Erythrocyte arginase, pyrimidine 5'-nucleotidase (P5N), and deoxypyrimidine 5'-nucleotidase (dP5N) as indices of lead exposure

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ABSTRACT The activities of three erythrocyte (rbc) enzymes, arginase, pyrimidine 5'-nucleotidase (P5N), and deoxypyrimidine 5'-nucleotidase (dP5N), were compared in 16 lead workers and 14 age matched controls as correlates of blood lead (PbB) and unextracted zinc protoporphyrin (EP) concentrations. Subjects with PbB of 0.9–2.5 μM (19–52 $\mu\text{g/dl}$) had 6.5 ± 0.6 IU of P5N activity with uridine monophosphate (UMP) as substrate, significantly less ($p < 0.001$) than the 12.0 ± 0.7 IU activity of controls with PbB 0.3–0.6 μM (6–12 $\mu\text{g/dl}$). The mean activity of rbc dP5N with either deoxyuridine monophosphate (dUMP) or thymidine monophosphate as substrate, and of rbc arginase, did not differentiate the two groups. The correlation coefficients of $\ln$ PbB with the selected substrates for P5N and dP5N were: UMP, $r = -0.75$; dUMP, $r = -0.61$; TMP, $r = -0.23$. The correlation coefficient of $\ln$ PbB and arginase activity was -0.03 . Rbc P5N (UMPase) is a significant correlate of PbB, equivalent to rbc protoporphyrin. HPLC assay of rbc UMPase activity is a sensitive and rapid assay that appears to meet criteria for a reliable clinical laboratory index of blood lead concentrations.

The analytical variability in blood lead determinations whether by atomic absorption, anodic stripping voltametry, or other available methods has provoked wide ranging searches for reliable biochemical correlates. After the first two months of lead exposure, erythrocyte δ -aminolaevulinic dehydratase (ALA-D) activity is considered the most sensitive correlate with an apparent zero threshold for inhibition by lead. ALA-D activity is also decreased by zinc deficiency and carbon monoxide or alcohol exposure but the mode of assay is a major deterrent to clinical use.¹ Extracted and unextracted erythrocyte protoporphyrins (EP) are widely used in clinical screening at low levels of PbB but are considered insufficiently sensitive to assess occupational toxicity. In a new approach Fukumoto *et al* reported the correlation of rbc arginase with PbB, $> 1.9 \mu\text{M}$ ($> 40 \mu\text{g/dl}$).² The stability of blood samples spotted on filter paper and an automated arginase assay suggested that this procedure had potential clinical use in evaluating exposure in lead workers.

The present study was designed to investigate correlations between PbB and EP concentrations with rbc arginase, pyrimidine 5'-nucleotidase (P5N), and deoxypyrimidine 5'-nucleotidase (dP5N). Numerous clinical studies have shown the correlation of P5N with PbB but most have used the radioimmunoassay of labelled phosphate liberated from the monophosphate.^{3–9} This technique is expensive for clinical use and has been less reliable in our laboratory than HPLC assay of the nucleoside uridine liberated from its monophosphate substrate, UMP. The clinical correlation of PbB with dP5N, assayed as dUMPase or TMPase, has not been examined, although both isozymes are sensitive to divalent metal ions *in vitro*.⁹ We have previously reported the effect of substrate and various cations on both nucleotidases and shown the feasibility of rapid assay of micro samples (50–200 μl) of blood by high performance liquid chromatography (HPLC).¹⁰

Subjects

Sixteen adult male lead workers and 14 adults without known exposure to excess lead provided informed

consent for the assays of PbB and the test enzymes. Heparinised venous blood, 5 ml, was collected in Vacutainer tubes containing $<0.0005 \mu\text{M}$ Pb/tube (Becton Dickinson, Rutherford, NJ).

Methods and materials

Blood lead concentrations (PbB) were determined in replicate in our laboratory using a Perkin Elmer 2380 atomic absorption spectrophotometer with a graphite furnace. Assays of the PbB of exposed workers were also performed by the employer's certified laboratory. The results from the two laboratories were comparable and average lead values are reported for the lead workers.

Unextracted zinc erythrocyte protoporphyrin (EP) concentrations were determined by direct reading fluorescence of a drop of heparinised blood in an ESA Model 4000 hematofluorometer (ESA Associates, Cambridge, MA).

Arginase activity was assayed by the method of Orfanos *et al* which is based on the arginase catalysed production of L-ornithine and urea from arginine¹¹; urea on incubation with urease at room temperature forms NH_4^+ . NH_4^+ is then reacted enzymatically with α -ketoglutarate in the presence of NADH to form L-glutamate. The decrease in fluorescence of NADH during this final step may be measured spectrophotofluorometrically. In our procedure 10 μl whole blood was spotted on filter paper (SS 903, Schleicher and Schnell, Keene, NH) and the 3 mm spots dried before use. Assays were performed in duplicate with a blank for each blood sample. An Aminco-Bowman spectrophotofluorometer set for excitation at 365 nm with emission at 460 nm was used for the assay of NADH. Total haemoglobin concentrations of sample spots were determined by the cyanmethaemoglobin procedure using Drabkin's reagent (Sigma Chemical Co, St Louis, MO). Arginase activity was determined from the micromoles of urea released per minute at 37°C per g haemoglobin. Like Orfanos, we found no decrease in arginase activity when spots were held at -20°C for up to one month before assay.

P5N and dP5N activities were determined by the method of Cook *et al*¹⁰ which measures the amount of nucleoside liberated from its monophosphate on incubation with rbc haemolysate. Red blood cells (rbc) were obtained from 50–250 μl of heparinised blood. The serum and buffy coat were aspirated and the rbc were washed 3x with 3 vols of cold phosphate buffered saline and haemolysed by the addition of 600–750 μl cold distilled H_2O . We omitted the initial elution with 5 ml saline through a 15 ml syringe packed with equal parts microcrystalline cellulose and α -cellulose at 4°C ¹² since the small amount of con-

tamination by residual white cells did not modify activity of UMPase, dUMPase, or TMPase. The total elimination of leukocytes is required to assay CMPase and dCMPase because of the relatively large amount of white cell cytidine deaminase which converts a fraction of the liberated cytidine to uridine. Haemolysates were kept on ice and enzyme assays were performed within four hours of venepuncture. Solutions containing 12 mM MgCl_2 or 12 mM substrate (UMP, dUMP, or TMP, Sigma Chemical Co, St Louis, MO) were prepared in 0.05M Tris, pH 7.5; 50 μl of each solution was added to 1.5 ml microcentrifuge tubes for preincubation at 37°C for five minutes. The reaction was initiated by the addition of 50 μl of haemolysate. After 30 minutes at 37°C the reaction tubes were immersed in boiling water for 1.5 minutes to denature protein. Reaction blanks were prepared by denaturation of haemolysate protein before addition of substrate and incubation. After denaturation, reaction tubes were centrifuged at 11 500 xg for three minutes. Nucleoside concentration in the supernatant was determined by isocratic high performance liquid chromatography (HPLC). The HPLC system consisted of a Model 110A reciprocating pump (Beckman-Altex, St Louis, MO), an LDC SpectroMonitor III variable wavelength detector (Laboratory Data Control, Riviera Beach, FL) at 280 nm, and a syringe-loading injector with a 10 μl sample loop, Model 7120 (Rheodyne, Berkeley, CA). An Altex Ultrasphere ODS column (25 cm $\times$ 4.5 mm ID, 5 μm particle diameter) was used with a Brownlee guard cartridge (RP-18 Spheri 5). Both column and guard cartridge were purchased from Rainin Instruments, Woburn, MA. The HPLC mobile phase contained 0.01M KH_2PO_4 , pH 4.0, with 4–10% methanol as described.¹⁰ Uridine was eluted in about six minutes using 5–6% methanol. Product areas were obtained using external standards and a Spectra-Physics 4270 integrating recorder (San Jose, CA). Haemolysate haemoglobin concentrations

Table 1 Effect of lead exposure on selected blood indices

	$\bar{X} \pm \text{SEM}$
PbB W (17)	$1.33 \pm 0.10^* \mu\text{M}$
C (14)	$0.37 \pm 0.02 \mu\text{M}$
EP W (16)	$1.2 \pm 0.2^* \mu\text{M}$ ($79 \pm 10^* \mu\text{g/dl}$)
C (13)	$0.3 \pm 0.05 \mu\text{M}$ ($16 \pm 3 \mu\text{g/dl}$)
ARG W (14)	$21 \pm 2 \text{ IU}$
C (10)	$23 \pm 2 \text{ IU}$
UMPase W (17)	$6.5 \pm 0.6^* \text{ IU}$
C (14)	$12.0 \pm 0.7 \text{ IU}$
TMPase W (17)	$7.3 \pm 0.3 \text{ IU}$
C (14)	$8.6 \pm 0.7 \text{ IU}$
dUMPase W (8)	$12.3 \pm 1.1 \text{ IU}$
C (8)	$12.3 \pm 0.9 \text{ IU}$

* $p < 0.001$ by Student's *t* test.

W = Workers; C = Controls.

Fig. 1 *1a* correlation between PbB and erythrocyte UMPase. *1b* Correlation between PbB and erythrocyte TMPase.

were determined by the cyanmethaemoglobin procedure. International units (IU) of activity were calculated as $\mu\text{mol nucleoside/h/g haemoglobin}$. All values are expressed as the mean $\pm$ the standard error of the mean (SEM).

We also examined the effect on arginase activity of in vitro lead addition by adding lead nitrate at 1.5, 3.0, and 6.0 $\mu\text{M Pb}^{2+}$ to control sera. These samples were spotted on filter paper and assayed. Lead addition was found to have no effect on arginase activity although 1.0 $\mu\text{M Pb}^{2+}$ reduced UMPase activity by at least 50%.¹⁰

Table 2 Correlation of PbB with indices of lead exposure ($n = 31$)

	EP	Arginase	UMPase	TMPase
ln PbB	0.78	-0.03	-0.75	-0.23
EP		0.05	-0.76	-0.42
Arginase			0.26	0.07
UMPase				0.61

Results

Blood lead, EP, arginase, P5N, and dP5N activities are summarised in table 1 for the 16 lead exposed subjects (PbB 0.9–2.5 μM or 19–52 $\mu\text{g/dl}$) and the 14 control subjects (PbB 0.3–0.6 μM or 6–12 $\mu\text{g/dl}$).

There were significant differences ($p < 0.001$ by Student's t test) between the groups for mean PbB, EP, and P5N activity with UMP as substrate (table 1). There were no significant differences in rbc arginase or dP5N activity with either TMP or dUMP as substrate. The linear regression coefficients determined by relations of ln in PbB with P5N as UMPase and dP5N as TMPase are illustrated in figs 1a and 1b. The intercorrelation of the four indices of exposure, listed in table 2, show significant and comparable correlation of ln PbB with EP and UMPase; UMPase correlates with TMPase although the latter, a substrate for dP5N, is not significantly correlated with PbB, EP, or arginase.

Discussion

The correlation of ln PbB with unextracted EP is as expected. Neither PbB or EP was significantly correlated with rbc arginase. The slope of the line relating ln PbB and rbc arginase is essentially zero with no correlation at PbB $< 1.9 \mu\text{M}$ ($< 40 \mu\text{g/dl}$) and no apparent trend for positive correlation at higher PbB. This is contrary to the report by Fukumoto *et al* of a tentative correlation of arginase with PbB $< 1.9 \mu\text{M}$ ($< 40 \mu\text{g/dl}$) and a significant correlation with PbB $> 1.9 \mu\text{M}$ ($> 40 \mu\text{g/dl}$).¹²

Chmielnicka *et al* examined serum, rather than rbc, arginase activity as an index of hepatic damage secondary to lead exposure.¹³ Sixty lead workers were divided into two groups based on PbB either greater or less than 1.9 μM (40 $\mu\text{g/dl}$). There was a significant increase in serum arginase in the high lead group. The mean PbB in our 16 workers of 2.2 μM (46 $\mu\text{g/dl}$) was considered too low to test for a hepatotoxic effect on serum arginase activity.

In this study it was not possible to correlate variations in PbB with arginase activity, the in vitro addition of Pb to blood samples did not affect red cell arginase activity, and the time required for the non-automated assay of arginase was clearly greater than

that for assay of UMPase. Erythrocyte arginase assay does not appear to be a potentially useful screening test at contemporary levels of industrial Pb exposure.

The correlation of PbB with P5N assayed by HPLC as UMPase is consistent with earlier reports by this laboratory and others.³⁻⁹ Erythrocyte UMPase activity, determined by a relatively rapid HPLC assay, appears to be a correlate of PbB at least equivalent to EP. The question of a threshold level of PbB for inhibition of UMPase is not resolved by the clinical data to date, although in vitro inhibition by lead is noted at 10^{-6} M.

The clinical correlation of PbB with rbc dP5N, assayed as dUMPase and TMPase, has not been previously investigated. This isozyme dephosphorylates the deoxymononucleotides and is similarly inhibited in vitro by Pb^{2+} and Cu^{2+} . Despite minimal differences in the in vitro effects of Pb^{2+} on P5N and dP5N,¹⁰ the in vivo correlation of rbc dP5N with ln PbB was not significant. The effect of lead on rbc P5N thus appears relatively specific for one isozyme. HPLC assay of rbc UMPase deserves wider clinical investigation as a reliable correlate of PbB.

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