, porphobilinogen synthase (EC 4.2.1.24) is the second enzyme in the heme biosynthetic pathway which catalyses the condensation of two molecules of ALA to form one molecule of porphobilinogen. Erythrocyte ALAD activity is rapidly inhibited by lead exposure. Determination of ALAD activity in erythrocytes is one of the most useful methods for evaluating lead exposure, because the activity is extremely sensitive to and specific for blood lead concentration. The activity is specifically inhibited by lead at concentrations between 5 and 50 µg/100 g blood. The highly purified enzyme from human erythrocytes is a homo-octomer with a molecular weight of 252 000 and is inhibited at 1 and 5 µM lead; the crude enzyme is also inhibited at a concentration exceeding 1 µM. Sakai et al. has suggested that certain factors involves in the hemoglobin fraction account for highly sensitive nature of the enzyme to lead.

For the monitoring purpose the activity is usually determined by the method of CEC (Committee of European Community). However there are two weak points in the determination of ALAD activity itself (ALAD(u)); wide range of normal activity and unstable nature of the activity during storage. The problems can be resolved by the activation methods by heating, zinc ion and/or dithiothreitol (DTT). Depressed ALAD activity in lead-exposed subjects is restored by heating the enzyme solution or by adding SH-compounds or zinc ion to the reaction mixture. The extent of restoration in activity is correlated with blood lead concentration and thus can be helpful in evaluating exposure to lead. The ratio (or percentage) of activity without activation to activated activity (ALAD(%) is rather stable during storage and varied in a narrow range in control subjects.

2) ALA-U, ALA-B, and ALA-P

ALA is synthesized in mitochondria from glycine and succinyl-CoA by ALA synthetase (ALAS), which is rate limiting enzyme in heme pathway. The decrease in ALAD activity and indirect activation of ALAS due to negative feedback regulation by lead exposure cause an increase in

Fig. 3. Effect of lead on heme biosynthesis and biomarkers of effect, which is indicated in italics.
ALA in various tissues and in plasma, and consequently excretion of ALA in urine is elevated\textsuperscript{56}. ALA in plasma or blood reflect the effect of lead on bone marrow more directly than ALA in urine (ALA-U), although ALA-U has been recommended as a measure of lead effect\textsuperscript{57}. An exponential relationship between urine and plasma ALA has been observed in wide range of Pb-B levels.

Since the assay of ALA-U was introduced in the monitoring of lead exposure in 1950s, several method of analyses were developed using ion exchange column chromatography or solvent extraction procedures with the combination of color reaction with Ehrlich’s reagent. In 1990, high-performance liquid chromatography (HPLC) method with sensitive fluorescence detection of ALAU was reported by Okayama et al.\textsuperscript{68}. Thereafter some investigators have tried to determine ALA in plasma or serum (ALA-P or ALA-Sr) for evaluation of the effects of lead\textsuperscript{60, 61}. Morita et al.\textsuperscript{62, 63} has improved the method, which is applicable to the determination of both ALA-P and ALA-B, and achieves a good recovery rate (almost 100\%) as well as high sensitivity. When plasma and blood samples is stored at 4°C, ALA-P and ALA-B levels do not decrease significantly for 6 days and 3 days, respectively. Almost all of ALA molecules in blood exist in plasma and not in blood cells, irrespective Pb-B, and that both values may be similarly useful as indices of lead exposure. However, ALA-P is more suitable than ALA-B for routine analysis, because the distribution of ALA in blood and the stability of sample storage.

The correlation coefficient of Pb-B vs logALA-P or logALA-B and that of Pb-B vs logALAD(u) or log ALAD(%) are in the same range\textsuperscript{64}. These correlation coefficients are higher than those Pb-B vs logALA-U-Re, logALA-U, or logZP. In the dose-effect relationship between the logarithms of Pb-B and ALA-P, a two-phase increase in ALA-P is observed as Pb-B increases. ALA-P levels increase slowly below 40 µg/dl (slow phase) and rapidly above 40 µg/dl (rapid phase). In the latter phase, ALA-P increases continuously up to 100 µg/dl of Pb-B. Significant correlations are found in both the slow and the rapid phase. The Pb-B of the intersecting point of the two lines is calculated as 42.9 µg/dl\textsuperscript{64}.

The relationship between ALA-P and ALAD(u) or ALAD(%) is also two phase in nature\textsuperscript{64}. The two regression lines intersect at 22.7u or 20.9% of ALAD activity. ALA-P increase slowly with the reduction of ALAD activity down to the intersecting point; this phase corresponds to the “slow phase” mentioned above. As the ALAD activity decrease to the intersecting point, ALA-P begin to increase sharply; this phase corresponds to the “rapid phase”. The slow phase in ALA-P or ALA-B increase may be explained by the linear inhibition of ALAD activity. When the inhibition of ALAD activity reaches a plateau at Pb-B levels between 40 and 50 µg/dl, the rapid phase may be accounted for the another mechanism, such as induction in ALAS. Meredith et al.\textsuperscript{65} has reported that severe depression of heme synthesis induces ALAS through negative feedback regulation at Pb-B levels more than 2 μM (41.4 µg/dl) and ALAD activity less than 18 units. These values are comparable to the intersecting points of the two regression lines at a Pb-B level of 42.9 µg/dl\textsuperscript{64}.

3) Coproporphyrin (CP)

Coproporphyrinogen I and III are metabolic intermediates of heme pathway that are easily oxidized to their respective coproporphyrins. The increased excretion of isomer III is observed in urine from lead workers at least 2 weeks after the commencement of exposure. In steady state exposure, CP excretion can be positively correlated with Pb-B, as well as with ALAU. CP levels are significantly increased at Pb-B levels above 70–80 µg/dl\textsuperscript{31}. Analytical techniques for CP are traditionally based on preliminary extraction and subsequent fluorometric or spectrophotometric determinations. Some disorders of porphyrin metabolisms produce unique patterns of CP excretion in urine\textsuperscript{57}. CP I is excreted in excess amounts in urine from patients with congenital erythropoietic porphyria. Hereditary coproporphyria is characterized by the excretion of large amounts of CP III, mainly in feces but also in urine.

Several workers\textsuperscript{58–72} have developed HPLC for the measurement of porphyrin derivatives in biological materials such as blood, urine and feces. Simultaneous separations of various derivatives of porphyrins are desirable for purposes of differential diagnosis. Sakai et al.\textsuperscript{73} have reported a simple method for separating and determining CP I and III in urine. The method is simpler with respect to pretreatment of samples, and CPs in concentrations as low as 10 µg/L of urine can be detected. Good correlations of CP vs Pb-B are obtained by the method\textsuperscript{74}.

4) Zinc protoporphyrin (ZP)

The final step of heme synthesis, introducing Fe\textsuperscript{2+} into protoporphyrin XI (PP), is also affected by lead. Inhibition of Fe\textsuperscript{2+} reduction by lead exposure causes the decreases in transportation of Fe\textsuperscript{2+} into mitochondria\textsuperscript{75}, resulting accumulation of PP in erythrocyte. PP is enzymatically or non-enzymatically chelated with Zn\textsuperscript{2+} to form zinc protoporphyrin (ZP). Thus accumulation of PP or ZP in erythrocytes provides an index of disturbance of the final
step in heme synthesis, suggesting biochemical effect of lead on bone marrow, and of active deposits of the metal in the tissue.

Several methods have been developed for the determining erythrocyte protoporphyrin (PP) concentrations; they include acid extraction, detergent dilution, neutral solvent extraction, hematofluorimetry and HPLC methods. The method most widely used is the acid extraction method, although a two-step extraction is complicated and the second extraction with dilute hydrochloric acid cleaves zinc from ZP molecules to form free PP. ZP concentrations are therefore measured as free PP fluorescence by the acid extraction method (FEP method). The detergent dilution method is subject to serious hemoglobin interference. The disadvantage of neutral solvent extraction is low extraction efficiency, variable recovery and interference of PP. The limitation of hematofluorimetry which is a simple and rapid technique for ZP detection, lies in weak quality control of standardization and in interference from bilirubin and PP.

In these three methods the fluorescence of ZP is usually measured.

The significant advantage of HPLC methods over other methods is that ZP and PP can be separately determined in a single run, eliminating the optical interference from co-extractable substances. ZP levels have been known to increase in iron deficiency other than lead poisoning, while PP levels in erythropoietic protoporphyrin.

By HPLC method ZP and PP can be separately determined and total protoporphyrin (TP=0.9ZP + PP) can be calculated. In acid extraction method ZP is converted into PP, and both porphyrins are measured as PP, which is commonly called FEP. TP by HPLC is comparable with FEP by acid extraction method, and ZP by HPLC is with that by hematofluorimetry. Consistently good agreement was found between the HPLC method and each of the two established methods.

The correlation coefficients between 0.9ZP or TP and Pb-B are significantly higher than that between PP and Pb-B, indicating that the concentrations of ZP and TP determined by HPLC method are useful indicators for the evaluation of occupational exposure to lead. The ratio of 0.9ZP to TP in the workers ranged from 0.53 to 0.99 (mean ± SD, 0.86 ± 0.085) and is independent on Pb-B levels. Reference values by HPLC method have been reported in blood from 34 male and 34 female subjects not exposed to lead. The mean ± SD concentrations of ZP, PP, and TP were 57.4 ± 14.7, 10.6 ± 8.6, and 62.2 ± 20.0 µg/dl of red blood cells in male and 69.5 ± 22.5, 10.0 ± 8.4, and 72.6 ± 26.4 µg/dl red blood cells in female, respectively.

5) Comparison of threshold levels of Pb-B for heme parameters

Threshold (no-effect) levels of Pb-B have been examined for the disturbances in heme metabolism in 191 lead workers, who are divided into 11 groups by Pb-B intervals of 5 µg/dl. Both the reduction in ALAD activity (u and %) and the elevation in ALA-P (also ALA-B) in each group (2–11) with a Pb-B level more than 5 µg/dl were significant in the comparison with group 1 (Pb-B<5 µg/dl). The no-effect level of Pb-B for ALAD activity has previously been reported to be 10 µg/dl. The threshold level for inhibition of ALAD is also coincident with that for increases in ALA-P levels. Thus the data indicate that the decrease in ALAD activity reflects the effect of active lead in bone marrow even at low Pb-B levels of ca. 5 µg/dl. By contrast, while a significant increase in ALA-U corrected for creatinine (ALA-Ucre) and ZP is continually found only in groups with Pb-B levels more than 30 µg/dl.

6) ROC analyses for the evaluation of diagnostic values of heme parameters

To compare the diagnostic efficiency of heme parameters, receiver operative characteristic (ROC) plots are adopted in 191 lead workers. The ROC curve for ALA-P is identical to that for ALAD(u) at the low to moderate Pb-B levels from 10 to 40 µg/dl, where the area under the ROC curves is between 0.81 and 0.94. The area indicates that ALA-P and ALAD(u) have moderate to high accuracy for the diagnosis of low lead exposure. The area under the ROC curve for ALAD(%) has the highest diagnostic efficiency among the parameters examined for low to moderate exposure.

However, ALAD activity cannot be used as the indicator for the highly exposed group because the decrease in ALAD activity reaches a plateau at a Pb-B level between 40 and 50 µg/dl. Furthermore, ALAD is extremely unstable after blood sampling, and the analytical procedure is complicated. Thus, determination of ALAD activity is not widely used for routine analyses in the biological monitoring of lead exposure. By contrast, ALA-P increases continuously even at Pb-B levels more than 40 µg/dl. ALA-P is stable for a long time after sampling and the procedure of ALA-P determination is also simple.

ROC curves for ALA-Ucre and ZP are essentially identical at every Pb-B level from 10–40 µg/dl. The area under the ROC curves gradually increases from 0.58 to 0.84 as Pb-B increases from 10 to 40 µg/dl, indicating that the parameters have low to moderate accuracy for the diagnosis of low to moderate exposure.

Based on the above findings, of the heme parameters ALA-
P can be considered the best discriminators of lead exposure over the wide range of Pb-B from baseline to high.

**Pyrimidinenucleotide metabolism**

In lead workers, erythrocyte enzyme pyrimidine 5'-nucleotidase (P5N) activity declines linearly with increasing Pb-B between 10 and 100 µg/100 g, and it can be used for an indicator of lead exposure. Congenital deficiency of this enzyme results in nonspherocytic hemolytic anemia in which the erythrocytes contain large amount of pyrimidine nucleotides and show pronounced basophilic stippling (BSE). The accumulated pyrimidine compounds appear to cause feedback inhibition of ribonucleic acid (RNA) catabolism. Undegraded ribosomes aggregate to produce BSE. Accumulation of pyrimidine compounds in erythrocytes also affects glucose-6-phosphate dehydrogenase and suppresses pentose phosphate cycle activity, which may result in hemolysis. The mechanism of hemolysis in lead poisoning appears similar to that postulated in hereditary deficiency (P5ND).

1) Pyrimidine 5'-nucleotidase (P5N) activity

P5N catalyses the hydrolysis of pyrimidine 5'-monophosphate to yield pyrimidine nucleoside and inorganic phosphate. Techniques for assay of P5N activity fall into two categories; one based on the determination of inorganic phosphate and the other on the determination of pyrimidine nucleosides. In the conventional method for determining P5N activity the amount of inorganic phosphate released is determined colorimetrically. Inorganic phosphate (and other sources of inorganic phosphate) are normally present in erythrocytes, so to reduce the blank values the phosphate must be removed by dialyzing the lysate before the enzyme assay. It is also necessary to incubate the samples for the relatively long periods to release sufficient phosphate. Because of these disadvantage the determination of P5N activity has not been used frequently in the routine monitoring of lead exposure. The other type of assay of P5N activity has been reported by and others. In this method radioactive CMP (14C-CMP, cytidine 5'-monophosphate) is used as the substrate, which is absorbed to on to barium sulfate after the reaction is stopped and the 14C-cytidine formed is counted with a liquid scintillation counter. This method eliminates the need for the dialysis of the enzyme solution and is also extremely sensitive. However, the method cannot easily be carried out in every clinical laboratory, because of radioactive method.

In the method reported by Sakai et al. P5N activity is also determined by measuring liberated pyrimidine nucleosides. The product is separated from the substrate and from red cell constituents by means of HPLC. In this method dialysis of the samples is not necessary and the incubation time is shortened because the amount of endogenous uridine is non-detectable and the intensity of the absorption of uridine at 254 nm is high. The method has been further improved using whole blood as the enzyme source with Con A which inhibits the serum nucleotidase activity. In the improved method Pb determination may be also omitted and the activity expressed as µmol/h/l blood or µmole/h/l RBC rather than µmole/h/g Hb. The activity may be determined even when the blood is partly hemolysed during storage at 4°C, which has little effect on the activity, and is expressed as µmole/h/l blood. reported that the reduction of P5N activity was only 1.4% after storage of samples for seven days at 4°C. As the assay of P5N can be carried out with stored samples, it seems to be more suitable than ALA-D for screening lead exposure.

In the HPLC method the geometric and arithmetic mean (± SD) of P5N activity are 17.4 (± 1.2) and 17.7 (± 2.95), respectively, in subjects whose Pb-B values are less than 10 µg/100 g (mean ± SD: 6.58 ± 1.6 µg/100 g). The mean activity of the group is similar to that reported by Sato et al. taking into account that the substrate used in HPLC method is UMP. Cook et al. reported normal values ± SD for P5N activity as 12.0 ± 0.71 (n=14), using an HPLC method with UMP as the substrate. The mean Pb-B ± SD in their control group was 7.6 ± 4.0 µg/100 g. The normal value for P5N activity by Cook et al. is similar to those reported by American and European investigators but somewhat lower than those reported by Sakai et al. or by Sato et al. It seems that the difference in P5N activity is not only due to the different concentrations of Pb-B but also to other factors, including genetic differences.

Significant correlation is reported between log P5N and Pb-B (r = -0.79) at Pb-B values between 3 and 80 µg/100 g. P5N activity is sensitive to lead in blood as low as 10–20 µg/100 g. The activity at Pb-B of 10–20 µg/100 g is reduced by 13.2% compared with that at Pb-B levels of less than 10 µg/100 g. Thus the threshold (no effect) value of Pb-B for P5N activity may be less than 10 µg/100 g. Based on these findings and the description by Paglia et al., it appears that P5N activity is progressively inhibited over a wide range of Pb-B values between 10 and 100 µg/100 g, and has a wide field of application in the monitoring of lead effects.

Highly significant correlation is found between P5N activity and other biological indicators of lead effect.
suggesting that the decrease in P5N activity is able to predict the metabolic disturbances in heme biosynthesis. In male workers exposed to lead and other heavy metals Mohammed-Brahim et al.\cite{107} have reported the validity of P5N and ZP as 1.71 and 1.73, respectively, when Pb-B values are greater than 40 µg/100 ml. A high validity for P5N (1.86 at a cut off of 10 unit) at Pb-B 40 µg/100 g is also reported by Sakai et al.\cite{97}, and it is higher than those of the other indicators examined. Thus it indicates that P5N is a good discriminator at moderate Pb-B values.

2) Accumulation of pyrimidine nucleotides in erythrocytes

Due to the decreased activity of erythrocyte P5N by lead exposure a large amount of pyrimidine nucleotides is accumulated in erythrocytes. The various kinds of pyrimidine nucleotides are observed in lead exposed subjects. The accumulated pyrimidine compounds appear to cause feedback inhibition of ribonucleic acid (RNA) catabolism\cite{93} and undegraded ribosomes aggregate to produce BSE, which is specifically observed in lead poisoned subjects. Thus the metabolic disturbances of red cell nucleotide pool are used for the biomarkers of lead effect instead of detection of BSE.

Angle et al.\cite{108} have found three nucleotides (i.e., UTP, CTP, and CDP) that are increased in blood cells of lead-treated animals using anion-exchange HPLC. Sakai et al. have reported HPLC separation of 14 nucleotides including CDPC and UDPG, which are major nucleotides found in erythrocyte from lead exposed workers. The pyrimidine diphosphodiesters, CDPC and UDPG, and CTP are the most prominent abnormal nucleotides in blood cells of lead-exposed subjects. The levels are well correlated with Pb-B and also P5N activity. These nucleotides increased in lead workers are similar to those found in P5N deficiency (P5ND)\cite{109-111}, but the concentrations are much lower than the massive amounts found in P5ND.

Sakai et al.\cite{112, 115} have found that the levels of purine nucleotides (other than pyrimidine nucleotides) are also affected by lead exposure, suggesting disturbances of the energy production system by lead. As Pb-B values rise, ATP levels show a slight reduction, whereas AMP levels are correlated positively with Pb-B. Energy charges are consequently have decreased in workers whose Pb-B is greater than 60 µg/100 g. Angle et al.\cite{108, 140} however, that purine nucleotides are unchanged in red cells from lead-poisoned rabbits with mean Pb-B levels between 30 and 72 µg/dl. The energy charge is also normal in two children with P5ND\cite{113}. The observed pattern of glycolytic intermediates in lead poisoning also suggests that the activity of the Embden-Meyerhof pathway is normal\cite{113, 114}. However, Torrance and Whittaker\cite{106} have found that the concentrations of ADP and AMP in red blood cells of P5ND are between 2 and 3 times of normal, and the distribution of adenine nucleotides is abnormal with the lower energy forms being favored.

**Nicotinamide adenine dinucleotide synthetase**

In the final step of the Preiss-Handler pathway for nicotinamide adenine dinucleotide (NAD) biosynthesis\cite{116}, NAD synthetase (NADS) transfers an amino group from glutamine (Gln) to nicotinic acid adenine dinucleotide (NAAD) to form NAD. NAD is a coenzyme for oxidoreduction, and considered to play an important role in preventing oxidative stress in tissues. NADH is regarded to supply reducing equivalents and to maintain normal levels of glutathione in tissues\cite{117}. Impaired rate of NAD synthesis in erythrocytes is reported in some disorders (e.g., pyruvate kinase deficiency\cite{118}, enolase deficiency\cite{119} and thalassemia\cite{120}).

The NADS activity is reduced in erythrocyte from lead exposed subjects\cite{21, 121, 122}. Zerez et al.\cite{121} firstly reported that NADS activity was markedly decreased in three workers exposed to lead. The World Health Organization (WHO) refers to the decrease in NADS activity as one of the important effects of lead on humans\cite{122}. To determine NADS activity, Zerez et al. use partly purified enzyme. Their method is almost impossible for routine analyses in the clinical laboratory because of the complicated procedures with purification steps. Morita et al.\cite{21} describe that whole blood can be simply used for NADS assay, and demonstrate the dose-effect relationship of NADS activity versus blood lead concentration (Pb-B), indicating that the activity could be useful as an biological effect marker. The Pb-B level inducing 50% inhibition of NADS activity is calculated to be 43 µg/dl of Pb-B.

Recently, a simple and reliable method has been further developed for the analysis of NADS activity in human blood, using HPLC\cite{22}. The concentrations of 0.5 mM ATP, 15 mM glutamine (Gln), and 0.5 mM NAAD are good compromise for the NADS determination by HPLC method. The chosen conditions are the most convenient and economical, without an inconveniently high concentration of NAAD. The concentrations are also enough to discriminate the decreased activity in lead exposed subject. HPLC method is more useful than spectrophotometric method for routine analyses in clinical laboratories and for biochemical research on the enzyme. Using HPLC method, the kinetics of the enzyme can be also examined. NADS activity is remarkably
decreased with blood from a lead worker even at the higher concentrations of ATP or Gln\textsuperscript{22}. However, the depressed activity of NADS in a lead worker tends to be partly recovered with increasing NAAD concentration.

**Biomarkers of Lead for Susceptibility**

The Biomarker of susceptibility is an indicator of an inherent or acquired ability of an organism to response to the challenge of exposure to specific xenobiotic substance\textsuperscript{2}; The biomarkers of susceptibility are concerned with factors in kinetics and dynamics of exogenous chemicals (Fig. 1). Under the similar exposure conditions, genetic differences of individuals in metabolisms or macromolecule components may produce markedly different doses at the target organs and thus a different level of response. Although most attention has been focused on genetic susceptibility, some of acquired factors, such as nutrition, disease, physiological changes, medication and exposure to other environmental agents may also affect individual susceptibility (Fig. 1). Biomarkers of susceptibility are refer to the indicator of interfering or confounding factors in BM, except interfering factors related to the determination or sampling procedures (e.g. effect of storage, time of sampling etc).

In lead exposure ALAD polymorphism is reportedly related in susceptibility of lead effect on heme metabolism. Recently, several groups have investigated on the relationships between ALAD polymorphism and susceptibility to lead toxicity\textsuperscript{123–125, 127–129}. In these studies, effect indices of lead exposure, such as ALAD, ALA-P, ALA-U and ZP, are examined whether ALAD genotype influences the values of the indices.

Human ALAD gene is located in chromosome 9q34\textsuperscript{130}. The gene frequencies of ALAD alleles 1 and 2 were reportedly about 0.9 and 0.1, respectively, in several Caucasian populations\textsuperscript{131–133}. In 1991, a simple method using polymerase chain reaction (PCR) is developed for the analysis of ALAD genotype\textsuperscript{134}. Using PCR method, the frequency of ALAD2 allele reported in various populations is 0.114 in 307 Korean\textsuperscript{124}, 0.074 in 691 American\textsuperscript{136}, and 0.085 in 205 Chinese\textsuperscript{141}. These frequencies are nearly the same as those (0.087) by Sakai et al.\textsuperscript{137} in 317 Japanese.

In comparison with the ALAD1 sequence, the only meaning difference in the ALAD2 cDNA is G-to-C transversion of nucleotide 177 in the coding region, which creates an MspI restriction site and causes the replacement of a lysine by a asparagine\textsuperscript{134}. The difference in amino acid might result in distinct charge isozyme, and ALAD2 protein may bind lead more tightly than ALAD1 protein\textsuperscript{135}.

Actually, ALAD2 carriers have reportedly higher Pb-B levels than ALAD1 homozygotes in the subjects exposed to lead occupationally or environmentally\textsuperscript{123, 124, 133, 138}. In the recent study using HPLC with ICP-MS, Bergdahl et al.\textsuperscript{139} indicate that ALAD2 carriers have higher percentage of lead bound to ALAD protein than ALAD1 homozygotes. However, there is no significant difference in ALAD activity between the two groups of ALAD genotypes when Pb-B levels are matched each other\textsuperscript{139}. In the report by Sakai et al., ALAD activity is not significantly different between two ALAD genotypes in each Pb-B group\textsuperscript{137}.

ALAD genotypes modify lead effects on heme metabolism in highly exposed workers, while ALAD genotypes do not affect the levels of heme precursors at low-Pb-B levels\textsuperscript{129, 135, 137}. Sakai et al. reported that ZP levels in ALAD1 homozygotes are significantly higher than that in ALAD2 carriers at the Pb-B levels of 20–59 µg/dL. At the Pb-B levels of 40–59 µg/dL, ALA-P levels in ALAD1 homozygotes are also significantly higher than that in ALAD2 carriers. Sithisarankul et al.\textsuperscript{128} have reported that ALA-P levels in ALAD1 homozygotes are higher than those in ALAD2 carriers, although ZP levels are not significantly different between both ALAD genotypes in lead workers. The difference may be partly explained by the insufficient number (n=65) of workers in the latter study. On the other hand, Schwartz et al.\textsuperscript{124} and Alexander et al.\textsuperscript{129} have reported that ALAD1 homozygotes show the higher ZP levels compared to ALAD2 carriers. In their study, the number of the subjects are sufficiently large (n=308 and 134, respectively), but ALA-P levels do not examined.

The differences in levels of heme precursors between two types of ALAD genotypes might be attributable to those in the affinity of different ALAD isozymes to lead. Because ALAD2 protein can more tightly bind lead in blood, the amounts of “biological active” lead in bone marrow tissue might be lowered in comparison with ALAD1 homozygotes. In the case, lead bound to the ALAD protein might be detoxified or inactive form, which prevents the lead inhibition of ferrochelatase and also of ferrous incorporation into mitochondria, resulting in relatively low levels of ZP in ALAD2 carriers\textsuperscript{124}. Reductive process of Fe\textsuperscript{3+} is reportedly inhibited by lead\textsuperscript{79}. By the mechanisms, incorporation of ferrous into protoporphyrin might be more highly inhibited by lead in ALAD1 homozygotes than ALAD2 carriers, resulting in the higher ZP and lower heme levels. Reduced levels of heme might induce ALA-S by negative feedback regulation and produce the large amount of ALA\textsuperscript{64, 65}. Thus, relatively higher levels of ALA-P can be explained in ALAD1 homozygotes in comparison with ALAD2 carriers.
In conclusion, ALAD1 homozygotes have higher levels of ZP and ALA in comparison with ALAD2 carriers at the high lead exposure, suggesting that ALAD1 homozygotes might be more susceptible for disturbance in heme biosynthesis by lead than ALAD2 carriers.

References


64) Sakai T, Morita Y (1996) δ-aminolevulinic acid in plasma or whole blood as a sensitive indicator of lead effects, and its relation to the other heme-related parameters. Int Arch Occup Environ Health 68, 126–32.


87) Nordman CH, Hernberg S (1975) Blood lead levels and erythrocyte δ-aminolevulinic acid dehydratase activity of selected population groups in Helsinki. Scan J Work Environ Health 1, 219–32.


100) Cook LR, Angle CR, Stohs SJ (1986) Erythrocyte arginase, pyrimidine 5'-nucleotidase (P5N) and deoxypyrimidine 5'-nucleotidase (dP5N) as an indices of lead exposure. Br J Ind Med 43, 387–90.


102) Paglia DE, Valentine WN, Fink K (1977) Lead poisoning. Further observations on erythrocyte pyrimidine nucleotidase deficiency and intracellular


capsule-type silica gels coated with silicone polymer. J Chromatogr 433, 73–9.


