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RECIPROCAL RELATIONSHIP BETWEEN ERYTHROCYTE ATP AND DEOXY-ATP LEVELS IN INHERITED ADA DEFICIENCY

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Abstract—A reciprocal relationship between erythrocyte ATP and deoxy-ATP levels has been noted in an immunodeficient child with adenosine deaminase (ADA) deficiency during therapy with red cell transfusions. The sum of red cell ATP plus deoxy-ATP equalled the normal complement of ATP prior to any form of therapy. dATP, dADP and dAMP levels were found in the same ratio (10:1:0.1) as the adenine nucleotides ATP, ADP and AMP. Red cell ATP levels were low, not high or normal as found by others in ADA deficiency, but no deoxyadenosine nucleotides could be found in peripheral blood mononuclear cells.

Erythrocyte ATP depletion has recently been identified as a serious consequence of anti-leukaemic therapy with ADA inhibitors; it may thus be an important but hitherto unrecognised contributing factor in the clinical expression of inherited ADA deficiency.

Severe combined immunodeficiency (SCID) is a heterogeneous syndrome in which affected children lack both cell-mediated and humoral immunity. Adenosine deaminase deficiency (ADA, EC 3.5.4.4) [1, 2], an enzyme of purine metabolism, is responsible for up to 20% of the recessively inherited variety of SCID. These children have repeated infections, failure to thrive, and unless treated by enzyme replacement (red cell transfusions, or bone marrow transplantation) die within the first few years of life.

Numerous hypotheses have been proposed for the severe lymphoid depletion in ADA deficient children. These are based on the original observations demonstrating the excretion of deoxyadenosine (dAR) in the urine [3], and the accumulation of dATP in erythrocytes [4, 5], or lymphocytes [6]. The hypothesis currently most favoured implicates intracellular deoxynucleotide accumulation, predominantly in T-cells [1–6] (Fig. 1). Alternatively inhibition of nucleic-acid methylation through inactivation of *S*-adenosylhomocysteine hydrolase (S-AHH) by deoxyadenosine has been proposed [1, 2]. S-AHH catalyses the removal of *S*-adenosylhomocysteine, a product and potent inhibitor of these vital reactions (Fig. 1).

The selective toxicity of ADA deficiency to lymphoid cells also stimulated investigation of the treatment of acute lymphoblastic leukaemias with the ADA inhibitor deoxycoformycin [7]. Studies in animals [8] and humans [9] have recently noted severe haemolysis during deoxycoformycin therapy, attributed to progressive reduction in erythrocyte ATP with increasing dATP levels [9].

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In contrast to other studies of ADA deficiency, where normal or high erythrocyte ATP levels have been described [1, 2], we previously noted low ATP levels in an ADA deficient SCID child [10]. We now confirm this finding in a second child, and suggest how this reciprocal relationship between erythrocyte ATP and dATP could also explain the observations in deoxycoformycin therapy of lymphoid malignancies.

MATERIALS AND METHODS

Subject. A male child, SY, with severe combined immunodeficiency was found to have extremely low levels of ADA in erythrocyte lysates. Full clinical details of this case and response to therapy will be the subject of a separate communication (Davies *et al.*, manuscript in preparation). The child was three months old when diagnosed as ADA deficient, and was studied prior to, and over a two month period of weekly and subsequently bi-weekly exchange transfusions (Fig. 1). Forty ml of blood were removed and replaced by 60 ml of compatible fresh irradiated blood. Thymosin injections, which had been given from the time of diagnosis of SCID (2 months), were continued twice weekly.

Separation of red cells. Fresh heparinised blood was centrifuged immediately. The plasma, buffy coat, and top fifth of red cells were removed. The remaining red cells were washed once with Earl's balanced salt solution, and the packed cell volume determined. Erythrocyte nucleotide extracts were made within 1 hr of venepuncture to minimise ATP breakdown [11]. Red cells were extracted with twice the volume of 10% trichloroacetic acid (TCA) and the extract brought immediately to neutrality by extraction with water-saturated diethyl ether. The

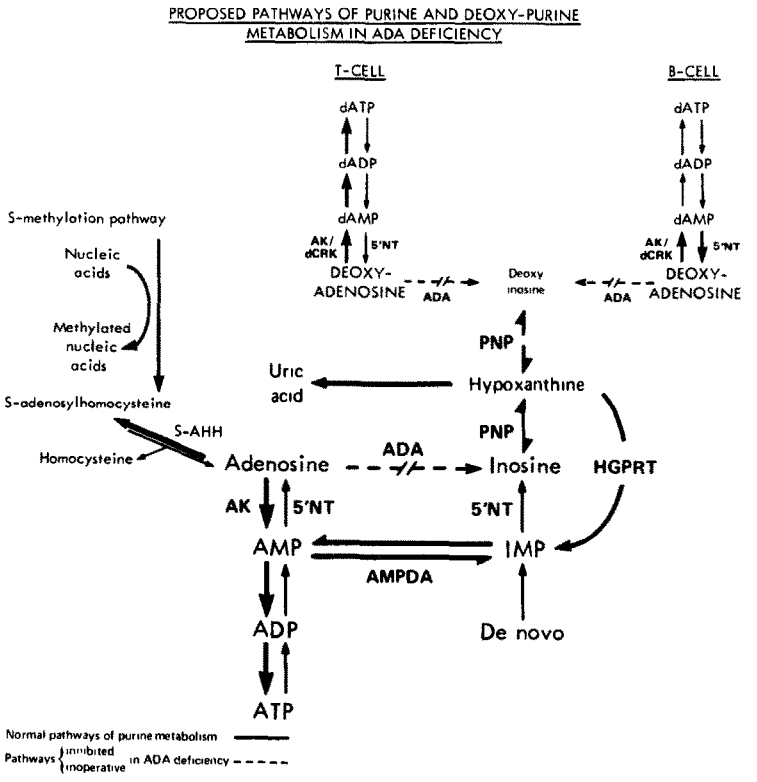

Fig. 1. Purine metabolic pathways indicating suggested mechanisms of toxicity in ADA deficiency, involving either accumulation of deoxy-ATP in T- as distinct from B-cells, or alternatively inhibition of the *S*-methylation pathway. Deoxyadenosine accumulates in ADA deficiency but uric acid production is not impaired. Abbreviations not listed in the text: dCRK, deoxycytidine kinase; AK, adenosine kinase; 5'NT, 5'nucleotidase; HGPRT, hypoxanthine–guanine phosphoribosyltransferase.

extracts were stored at −20° until analysed. For the peripheral blood mononuclear cell (lymphocyte) extracts, heparinised blood taken at the time of exchange transfusion was separated by Ficoll–Triosil gradient and washed twice with buffered medium. One ml containing 1×10^6 cells was centrifuged for 5 min at 3500 rpm (600 *g*) re-suspended in 10 μl saline and nucleotides extracted with 100 μl 10% TCA and ether, as for red cells.

Enzyme activities in erythrocyte lysates [12] and intact cells [13] were determined using radiochemical methods as described previously.

High performance liquid chromatography (HPLC) was used for the determination of erythrocyte and lymphocyte nucleotide [14] and nucleoside levels [15].

RESULTS

The ADA activity in erythrocyte lysates of the patient before treatment was <1.0 nmole/mg Hb/hr. The pre-treatment red cell nucleotide levels are given in Table 1. The nucleotide levels in an earlier case, LS [10], with SCID and ADA deficiency are listed for comparison. The similarity between the two sets of data is obvious. The low ATP levels in SY were also compensated for by an equal increment in dATP, the sum of the two equalling the upper normal level for erythrocyte ATP. As for ATP, ADP, and AMP, the three deoxynucleotides were also present in the approximate proportion of 10:1.0:0.1. The finding of these high ratios demonstrates satisfactory sample preparation. dAMP was again found consistently [10, 13]; its identification making possible the confirmation that dATP is maintained at a high ratio of the triphosphate to the other two nucleotides, as for ATP in the human red cell.

We found adenine, but no adenosine or deoxyadenosine in the erythrocyte extracts prior to, but not after, red cell transfusion (Fig. 2). In separate experiments we confirmed that this adenine represented deoxyadenosine degraded to adenine in the acid extraction step.

Curiously (Table 1), we have been unable to identify dATP, dADP, or dAMP in lymphocyte extracts from this child, either pre- (Table 1) or post-transfusion, despite the fact that the child's intact lymphocytes contained no measurable ADA activity (results not shown).

Following the first exchange transfusion there was an immediate reduction in red cell dATP levels with a concomitant increment in ATP levels (Fig. 3). This reciprocal relationship was maintained throughout the subsequent period of study, with the sum of the ATP plus dATP generally falling within the normal range for ATP alone: dATP dropped to very low levels (more than could be accounted for by dilution with normal red cells—each exchange transfusion

Table 1. Nucleotide levels obtained by HPLC on two occasions prior to therapy in the erythrocytes of an immunodeficient child (SY) with ADA deficiency—compared with results in a previous case (LS)—demonstrating the low ATP levels together with an equal amount of dATP

Date	ATP	ADP	AMP	dATP	dADP	dAMP	GTP	GDP	
ERYTHROCYTE NUCLEOTIDE LEVELS IN AN ADA DEFICIENT CHILD (SY)* (nmol/ml packed red cells)									
22.1.81	748	150	5.5	**904**	**98**	**5**	50	18	
28.1.81	823	135	7.0	**946**	**114**	**4**	20	-	
Controls	1278 ± 127	114 ± 24	10 ± 3	Not normally detectable			60 ± 10	15	(n = 9)
LYMPHOCYTE NUCLEOTIDE LEVELS (nmol/10^8 cells)*									
28.1.81	3.34	0.98	0.16	-	-	-	0.46	0.07	
Controls	2.13-3.09	0.79-1.39	0.1-0.18	-	-	-	0.29-0.55	0.14-0.23	(n = 5)
PRETREATMENT RED CELL NUCLEOTIDE LEVELS PREVIOUS CASE OF ADA DEFICIENCY (nmol/ml packed red cells)									
LS	760	135	30	**750**	**116**	**15**	56	11	

* pretreatment
- not measurable

By contrast, no deoxyadenosine nucleotides could be identified in the lymphocyte extracts from SY made at the same time, on 28 January 1981.

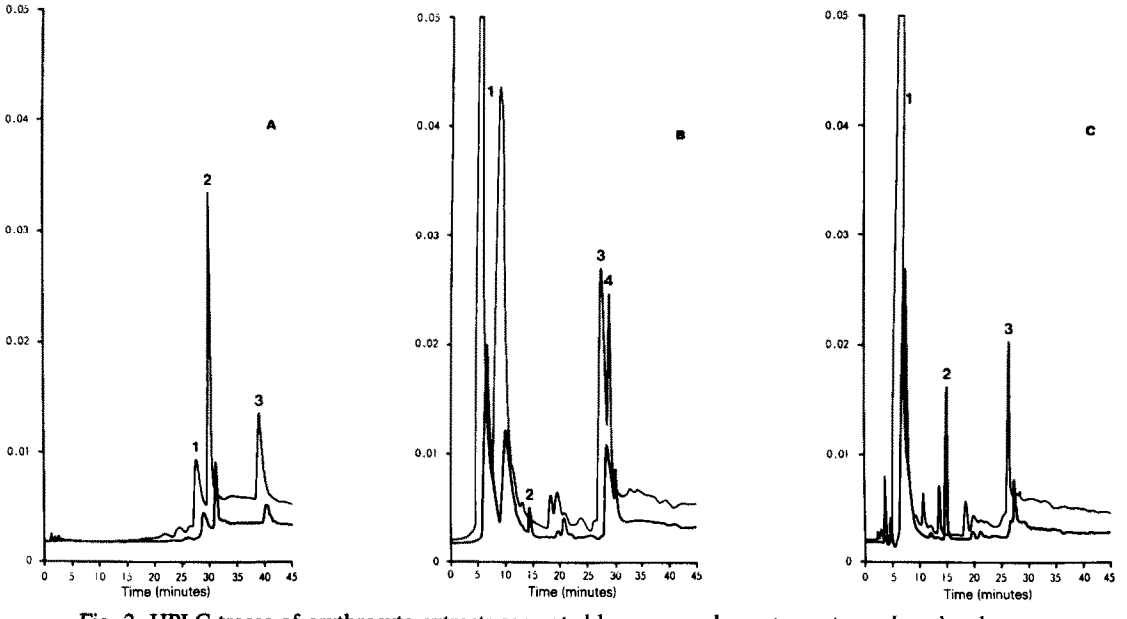

Fig. 2. HPLC traces of erythrocyte extracts separated by reverse phase chromatography; absorbance at 280 nm —, and 254 nm —. (A) A 25 μl injection of a standard mixture containing: 1 = adenine, 2 = adenosine, and 3 = deoxyadenosine. (B) A 25 μl injection of an extract of erythrocyte prior to treatment from patient SY. 1 = nucleotides, 2 = uric acid, 3 = adenine, and 4 = NAD^+. (C) A 25 μl injection of an extract from control red cells. 1 = nucleotides, 2 = uric acid, and 3 = NAD^+. Figures on the ordinate represent absorbance units compared with the elution time at a flow rate of 1 ml/min. Adenine in the ADA deficient extract (SY) has been derived from dAR degraded during the extraction step. (Note double nucleotide peak in B.)

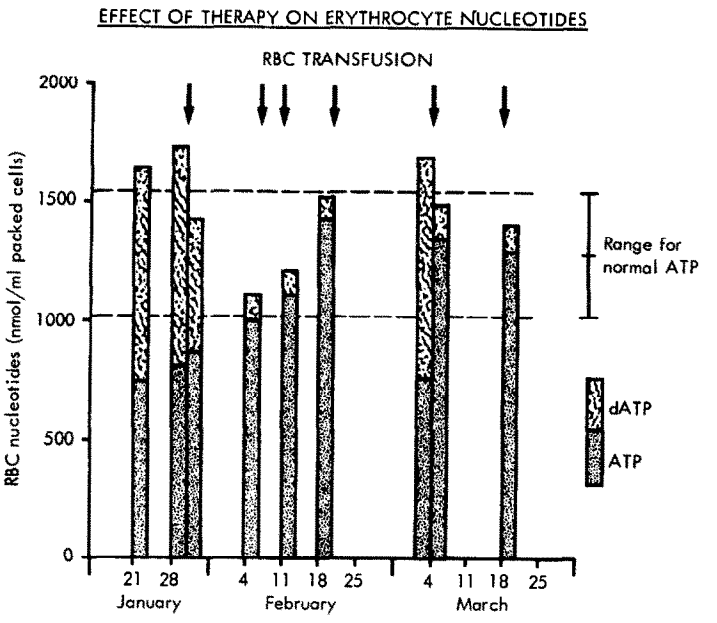

Fig. 3. Histogram showing reciprocal relationship between erythrocyte ATP and dATP levels (compared with the normal range for ATP—dotted lines) in cells from SY before and after treatment by exchange transfusion with irradiated erythrocytes at the points indicated.

only replaced 14% of the circulating blood volume), but rose before the next transfusion when the transfusion interval period was extended to two weeks. The approximate ratio 10:1.0:0.1, was maintained throughout for all the three adenosine and deoxyadenosine nucleotides.

DISCUSSION

We have again found low erythrocyte ATP levels in a child with inherited ADA deficiency, there being a similar amount of dATP present, the two together equalling the normal ATP complement [10]. Both were maintained at the same high ratio ~10:1.0:0.1 (Fig. 1) to the other two nucleotides. Our findings are in contrast to the normal to high ATP values, associated with variable dATP levels, in other reports in which no dAMP and frequently no dADP levels have been given [1, 2, 4, 5, 16–18]. Consequently the results in this report provide the first clear evidence that the same important relationship exists between the three deoxyadenosine nucleotides, as for the adenosine nucleotides, in the ADA deficient erythrocyte.

Likewise, although reduction in dATP levels following exchange transfusion is a well documented finding [1, 2, 16, 17], our observation that this reduction was accompanied by an increment in ATP levels, with a reciprocal relationship between the two, has not been noted previously. The reciprocal relationship between erythrocyte ATP and dATP levels following exchange transfusion is the mirror image of that noted during therapy to induce ADA inhibition with deoxycoformycin in the lymphoid malignancies [9]. Nevertheless the maximal dATP levels attained were very much higher, and the ATP levels correspondingly very much lower [9] than in this report.

We do not know to what level erythrocyte ATP can fall in inherited ADA deficiency before haemolysis occurs. Inherited enzyme defects in the glycolytic pathway can produce a variety of haemolytic processes associated with low ATP levels [19]. It is thus not surprising that such side-effects have accompanied the severe ATP depletion induced by deoxycoformycin therapy [9], and that concern is being expressed about its clinical use [9]. Deoxycoformycin, like its analogue coformycin, also may be a potent inhibitor of AMP deaminase (AMPDA, EC 3.5.4.6, Fig. 1; 10^{-5}, compared to 10^{-7} M for ADA inhibition [20]). AMPDA is an enzyme vital for the maintenance of nucleotide pools and is the preferred route of AMP deamination in most body tissues [21]. It should therefore be borne in mind that such inhibitors may have more than one mode of action *in vivo* and other serious side effects could develop in the long term.

The reciprocal relationship between the erythrocyte deoxyadenosine and adenosine nucleotides is also of considerable importance. One interpretation of these results is that the erythrocyte is normally dependent, at least in part, on a supply of adenosine from the *S*-methylation pathway for the maintenance of its ATP pools (Fig. 1). Adenosine production via this route may well be low in ADA [1, 2] (or simulated ADA [9]) deficiency, and the deoxyadenosine (dAR) which accumulates in either situation [3, 9, 14] could thus become an effective substrate for erythrocyte adenosine kinase (AK). Several observations would support this hypothesis: (1) The erythrocyte ATP pool turns over rapidly [13]. The mechanism has puzzled investigators since the mature erythrocyte lacks adenylosuccinate synthetase, an enzyme essential for the conversion of IMP to AMP [22]. Hence it cannot normally make ATP

from IMP as do other tissues by purine 'salvage', or *de novo* synthesis [21] (Fig. 1). (2) The rate constant, K_m, for the reaction of adenosine with adenosine kinase is several orders of magnitude lower (10^{-7} compared with 10^{-4} M for ADA) [13] and adenosine is thus phosphorylated and not deaminated at physiological substrate concentrations by intact erythrocytes [13] (Fig. 1). (3) Adenosine is a by-product of the *S*-methylation pathway vital for nucleic acid synthesis [23]. Its continued removal is essential for the further metabolism of *S*-adenosyl homocysteine (SAH) because the equilibrium for this reaction, catalysed by *S*-adenosylhomocysteine hydrolase (S-AHH), favours SAH formation [23] (Fig. 1).

Consequently, if the *S*-methylation pathway normally plays an important part in the maintenance and turnover of erythrocyte ATP, the low ATP levels in inherited and simulated ADA deficiency would be anticipated. This hypothesis also suggests that the erythrocyte may play an extremely vital role in removing, as well as supplying, purines such as adenosine in different body tissues [21].

The higher dATP levels attained during deoxycoformycin therapy, compared to the levels we noted in ADA deficiency, could also be explained on this basis. In *in vitro* studies we found that, even with ADA completely inhibited, the normal erythrocyte can make dATP from dAR only at extremely high unphysiological phosphate levels (unpublished observations). However, high phosphate levels would be anticipated, and have been recorded [1], following rapid cellular breakdown during deoxycoformycin therapy. Furthermore, in one such study S-AHH inactivation and dAR accumulation both preceded the rise in dATP levels [9]. This sequence of events would set the scene for deoxyadenosine phosphorylation; the higher phosphate and falling adenosine production allowing the attainment of the much higher dATP values noted [9]. The existence of non-metabolised deoxyadenosine in the erythrocyte before treatment in our case indicated saturation of this process at the physiological phosphate levels present in the patient.

Our inability to detect dATP, dADP or dAMP in the ADA deficient peripheral blood mononuclear cells, either pre- or post-transfusion, differs from earlier reports where high dATP levels [1, 2, 6] increasing after transfusion to as much as a third ATP levels [6] were noted. The HPLC system used here was clearly capable of separating all three deoxyadenosine nucleotides from ATP, ADP and AMP since the red cells taken from the patient at the same time contained high levels of all three deoxyadenosine mucleotides. Obviously further studies will be necessary in future ADA deficient subjects at a much younger age. Nevertheless the results do suggest that it may be the erythrocyte rather than the lymphocyte [24, 25], which in these unusual circumstances has the capacity to make large quantities of dATP from dAR.

The questions raised by these findings have important implications for understanding the associated immunodeficiency, as well as for extending our knowledge of the biochemical processes involved, in this inherited abnormality of purine metabolism. They also suggest that the two opposing hypotheses of deoxyadenosine toxicity may both be compatible with the clinical expression in ADA deficiency.

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