Identification of Cytidine Diphosphodiesters in Erythrocytes From a Patient With Pyrimidine Nucleotidase Deficiency

By M. S. Swanson, R. S. Markin, S. J. Stohs, and C. R. Angle

Pyrimidine 5'-nucleotidase deficient (PND) erythrocytes contain elevated levels of pyrimidine nucleotides and relatively normal purine nucleotide levels. The composition of this nucleotide pool has been examined by others, but not all of the abnormal red cell metabolites in this disorder were identified. We have isolated and positively confirmed the identity of cytidine diphosphate (CDP)-choline and CDP-ethanolamine from PND red cells using methods including proton FT-NMR, spectroscopy, and comparative mass spectrometry. The concentrations of these and other pyrimidine nucleotidase-deficient erythrocyte nucleotides were determined using anion-exchange high performance liquid chromatography and ultraviolet (u.v.) detection. The pyrimidine phosphodiesterases appear to be the most prominent abnormal pyrimidine nucleotides in PND red cells, accounting for 55% of the total red cell pyrimidine nucleotides in this disorder. It is proposed that these abnormal phosphodiesterases may be related to the accelerated hemolysis in PND.

ERYTHROCYTE PYRIMIDINE 5'-nucleotidase deficiency (PND) was first described by Valentine et al.12 as a chronic nonspherocytic hemolytic anemia associated with increased erythrocyte adenine nucleotides, prominent basophilic stippling, and an elevation in reduced glutathione. These investigators then found the increase in red cell nucleotides to be predominantly pyrimidines due to the deficiency of a 5'-pyrimidine-specific nucleotidase normally present in the red cell.1 Paglia6 suggested that the abnormal accumulation of red cell pyrimidines occurred secondary to ribosomal RNA degradation, whereas Harley et al.5 demonstrated plasma ureide salvage as a more likely source of red cell pyrimidine accumulation. The composition of this abnormal red cell nucleotide pool was examined by Torrance and Whittaker,4 who found that it consisted primarily of cytidine triphosphate (CTP), uridine triphosphate (UTP), and uridine diphosphate (UDP) glucose, with relatively normal levels of purine nucleotides. In addition, they reported large levels of an unidentified cytidine diphosphate (CDP) compound. Swanson et al.7 recently suggested that this cytidine diphosphate compound was a mixture of CDP-choline (CDP-C) and CDP-ethanolamine (CDP-E) based on 31P NMR studies of pyrimidine 5'-nucleotidase deficient (PND) red cell extracts.

In this study, we definitively confirm the identity of CDP-choline and CDP-ethanolamine in erythrocytes obtained from a patient with PND by means of 1H nuclear magnetic resonance (NMR) spectroscopy. The concentrations of these and other PND erythrocyte nucleotides are determined by anion-exchange liquid chromatography.

MATERIALS AND METHODS

The pyrimidine 5'-nucleotidase deficient (PND) subject is a 26-yr-old white male previously studied by our group1 and by Beutler.8 After obtaining informed consent, venous blood from the PND subject and normal subjects was collected using heparinized tubes. Red cell suspensions were prepared from whole blood by washing 3 times in 3 volumes of phosphate-buffered saline (pH 7.4), with removal of the buffy coat following each centrifugation at 3,000 g for 10 min. Phosphate metabolites were extracted by the method of Chen et al.,4 in which packed red cells were hemolyzed in 4 volumes of water and made 7% in trichloroacetic acid (TCA). The acid extracts were neutralized using tri-N-octylamine. Erythrocyte nucleotide concentrations were determined using anion-exchange high pressure liquid chromatography (HPLC) and ultraviolet (u.v.) detection at 280 nm, as described previously.10

Milligram quantities of the previously unidentified diphosphate diester nucleosides were collected from PND red cell extracts using a preparative anion-exchange HPLC column (M 9 SAX, Whatman Inc., Clifton, NJ). The resulting fractions were lyophilized and desalted using a reverse-phase preparative column (M 9 ODS, Whatman Inc.). The fractions collected were analyzed using four methods. Proton FT-NMR spectra were obtained using a 360 MHz Bruker Superconducting spectrometer operating in the Fourier transform mode. Mass spectral analysis was carried out by the fast atom bombardment technique, using a Hitachi MS-10 mass spectrometer at the Midwest Center for Mass Spectrometry in Lincoln, NE. Infrared spectra were obtained using a Perkin-Elmer 411 spectrophotometer. Ultraviolet-visible spectra were determined by a Cary Model 15 spectrophotometer.

The relative percent composition of erythrocyte membrane phospholipids was determined using the methods of Chapelle11 and Fenster et al.12 with some modifications. Lipid extractions were carried out using hemoglobin-free ghosts prepared by the method described by Salhany and Swanson.13 Lipid extracts were chromatographed using high resolution thin-layer chromatography (TLC) plates (HP-TLC Silica Gel 60. E. Merck). Plates were developed using a solvent system consisting of chloroform/methanol/acetic acid/water (25:15:4:2 v/v).

RESULTS AND DISCUSSION

Positive identification of CDP-choline and CDP-ethanolamine in PND red cell extracts was accom-

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665
accomplished using proton NMR, operating in the Fourier-transform mode. Proton NMR spectra were collected from samples dissolved in D$_2$O, thus, the ribosyl hydroxyl protons and amine protons exchanged with deuterium. Chemical shift assignments were made with respect to HDO.

The spectrum of CDP-C isolated from PND erythrocytes (Fig. 1) is similar to the reference compound (Fig. 2), but some differences are noted. All of the signals in the CDP-C isolate spectrum (Fig. 1) show a downfield shift of roughly 0.1 ppm as compared to the reference standard (Fig. 2), due to the position of HDO in this spectrum at 4.74 ppm (not visible in spectrum), which is 0.1 ppm downfield of the signal for HDO in Figs. 2 and 3. This explains the discrepancy in the signals corresponding to the equivalent protons of the choline methyl groups at 3.05 ppm for the reference compound (Fig. 2) as compared to 3.15 ppm for the isolated CDP-C (Fig. 1). The triplets at 3.65 ppm and 3.55 ppm in Figs. 1 and 2, respectively, representing ethylene protons adjacent to choline, can also be explained by the relative position of HDO in these spectra. The 2′-5′-ribosyl and remaining ethylene protons of both the isolate and reference standard for CDP-C show signals from roughly 4.1 ppm to 4.5 ppm. The remaining 1′-ribosyl protons of isolated and reference CDP-C give doublets at 5.89 ppm and 5.85 ppm, respectively, which are very nearly equivalent. Other spectral differences in the isolate of CDP-C and the corresponding reference standard are observed with the cytosine 5-H and 6-H protons. These protons give doublets at 7.82 ppm and 5.98 ppm, respectively, for the CDP-C reference standard (Fig. 2) as compared with an approximate downfield shift of 0.2 ppm for these protons in the CDP-C isolate in Fig. 1, considering the relative positions of HDO in these spectra. Several explanations for these chemical shifts are possible. The reference standard for CDP-C was obtained from Sigma Chemical Co. (St. Louis, MO) as the sodium salt. The salt form of the isolated CDP-C is not known and may include ions such as phosphates and halides, which could alter pH during collection of the spectra. The degree of protonation of the cytosine base amino groups can affect the 5-H and 6-H proton signals, with deshielding shifting these signals downfield. These protons are also sensitive to temperature, with both the 5-H and 6-H cytosine protons shifting downfield at elevated temperature. It is also possible that the much lower concentration of the isolated CDP-C could have affected these ring protons. This is a well known phenomenon with purines (base stacking). In addition, published (Sadtler) spectra of cyti-
Fig. 3. A 360-MHz proton NMR spectrum for cytidine diphosphoethanolamine isolated from PND erythrocytes.

Cytidine monophosphate show 5-H and 6-H protons of cytosine at 8.08 ppm and 6.13 ppm, respectively. In comparison to such spectra, the 5-H and 6-H protons for the CDP-C isolate (Fig. 1), after adjusting for the relative position of HDO, are nearly identical (8.05 ppm versus 8.08 ppm and 6.15 ppm versus 6.13 ppm). On the basis of these observations, the compound isolated from PND erythrocytes as CDP-C can be positively identified.

The spectra for the isolate of CDP-E is shown in Fig. 3. This spectrum differs from the reference CDP-C spectrum (Fig. 2) with respect to two major resonances. The prominent singlet at 3.05 ppm, representing the methyl protons of choline, is missing in Fig. 3, enabling us to make the differentiation between choline and ethanolamine. The CDP-E triplet signal (expanded, not shown) at 3.15 ppm represents the two ethylene protons adjacent to the amino group of ethanolamine. The remaining downfield resonances for CDP-E (Fig. 3) are nearly identical to those for the reference compound CDP-C (Fig. 2), as would be expected. These spectra confirm the presence of CDP-E in PND erythrocytes. Mass spectral data of these compounds, obtained by the fast atom bombardment technique, revealed major peak molecular ions to be the same as those of the reference isolated compounds. Minor differences in the mass spectral patterns of the isolates from red blood cells were attributed to contamination during preparation. Ultraviolet spectra of these compounds revealed an $E_{\text{max}}$ of 284 nm, confirming the presence of a cytidine nucleus as compared to a purine nucleus.

Concentrations of the major nucleotide metabolites identified in the PND and normal erythrocyte TCA extracts are summarized in Table 1. Chromatographic separations of these metabolites from PND and normal erythrocyte extracts are illustrated in Fig. 4.

The most prominent abnormal pyrimidine nucleo-

tides in PND red cell extracts were the cytidine diphosphate diesters, CDP-choline (CDP-C) and CDP-ethanolamine (CDP-E), which we determined to be 0.94 mM and 0.41 mM, respectively. In addition, high levels of CTP (0.77 mM), UTP (0.38 mM), and UDP-glucose (0.31 mM) are also present in the PND red cell extracts. It is evident in Fig. 4 that some nucleoside monophosphates (AMP, UMP) in the PND red cell extracts are obscured by coelution of the large peak that we have identified as CDP-C and were therefore not measurable. Concentrations of the previously identified nucleotides, including UDP-glucose and the nucleoside triphosphates, compare favorably with those reported by Torrance and Whittaker. The corresponding pyrimidine mono- and diphosphate nucleosides are also present in PND red cell extracts, but at lower concentrations than those reported previously by Torrance and Whittaker. In general, we found lower levels of both purine and pyrimidine

<table>
<thead>
<tr>
<th>Compound</th>
<th>PND</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDP-E</td>
<td>410</td>
<td>—</td>
</tr>
<tr>
<td>CMP</td>
<td>25</td>
<td>—</td>
</tr>
<tr>
<td>CDP-C</td>
<td>930</td>
<td>—</td>
</tr>
<tr>
<td>AMP</td>
<td>*</td>
<td>140</td>
</tr>
<tr>
<td>UMP</td>
<td>*</td>
<td>—</td>
</tr>
<tr>
<td>UDP-G</td>
<td>310</td>
<td>190</td>
</tr>
<tr>
<td>UDP</td>
<td>24</td>
<td>—</td>
</tr>
<tr>
<td>CDP</td>
<td>130</td>
<td>—</td>
</tr>
<tr>
<td>ADP</td>
<td>260</td>
<td>—</td>
</tr>
<tr>
<td>GDP</td>
<td>17</td>
<td>23</td>
</tr>
<tr>
<td>UTP</td>
<td>380</td>
<td>—</td>
</tr>
<tr>
<td>CTP</td>
<td>770</td>
<td>—</td>
</tr>
<tr>
<td>ATP</td>
<td>1,270</td>
<td>1,160</td>
</tr>
<tr>
<td>GTP</td>
<td>60</td>
<td>80</td>
</tr>
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</table>

All values are expressed as mM concentration.

*Not determined due to coelution with CDP-C.
Fig. 4. Liquid chromatograms of erythrocyte nucleotides from pyrimidine nucleotidase deficient (upper) and normal subjects (lower). Neutralized trichloroacetic acid extracts of washed erythrocytes were separated using anion-exchange liquid chromatography and ultraviolet detection at 280 nm.

Table 2. Percentages of Total Membrane Phospholipid in Normal and PND Erythrocytes

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Normal</th>
<th>PND</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysophosphatides</td>
<td>1.9 ± 0.5</td>
<td>1.7</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>20.6 ± 2.2</td>
<td>18.4</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>31.2 ± 3.4</td>
<td>31.6</td>
</tr>
<tr>
<td>Phosphatidylserine*</td>
<td>15.2 ± 1.7</td>
<td>16.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>29.9 ± 3.6</td>
<td>32.0</td>
</tr>
</tbody>
</table>

Normal values are the mean ± SD for 9 subjects. PND values are the mean of 4 determinations. The highest cell volume for the PND values did not exceed 12%, with the exception of the lysophosphatides (35%).

*Phosphatidylserine values include a small percentage of phosphatidylinositol.

... mono- and diphosphates than were detected by these investigators, possibly due to the age or metabolic condition of the erythrocytes prior to extraction or to differences in the red cells of the subjects. The unidentified CDP compound reported by Torrance and Whittaker to be present at a concentration of about 1 mM is probably CDP-C, or perhaps a combination of CDP-C and CDP-E. Our values for the concentrations of UTP, CTP, and ATP (1.27 mM) in PND red cells are also consistent with our earlier data. We reported total nucleoside triphosphate levels at roughly twice the normal levels in intact PND red cells, with the concentration of the purine triphosphates equal to the concentration of the pyrimidine triphosphates.

The percent composition of major normal and PND red cell membrane phospholipids is presented in Table 2. No significant differences are evident in this relative phospholipid composition of normal and PND red cells.

The identification of large levels of CDP-C and CDP-E in PND red cells is of interest, as these compounds are not present at measurable levels in normal circulating reticulocytes or erythrocytes. Their presence in the PND red cell may only be epiphenomenal or may contribute to the mechanism of shortened erythrocyte lifespan. One other case in which a high level of red cell CDP-C was found in a patient with normal P5N activity was reported by Paglia and coworkers. This patient exhibited chronic hemolytic anemia and basophilic stippling with no other apparent enzymopathies or abnormalities, suggesting that the presence of red cell CDP-C is related to hemolysis.

Several mechanisms have been suggested for the hemolytic process in PND. Oda and Tanaka analyzed glycolytic intermediates and enzymes in PND red cells and found that the inhibitory effect of pyrimidine nucleotides on ATP-dependent glycolytic enzymes was not prominent. They did observe, however, that glycolysis was decreased, with an increase in glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and pyruvate. Paglia et al. also reported increased G6P levels. We have suggested that the decreased intracellular pH of PND red cells, consequent to the Donnan-related ion shifts of accumulated fixed negative charges, is a cause of impaired glycolysis in these cells.

Torrance and Whittaker have suggested that erythrocyte ATPases could be inhibited by pyrimidine nucleotides, thus disturbing the ionic balance of the cell and causing hemolysis. It is known that pyrimidine triphosphates are ineffective substrates for both Ca++ ATPase and Na+/K+ ATPase, but it has also been shown that they are not inhibitors of red cell ATPase activity in the presence of Mg ATP or ATP-generating systems. Swanson et al. observed that PND red cells contain a normal level of Mg ATP despite an equal amount of CTP and UTP, due to an increase in the total PND red cell Mg++ concentration. In addition, normal ouabain-sensitive and non-ouabain-sensitive ATPase activity and normal Na+ and K+ concentrations were reported in PND erythrocytes by Valentine et al. These findings suggest that the excess pyrimidines present in PND erythrocytes do not directly affect energy-dependent cation exchange or transport systems.
Tomoda et al. have recently demonstrated inhibition of glucose-6-phosphate dehydrogenase (G6PD) by CTP and UTP (as well as ATP). They hypothesized that increased CTP and UTP concentrations in PND red cells specifically inhibit G6PD by competitive inhibition of G6P and noncompetitive inhibition of NADP for G6P. They further proposed that the decrease in pentose-phosphate shunt activity renders the PND red cells more susceptible to oxidative stress and hemolysis. Tomoda and coworkers used high NADP for G6P. They further proposed that the inhibition of G6P and noncompetitive inhibition of CTP and UTP concentrations in PND red cell extracts and may indicate shifts of crude PND red cell extracts and may reflect large levels of diphosphodiesterase (CDP-C, CDP-E, UDP-G) as well as CTP and UTP. NTP-mediated control of G6PD is an interesting hypothesis for decreased pentose-phosphate pathway activity, but, at a NTP level of 2–2.5 mM, significant inhibition does not seem likely. Normal red cell ATP levels range up to nearly 2 mM. Tomoda and coworkers also observed G6PD inhibition with pyrimidine diphosphate and monophosphate nucleosides, but these were less effective inhibitors. It should be noted that chronic hemolytic anemia in the absence of exogenous stress (primaquine) is observed only in rare forms of G6PD deficiency, such as the Chicago and Oklahoma variants. In general, chronic hemolytic anemia associated with G6PD deficiency relates to excessive NADPH inhibition or a high $K_m$ for NADP. In the absence of such an enzyme variant, no more than 30% G6PD activity is necessary to maintain erythrocyte integrity. Therefore, a 40%–80% inhibition of pentose shunt activity, as determined by Tomoda and coworkers using hemolysates incubated with NTP of 6.5 mM, does not adequately explain the chronic hemolysis exhibited by patients with PND. The sensitivity of G6PD to inhibition by cytidine diphosphodiesterase should be measured to clarify this question.

The normal red cell is capable of CDP-C- and CDP-E-dependent synthesis of phospholipids during the reticulocyte stage, but these compounds are not found in measurable amounts in normal red cells or reticulocytes. As patients with PND exhibit marked reticulocytosis, indicating a young red cell pool, CDP-C-dependent phospholipid synthesis may be a significant pathway in these erythrocytes. However, our findings indicate that PND red cell membrane phospholipid composition is unaffected by high CDP-C and CDP-E levels. A case of non-PND hemolytic anemia, cited by Paglia et al., in which high red cell CDP-C was the only identified erythrocyte defect is of interest. Red cell phospholipids and phospholipid-related enzyme activity were normal despite high levels of CDP-C. This suggests that the relationship between high CDP-C and shortened red cell lifespan may be the inhibition of critical red cell enzymes, rather than altered phospholipid metabolism; but a more complete investigation of membrane phospholipid and phospholipid fatty acid metabolism is needed to rule out hemolysis by this mechanism.

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