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D E Paglia, W N Valentine, J G Dahlgren

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Research Article

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Effects of Low-Level Lead Exposure on Pyrimidine 5'-Nucleotidase and Other Erythrocyte Enzymes

POSSIBLE ROLE OF PYRIMIDINE 5'-NUCLEOTIDASE IN THE PATHOGENESIS OF LEAD-INDUCED ANEMIA

DONALD E. PAGLIA, WILLIAM N. VALENTINE, and JAMES G. DAHLGREN

From the Division of Surgical Pathology, Department of Pathology, and the Department of Medicine, University of California School of Medicine, Los Angeles, California 90024, and Wadsworth General Hospital, Veterans Administration Center, Los Angeles, California 90073

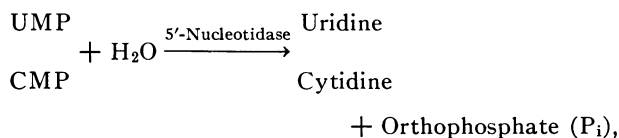
ABSTRACT Similarities between lead-induced anemia and a new hereditary erythroenzymopathy involving pyrimidine-specific 5'-nucleotidase prompted studies of the effects of lead on this and other erythrocyte enzymes. In vitro incubations of normal mature erythrocytes demonstrated that significant inhibition of pyrimidine 5'-nucleotidase occurred in the presence of lead at concentrations that had minimal effects on many other erythrocyte enzymes assayed simultaneously. Similarly, subjects with chronic lead intoxication secondary to industrial exposure exhibited substantial and consistent impairment of erythrocyte pyrimidine-5'-nucleotidase activity. Results suggest that lead-induced deficiency of this enzyme in maturing erythroid elements could, if sufficiently severe, result in induction of basophilic stippling and premature erythrocyte hemolysis analogous to that encountered in the genetically induced enzyme-deficiency syndrome.

INTRODUCTION

Laënnec has been credited with the first description of anemia in association with lead poisoning (1). Numerous attempts to define the responsible pathogenetic mechanisms have shown that no single factor is completely culpable, since the demonstrable deleterious effects of lead on erythrocytes in vitro and in vivo are multiple and varied (1, 2). These include defects both in globin (3, 4) and in heme (3, 5, 6) synthesis with resultant retardation of erythrocyte maturation and di-

rect membrane effects with impaired cation pumping (7-11), altered fragilities (12), and consequent hemolysis.

The present studies evolved from recent recognition of an hereditary hemolytic syndrome in which erythrocytes were characterized in part by prominent basophilic stippling and a specific enzyme deficiency (13). The defective enzyme was a pyrimidine 5'-nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5) not known to exist in other tissues. In normal hemolysates, it hydrolytically dephosphorylates uridine and cytidine 5'-monophosphates (UMP and CMP):



but it is ineffective with AMP, other purine ribonucleotides, or with substrates commonly used for assay of nonspecific acid or alkaline phosphatases. Enzymatic activity was found to be markedly sensitive to heavy metal inhibition (14). The latter characteristic and the occurrence of prominent basophilic stippling in both the hereditary deficiency state and in severe plumbism prompted the present studies to investigate a possible causal relation between lead-induced anemia and this metal's specific effect on erythrocyte pyrimidine 5'-nucleotidase.

METHODS

Venous blood, anticoagulated with heparin, was obtained from normal healthy volunteers and, in a single-blind study, from subjects accidentally exposed to lead as a consequence

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of their occupations. Erythrocytes were separated from leukocytes and platelets by sedimentation in Plasmagel or by cotton filtration followed by multiple saline washes. Activities of enzymes of the Embden-Meyerhof pathway, the pentosephosphate pathway, and glutathione and nucleotide metabolism and of certain nonglycolytic enzymes were measured by methods noted in earlier reports (13, 15-20). Erythrocyte glutathione concentration was determined by the method of Beutler et al. (21).

Hemolysates prepared by alternate freezing and thawing were dialyzed free of endogenous phosphates before assaying 5'-nucleotidase activity as detailed previously (13, 14). The assay system employed aliquots of dialyzed hemolysate incubated at 37°C with 2.3 mM UMP or CMP as substrate in 25 mM Tris buffer with 10 mM MgCl₂ at a final pH of 7.8. Inorganic phosphate (P_i) liberated by nucleotidase activity was determined by the Fiske and SubbaRow technique (22) after terminating the reaction by deproteinization with trichloroacetic acid. Activities were expressed as micromoles of P_i evolved per hour per gram of hemoglobin. Duplicate determinations with each substrate were performed, providing a minimum of four nucleotidase assays on each subject. Nucleotide ultraviolet absorption spectra were determined on acid extracts of washed erythrocytes as described previously (13).

Appropriate specimens from individuals subjected to lead overburden were also sent to a commercial laboratory for routine hematologic measurements and for assays of blood and urinary lead concentrations and urinary δ -aminolevulinic acid excretion. These findings were not reviewed until completion of erythrocyte enzyme studies.

RESULTS

Effect of lead on pyrimidine-5'-nucleotidase activities in normal hemolysates. In vitro effects of lead on erythrocyte nucleotidase were assessed by addition of the divalent acetate salt directly to buffered hemolysate during the 1-2-h incubation phase of the assay. Results in Fig. 1 are presented relative to appropriate controls run in parallel and show the marked inhibitory effect of this cation on enzymatic liberation of P_i from pyrimidine nucleotides. Measurements with either UMP or CMP as substrate were combined, since relative values were essentially identical. 50% inhibition was consistently observed when lead concentration was in the order of 10 μ M. Inhibition was clearly detectable in some experiments even with 0.1 or 1 μ M lead acetate and in most experiments was virtually complete above 0.1 mM.

Shortening the period of lead exposure and subsequent dialysis of the hemolysate did not appreciably alter the inhibitory effect on pyrimidine 5'-nucleotidase. An hemolysate incubated for only 10 min at 37°C with 0.1 mM lead acetate was dialyzed for 18 h at 4°C against 1,500 vol 10 mM Tris buffer containing 10 mM MgCl₂ at pH 8.0 and then assayed for nucleotidase activity. Samples exposed to lead before dialysis retained only 10 and 23% of unexposed control nucleotidase activities when measured with UMP and CMP, respectively.

Results of a similar experiment are also presented in Fig. 1. Hemolysates were divided into aliquots that

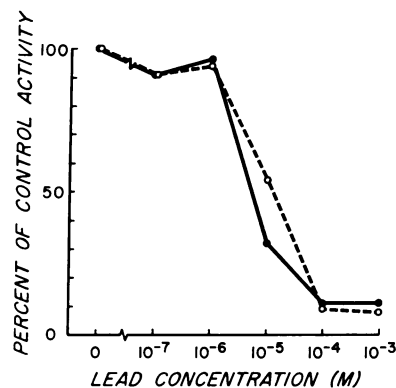


FIGURE 1 Effect of lead concentration on pyrimidine-5'-nucleotidase activity in normal hemolysates. The solid curve depicts values obtained with lead added directly to hemolysate in the nucleotidase assay system. Values are means of 10 determinations on four separate hemolysates except for the value at 0.1 μ M lead acetate, which is the mean of four assays on two samples. The broken curve was obtained from two determinations on a single hemolysate dialyzed free of lead after a 20-min exposure (see text for details).

were incubated at 37°C for 20 min with varying concentrations of lead acetate, then dialyzed at 4°C against two 1,500-vol changes of the same dialysis medium for 3½ and 17½ h before assaying nucleotidase activities. Inhibitory effects were similar in magnitude to those observed with lead present directly in the assay medium (solid curve in Fig. 1).

Effect of lead on intact normal erythrocytes. Washed erythrocytes were incubated at 37°C for 30-60 min in isotonic saline containing 1 mM glucose and 0.1 mM lead acetate, then rewashed immediately with two changes of at least 25 vol isotonic saline. Cells were resuspended in saline to approximately $3 \times 10^6/\mu$ l and counted electronically before assaying various enzyme activities. Results, presented in the first part of Table I, indicated that no significant impairment of glycolytic or other erythrocyte enzymes, aside from pyrimidine 5'-nucleotidase, occurred under these conditions. Several kinases were inhibited about 15%, but most other enzymes appeared slightly stimulated by lead treatment.

Aliquots of the same erythrocyte suspensions, hemolyzed by alternate freezing and thawing, were immediately dialyzed at 4°C for 2 h and 18 h against two changes of 1,500 vol each of the dialysis medium defined above. Control preparations were treated identically except for the absence of lead. Residual nucleotidase activity in lysates of lead-exposed cells averaged 15% of controls when measured with UMP as substrate, and 37% when assayed with CMP. Residual activities were approximately twice as much when 0.4 mM 2-mercaptoethanol was added to hemolysates as a protector of sulfhydryl groups. The more pronounced

TABLE I
Enzyme Activities and Glutathione Content in Normal Erythrocytes Exposed to Lead In Vitro and in Erythrocytes from Subjects with Occupational Lead Overburden

	Erythrocytes loaded in vitro	Subjects with lead overburden	Normal controls	
	% of control*		Enzyme U‡	
Hexokinase	85	0.28	0.25	(0.05)§
Glucosephosphate isomerase	106	15.5	12.1	(1.5)
Phosphofructokinase	87	3.3	3.25	(0.45)
Fructosediphosphate aldolase	119	0.9	0.92	(0.09)
Triosephosphate isomerase	107	136	156	(10)
Glyceraldehyde-3-phosphate dehydrogenase	115	28.0	31.8	(3.7)
Phosphoglycerate kinase	104	30.9	28.4	(1.4)
Phosphoglyceromutase	106	7.8	7.1	(0.9)
Phosphopyruvate hydratase (enolase)	103	4.7	4.0	(0.5)
Pyruvate kinase	114	4.3	4.8	(0.7)
Lactate dehydrogenase	106	52.2	53.1	(14.4)
Glucose-6-phosphate dehydrogenase	94	2.5	2.65	(0.5)
Phosphogluconate dehydrogenase	102	1.9	1.94	(0.4)
Terminal pentosephosphate pathway	118	14.8	11.4	(2.1)
Glutathione reductase	103	2.7	2.15	(0.3)
Glutathione peroxidase	104	6.6	6.8	(1.2)
Reduced glutathione	98	721	739	(111)
Glyoxalase I	83	58.0	46.3	(4.4)
Glyoxalase II	111	13.0	12.9	(2.9)
Malic dehydrogenase	112	4.5	4.7	(0.4)
Glutamic-oxalacetic transaminase	115	—	—	—
Transulferase	104	81	86	(11)
Adenylate kinase	101	89	85.8	(12.3)
Ribosephosphate pyrophosphokinase	86	37.6	30.5	(3.8)
Adenine phosphoribosyltransferase	102	3.2	3.4	(0.4)
Nucleosidemonophosphate kinase	87	3.2	—	—
Pyrimidine 5'-nucleotidase (UMP)	26	2.6¶	6.6	(2.0)¶
(CMP)	36	2.4¶	5.5	(1.4)¶

* Values are means of separate determinations on washed erythrocytes from five normal subjects and are expressed as percent of activities in cells from the same specimens treated identically except for exposure to lead.

‡ Micromoles of substrate converted per minute by 10^{10} erythrocytes at 37°C, except for glyoxalase which was measured at 25°C.

§ Normal laboratory means with standard deviations in parentheses.

|| Micrograms per 10^{10} erythrocytes.

¶ Micromoles P_i liberated per hour per gram hemoglobin.

effects on dephosphorylation of UMP relative to CMP have been noted previously under other deleterious conditions, such as thermal stress (14).

Studies on subjects with lead overburden. Most of the exposed individuals had subjective symptoms referable to lead toxicity, such as irritability, difficulty sleeping, gastrointestinal complaints including crampy abdominal pain, and other signs of peripheral or central nervous system effects. At the time of study, only one subject had anemia, and this was mild. Hemoglobin levels averaged 15.2 g/100 ml, and the lowest value was

12.7 g/100 ml with a reticulocyte count of 2.1%. Basophilic stippling was not observed in peripheral erythrocytes from any subject. Results of routine hematologic studies and lead toxicity assays are presented in Table II.

Erythrocyte pyrimidine-5'-nucleotidase activities were below normal control values in all 15 subjects with lead overburden, averaging 2.6 and 2.4 U, respectively, with UMP and CMP as substrates. Corresponding normal laboratory values are 6.6 U (SD, 2.0 U) and 5.5 U (SD, 1.4 U). Mean activities in these subjects were

equivalent to 39% (with UMP) and 44% (with CMP) of normal controls and ranged from 11 to 70% (UMP) and from 12 to 82% (CMP) of normal means.

The lowest nucleotidase activity observed (< 0.7 U) occurred in the only subject with mild anemia (hemoglobin, 12.7 g/100 ml; reticulocytes, 2.1%). This was equivalent to 11–12% of normal mean values, but was effectively somewhat lower, since this nucleotidase is characteristically more active in younger erythrocytes and reticulocytes (14). Blood lead concentration and urinary lead excretion in this patient were 80 $\mu\text{g}/100$ ml and 218 $\mu\text{g}/24$ h, respectively, 4 days before performance of enzyme assays.

The subject with the greatest overburden on the basis of blood lead concentration (109 $\mu\text{g}/100$ ml, 18 days previously) had the second lowest nucleotidase activities recorded, 1.35 and 1.45 U with UMP and CMP, respectively.

Intervals between lead toxicity analyses recorded in Table II and assays of erythrocyte enzyme activities varied from 0 to 41 days, averaging 15 days, so direct temporal relations could not be established. Despite this, blood lead concentrations in these patients exhibited general correlations with pyrimidine-5'-nucleotidase activities in their erythrocytes (Fig. 2). There was no apparent correlation between nucleotidase activities and either lead or δ -aminolevulinic acid excretion in the urine.

Specimens from 12 subjects were also assayed for activities of other erythrocyte enzymes. Results presented in the second part of Table I demonstrate the selective depression of pyrimidine-5'-nucleotidase activities in these patients. In some individuals, slightly lowered values were found for phosphoglycerate kinase, triosephosphate isomerase, phosphofructokinase, and

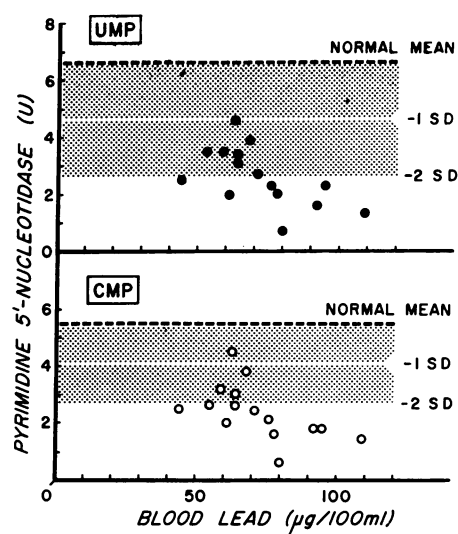


FIGURE 2 Erythrocyte pyrimidine-5'-nucleotidase activity as a function of blood lead concentration in subjects with lead overburden. Solid and open symbols are values obtained with UMP and CMP, respectively, and each is the mean of at least two determinations. Correlation coefficients were approximately -0.6 .

some dehydrogenases, but even these occasionally low values remained within 70–80% of normal mean activities. Two subjects had approximately 55% of normal pyruvate kinase activity, consistent with the heterozygous deficiency state, but additional studies to confirm an hereditary defect were not performed. Glutathione content was not significantly altered.

Ultraviolet absorption spectra of erythrocyte extracts revealed only the characteristics of adenine nucleotides. There was no evidence of intracellular accumulation of uridine or cytidine nucleotides.

DISCUSSION

The precise pathogenesis of lead-induced anemia remains indefinite despite numerous investigations directed toward its elucidation (1, 2). It seems clear that lead affects both erythroid precursor cells and mature circulating erythrocytes, resulting in impaired erythroid maturation, defective heme synthesis, sideroblastosis in some instances, and distinct hemolytic tendencies. The latter may be due in part to slight but specific effects on membrane components, including ATPase (9, 10, 23), which cause affected cells to be selected for early removal by a discriminating reticulo-endothelial system or which induce premature osmotic lysis.

As noted previously, defective hemoglobinization is probably the consequence of impaired globin formation

TABLE II
Clinical Laboratory Measurements on Subjects Exposed to
Occupational Lead Overburdens

Determination	Mean	Range	Normal values
Hemoglobin, g/100 ml	15.2	12.7–16.9	14.4–16.6
Packed cell volume, %	46	38–52	43–49
Reticulocytes, %	1.2	0.4–2.1	0.6–1.7
Blood lead, $\mu\text{g}/100$ ml	72	44–109	< 40
Urine lead, $\mu\text{g}/24$ h	409	40–3,638	< 80
	178*	40–321*	
Urine δ -aminolevulinic acid, \dagger mg/24 h	19.3	12.6–27.6	7.0
	4.5	0.9–7.1	0.6

* Values obtained if one patient with markedly elevated urinary lead is excluded.

\dagger Specimens assayed by two different analytic procedures.

combined with specific enzymatic defects in protoporphyrin and heme synthetic pathways. The latter include variable inhibitions of heme synthetase (24) and of δ -aminolevulinic acid synthetase (24-26) and dehydratase (25-28), accounting for the elevated blood and urine concentrations of those intermediates of porphyrin and heme metabolism that are characteristic of lead toxicity.

These inhibitory effects on multiple enzyme systems (and/or structural proteins) are presumably due to effects on critical protein sulfhydryl groups. Sulfhydryl-rich enzymes, such as those of heme biosynthesis, are thus especially susceptible (1, 6). Waldron suggested that anemia might be due not only to effects on heme synthesis but also on enzymes concerned with glycolysis (1). Glycolytic impairment, however, does not appear to be a significant factor under conditions of this investigation. Our observations support those of Rogers et al. (27), who did not measure activities of individual glycolytic enzymes but found lactate and CO_2 production from glucose to be normal in erythrocytes from rabbits with experimentally induced lead poisoning (blood lead levels, $117 \pm 14 \mu\text{g}/100 \text{ ml}$). Our observations regarding malic dehydrogenase, acetylcholinesterase (measured on only three subjects), acid phosphatase, and glucose-6-phosphate dehydrogenase are also in accord with theirs, although their report cites conflicting data on the latter (27). Glutathione content, usually thought to be decreased in lead toxicity (2), was not significantly altered, a finding supported by that of Angle and McIntire (23).

The present studies demonstrate a manifestation of lead toxicity that may relate to the occurrence of basophilic stippling and premature hemolysis in some cases of plumbism. Mature erythrocytes exposed to low concentrations of lead, either in vivo or in vitro, exhibited substantial, sometimes marked, decreases in activity of pyrimidine 5'-nucleotidase. This enzyme was inhibited at lead concentrations that exerted minimal effects on a wide variety of other erythrocyte enzymes assayed simultaneously. Such lead-induced impairment of pyrimidine-5'-nucleotidase activity prompts comparison with the genetically induced, severe deficiency state involving this same enzyme (13, 29, 30). The latter is characterized clinically by severe chronic hemolytic anemia with pronounced basophilic stippling of the erythrocytes that uniquely contain large amounts of uridine and cytidine nucleotides. The presence of high concentrations of pyrimidine nucleotides may secondarily affect the activities of critical glycolytic enzymes, such as the kinases, by competition with the preferred and far more efficient adenine nucleotide cofactors (13) and possibly by other mechanisms such as feedback inhibition. It should be emphasized that these manifestations

were not observed in individuals heterozygous for the deficiency, who had approximately half-normal nucleotidase activities, but only in homozygotes with residual nucleotidase activities that were 10% of normal or less, even in the presence of marked reticulocytosis. Normal reticulocytes and young erythrocytes exhibit nucleotidase activities considerably increased above those in more mature cell populations (13, 14).

Both the basophilic reticulum of normal reticulocytes and the basophilic granules of stippled erythrocytes are composed of RNA (31, 32) which may be in the form of aggregated ribosomes in lead poisoning (33). In patients with severe hereditary nucleotidase deficiency, the granules presumably result from retarded RNA degradation secondary to inability to dephosphorylate and render diffusible the pyrimidine degradation products of RNA (13). In the present study, despite clear evidence of lead overburden, neither increased intracellular pyrimidine nucleotides nor basophilic stippling were evident, and anemia was absent in virtually all subjects. Absence of frank basophilic stippling and anemia in many adults with chronic lead intoxication has been documented in other studies (1, 2). Blood lead levels must exceed about $110 \mu\text{g}/100 \text{ ml}$ before clinically evident depression of hemoglobin concentration generally occurs (34).

The present studies suggest that with greater degrees of lead overburden, such as in cases of severe acute poisoning, erythrocyte nucleotidase activity might well be more deficient and uniformly distributed and thereby more closely resemble the genetically determined, homozygous deficiency state. If such were the case, anemia, demonstrable hemolysis, increased basophilic stippling, and elevated intracellular concentrations of uridine and cytidine nucleotides would be expected. Lack of appropriate clinical material has thus far precluded adequate assessment of this possibility.

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