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HUMAN B LYMPHOCYTES AND THYMOCYTES BUT NOT PERIPHERAL BLOOD MONONUCLEAR CELLS ACCUMULATE HIGH dATP LEVELS IN CONDITIONS SIMULATING ADA DEFICIENCY

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Abstract—Inherited adenosine deaminase (ADA) deficiency is associated with a lymphospecific cytotoxicity affecting both dividing and non-dividing cells. The metabolic basis for this was investigated using different cell types and the potentially toxic metabolite 2′-deoxyadenosine (dAR) in short-term experiments under physiological conditions simulating ADA deficiency (1 mM P_i 8.7 μM dAR).

In the uncultured cells, [8-^{14}C] dAR alone was metabolized almost completely only by thymocytes and tonsil-derived B-lymphocytes. The greater percentage of counts (>75%) were in the medium (deoxyinosine, hypoxanthine). Cellular counts were predominantly in adenine nucleotides, and to a lesser extent guanine nucleotides. Interestingly, both thymocytes and tonsil-derived B-lymphocytes, and a partially ADA deficient B lymphoblast line, accumulated detectable amounts of dATP even in the absence of ADA inhibition. Peripheral blood lymphocytes (PBMs) did not, and showed little dAR metabolism.

In experiments simulating ADA deficiency varying amounts of 2′-deoxycoformycin (2′dCF) were needed to completely inhibit ADA (20–60 μM), with thymocytes requiring the highest amount. ADA inhibited thymocytes and tonsillar B-lymphocytes accumulated very high dATP levels, which were sustained to an equal extent by both over a 60-min period; PBMs accumulated the lowest values. Results in cultured cells reflected findings in previous studies. Some counts were also found in ATP by a route excluding ADA or PNP.

These results again question the hypothesis that B-cells are more resistant than T-cells to the toxic effects of dAR because of an inability to accumulate and sustain elevated dATP levels and underline the lack of comparability between enzyme activity in intact as distinct from lysed cells. They cast doubt on the validity of cultured cells as a model for ADA deficiency and suggest the observed toxicity in some instances might result from altered ATP or GTP pools through inadequate ADA inhibition. They indicate that combined immunodeficiency in ADA deficiency could relate to an equal sensitivity of B-cells and T-cell precursors to the toxic effects of dATP accumulation.

Adenosine deaminase (ADA: EC 3.5.4.4) deficiency is a potentially lethal inherited disorder associated with severe combined immuno-deficiency (SCID). Despite more than a decade of intensive research, summarized in recent reviews [1–3], the mechanism of the lymphocyte depletion remains undefined. It is still not certain whether the problem lies in the absence of the enzyme itself, or is related to the toxicity of 2′-deoxyadenosine (dAR) or its phosphorylated derivative dATP, both of which accumulate in cells and body fluids of affected infants [1–5].

Many hypotheses have been advanced, supported by different *in vitro* models, which involve either resting lymphocytes [6–8], early events in the cell cycle [9–11], or inhibition of ribonucleotide reductase and DNA synthesis [12, 13]. Support for the reductase model comes from studies in cultured lymphocytes which have demonstrated that T-cells are more sensitive than B-cells to the toxic effects of dAR in simulated ADA deficiency, ostensibly because of a greater ability to accumulate and sustain elevated dATP levels [1–3, 12, 13]. This model was questioned by reports demonstrating that resting peripheral blood lymphocytes (PBMs), both T and non-T, could accumulate and sustain elevated dATP levels and were also susceptible to dAR toxicity [10, 11]. Others have confirmed the toxicity to early events in lymphocyte activation, but in the absence of significantly elevated dATP levels [9].

The severe lymphopenia in ADA deficient SCID infants [2, 3, 14] would support the contention that more than one mechanism of toxicity may be involved [15, 16]. Most studies have looked only at dAR toxicity in relation to dATP accumulation. The possibility that dAR might be metabolized by alternative routes has not been investigated. Many studies also equate results using cells from different animal species to the human situation. Cultured cells may likewise be a poor model for the normal mature human lymphocyte.

For this reason the same techniques previously applied to the study of cultured lymphocytes [17, 18] were used to investigate the metabolism of dAR in human cells of either T or B lineage. The results do

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not support the contention that B-cells are more resistant than T-cells to the toxic effects of dAR in ADA deficiency, becsuse of an inability to accumulate and sustain elevated dATP levels.

MATERIALS AND METHODS

Materials

[8-^{14}C]-deoxyadenosine (55 mCi/mmole) was purchased from New England Nuclear and 2′-deoxycoformycin (Pentostatin) was a gift from Dr R. Payne (Institute for Cancer Research, Sutton, Surrey, U.K.).

For the cell culture and incubation experiments Iscove's serum-free medium, foetal calf serum (FCS: Gibco, Europe) and horse serum (Sera Labs) were employed.

The nylon fibres used in the preparation of the enriched B-lymphocyte suspensions were purchased from Travenol Labs (Norfolk, U.K.), the sheep red blood cells (SRBC) from Flow Labs (Scotland, U.K.), the Ficoll/Histopaque (1.077 g/ml) and Neuraminidase Type V, from Sigma (U.K.).

Methods

Preparation of enriched B-lymphocyte populations. Human tonsils were obtained from children undergoing routine tonsillectomy. The glands were minced in medium plus 10% FCS and, after brief sedimentation, the supernatants were collected. B-lymphocytes were obtained from this cell suspension by adherence to nylon fibre columns and were further purified by eliminating any remaining T-cells by rosetting with neuraminidase-treated SRBC followed by Ficoll/Hystopaque density gradient centrifugation [19, 20]. Cells collected from the interphase were washed twice in medium supplemented with 10% horse serum and viability determined by trypan-blue exclusion (>95%). Cell numbers were adjusted by counting in a haemocytometer.

The final cell suspensions obtained with this separation procedure consisted of 95% B-lymphocytes as assessed by indirect membrane immunofluorescence with mouse monoclonal antibodies specific for B-cells (RFB4, RFB6: courtesy of Professor G. Janossy, Immunology Department, Royal Free Hospital, London) and subsequent analysis using an Ortho 50H Flow Cytometer.

Preparation of human thymocyte suspension. Human thymuses were obtained from children undergoing cardiac surgery. The tissue was cut into small pieces with forceps in Iscove's medium plus 10% FCS, and after brief sedimentation the cells in the supernatant were collected and thymocytes isolated after a standard Ficoll/Histopaque gradient centrifugation (20 min, 450 *g*) [20]. The cell suspension was washed twice in medium plus 10% horse serum, cell numbers adjusted and viability determined as described previously.

Separation of peripheral blood mononuclear cells (PBMs). Samples of 20 ml of venous blood from healthy control donors were defibrinated and diluted with a solution containing phosphate buffered saline/5% EDTA, pH 7.2, in the proportion of 2:1. The mononuclear cells were separated by Ficoll/Histopaque gradient centrifugation (20 min, 450 *g*) [20]. The collected cells were then washed twice in medium supplemented with 10% horse serum, and when necessary contaminating erythrocytes lysed by hypotonic shock [21]. Cell numbers were adjusted and viability determined as above.

Cell lines. The cells in long-term culture used in this study were routinely growing in Iscove's medium supplemented with 10% FCS and were subcultured every 3–4 days.

The lymphoblastoid (EBV$^+$) line (KBS-2), kindly supplied by Dr P. Shepherd, was derived from a patient with plasma cell leukaemia. Another cell line, partially deficient in adenosine deaminase (ADA), was EBV$^+$ and was derived from a patient W.D. with no ADA activity in the erythrocytes, but residual activity in lymphocytes (provided by Dr S. Pereira). The lymphoblasts (EBV$^+$) deficient in purine nucleoside phosphorylase (PNP) have been previously described [17].

The cells were routinely checked for mycoplasma contamination using the fluorescent DNA-binding dye (Hoechst/33256) and the 6-methyl-purine deoxyriboside test [22].

For the metabolic studies exponentionally growing cells (48 hr after subculture) were washed twice in medium plus 10% horse serum, and cell numbers adjusted and viability determined.

Incubation with [8-^{14}C]-deoxyadenosine. In all experiments duplicate samples containing 2×10^6 cells resuspended in Iscove's medium plus 10% horse serum were incubated with [8-^{14}C]-deoxyadenosine (dAR), at a final concentration of 8.7 μM (0.48 μCi/ml), for 2 hr at 37° in an atmosphere of 5% CO_2/95% air. The final incubation volume per sample was 205 μl. Before the addition of the radiolabelled substrate there was always a pre-incubation of 20 min, with or without the appropriate concentration of the adenosine deaminase inhibitor, 2′-deoxycoformycin (dCF). The latter was determined in pilot experiments for all cell types over a concentration range from 10 to 100 μM.

The incubations were terminated by centrifugation (450 *g* for 10 min at 4° and the supernatants containing the incubation mediums were separated from the cell pellets. In both cases protein was removed by precipitation after the addition of trichloroacetic acid (TCA) (10% final concentration) followed by centrifugation at 12,000 *g*, 1 min (Beckman, microcentrifuge). The TCA was extracted with water-saturated ether to a pH above 5.0. Samples were stored at −20° if not analysed immediately.

dATP stability experiments. Samples containing 2×10^6 thymocytes or tonsillar B lymphocytes were incubated with [8-^{14}C]-dAR (8.7 μM) as described above, in the presence of 60 μM dCF or 20 μM dCF, respectively, for 2 hr. The cells were then sedimented and washed twice (460 *g* 10 min, 4°) and placed in fresh medium at 37°. At varying points (from 0 to 60 min) duplicate samples were withdrawn and the incubation terminated as described.

Measurement of radioactivity in nucleotides, nucleosides and bases. A Waters Associates (Waters, Harrow, U.K.) high-pressure liquid chromatography (HPLC) trimodule fully-automated system, coupled to a precision radioactivity monitor (Reeve Analytical, Glasgow, U.K.) was used to measure incor-

poration of [8-^{14}C] into nucleotides, nucleosides and bases [23]. For the nucleotides a 5 μ APS-Hypersil column (Hichrom, U.K.) was used (25 cm × 4.9 mm internal diameter) with a linear phosphate gradient (gradient 6) at a flow rate of 1 ml/min, increasing to 100% buffer B in 10 min. Buffer A contained KH_2PO_4 (5 mM) at pH 2.50. Buffer B contained KH_2PO_4 (68 g/l) plus KCl (68 g/l) at pH 5.0.

The same equipment was used for the separation of nucleosides and bases in the medium, but with a Spherisorb 5 ODSI (25 cm × 4.9 mm i.d.) column (Hichrom, U.K.) The column was eluted with a linear gradient (Waters gradient 6) at a constant flow rate of 1 ml/min, from an initial buffer A, consisting of 40 mM ammonium acetate (adjusted to pH 5.0 with chromatographic grade acetic acid), to a final eluent composition of 70% buffer A, 30% buffer B over 30 min. Buffer B consisted of 77% methanol, 13% acetonitrile and 10% tetrahydrofuran.

RESULTS

Metabolism of [8-^{14}C]-deoxyadenosine (dAR) under normal and ADA inhibited conditions

Viability remained constant during the 2-hr incubation period and was greater than 95% for all the different cell types studied. All incubations were carried out in medium plus 10% horse serum because it lacks ADA activity. In all experiments the highest percentage of counts was in the medium. Incorporation into cellular nucleotides was always less than 25%.

A. *Metabolism of [8-^{14}C]-deoxyadenosine (dAR) by T-cell types under physiological conditions*

In the absence of ADA inhibition (Table 1) the normal route of dAR metabolism for both thymocytes and PBMs was deamination via deoxyinosine (dHR) to hypoxanthine (H), with subsequent conversion within the cell to ATP and GTP, in the ratio of approximately 4:1, confirming active incorporation of dAR into both the adenine and guanine nucleotide pools via IMP (Figs 1a and c). However, incorporation into cellular nucleotides by PBMs was only 50% that for thymocytes. Thymocytes also formed some inosine (HR) suggesting saturation of these routes under the physiological conditions used, with subsequent degradation of IMP and accumulation as HR because of the lack of sufficient phosphate for further metabolism by PNP at 1 mM P_i.

The complete disappearance of dAR in the thymocyte experiments, contrasts with the presence of 20% of counts in unmetabolized substrate [dAR plus adenine (A) derived from dAR degradation during TCA extraction] in the PBM sutdies (Table 1) and confirms that intact thymocytes have a much higher ADA activity than peripheral blood T-cells. The fact that no dATP was formed, despite the presence of unmetabolized dAR in the PBM experiments, while thymocytes formed detectable amounts of dATP although no dAR remained unmetabolized (Table 3), demonstrates a much higher activity of the kinase responsible for converting dAR to dATP in thymocytes.

Table 1. Route and extent of incorporation of [8-^{14}C] dAR (8.7 μM) into the cellular nucleotides of thymocytes or PBMs in 2 hr at 37°, with or without added dCF at 60 and 40 μM, respectively

			Cellular nucleotides (pmoles/10^6 cells/2 hr)						Incubation medium (pmoles/10^6 cells/2 hr)				
	(N)		ATP	ADP	dATP	dADP	GTP	IMP	H	dHR	HR	dAR/A*	
Thymocytes	5	—	114 (9–141)	22 (10–35)	14 (11–24)	—	28 (18–48)	13 (6–21)	450 (306–546)	390 (280–487)	64 (52–84)	—	—
	5	60 μM	43 (29–56)	5 (1–9)	105 (76–120)	8 (3–13)	—	—	—	—	—	483 (446–553)	241 (191–299)
PBMs	6	—	56 ([illegible]37)	16 (9–19)	—	—	17 (5–23)	8 (3–13)	437 (358–503)	374 (243–435)	—	122 (52–171)	52 (29–69)
	8	40 μM	35 (29–49)	2 (1–4)	12 (6–24)	3 (1–6)	—	—	—	—	—	524 (427–589)	318 (267–445)

Results are the mean plus the range for the number of duplicate experiments (N) indicated.
—, below the limits of detection (<1 pmol/10^6 cells).
* Counts in adenine (A) derived from dAR in the acid extraction step.
H, dHR, HR, hypoxanthine, deoxyinosine, inosine, respectively.
dCF, 2′-deoxycoformycin.

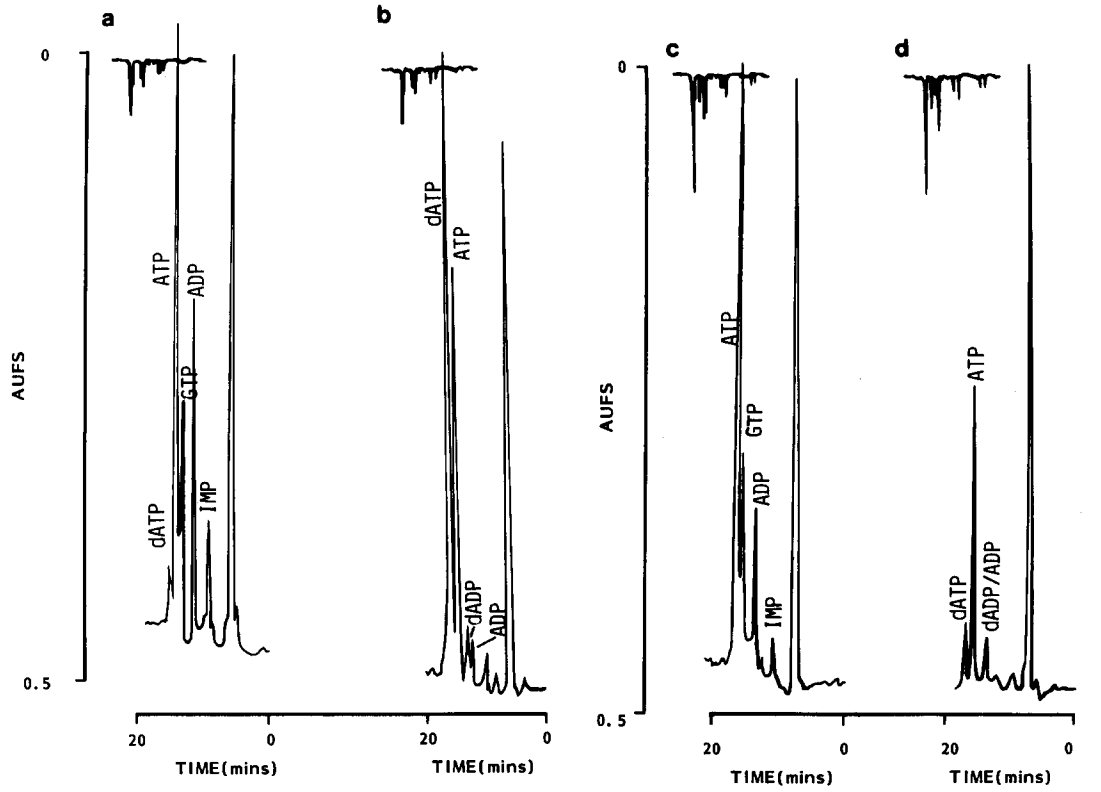

Fig. 1. Chromatograms recorded at 245 nm (upper trace), a chart speed of 0.1 cm/min and 0.5 absorbance units full scale (AUFS), showing the incorporation of radiolabel (lower trace) from [8-^{14}C] dAR into the different cellular nucleotides as indicated. 75 μl of extract of thymocytes (a and b), or peripheral blood mononuclear cells (c and d), incubated alone (a and c) or in the presence of 60 or 40 μM dCF (b and d), respectively, were injected. The radiolabelled peak eluting in the first five minutes represents counts in deoxynucleosides and bases.

B. *Metabolism of [8-^{14}C] dAR by T-cell types under conditions simulating ADA deficiency*

2′-Deoxycoformycin (dCF) completely inhibited ADA activity at a concentration of 60 μM and 40 μM for thymocytes and PBMs, respectively. ADA inhibition was considered to be complete when no counts could be detected in dHR and H in the medium, or in GTP and IMP within the cell. Higher concentrations of dCF gave identical results.

The requirement for higher dCF concentrations to inhibit dAR deamination is in accordance with the higher specific activity of ADA reported for thymocytes in comparison to other lymphoid cell types [24, 25]. Under these ADA inhibited conditions there was a sevenfold increase in the dATP formed by thymocytes (mean of 105 pmoles/10^6 cells) (Fig. 1b) while in PBMs the dATP formed (Fig. 1d) was much lower (mean 12 pmol/10^6 cells), and similar to levels found for the thymic cells with no dCF added.

An interesting feature (which has been noted previously in cultured B and T lymphoblasts [17, 18]) was the finding of some counts in ATP, despite the complete absence of any detectable ADA activity at the dCF concentrations used. The origin of this ATP formation is unclear, since increasing dCF concentrations (up to 90 μM—results not shown) did not reduce ATP formation.

Stability of dATP in human thymocytes. Duplicate experiments, carried out in thymocytes (Table 2) to determine the stability of dATP accumulated from dAR in the above experiments simulating ADA deficiency, confirmed the high degree of stability noted by others for T lymphoblastoid cell lines [2]. Human thymocytes washed and resuspended in fresh medium showed only a slow decrease in dATP levels with time (21% in 60 min). Addition of 60 μM dCF to the incubation medium in identical experiments, did not improve the results. Release of radioactivity into the medium was minimal in all instances.

Table 2. Stability of [8-^{14}C] incorporated from dAR (8.7 μM) into dATP by thymoctyes and tonsillar B lymphocytes during incubation for 2 hr at 37° in the presence of 60 and 20 μM dCF, respectively, and subsequently washed and reincubated in fresh medium for the times indicated

Time (min)	Thymocytes (pmoles/10^6 cells dATP)	B-Lymphocytes
0	109	97.9
15	104	86.8
30	92.6	92.1
60	86.3	77.4

Results are the mean of two experiments.

Table 3. Route and extent of incorporation of [8-^{14}C] dAR (8.7 μM) into cellular nucleotides of different B cell types, incubated alone, or with 20 μM dCF as indicated for 2 hr at 37°

			Cellular nucleotides (pmoles/10^6 cells/2 hr)						Incubation medium (pmoles/10^6 cells/2 hr)				
Cell type	dCF	(N)	ATP	ADP	dATP	dADP	GTP	IMP	H	dHR	HR	dAR/A*	
B-lymphocytes (tonsil-derived)	—	5	93 (76–131)	43 (28–61)	4 (1–10)	—	35 (26–49)	38 (33–43)	309 (193–400)	259 (139–320)	56 (37–73)	35 (15–59)	†
	20 μM	5	81 (68–103)	18 (11–29)	68 (52–103)	16 (11–23)	—	—	—	—	—	372 (286–460)	300 (199–360)
Plasma cells (KBS-2)	—	3	96 (86–107)	55 (53–59)	—	—	47 (45–46)	40 (34–47)	603 (489–726)	225 (201–245)	79 (41–127)	—	—
	20 μM	3	41 (23–73)	9 (3–19)	19 (10–30)	3 (1–4)	—	—	—	—	—	519 (213–679)	401 (362–465)
B-lymphoblasts (WD:ADA$^-$)	—	4	73 (68–81)	14 (9–18)	28 (24–32)	2 (0.5–4)	23 (19–26)	13 (9–17)	394 (363–425)	39 (22–57)	—	277 (240–314)	211 (187–236)
	20 μM	4	27 (21–35)	—	36 (28–50)	1.5 (1–2.5)	—	—	—	—		519 (481–558)	355 (320–386)
B-lymphoblasts (SB:PNP$^-$)	20 μM	3	39 (36–42)	7 (6–9)	47 (20–60)	7 (2–10)	—	—	—	—	—	455 (420–493)	267 (276–259)

Results are the mean plus the range for the number of duplicate experiments (N) indicated.
—, below the limits of detection (<1 pmole/10^6 cells).
* Counts in adenine (A) derived from dAR in the acid extraction step.
† Trace.
H, dHR, HR, hypoxanthine, deoxyinosine and inosine, respectively.
dCF, 2′-deoxycoformycin.

C. *Metabolism of [8-^{14}C]-dAR under physiological conditions by B cell types*

The metabolism of dAR was again complete, or almost complete, for KBS-2, the plasma cell line, and tonsillar B-lymphocytes, respectively (Table 3), the main metabolic route being deamination to deoxyinosine (dHR) with cleavage to hypoxanthine (H) and subsequent incorporation into the nucleotides ATP and GTP, via IMP. Fifty per cent of the radioactivity was also metabolized via this route by the line from the ADA deficient infant, confirming a partial defect of ADA in the lymphoid cells of this child.

The ATP/GTP ratio (2–3:1) was lower than for T-cell types. Counts in inosine (HR) in the plasma cells and tonsillar B-lymphocytes, but not the ADA deficient lines, were again considered to reflect the increased flux through these pathways, associated with HR accumulation due to depletion of available phosphate following IMP breakdown. Some dATP formation was noted in the partially ADA deficient line, but, surprisingly, detectable amounts of dATP were also formed by the tonsil-derived B-cells even in the absence of any ADA inhibitor (Fig. 2a).

D. *[8-^{14}C] dAR metabolism by B-cell types under ADA inhibited conditions*

Lower levels of dCF (20 μM) were sufficient to completely inhibit ADA activity in all B-cell types (Table 3) as assessed by the absence of counts in dHR and H in the medium, or GTP and IMP in the cells. Increased concentrations of dCF gave identical results (not shown). Intact B-cells thus appear to have a lower ADA activity than thymocytes or PBMs.

In these ADA inhibited conditions all B-cell types formed detectable amounts of dATP. The lowest dATP levels (little higher than that accumulated by PBMs) were formed by the KBS-2 line under these ADA inhibited conditions and only unmetabolized substrate was found in the medium for all the B-cell types. The unexpected finding was that the highest dATP levels were formed by the tonsil-derived B-lymphocytes (mean 68 pmoles/10^6 cells), reaching values within the range for the ADA inhibited thymocytes. Intermediate values were formed by the ADA and PNP deficient lymphoblast lines.

As noted previously, counts also accumulated in ATP (Figs 2, b and c) under these ADA inhibited conditions but were less than 50% of the values attained in the absence of dCF (Table 3) for both the KBS-2 and ADA deficient line (the PNP deficient line was not incubated with dAR alone since previous experiments [17] confirmed these cells were completely PNP deficient and could not metabolize dAR beyond dHR, thus cellular nucleotide incorporation was minimal). By contrast the ATP formed by the tonsil-derived B-lymphocytes was only slightly reduced with respect to the levels formed under normal conditions. The route of formation of this ATP is not yet clear. However, since ATP levels in the PNP deficient (Fig 2c) and ADA deficient dCF

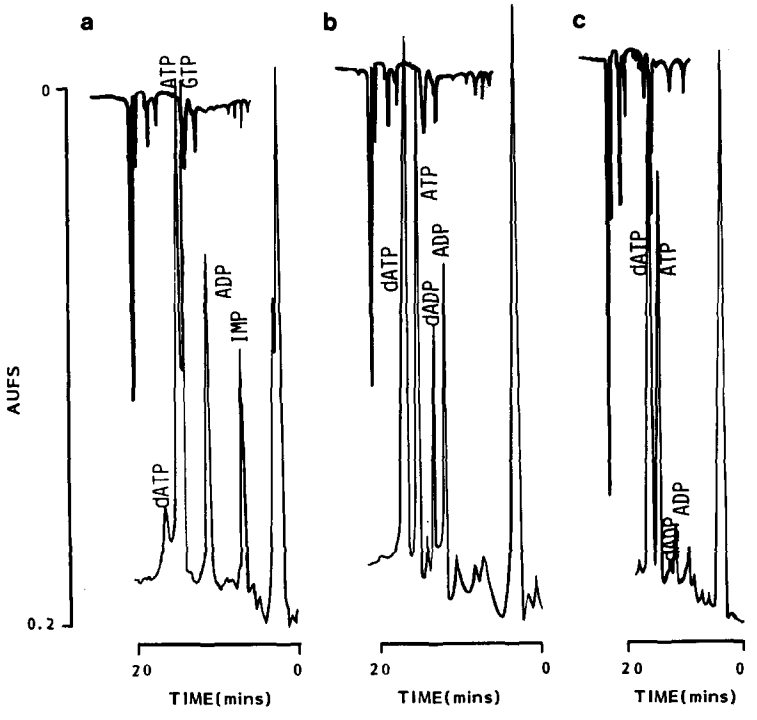

Fig. 2. Chromatograms recorded at 254 nm (upper trace), a chart speed of 0.15 (a, b) or 0.1 (c) cm/min and 0.2 absorbance units full scale (AUFS), showing the incorporation of radiolabel (lower trace) from [8-^{14}C] dAR into the different cellular nucleotides as indicated. 75 μl of extract of tonsil-derived B-lymphocytes (a and b), or a PNP deficient lymphoblast line (c), were injected following incubation with dAR alone (a) or together with 20 μM dCF (b and c). The radiolabelled peak eluting in the first five minutes represents counts in deoxynucleosides and bases.

inhibited line were similar to those for the other dCF inhibited cell types (Tables 1 and 3), a route via ADA, PNP and consequently HGPRT can be excluded.

Stability of the [8-^{14}C] dATP accumulated by tonsil-derived B-lymphocytes. The stability of the intracellular dATP levels accumulated by the tonsillar B-lymphocytes after washing and reincubation in fresh medium for up to 60 min is compared with that in thymocytes in Table 2. There was a very gradual decrease in dATP levels, identical with that for the thymocytes, with dATP levels at 60 min being reduced by 21%. This result contrasts with the high level of dATP breakdown (approximately 50% in 25 min) which has been reported for B-lymphocytes in long-term culture [12].

DISCUSSION

The toxicity to the immune system in inherited ADA deficiency involves both dividing and non-dividing cells. The favoured hypothesis has focused on dATP itself, a potent inhibitor of ribonucleotide reductase and hence DNA synthesis in dividing cells [1–3]. Since T-cells exhibited a much higher activity of the kinase responsible for dATP accumulation compared with lower activities of the three different enzymes capable of degrading dATP, with the reverse for B-cells [6, 12, 13, 31], the enhanced *in vitro* toxicity of dAR to T-cells was ascribed to their ability to accumulate and sustain much higher dATP levels under ADA deficient conditions [1]. Others have shown that dAR is not inhibitory of mitogen-stimulated lymphocyte transformation unless added initially, which argued against the reductase hypothesis [1, 37]. A wealth of data now exists confirming dAR toxicity to early phases of the cell cycle, associated with DNA strand breaks, or inhibition of RNA synthesis or transcription. Putative mechanisms include the main alternative hypotheses of ATP depletion and/or inhibition of vital methylation reactions by dAR directly [1–9, 15, 16, 21, 27–30].

Our earlier studies in cultured lymphocytes did not support the hypothesis that B-cells had an inherent inability to sequester dATP. We found that B-cells of malignant origin could accumulate dATP as efficiently as T-cells, which appeared to relate to their malignant transformation rather than their original cell type [17, 18]. Others have shown that some B-cells do not accumulate dATP despite low ecto-ATPase levels [3] whilst null cells accumulate high dATP levels despite high 5′-nucleotidase levels [6]. Additionally, recent studies demonstrated dAR toxicity to resting lymphocytes associated with an equal ability of both T and non-T peripheral blood lymphocytes (PBMs) to accumulate dATP [6, 10, 11], or alternatively toxicity to early events in lectin-stimulated lymphocytes in the absence of dATP accumulation [9], which again questioned the reductase model.

One of the main problems relates to the fact that a large number of hypotheses are based on enzyme levels in cell lysates, not intact cells. Many believe a high ADA activity (as in immature cortical thymocytes—also found in cultured T-lymphoblasts and other cells of malignant origin) renders a cell peculiarly sensitive to ADA deficiency [32]. ADA activity is low in mature medullary thymocytes, bone marrow and peripheral blood thymocytes [24, 25]. Consequently, the report that mature thymocytes as well as PBMs were equivalent to cortical thymocytes in their capacity to elevate dATP to potentially toxic levels was at odds with results anticipated from lysate activity [6]. The existence of at least eleven people who are not immunodeficient, with low but detectable ADA activity in some cell types including PBMs [3] likewise suggests that studies in lysed cells may not be a good guide to the *in vivo* situation.

The results in this paper, employing sensitive methods to follow the metabolism of dAR under physiological conditions in different intact human cells of T or B lineage, also question the validity of studies in cultured lymphocytes and lysed cells as appropriate models for the *in vivo* situation [17, 18]. They indicate that unseparated PBMs (more than 80% T-cells) have a poor capacity to either deaminate or phosphorylate dAR under any circumstances. By contrast, unseparated thymocytes (predominantly immature cortical cells) and tonsil-derived B-lymphocytes, have a much greater and comparable ability to accumulate dATP, and moreover sustain these elevated dATP levels to an equal extent. More importantly, the capacity to do this exists in normal cells, despite high ADA activity. This ability to accumulate dATP in the absence of ADA inhibition may be relevant to the large number of thymocytes destroyed daily in the thymus, particularly since dAR levels could rise significantly in such a close environment [33, 34].

The observation that B-lymphocytes resembled thymocytes in their ability to elevate dATP under ADA deficient conditions was unexpected and contrary to current thinking; particularly since kinase activity in lysed thymic cells is reportedly threefold that in tonsils or PBMs, with the reverse for the 5′-nucleotidase [35]. However, the results accord with recent data providing no evidence that T-lymphocytes were more sensitive than B-lymphocytes to ADA deficiency [36]. Redelman *et al.* [9] also found that although unseparated human PBMs accumulated some dATP, T-cells alone cultured with dAR accumulated virtually no dATP while non-T cells accumulated sufficient dATP to account for the raised dATP in unseparated PBMs. The accumulation of ATP as well as dATP under ADA deficient conditions in our present as well as previous studies [17, 18]—by a route as yet undetermined, but excluding ADA and PNP—could explain the inability of some to demonstrate ATP depletion *in vitro* [9].

Our data indicate that B-cells may also accumulate dATP to levels which could significantly affect cell viability. This might explain the reported variability in sensitivity of B-cells to dAR [37, 38]. The results suggest that a 40% contamination of non-T cells by monocytes may have been a contributory factor in other studies demonstrating that non-T cells were an order of magnitude less sensitive than T-cells to dAR [6, 10]. The same workers found that a monocyte-depleted non-T lymphocyte population accumulated similar dATP levels to T-cells. We recently showed dATP accumulation by macrophages was minimal [39]. The results in the present study question

whether the severe combined immunodeficiency in inherited ADA deficiency may not be due to an equal sensitivity of B-cells to dAR through its phosphorylated derivative dATP.

The ability of the different cell types in this study to accumulate dATP is at variance with a number of reports and may relate to several factors: (a) the type and concentration of ADA inhibitor used; (b) the use of non-human cells in some studies, since important species differences in purine enzymes exist [39]; (c) the specificity and sensitivity of the method used for measuring dATP (the sensitive HPLC method, coupled to radiodetection, used in the present study [17] enabled unequivocal determination of the total fate of dAR and its effect on all the different nucleotide pools, not just dATP levels as in most studies); (d) the use of differing time scales and high unphysiological phosphate levels would stimulate nucleotide synthesis—particularly in cultured cells. The short time interval, physiological levels of substrate and phosphate in this study were chosen to determine whether significant dATP accumulation could occur in uncultured human T and B-cells, to explain the toxicity of dAR to resting cells and/or lymphocyte triggering within the first few hours.

The lack of effective ADA inhibition could be important. We and others found that EHNA (erythro-9-(2-hydroxy-3-nonyl) adenine, or dCF, at low concentrations inhibit ADA completely only in lysed cells. Much higher concentrations were needed for intact cells—due possibly to transport effects or the varying ADA levels [15, 17, 18]; in fact, EHNA is ineffective in intact cells even at the limit of its solubility [17, 18]. Consequently, as demonstrated here in both uncultured and cultured cells, inadequate ADA inhibition could produce expansion of both the ATP and GTP pools and thus significantly alter the extent of dATP accumulation and/or cell viability *in vitro*. Several studies now relate deoxyguanosine toxicity *in vitro* to effects on GTP and ATP, not dGTP pools [18, 40, 41]. Although our studies may be criticized on the grounds that dCF at higher concentrations in some cells is an inhibitor of adenylate deaminase (AMPDA), this would not have affected the accumulation of dATP by the different cell types, since dAMP is not a substrate for AMPDA [4], nor is the AMPDA inhibitory dCF concentration known for all these cell types.

The present studies demonstrate that while dATP accumulation may be associated with toxicity to thymocytes and B-lymphocytes in inherited ADA deficiency, this is unlikely to be of significance to peripheral blood T-cells. The limited ability of the latter to metabolize dAR by any route suggests that inhibition of vital methylation reactions, or alternative hypotheses, require further investigation. The ability of B-cells to accumulate high dATP levels could explain the unexpected success of dCF in some lymphoid malignancies of the B-cell type [42]. The combined results underline the problems inherent in any *in vitro* model, particularly when using cultured cells, as well as the difficulty of extrapolating directly to the *in vivo* situation from lysed cell studies. The findings support the existence of more than one mechanism of toxicity in inherited ADA deficiency.

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