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GTP DEPLETION AND OTHER ERYTHROCYTE ABNORMALITIES IN INHERITED PNP DEFICIENCY

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Abstract—GTP levels were low and NAD^+ levels high in purine nucleoside phosphorylase (PNP) deficient erythrocytes, in addition to the raised deoxy-GTP (dGTP) levels previously noted by others. dGTP was also identified in the PNP deficient child's lymphocytes.

A further novel finding was the conversion of hypoxanthine to inosine by the PNP deficient red cells, as compared to inosine monophosphate (IMP) in controls. This has been attributed to IMP formation with subsequent breakdown, and raises interesting questions regarding the controls which normally maintain erythrocyte nucleotide pools.

These findings may also explain the gross purine overproduction seen in this defect; they may likewise be related to the associated immunodeficiency, anaemia, and other clinical manifestations. The results may also have important implications for the development and clinical use of PNP inhibitors.

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) deficiency is the second inborn error of purine metabolism to be identified associated with severe immunodeficiency [1]. Unlike its companion enzyme defect, adenosine deaminase (ADA) deficiency [1], only T lymphocytes are significantly affected [1]. The reason for the differential lymphocyte toxicity in these two enzyme defects has been the subject of extensive investigation summarised in some excellent reviews [2–4]. The favoured hypotheses centre around the ability of T-cells, as distinct from B-cells, to accumulate toxic deoxynucleotide triphosphates [5, 6] (Fig. 1). In PNP deficiency deoxyguanosine triphosphate (dGTP) has been implicated, based on the original observations that deoxynucleosides are found in body fluids [7, 8] and dGTP accumulates in PNP deficient red cells [8]; observations which have subsequently been made in other PNP deficient subjects [9, 10].

This study reports observations in the first surviving case of PNP deficiency in the U.K., a male infant confirmed as being totally PNP deficient shortly after birth. The only other sibling had been found to have PNP deficiency during a terminal illness one year previously [11, 12]. We have confirmed the raised erythrocyte dGTP levels found by others [8–10], but have also noted a profound depletion of GTP and other abnormalities which may help explain some of the clinical features in this disease [2, 3, 7–12].

MATERIALS AND METHODS

Subject. A male infant, SB, born 19 June 1980, was found to have no detectable purine nucleoside phosphorylase activity in peripheral or cord blood red or white cells shortly after birth [12].

The clinical course in a previously affected sibling has already been reported [11]. The parents were distantly related and the defect shown to be inherited in an autosomal recessive manner in a large kindred [11, 12].

The subject had not received any medication at the commencement of the study but from 17 September 1980 onwards was being given a trial of parenteral deoxycytidine, in doses increasing from an initial 15 mg/kg through 25, 50–100 mg/kg (as indicated by successive arrows in Table 1). Preliminary results have appeared in abstract [13]. The child has thrived and recovered successfully from urinary tract and whooping cough infections. Full details will be given in a clinical evaluation at the completion of twelve months of therapy (Watson *et al.*, manuscript in preparation).

Erythrocyte studies. Fresh heparinised blood was centrifuged and separated immediately. The plasma, buffy coat and top fifth layer of cells were removed, the remaining cells washed once with Earl's balanced salt solution, and the packed cell volume determined. Erythrocyte nucleotide extracts were made within 45 min of venepuncture and all intact cell incubations also commenced within 1 hr to minimise ATP breakdown [14].

Lymphocyte extracts could not be made until nine months of age (7 April 1981), due to a sustained lymphopenia. 2×10^6 cells, previously separated by Ficoll–Triosil gradient and washed twice, were extracted with 200 μl 10% TCA, and the extract brought to neutrality with water-saturated ether as previously described [15].

Enzyme activities in intact and lysed cells were determined by radiochemical methods as described in detail in earlier publications [15, 16]. High per-

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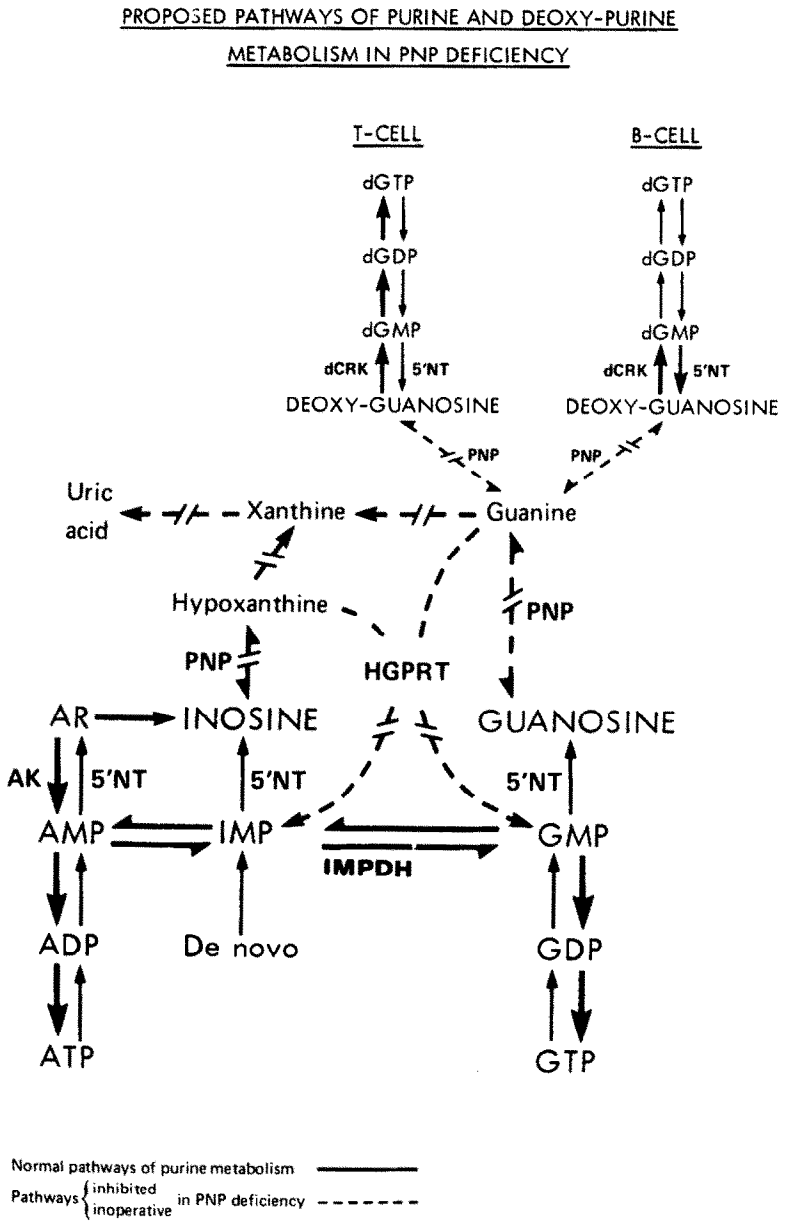

Fig. 1. Purine metabolic pathways, including hypothetical mechanisms of toxicity in PNP deficiency, based on dGTP accumulation, preferentially in T- as distinct from B-cells. In this defect inosine, guanosine, deoxyinosine and deoxyguanosine replace uric acid as the end product of purine metabolism. Abbreviations other than those defined in the text: dCRK, deoxycytidine kinase; AK, adenosine kinase; 5'NT, 5'nucleotidase; HGPRT, hypoxanthine–guanine phosphoribosyltransferase; AR, adenosine.

formance liquid chromatography (HPLC) was used both for the determination of erythrocyte and lymphocyte nucleotide [16] and nucleoside [17] levels.

RESULTS

PNP activity in lysed erythrocytes from patient SB was negligible with inosine or hypoxanthine as substrate, at either saturating or sub-saturating concentrations (Table 1 or not shown), confirming the defect in both the degradative and synthetic directions [12].

Studies in intact erythrocytes also demonstrated the defect in SB in the degradative direction (inosine to hypoxanthine, Table 1). There was very little hypoxanthine or IMP formation in the patient's cells; over 90% of the inosine remaining completely unmetabolised. The lack of IMP formation from inosine in the PNP deficient cells also indicates that direct phosphorylation of this nucleoside does not occur to any significant extent in the human erythrocyte. In contrast, control cells converted a large proportion of inosine to hypoxanthine and IMP,

Table 1. Inosine and hypoxanthine metabolism in intact and lysed erythrocytes from a PNP deficient child (SB) and a control (C)

METABOLISM IN ERYTHROCYTES DEFICIENT IN NUCLEOSIDE PHOSPHORYLASE
(% of substrate supplied)

Substrate : Inosine					Hypoxanthine					
	(40 minutes, 18 mM Pi)				(40 minutes, 18mM Pi)				(5 minutes, 1 mM Pi)	
Concentration (μmol/l)	5.0		50.0		5.0		50.0		50.0	
Subject	SB	C	SB	C	SB	C	SB	C	SB	C
% conversion to:-										
Nucleotide	2.2	81.9	2.4	18.9	26.1	96.0	10.8	53.6	6.6	1.5
Nucleoside	90.4	3.1	94.6	13.3	72.1	<1.0	15.6	<1.0	7.3	1.0
Base	1.7	14.1	0.9	66.8	1.0	2.0	72.6	46.0	85.0	96.8

PNP ACTIVITY IN LYSED ERYTHROCYTES (nmol/mg Hb/h)

Substrate :	Inosine (1.4 mM)	Hypoxanthine (0.11 mM)
SB	Not detectable	Not detectable
Control	4194	5633

Cells were incubated with ^{14}C-labelled substrates in medium containing either 18 mM or 1 mM phosphate (P_i) as indicated.
Reaction products were separated by electrophoresis as previously described.
Nucleotide = IMP, nucleoside = inosine, base = hypoxanthine.

Table 2. Nucleotide levels measured by HPLC on the days indicated in PNP deficient erythrocytes of SB prior to and during therapy with deoxycytidine in increasing doses

ERYTHROCYTE NUCLEOTIDE LEVELS IN PNP DEFICIENT CHILD (SB) (nmol/ml packed red cells)

Date	ATP	ADP	AMP	GTP	GDP	dGTP	
22.7.80	750	92	7.5	1.5	-	2.8	
16.9.80	745	111	8.5	4.0	-	6.0	←
23.9.80	1140	170	8.0	6.0	-	6.8	←
13.1.81	1120	160	6.0	4.2	-	2.5	←
31.3.81	1234	176	-	24.0	-	11.0	←
21.4.81	1327	103	7.7	14.0	-	8.0	
Controls	1278 ± 127	114 ± 24	10 ± 3	60 ± 10	15	-	(n = 9)

LYMPHOCYTE NUCLEOTIDE LEVELS (nmol/10^8 cells)

7.4.81	3.68	0.91	0.31	0.35	*	0.21	
Controls	2.13-3.09	0.79-1.39	0.1-0.18	0.29-0.55	0.14-0.23	-	(n = 5)

← represents change in treatment
- not measurable
* impure peak

Lymphocyte nucleotide levels on a single occasion during deoxytytidine therapy are also listed.
dGTP was found in lymphocyte as well as erythrocyte extracts of patient SB.

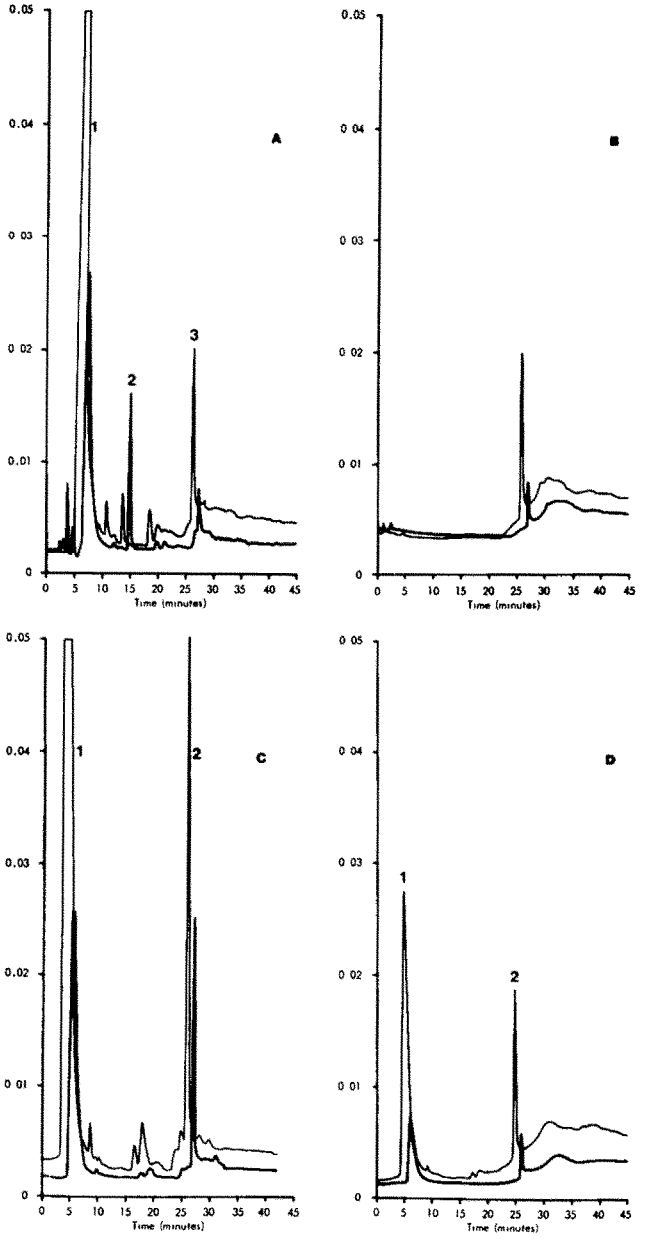

Fig. 2. HPLC traces of erythrocyte extracts separated by reverse-phase chromatography; absorbance at 280 nm (—), and 254 nm (—). (A) A 25 μl injection of an extract from control red cells (1 = nucleotides, 2 = uric acid, 3 = NAD^+). (B) A 25 μl injection of an NAD^+ standard. (C) and (D) 25 and 5 μl injections, respectively, of an extract from patient SB (1 = nucleotides, 2 = NAD^+—note the absence of uric acid in PNP deficiency). Figures on the ordinate represent absorbance units compared with elution time at a flow rate of 1 ml/min.

which (from the above observation) must have occurred by the sequential action of PNP and HGPRT (Fig. 1). The latter step requires phosphoribosylpyrophosphate (PP-ribose-P) which is generated at 18 mM P_i, but not at physiological phospate levels (1 mM P_i). (Table 1).

The metabolism of hypoxanthine by the erythrocytes is also shown in Table 1. Whereas control cells at 18 mM P_i converted very little hypoxanthine to inosine (~ 1%) but almost exclusively to IMP, there was a substantial conversion to inosine by the patient's cells. This was completely unexpected in view of the observation that PNP activity in the synthetic direction (hypoxanthine to inosine) was undetectable in the patient's haemolysate. These results indicate that in the PNP deficient erythrocytes the inosine was formed indirectly via IMP.

Nucleotide levels in the PNP deficient erythrocytes prior to therapy (Table 2) showed the presence of detectable amounts of dGTP, as noted in other PNP deficient cases [8, 9]. Therapy had little effect on this. dGTP was also identified in significant quantity

in the PNP deficient peripheral blood mononuclear cells (lymphocytes) but was undetectable in control lymphocytes (Table 2).

The most remarkable finding in the PNP deficient erythrocytes was the severe depletion of GTP and GDP, with the former levels rising slightly during deoxycytidine therapy (Table 2). Although ATP:ADP:AMP ratios were normal, ATP levels were also consistently low prior to therapy but improved subsequently (Table 2).

Erythrocyte extracts examined for purine nucleosides and bases using a different HPLC method [17] (Fig. 2), showed a large peak eluting at approximately 26 min which was formed in all extracts, but the values in SB were consistently three-fold that in controls (Fig. 2). This peak was identified by elution time, 280 nm/254 nm ratio, and co-chromatography as nicotinamide adenine dinucleotide (NAD^+), a normal red cell constituent. NAD^+ was also found to be raised in the nucleotide HPLC analyses [16] of SB extracts. Results from the nucleoside separation system gave a normal range of 48–95 nmole/ml packed cells ($n = 20$), similar to that reported by conventional methods [18]. The values in SB ranged from 208–302 nmole/ml.

DISCUSSION

Increased erythrocyte dGTP levels, of the order found in the present case, have been reported previously in PNP deficiency [8, 9]. However, in this report they are associated with a consistent reduction in both GTP levels and GDP levels of considerable magnitude. In previous reports GTP levels have been below normal but have not been considered remarkable, possibly because the reduction was not of this degree, or the systems used did not measure GDP [2, 3, 8, 9]. The severe GTP and GDP depletion in this PNP deficient child's erythrocytes may be important. If it occurs in other tissues it could well explain the gross purine overproduction and other clinical manifestations in the defect [2, 3].

GTP plays a vital role in many metabolic processes. Of particular relevance to this paper are studies which demonstrated reduced GTP levels, associated with increased *de novo* purine synthesis and total purine overproduction, in cultured cells where the conversion of IMP to GMP was inhibited [19]. Severe GTP depletion was also associated with inhibition of DNA synthesis in similar studies in neuroblastoma cells [20], while leaving RNA and protein synthesis relatively intact. This could have serious consequences for a cell suddenly called upon to proliferate rapidly, such as a lymphocyte.

Other workers have also demonstrated conversion of hypoxanthine to inosine during studies in cultured cells incubated with inhibitors of IMP dehydrogenase (IMP:NAD oxidoreductase, EC 1.2.1.14) [21]. As with the PNP deficient erythrocytes in this report, this conversion was considered to occur via prior nucleotide formation rather than direct base-nucleoside interconversion—due to blockage of the further metabolism of IMP to GMP in that instance [21]. IMP dehydrogenase (IMPDH, Fig. 1) catalyses the first step in this conversion [19–21]. The similarity between the findings by the above workers and our own results in this PNP deficient child, strongly implicate a failure of IMP dehydrogenase to generate GMP.

For this reason, possibly the most important observation is the raised level of NAD^+ found in these PNP deficient erythrocytes. The basis for this is not clear but cannot relate to an altered NAD^+/NADH ratio. The absolute total was more than three-fold normal and NADH levels are reputedly ten to one hundred-fold lower than NAD^+ values [18].

It is conceivable that an important reciprocal relationship exists between NAD^+ and GTP in the red cell, particularly since low total erythrocyte NAD^+/NADH levels were noted, together with raised GTP levels, in an early study in pyruvate kinase deficiency—the converse of our findings [22]. Pyruvate kinase is one of several enzyme defects of the glycolytic pathway associated with severe anaemia [18]. Anaemia has been a prominent feature in five of nine PNP deficient subjects [1–3], and profound stimulation of glycolysis was noted in one PNP deficient case post-transfusion [23]. Unfortunately NAD^+ levels were not measured prior to treatment [23].

Of greater significance however, are reports demonstrating that: (1) while NAD^+ is an essential coenzyme in the conversion of IMP to GMP by IMP dehydrogenase, levels in excess of 250 μM strongly inhibit this enzyme [24]; (2) IMP dehydrogenase is an enzyme linked with proliferation and malignancy—increases in specific activity being an indicator both of malignant transformation and growth rate [24]; (3) highest activites of this enzyme are found in thymus, spleen and bone marrow [24].

The NAD^+ levels in these PNP deficient erythrocytes were generally above 250 μM. It will thus be important to establish how these findings apply to the *in vivo* situation in PNP deficiency, particularly in relation to other tissues.

As discussed above, the possibility that our results also relate to some alteration in anaerobic glycolysis which regenerates erythrocyte NAD^+ must be considered. The 40% reduction in erythrocyte ATP levels prior to treatment would support this view. A similar explanation to that proposed for the low ATP levels in the erythrocytes of an ADA deficient child also could be advanced [26]. This involves dependence on a continuing supply of adenosine from the *S*-methylation pathway for the maintenance and turnover of the erythrocyte ATP pool. Inhibition of *S*-adenosylhomocysteine hydrolase (S-AHH) [25], the enzyme vital for this reaction, could result in reduced ATP levels [26]. *S*-AHH activity is also low in PNP deficiency (12–19% normal), and seemingly inosine is the agent toxic to S-AHH in this situation [25]. How these findings could relate to the improved ATP levels post-therapy, or the anaemia in some PNP deficient subjects [1–3], must await further study.

This is the first case in which measurable amounts of dGTP have been found in PNP deficient mononuclear cells (lymphocytes). The only other study failed to detect dGTP in this cell type, but the child had received multiple transfusions [10]. In a comparison paper [26], by contrast, we failed to detect

significant amounts of dATP in peripheral blood mononuclear cells from an ADA deficient child whose erythrocytes contained extremely high dATP levels [26]. These observations, although in conflict with earlier reports, are difficult to interpret at present and may simply relate to the remaining lymphocytes being predominantly non-T or immature B-cells. They must await confirmation in lymphocytes from further cases of ADA and PNP deficiency, preferably in perinatal or cord blood.

The severe dGTP depletion and other erythrocyte abnormalities in this PNP deficient child may have wider implications. Attention is currently being focussed on the development of PNP specific inhibitors as a potential therapy in T-cell disorders or for immunosuppression. The abnormalities documented in this report suggest caution in such an approach. There is a striking similarity between our observations in the erythrocytes of ADA deficient children [16, 26], and the severe erythrocyte ATP depletion, haemolytic anaemia, and other adverse clinical effects noted in humans with lymphoid malignancies treated with the ADA inhibitor, deoxycoformycin: results which have already aroused concern in the U.S.A. [27]. By analogy PNP inhibitors could likewise profoundly affect erythrocytes as well as lymphoid cells *in vivo*, which would seriously restrict their clinical usefulness.

The findings in this PNP deficient child also raise important questions as to the mechanisms which maintain normal nucleotide pools, and how these nucleotides are broken down *in vivo*, particularly in the erythrocyte which lacks 5′-nucleotidase [28]. Clearly much more work involving a new approach is needed to answer these different points—including their significance for the associated immunodeficiency in inherited T-cell defects.

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