CONCISE REPORT

Pyrimidine Nucleotidase Deficiency With Active Dephosphorylation of dTMP: Evidence for Existence of Thymidine Nucleotidase in Human Erythrocytes

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Erythrocytes from a patient with classical pyrimidine nucleotidase (PyN) deficiency had less than 10% residual PyN activity with uridine 5'-monophosphate (UMP) or cytidine 5'-monophosphate (CMP) as substrate, but exhibited brisk nucleotidase activity with thymidine 5'-monophosphate (dTMP). This strongly suggests the existence of separate enzymes or isozymes of PyN in normal human erythrocytes—an hypothesis that should be tested by similar studies in other cases of severe PyN deficiency, whether induced by genetic defects or lead toxicity.

PROCEDURES

Blood specimens anticoagulated with heparin were air expressed under refrigeration to Los Angeles for processing within 24-36 hr. Leukocyte-free suspensions of saline-washed erythrocytes were prepared and assayed for enzymes of oxidative and anaerobic glycolysis and glutathione and nucleotide metabolism, according to standard procedures. Ribosephosphatopyrophosphokinase (RPPK) was measured by the technique of Valentine and Kurschner. Glycolytic intermediates were assayed by the method of Minakami et al., except for 2,3-DPG, which was measured by the method of Keitt. Reduced glutathione was measured by the method of Bestler et al.

Hemolysates prepared by sonication were cleared of endogenous phosphates by dialysis against at least 200 volumes of isotonic saline buffered to pH 8.0 by 0.01 M Tris-HCl, containing 10 mM MgCl₂, 0.02 mM EDTA, and 1 mM mercaptoethanol. Dialyzed hemolysates were then assayed for PyN activity as described previously. The standard assay employed 0.5-mI aliquots of dialyzed hemolysate incubated at 37°C in 25 mM Tris-HCl buffer with 8.5 mM MgCl₂ and 0.5 mM dithiothreitol at a final pH of 7.4. The substrates used were UMP, CMP, dTMP, and AMP at a final concentration of 2.3 mM. Inorganic phosphate (P_i) liberated during the 2-hr incubation was measured by the Fiske and SubbaRow technique, after terminating the PyN reaction by deproteinization in trichloroacetic acid. Nucleotidase activities were expressed as micromoles P_i liberated per hour per gram of hemoglobin.

Ultraviolet absorption spectra were determined on acid extracts as described previously. Chromatographic and electrophoretic procedures to identify intracellular nucleotides are detailed in the same report. Assays for conjugated nucleotides were performed on neutralized extracts treated with nucleotide pyrophosphatase and/or alkaline phosphatase. Choline was assayed by the choline kinase

MATERIALS AND METHODS

Clinical Summary

The proband was a 45-yr-old female of Italian ancestry. She had persistent jaundice, splenomegaly, and chronic hemolysis, with Hb as low as 5.5 g/dl. Because of her transfusion requirement and incipient heart failure, she had porcine valve-leaflet prostheses inserted at age 44 for repair of severe rheumatic mitral and aortic valvular incompetence. Splenectomy performed at the same time was followed by stabilization of her hemolytic process, with 24% hematocrit (Hct), 7.9 g/dl Hb, and 19% reticulocytes. Follow-up studies at the University of Florida, Gainesville, indicated the presence of an intrinsic cell defect with basophilic stippling, echinocytosis, and decreased erythrocyte concentrations of 2,3-diphosphoglycerate (2,3-DPG) and adenine nucleotides. The proband's mother and four other siblings had no known hematologic abnormality.
reaction" and in the UCLA laboratory of Dr. Donald Jenden by gas chromatographic/mass spectrographic techniques."

RESULTS

As shown in Table 1, several enzymes (hexokinase, pyruvate kinase, glutamic oxalacetic transaminase) that are sensitive erythrocyte age markers were significantly elevated in activity, reflecting the young mean cell age of proband erythrocytes. Normally, RPK and PyN are also consistently and prominently more active in reticulocytes and young erythrocytes, so their low activities in proband cells were even more abnormal than apparent from the absolute values in Table 1.

Despite having less than 10% of expected PyN activity with UMP and CMP as substrates, proband hemolysates readily dephosphorylated dTMP. Thymidine nucleotidase activity was comparable to normal controls. Under these assay conditions, there was no apparent increase in thymidine nucleotidase activity due to reticulocytosis per se. AMP was not a substrate for PyN activity in proband, maternal, or control hemolysates.

The mother, who was the only relative available for study, was hematologically normal, and her erythrocytes had approximately half normal PyN activities, consistent with heterozygosity for the defect. Basophilic stippling and pyrimidine compounds were not detectable in maternal cells.

Perchloric acid extracts of proband erythrocytes exhibited pronounced bathochromic shifts in ultraviolet absorption spectra induced by high intracellular concentrations of uridine and cytidine compounds that invariably accumulate in severe PyN deficiency. Maximal absorbance was 273 nm, rather than the adenine peak of 257.5 nm that is characteristic of normal or reticulocyte-rich blood.

The area and amplitude of spectral curves were also increased, reflecting an elevation in total intracellular nucleotides. Adenine compounds (ATP, ADP, and AMP) normally constitute 97% or more of erythrocyte nucleotides, with a total mean concentration of 1.4 mM. Young erythrocytes have increased concentrations, averaging 1.9 mM in 15 subjects with 8% mean reticulocyte counts. By contrast, nucleotides in proband erythrocytes totaled 9.3 mM, of which 14% were nucleotides of adenine (or inosine), 63% of cytidine, and 23% of uridine. In absolute terms, therefore, total adenine nucleotides were reduced to 1.3 mM, about 60% of expected concentration.

When trichloroacetic acid extracts prepared by the alamine/freon method were treated with barium and zinc to precipitate unconjugated nucleotides, a large nucleotide-containing component remained in the supernate, with a peak absorbance at 280 nm. Further treatment with nucleotide pyrophosphatase and/or alkaline phosphatase and analysis of the resultant products indicated that this consisted of CDP-choline, which was present in proband erythrocytes at a concentration of approximately 1.1 mM.

DISCUSSION

The proband in this study exhibited the classic syndrome of severe hereditary PyN deficiency: chronic hemolytic anemia with erythrocytes that contained markedly increased concentrations of uridine and cytidine compounds, decreased adenine nucleotides, basophilic stippling, markedly impaired PyN activity, elevated glutathione, and intermediate activity of RPK. The mother had the phenotypic pattern of heterozygosity for PyN deficiency. The father was not available for study. In addition to high concentrations of uridine and cytidine phosphates in proband erythrocytes, large amounts (1.1 mM) of a conjugated form, CDP-choline, were also found, confirming the observations of deVerdier et al. and Swanson et al. in other cases of PyN deficiency. Either CTP or CMP, which accumulate in PyN deficiency, might be converted into CDP-choline by CDP-choline synthetase or choline phosphotransferase if phosphorylcholine and lecithin are available. We have previously observed CDP-choline synthetase activity in erythrocytes, but choline phosphotransferase has not as yet been demonstrated.

When PyN in normal erythrocytes was originally characterized, dTMP, dUMP, and dCMP were all found to be effective substrates in dialyzed hemolysates. The many subsequent studies of normal or deficient PyN performed on hemolysates or partially purified preparations have neglected dTMP and other deoxynucleotides as potential substrates, often because radioactive techniques utilizing labeled CMP or UMP were employed and because a single PyN enzyme was presumed to exist.
The presence of normal thymidine nucleotidase in red cells with severely deficient PyN activity for UMP and CMP is most likely explained by the existence of separate enzymes or isozymes with different substrate specificities. Alternatively, but less likely, the data may be explained by structural alteration of a single PyN enzyme protein that primarily affects substrate specificity or avidity. This seems unlikely, however, in view of the marked quantitative differences in relative activities with these three pyrimidine substrates and because of their close structural similarities.

It is also unlikely that nonspecific phosphatase could account for the phosphohydrolase effect of proband hemolysates on dTMP, since dephosphorylation of UMP and CMP is poor at acid or alkaline pH. Preliminary studies with other cases of PyN deficiency indicate that dephosphorylation of dTMP as a function of pH differs significantly both from UMP and CMP and from alpha- and beta-glycerophosphates, substrates for acid and alkaline phosphatases. In those cases, dUMP and dTMP (which is 5-methyl dUMP) were equally effective as PyN substrates, but UMP, CMP, and AMP were not, lending further support to the presence of distinct enzymes or isozymes.

Cases of PyN deficiency, both hereditary and acquired from lead toxicity, are now being studied with dTMP as well as other pyrimidine and purine substrates. Such cases provide the natural experiments necessary to unmask existence of separate enzymes or isozymes of different properties, as well as their corresponding deficiency states.

The present study indicates that a distinct thymidine nucleotidase may exist in human erythrocytes, the activity of which is dependent on the deoxyribose moiety and the nature of the substitution on carbon-4 of the pyrimidine base. Such a highly specific enzyme might be functional in DNA degradation in a manner analogous to that presumed for PyN in ribosomal RNA catabolism. This raises the possibility that pyknosis and nuclear pitting in maturing erythroblasts may be accompanied by a degree of karyolysis that requires clearance of DNA degradative products from the cytosol.

NOTE ADDED IN PROOF

Swallow et al. recently reported similar findings in a Japanese patient with homozygous PyN deficiency. They measured low activities with UMP and CMP as substrates, normal activities with dUMP and dTMP, but detected no activity in electrophoretic gels stained with dCMP. By contrast, we have observed moderate residual activity with dCMP in other subjects with PyN deficiency, if assayed at the lower pH optimum of the thymidine nucleotidase isozyme. This isozyme, therefore, may prove to be a deoxyxymidine nucleotidase with variable substrate preferences.

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REFERENCES

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