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METABOLISM OF DEOXYNUCLEOSIDES BY LYMPHOCYTES IN LONG-TERM CULTURE DEFICIENT IN DIFFERENT PURINE ENZYMES

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Abstract—The metabolism of 8-^{14}C-labelled 2′-deoxyadenosine (dAR) and 2′-deoxyguanosine (dGR) has been investigated using lymphocytes in long-term culture transformed by Epstein–Barr (EB) virus (B-cells) from eight patients with different inherited purine enzyme defects. The use of such lines enabled accurate mapping of the route of metabolism by acting as a 'trap' for the radiolabel at specific points. With either substrate (25 μM) most of the label was recovered in the medium. Using dAR, less than 30% of the radiolabel was incorporated into cellular nucleotides. For dGR, values were less than 18%.

Studies with dAR alone confirmed the principal route of metabolism was to hypoxanthine, with further metabolism (by lines with intact salvage pathways) to ATP and GTP in the ratio of approximately 4:1. Lack of accumulation of deoxyinosine in the purine nucleoside phosphorylase (PNP) deficient line, or hypoxanthine in the hypoxanthine guanine phosphoribosyltransferase (HGPRT) deficient line, using dAR together with the adenosine deaminase (ADA) inhibitor 2′-deoxycoformycin (dCF) at 10 μM, confirmed the effectiveness of ADA inhibition. Nevertheless, some ATP was still formed by all lines in the presence of dCF by a route as yet unknown. Only the ADA deficient lines formed dATP with dAR alone. However, some dATP was formed by all lines in the presence of dCF. A partially HGPRT deficient line formed extremely high dATP levels, well in excess of those formed by the T-cell line CEM.

Studies with dGR revealed some interesting differences, a large proportion of the substrate being metabolized predominantly to xanthine by most enzyme deficient lines. In the PNP deficient line most of the substrate remained unmetabolized, but some dGTP was formed. No other enzyme deficient line formed any dGTP—with or without the PNP inhibitor 8-aminoguanosine (8-NH_2GR)—with one exception. Again this was the partially HGPRT deficient line, which with the inhibitor again formed more dGTP than the T-cell line. Within the cells most of the substrate was metabolized to GTP, except in the PNP, and totally HGPRT deficient lines. Levels of GTP formed were not altered by the inhibitor, reflecting the lack of effective PNP inhibition by 8-NH_2GR. Some counts were also found in ATP and IMP, confirming the existence of this route in mammalian cells of lymphoid origin.

The results also support previous studies by us using cell lines with intact purine pathways, which demonstrated that, contrary to current beliefs, some B-cell lines are capable of accumulating high levels of deoxynucleotides. They indicate that the levels of substrate used in many studies are generally well in excess of those either desirable or necessary. They suggest that in any such *in vitro* studies in cultured lymphocytes—using either EHNA, dCF or 8-NH_2GR to inhibit the degradation of dAR or dGR—toxicity demonstrated could equally relate to either the accumulation of large amounts of unmetabolized substrate, or metabolism and accumulation of the substrate in other purine, not deoxypurine, pools.

Of the known inherited disorders of purine metabolism only two are associated with immunodeficiency. In adenosine deaminase (ADA, EC 3.5.4.4) deficiency both T- and B-cell function are defective. In purine nucleoside phosphorylase (PNP, EC 2.4.2.1) deficiency, T-cells are affected. Since these are also the only purine disorders in which the accumulation of deoxynucleosides has been demonstrated, a cause and effect relationship has been postulated [1–7].

Many *in vitro* studies in cultured lymphocytes have found that T-lymphoblasts are much more sensitive than B-lymphoblasts to the cytotoxic effects of 2′-deoxyguanosine (dGR) or 2′-deoxyadenosine (dAR) in the presence of an ADA inhibitor such as erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) or 2′-deoxycoformycin (dCF) [8–19]. The accumulation of high levels of dATP, or dGTP, in such studies in T-, but not B-cells, has been related to an enzyme pattern peculiar to the former [2, 8–13]. These deoxynucleotides are potent inhibitors of the ribonucleotide reductase (EC 1.17.4.1) essential for DNA synthesis, which could explain the preferential toxicity of dAR or dGR to T-cells *in vivo* [20, 21].

Assumptions based on the above *in vitro* experiments may be questioned because studies of deoxynucleoside toxicity have been confined almost exclusively to measurement of deoxynucleotide levels, and/or viability of cells in long-term culture [20]. The toxicity in different studies could well relate to metabolism of the substrate by other routes—particularly in those studies relating to dGR where no PNP inhibitor has been available until recently.

The present report details metabolic studies in cultured lymphoblasts deficient in enzymes which catalyse sequential steps in the synthesis and degradation of different purine nucleotides. The purpose was to investigate findings during similar studies in

cells with intact purine pathways.* They have provided an accurate map of the principal routes of metabolism of both dAR and dGR. The combined results do not accord with published work and indicate that new experiments employing much lower substrate levels must be devised.

MATERIALS AND METHODS

Materials. [8-^{14}C]Deoxyadenosine (40.9 mCi/mmole) and [8-^{14}C]deoxyguanosine (56.6 mCi/mmole) were purchased from New England Nuclear. Unlabelled 2′-deoxyadenosine (Sigma Chemical Co., St. Louis, MO) and 2′-deoxyguanosine (BDH Chemicals Ltd., Poole, Dorset, U.K.) were used to dilute the labelled substrates. Mediums used were either Iscoves or RPMI-1640 (Flow Laboratories) with appropriate additives to ensure viability and growth.

2′-Deoxycoformycin (pentostatin for injection) was a gift from Professor A. V. Hoffbrand, Royal Free Hospital and 8-aminoguanosine (8-NH_2GR) a gift from Dr. J. Wilson, Department of Medicine, Ann Arbor, MI.

Cell lines. The cell lines used in this study were obtained from patients with different purine enzyme defects during collaborative studies of purine metabolism. ADA_1 and ADA_2 were from patients SY and KA [22], studied in collaboration with Dr. R. Levinsky, Institute for Child Health, London. PNP was from patient SB [23], studied in collaboration with Dr. A. Watson, Royal Manchester Children's Hospital. $HGPRT_1$ was from GK, a Lesh–Nyhan patient; $HGPRT_2$ from CL with partial hypoxanthine guanine phosphoribosyltransferase, (EC 2.4.2.8) deficiency [24], a patient of Professor J. S. Cameron, Renal Unit, Guy's Hospital PPRPS (aberrant phosphoribosylpyrophosphate synthetase, EC 2.7.6.1) was from NB [25], a patient of Dr. J. Wilson, Great Ormond Street Hospital for Sick Children. $APRT_{1\&2}$ [adenine phosphoribosyltransferase (EC 2.4.2.7) deficiency] were from B D'H, and F D'H [26], patients of Professor K. J. Van Acker, University of Antwerp, Belgium. The lines were transformed by Epstein–Barr (EB) virus infection and made available to us through the courtesy of Dr. S. Pereira, Northwick Park; Dr. M. Greaves, Imperial Cancer Research Fund; and Dr. J. E. Seegmiller, La Jolla, CA. The T-cell line CEM was kindly supplied by Professor M. A. Epstein, University of Bristol.

Cell lines were checked monthly for mycoplasma contamination using two separate methods: a fluorescent DNA-binding dye (Hoechst 33256) method, and the 6-methyl-purine deoxyriboside (6-MeP-dR) test of McGarrity and Carson [27]. All lines used were checked initially by culture and found to be mycoplasma-free. We are greatly indebted to Dr. D. A. Carson, La Jolla, CA for kindly donating the 6-MepdR, and to Dr. Windsor and staff of the Mycoplasma Unit, Wellcome Research Laboratories, Beckenham, U.K. for the mycoplasma culture testing.

Metabolism of deoxyadenosine and deoxyguanosine in intact cells. Cells in logarithmic growth phase were harvested 48 hr after subculture and resuspended in fresh complete medium (Iscove's or RPMI-1640 plus 10% heat-inactivated foetal calf serum) and incubated for 2 hr at 37° in an atmosphere of 95% air 5% CO_2, with [8-^{14}C] dAR or [8-^{14}C]dGR, at a final concentration of 25 μM. The incubation volume was 100 μl in total. The conditions chosen were selected to be as close to physiological as possible (i.e. physiological phosphate, plus final substrate concentration of 25 μM). The latter was selected as being comparable to the lower concentration used in most published studies [20]. Viability over 2 hr was greater than 90% (trypan blue exclusion). Densities of the different cell lines studied were 1×10^7/ml. In each experiment quadruplicate samples were analysed as follows:

(i) When the incubations were carried out in the presence of the ADA inhibitor (10 μM) dCF or the PNP inhibitor (210 μM) 8-NH_2GR, 40 μl of cell suspension (containing 1×10^6 cells) was preincubated for 10 min together with 10 μl of inhibitor (of the appropriate molarity to give the above final concentration) prior to addition of labelled substrate.

(ii) Duplicates containing 40 μl of cell suspension plus 10 μl medium (or inhibitor, as above) were preincubated for 10 min and the reaction was started by the addition of 50 μl of labelled substrate (dAR 1 μCi/ml or dGR 1.4 μCi/ml). After 2 hr the incubations were terminated by centrifugation at 400 *g* (1500 rpm) for 5 min at 4°. Supernatants containing the incubation medium were transferred to a tube containing 25 μl of ice-cold trichloroacetic acid (TCA) (40%) and the cell pellet was suspended in 100 μl of cold TCA (8%). The protein precipitate was removed at 12,000 *g* (Beckman microcentrifuge) for 1 min and both the above supernatants were immediately extracted with water-saturated diethyl ether to pH 5.0. All manipulations were done on ice to minimize the acid breakdown of deoxy compounds. Samples were then stored at −20° until analysed by high performance liquid chromatography (HPLC).

(iii) The remaining duplicates, containing isotope diluted with cold substrate to a final radioactive concentration of 0.25 μCi/ml dAR (or 0.28 μCi/ml dGR), were processed to quantify total radioactivity in the soluble and insoluble cellular fractions, and in the medium. Two hours after the start of the incubation, the cells were centrifuged at 400 *g* (1500 rpm) for 5 min at 4° and the supernatant was removed by aspiration. A wash with 200 μl of cold isotonic saline followed (5 min at 400 *g* at 4°). Both supernatants were transferred to a counting vial to quantify total radioactivity in the medium. The cell pellets were then extracted with 200 μl 8% TCA for 20 min at 4° and centrifuged at 600 *g* (2000 rpm) for 20 min at 4°. The supernatant was removed and the pellet washed with 100 μl of cold 8% TCA and spun again for 10 min (600 *g*, 4°). Both supernatants were transferred to a counting vial for acid-soluble fraction quantification. The remaining pellets were resuspended in 100 μl TCA (8%) and incubated at 70° for 40 min. The suspension plus 200 μl H_2O used to wash the tube

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were transferred to a scintillation vial and quantified as the cellular insoluble fraction. Scintillant (3 ml) was added to each vial and radioactivity determined by liquid scintillation counting (1215 Rachbeta, LKB Wallac).

Chromatography. The HPLC system used has been described [28]. A reverse-phase system was employed to separate nucleosides and bases in the medium. For the dAR incubations, buffer A contained KH_2PO_4, 2.7 g/l. (20 mM), pH 4.45; buffer B consisted of 60% methanol, 40% water (v/v). A linear gradient (gradient 6) increasing to 40% B in 20 min was used. For studies using dGR the pH of buffer A was 3.0. For the nucleotides in the cell extracts, an anion exchange system employing a phosphate gradient was used. Buffer A contained KH_2PO_4, 0.68 g/l. (5 mM), pH 2.65; buffer B, 25 g KH_2PO_4/l. plus 25 g KCl/l., pH 3.85. The flow rate was 3 ml/min using a linear gradient (gradient 6) increasing to 100% B in 20 min (Tri-modular system, Waters Associates, Cheshire, U.K.

The phosphate was Aristar grade, the potassium chloride Analar grade, from BDH Chemicals Ltd. (Poole, Dorset, U.K.). The methanol was a special HPLC grade from Rathburn Chemicals (Walkerburn, Scotland, U.K.). For all studies, the 280 nm channel recorder was disconnected and the radioactivity monitor attached in its place for continuous monitoring of radioactivity in parallel with the UV absorbance at 254 nm. The radiodetector contained a flow cell (200 μl void volume) packed with solid scintillant (99/3811 GSI glass scintillant powder, Grade W, 63–80) obtained from Koch-Light Ltd. The radiodetector was a Precision Radioactivity Monitor supplied by Reeve Analytical Ltd. (Chapel Street, Glasgow, U.K.).

RESULTS

Metabolism of dAR with and without 10 μM dCF

Figure 1 shows that of the enzyme deficient lines only those with intact pathways for both catabolism and salvage of dAR ($APRT_{1\&2}$, PPRPS) incorporated significant amounts (up to 23%) of radioactivity into the cell, predominantly into the soluble fraction. As expected, the ADA deficient line ADA_1 showed no change in incorporation in the presence of dCF, while incorporation if anything increased slightly in the PNP and completely HGPRT deficient line $HGPRT_1$. Surprisingly, incorporation by the partially deficient line $HGPRT_2$ was trebled in the presence of dCF. The T-cell line incorporated approximately 30%, which was reduced to 12% by dCF. In all lines incorporation into the insoluble fraction was proportional to the incorporation into the soluble fraction and was reduced by up to 90% by dCF in lines with intact salvage pathways.

Table 1 demonstrates the reason for the differences observed in Fig. 1 and shows that the majority of counts were in the medium. The results are expressed on a protein basis because of considerable variation in cell size. Lines $APRT_{1\&2}$ $HGPRT_1$, PNP and ADA were relatively uniform in size and contained from 0.14 to 0.18 mg protein/10^6 cells (mean of seven determinations per cell type). Lines ADA_2, $HGPRT_2$ and CEM were much larger, with a mean protein content of 0.317, 0.244 and 0.292 mg protein/10^6 cells, respectively.

As anticipated, in the two ADA deficient lines most of the substrate was recovered as unchanged dAR. (The small amount of radiolabel in adenine was found, by experiments at zero time, to be due to the instability of dAR in the acid extraction conditions.) These lines were the only lines (apart from the T-cell line CEM) to form significant amounts of dATP without dCF, but surprisingly counts were also found in ATP. In the other lines counts in the medium were found predominantly in hypoxanthine (H), except in the PNP deficient line where they were found almost exclusively in deoxyinosine (dHR). (Again in the PNP deficient line the small fraction

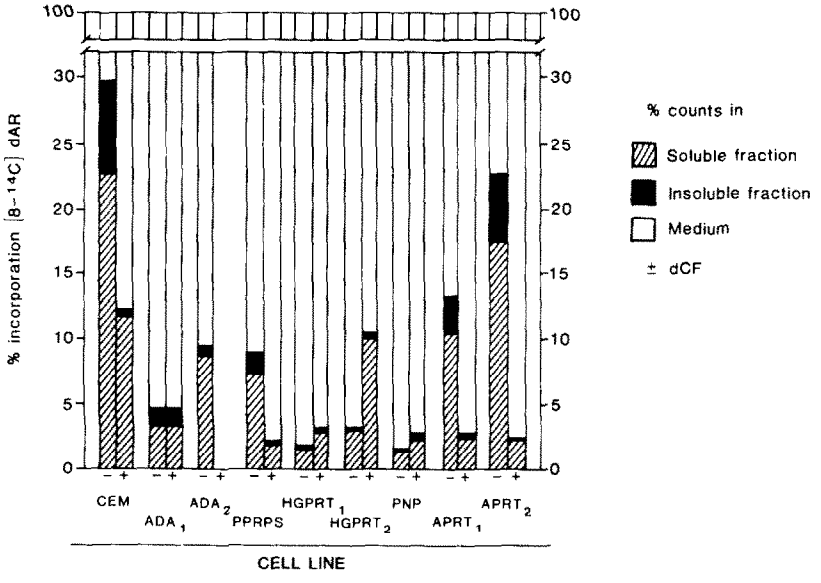

Fig. 1. Percentage incorporation of radiolabelled dAR (25 μM) in 2 hr into the soluble (hatched bars) and insoluble (closed bars) cellular fractions relative to the amount of label remaining in the medium (open bars), using nine different cell lines with (+) and without (−) the ADA inhibitor dCF (10 μM).

Table 1. Metabolism of radiolabelled dAR (25 μM) by eight different cell lines derived from peripheral blood lymphocytes of patients with five different inherited purine disorders by EB virus transformation

		Soluble fraction (pmole/mg protein per 2 hr)								Medium (nmole/mg protein per 2 hr)			
Cell line	dCF (*n*)	ATP	ADP	dATP	dADP	NAD^+	IMP	GTP	GDP	dAR	dHR	H	A
ADA_1	±(9)	75.7 (41–100)	—	62.8 (19–100)	2.8 (1.2–11)	—	—	—	—	16.2 (14.8–17.9)	—	—	1.2 (0.8–24.8)
ADA_2	−(4)	243.9 (182–262)	10.7 (11–19)	57.3 (43–67)	—	11.4 (7–15)	—	—	—	6.1 (5.2–7.1)	—	—	0.82 (0.72–1.22)
PRPPS	−(6)	485 (373–625)	132 (88–199)	—	—	34.1 (7–56)	49.7 (9–76)	103 (77–156)	27 (18–48)	—	5.9 (5.0–6.9)	7.4 (7.1–7.6)	—
	+(6)	98 (79–122)	—	—	—	—	—	—	—	13.2 (11.9–15.2)	—	—	2.4 (1.3–3.2)
$HGPRT_1$	−(4)	—	—	—	—	—	—	—	—	—	1.4 (1.1–2.1)	10.9 (10.7–11.2)	—
	+(6)	99 (92–103)	—	22 (4–42)	—	—	—	—	—	11.9 (10.3–12.7)	—	—	0.79 (0.60–1.2)
$HGPRT_2$	−(5)	116 (100–128)	6.4 (4–8)	—	—	—	—	13.9 (9–24)	—	—	3.6 (2.9–5.0)	8.2 (6.7–9.1)	—
	+(6)	72 (50–100)	—	535 (491–582)	77 (60–102)	—	—	—	—	7.3 (7.2–7.4)	—	—	1.04 (0.63–1.27)
PNP	−(6)	9.1 (4–15)	—	—	—	—	—	—	—	—	11.6 (13.9–15.3)	1.1 (0.81–1.63)	—
	+(6)	75 (68–83)	—	4.8 (2–8)	—	—	—	—	—	13.1 (11.3–14.5)	—	—	1.7 (0.48–3.66)
$APRT_1$	−(7)	832 (696–968)	119 (57–181)	—	—	14.3 (5–40)	44 (14–84,	159 (84–242)	13.6 (8–37)	—	2.7 (1.8–3.4)	7.8 (7.5–8.1)	—
	+(4)	27.5 (7–51)	—	42 (23–62)	—	—	—	—	—	9.9 (8.9–10.8)			1.8 (1.7–2.0)
$APRT_2$	−(6)	1382 (1263–1793)	157 (97–218)	—	—	—	22.5 (11–34)	424 (367–482)	14 (10–28)	—	3.1 (1.8–4.7)	7.7 (6.1–9.3)	—
	+(6)	17.3 (7–27)	—	30 (18–42)	—	—	—	—	—	13.9 (12.8–14.8)	—	—	1.3 (0.78–1.9)
CEM	−(6)	1102 (904–1212)	117 (79–144)	107 (58–288)	—	—	84 (28–150)	215 (190–263)	—	0.32 (0.2–0.44)	—	5.4 (4.9–5.6)	—
	+(6)	199 (101–366)	14 (6.7–55)	257 (160–359)	16 (2.8–31)	—	—	—	—	6.8 (6.5–7.3)	—	—	0.51 (0.38–0.98)

Results are the mean plus the range for the number of experiments (*n*) indicated. A, Adenine (derived from dAR degradation in the acid extraction step).

+, Incubated with 10 μM dCF; −, no inhibitor; —, below the limits of detection for the method.

The results are compared with a T-cell line CEM. Note the different units (pmole/mg protein per 2 hr) for the cells, compared with nmole/mg protein per 2 hr for the medium, reflecting the low incorporation into the cellular fraction seen in Fig. 1.

of radiolabel in H was due to the lability of dHR in the acid extraction conditions.) The absence of counts in H or dHR in all lines using dCF, and the accumulation of label in the medium exclusively in unmetabolized dAR, confirmed the effectiveness of dCF as an ADA inhibitor.

Within the cells with intact pathways for the catabolism and salvage of dAR, counts were found predominantly in ATP or GTP (ratio 4:1 approximately), but some counts were also found in IMP and NAD^+. The virtual absence of counts in either IMP or GTP in the PNP and completely HGPRT deficient line, and their accumulation in dHR or H respectively, confirmed dAR degradation via ADA and PNP, with recycling via HGPRT, as the route of origin of the label in ATP and GTP in cells with intact pathways.

This also explains the almost complete disappearance of label from these nucleotides following ADA inhibition with dCF. Nevertheless, in all lines some counts were still found in ATP. The fact that similar amounts of ATP were formed by the two APRT as well as the PNP deficient lines would exclude the potential degradation of dAR via adenine by a nucleoside phosphorylase with subsequent conversion to ATP by APRT. These results, together with the finding of significant amounts of label in ATP in the ADA deficient and completely HGPRT deficient line, would appear to exclude known routes of ATP formation.

Most lines synthesized some dATP from dAR with the ADA inhibitor dCF, although in the case of the PNP deficient line the level was extremely low. Curiously, the partially deficient HGPRT line formed more dATP than any other cell line, even the T-cell line, with dCF.

Metabolism of dGR with and without the PNP inhibitor 8-NH_2GR

Studies were restricted to five enzyme deficient lines compared with one T-cell line (CEM) because of the limited availability of [8-^{14}C]dGR. However, using dGR, even in cell lines with normal pathways of deoxynucleoside catabolism and salvage total cellular incorporation of radiolabel was much lower than with dAR (Fig. 2). The inhibitor did not alter this pattern significantly, except in the case of the partially deficient line $HGPRT_2$ which showed increased incorporation of radiolabel with the inhibitor. In all lines the % incorporation into the insoluble fraction (6.4–37%) was not altered greatly by the presence of the inhibitor (Fig. 1).

The metabolic studies (Table 2) again help to provide a picture of the above sequence of events. In cells with intact pathways, counts within the cell were found mostly in GTP, and this pattern was not changed by the inhibitor. In the medium, counts in CEM were predominantly in guanine (G) with dGR alone, but predominantly in dGR with the inhibitor. For the PNP deficient line, counts were found almost exclusively in dGR and the only counts in any nucleotide were found in dGTP. (The very small number of counts in guanine was due to dGR degradation in the acid extraction step.) CEM was the only other line to form significant amounts of dGTP without inhibitor.

It is noteworthy that in all the enzyme deficient lines (apart from $HGPRT_2$ where the ratio was reversed) counts in the medium were found predominantly in xanthine, as compared with guanine for CEM. Counts in xanthine were reduced by the inhibitor, with the majority now being found in unmetabolized dGR. Nevertheless, the nucleotide pattern did not

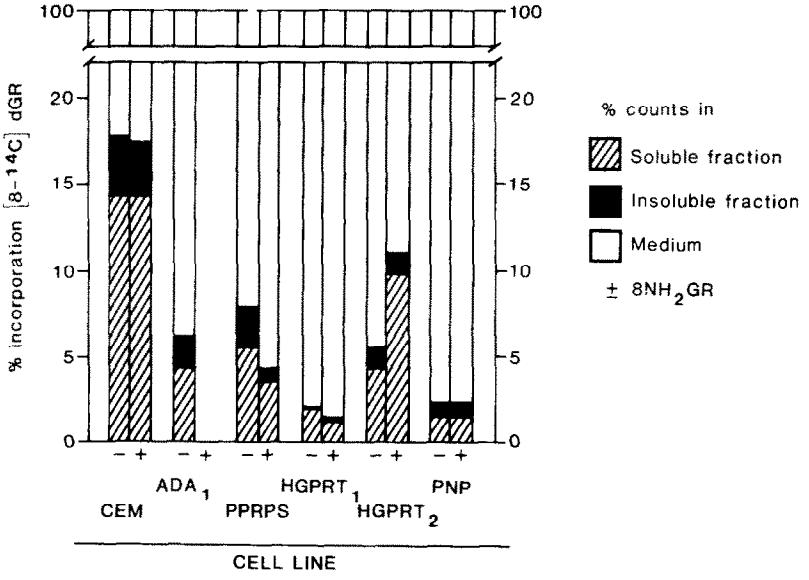

Fig. 2. Percentage incorporation of radiolabelled dGR (25 μM) in 2 hr into the soluble (hatched bars) and insoluble (closed bars) cellular fractions relative to the counts remaining in the medium (open bars) in six different cell lines with (+) and without (−) the PNP inhibitor 8-NH_2GR (210 μM).

Table 2. Metabolism of radiolabelled dGR by five of the different enzyme deficient lines described in Table 1 compared with the T-cell line CEM

Cell line	*n*	Soluble fraction (pmole/mg protein per 2 hr)					Medium (nmole/mg protein per 2 hr)		
		GTP	GDP	dGTP	IMP	ATP	dGR	G	X
PNP	±(10)	—	—	22 (7–30)	—	—	14.1 (12.8–15.3)	0.81 (0.93–1.24)	—
$HGPRT_1$	−(6)	—	—	—	—	—	3.1 (2.5–4.08)	4.1 (3.4–5.2)	5.0 (3.6–6.9)
	+(5)	—	—	—	—	—	10.7 (9.9–11.4)	0.80 (0.23–0.99)	2.3 (1.4–3.3)
$HGPRT_2$	−(8)	155 (22–194)	20 (17–24)	—	—	11 (6.1–17.0)	2.8 (2.7–3.2)	5.4 (4.8–6.2)	1.4 (1.2–1.5)
	+(6)	430 (325–671)	34 (17–62)	166 (105–251)	—	40 (2.5–83)	7.6 (7.4–7.9)	1.8 (1.6–2.1)	—
PPRPS	−(8)	357 (304–426)	84 (31–94)	—	22 (9.0–56)	7.0 (3.9–12)	3.3 (2.9–3.8)	1.5 (1.1–1.7)	7.6 (5.7–8.6)
	+(4)	141 (122–170)	—	—	—	10.1 (0.9–11.1)	0.52 (0.34–0.69)	2.6 (2.1–2.9)	+(4)
ADA	−(6)	381 (275–507)	50 (39–86)	—	10.3 (5.7–12.1)	6.6 (0.8–13)	2.6 (2.4–2.8)	1.4 (0.86–2.2)	12.0 (11.0–12.6)
CEM	−(7)	622 (422–822)	138 (83–223)	61 (15–172)	—	47 (12–100)	—	7.1 (6.8–7.5)	—
	+(5)	612 (388–919)	(34–120)	110 (33–172)	—	58 (15–96)	3.7 (3.2–4.7)	2.3 (1.1–2.5)	—

The results given are the mean (and range) for the number of experiments (*n*) indicated. +, In the presence of 210 μM 8-NH_2GR: −, no inhibitor; —, below the limits of detection for the method.

Note the different units for cells (pmole) as compared with medium (nmole) reflecting the small percentage of counts found within the cell (Fig. 2).

alter significantly with the inhibitor, except in $HGPRT_2$ which again showed a curious pattern in that it now incorporated counts into dGTP but formed no xanthine. The levels of dGTP attained were even higher than those formed by the T-cell line CEM. However, none of the other enzyme deficient lines formed any dGTP, with or without inhibitor, apart from the PNP deficient line.

The fact that (except in the PNP and HGPRT deficient lines), most of the counts were still found in GTP, with or without inhibitor, confirmed our previous studies in normal cell lines showing 8-NH_2GR to be a poor PNP inhibitor.* The results in the PNP and HGPRT deficient lines demonstrated that the GTP is derived from dGR by prior degradation to guanine. However, the presence of some counts in ATP and IMP also indicate the existence of a route for the conversion of guanine to adenine nucleotides in mammalian cells.

DISCUSSION

The present studies were undertaken to determine whether further insight into the underlying mechanisms of lymphospecific cytotoxicity associated with the absence of two sequential enzymes of purine catabolism [8–19] could be gleaned from detailed metabolic studies using cultured lymphocytes. Current concepts based on such studies favour the accumulation of dATP or dGTP in T- as distinct from B-lymphocytes, with subsequent arrest of cell division due to inhibition of ribonucleotide reductase [21].

Recent studies *in vivo* by us [22, 23] together with *in vitro* studies by others [29–32] have questioned these concepts. Many such *in vitro* studies may be criticized in that they have focused almost exclusively on cell viability in the long-term (72 hr) relative to dATP (or dGTP) accumulation in the short-term, sometimes only the former [20]. Moreover, there is considerable inconsistency in the results obtained [8–20] and frequently as few as two lines have been compared. For example, dATP levels of 600 pmole/10^6 cells were noted in 30 min using EHNA to inhibit dAR metabolism [10], but only after 24 hr [9] by others using 5 μM dCF. Substrate levels, using either dAR or dGR, have also varied widely (range 1 ≈ 750 μM [20]. Only one study with dGR has used a PNP inhibitor and again only one T- and B-cell lines were compared [33]. The possibility that any toxicity demonstrated could equally result from metabolism of either dAR or dGR by other routes has not been considered in most cases.

For the above reasons, we carried out detailed metabolic studies in cultured lymphocytes with intact purine pathways*, utilizing sensitive methods coupling simultaneous radiodetection with rapid and efficient separation of nucleotides, nucleosides and bases [28]. The first problem encountered was that only dCF completely inhibited ADA. With EHNA at concentrations normally employed (10 μM) 50% of dAR was still metabolized by ADA, predominantly to ATP. The PNP inhibitor 8-NH_2GR likewise in this intact cell system did not alter the amount of GTP formed from dGR, and was seemingly an ineffective inhibitor.* There were other inconsistencies, such as the fact that two of the four B-cell lines studied formed amounts of dATP or dGTP comparable to those formed by a T-cell line.* Since these two lines were of malignant origin, we speculated that the ability of cells to accumulate toxic deoxynucleotides could be related to an altered metabolism, associated with the process of malignant transformation, rather than the original cell type.* This would also explain the finding by others that null cells of leukaemic origin, with an enzyme pattern quite different from T-cells, also accumulate high dATP levels [31].

In the present report we have utilized lines from our patients deficient in several steps of the purine recycling process [22–26] in an attempt to answer some of the questions posed by the above studies. We now confirm the ability of a third B-cell line (which is also clearly unusual) to accumulate high levels of dATP and dGTP. The high level of counts found in ATP and GTP (ratio approximately 4:1), or in H or dHR in lines incapable of metabolizing these purines further, confirmed deamination of dAR and metabolism via these intermediates as the principal route of ATP formation. It also demonstrates that hypoxanthine salvage is a significant source of GTP as well as ATP in both T- and B-cell lines. The finding of some counts in NAD^+ indicates active synthesis from ATP. However, the fact that in the presence of dCF some ATP was still formed by *all* the different enzyme deficient lines would exclude known routes of ATP formation, and other explanations must be sought.

The finding of low percentage of counts in ATP and IMP using dGR confirms the existence of pathways for the conversion of GTP to ATP via IMP in mammalian cells, but this pathway does not seem to be of significance in T- or B-cells. It is noteworthy that in lines with intact salvage pathways counts in GTP were again not reduced by the inhibitor, which is thus an inappropriate model for the inherited disorder. The above observations relating to nucleotide levels were confirmed in all instances by the accumulation of the substrate in the appropriate intermediate in the different lines deficient in specific enzymes.

These studies in the enzyme deficient lines have also provided interesting data regarding nucleoside and base metabolism. The fact that dAR is rapidly taken up (except by the ADA deficient lines), completely metabolized, with 70% or more being excreted back into the medium—partly as dHR, while some unmetabolized dGR was always recovered in the medium using the same lines—confirms that ADA is more active than PNP in these B-cell lines given the same amount of substrate. The behaviour of some of the enzyme-deficient lines in forming significant amounts of xanthine from dGR is curious, particularly in view of the report that human lymphocytes apparently lack the enzyme guanase [34]. However, it is noteworthy that xanthine formation occurred only in those lines where, or when, no detectable amounts of dGTP were formed.

The present studies demonstrate that even using as little as 25 μM substrate, a maximum of ≈ 30% of dAR (and less than this for dGR) is utilized by the

* Submitted for publication.

cell for nucleotide formation. The remainder will remain in the medium, or be metabolized by other routes to form purines, not deoxypurines, depending on the effectiveness of the inhibitor used to simulate inherited ADA or PNP deficiency. Clearly, substrate levels used in many studies have been well in excess of those either desirable or necessary. Moreover, the significance of studies comparing dAR toxicity alone or together with dCF must be questioned, since they could merely relate to the enhancement of ATP pools by dAR in the uninhibited situation. This is particularly relevent to the reciprocal relationship demonstrated between ATP and dATP if, as currently believed, the maintenance of adequate ATP levels is essential for the survival of resting, as well as dividing, lymphoid cells [35]. The effect of dGR on GTP levels likewise cannot be disregarded, especially when GTP formation was not influenced by 8-NH_2GR.

Further studies using lower substrate levels are planned to resolve the many points raised by these studies. Nevertheless, the observations are important for several reasons. First, they demonstrate the major routes of purine metabolism in T- and B-cells. Second, they confirm there is considerable variation in the ability of B-cells to form dATP and dGTP, and indicate that too few lines have been investigated in the past. Such limited studies as have been done, by chance, have been carried out in EB virus transformed normal lines where purine metabolism is different from that of malignant lines.

The fact that substrate levels have often been too high and consequently have significantly altered cellular ATP or GTP levels is particularly important when comparing deoxynucleoside toxicity over a 72 hr period with the ability to accumulate deoxynucleotides in short-term metabolic experiments. Our studies confirm that only dCF will effectively simulate the *in vivo* situation in ADA deficiency and there is no effective PNP inhibitor yet available—findings pertinent to all reports employing dGR, and to those with dAR using EHNA to inhibit ADA activity [10, 13, 35, 36].

The results concur with our unsuccessful attempts to alter the clinical course, or improve immunological parameters, in children with either ADA or PNP deficiency [22, 23] and cast further doubt on the ribonucleotide reductase hypothesis. New experiments may need to be devised to resolve many of these points and more studies in T-cells are essential.

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