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EHNA is a Poor Inhibitor of Deoxyadenosine Catabolism in Cultured Human Lymphocytes

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Erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) has been used by many workers as enzyme inhibitor *in vitro* to simulate the *in vivo* situation in inherited adenosine deaminase (ADA) deficiency. In this study the metabolism of 8-^{14}C deoxyadenosine (dAR) has been followed in cultured lymphocytes from patients deficient in enzymes associated with the catabolism and salvage of dAR, in the absence and presence of 10 μM EHNA. The results show that EHNA, at these concentrations, does not prevent the catabolism of dAR and thus does not provide a valid model for investigating the toxicity to the immune system in inherited ADA deficiency.

Key words: ADA, EHNA, Deoxyadenosine catabolism.

The genetic deficiency of adenosine deaminase (ADA, EC 3.5.4.4) is associated with impairment of both T- and B-cell functions (6). The reason for this selective toxicity to the immune system has been investigated by many workers using different *in vitro* systems. Most studies have focused on the potential toxicity of deoxyadenosine (dAR) which accumulates in this disorder (2) and is excreted in the urine (12).

This genetic defect has been simulated in various *in vitro* studies using two different ADA inhibitors: erythro-9-(2-hydroxy-3-nonyl)-adenine (EHNA) and 2′-deoxycoformycin (3, 7). The latter is currently being investigated *in vivo* in the treatment of patients with leukaemic disorders refractory to other forms of treatment (10). EHNA, a semi-tight inhibitor of ADA with a Ki for the human erythrocytic enzyme of 1.6×10^{-9}M (1) has been extensively used at low concentrations in studies related to dAR toxicity (5, 8, 15).

In the present study the effects of EHNA have been investigated at the concentration used by most workers (10 μM) in an intact cell system of cultured lymphocytes. The results show that in the different cell lines studied, 10 μM EHNA

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does not inhibit effectively the deamination of dAR by ADA.

Materials and Methods

Cell lines. The cell lines used in this study were derived from peripheral blood lymphocytes by EB virus transformation. They were obtained from patients with different inherited purine disorders during collaborative metabolic studies as indicated in parenthesis. PPRPS was from patient NB with aberrant PP-ribose-P synthetase activity (Dr. J. Wilson, Gt. Ormond Street, Hospital for Sick Children, London); $HGPRT_1$ was from GK a Lesh-Hyhan patient with complete hypoxanthine guanine phosphoribosyltransferase deficiency; $HGPRT_2$, from a partially HGPRT deficient patient CL (Prof. J. S. Cameron, Guy's Hospital, London); PNP was from SB with complete purine nucleoside phosphorylase deficiency (Dr. A. R. Watson, Royal Manchester Children Hospital); $APRT_1$ was from B. D'h with complete adenine phosphoribosyl transferase deficiency (Prof. K. J. Van Acker, University of Antwerp, The Netherlands). All lines were routinely checked, at monthly intervals, for mycoplasma contamination and were mycoplasma free.

Metabolism of deoxyadenosine by intact cells. For all studies cells in logarithmic growth phase were harvested 48 h after subculture and were resuspended in fresh complete medium (Iscove's or RPMI-1640 plus 10 % heat inactivated foetal calf serum).

Conditions were chosen to approach the physiological conditions as close as possible: 1 mM phosphate, plus substrate concentration of 25 μM; the latter was selected as being comparable to the lowest concentration used in most published studies. 8-^{14}C dAR (40.7 mCi/mmol) was purchased from New England Nuclear.

For the incubation studies, 40 μl of cell suspension containing 1×10^6 cells were preincubated in duplicate with 10 μl of fresh medium or of the ADA inhibitor EHNA (final concentration 10 μM) for 10 min prior to the addition of the radiolabelled substrate, in a 5 % CO_2/95 % air atmosphere at 37 °C; 50 μl of 8-^{14}C dAR were then added to produce a final concentration of 25 μM in the incubation medium (1 μCi/ml). Incubations were carried out for 2 h. Viability over the 2 h period was greater than 90 %.

Incubations were terminated by centrifugation at 400 *g* (1500 rpm) for 5 min at 4 °C. The 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 resuspended in 100 μl of cold 8 % TCA. Protein precipitate was removed at 12.000 *g* (Beckman Microcentrifuge) for 1 min and the two above mentioned supernatants were immediately extracted with water-saturated diethylether to a pH above 5. All manipulations were done on ice to minimize the acid breakdown of deoxycompounds. Samples were then stored at — 20 °C until analysed by high performance liquid chromatography (HPLC).

Chromatography. The HPLC system used has been described in detail elsewhere (4). For the separation of nucleosides and bases in the medium a reverse phase system was employed. 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 17 min was used. 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 lin-

ear gradient (gradient 6) increasing to 100 % B in 20 min.

The phosphate used was Aristar grade, the KCl, Analar grade, from BDH Chemicals. The methanol used was a special HPLC grade from Rathburn Chemical (Walkerburn, Scotland).

The HPLC system was a Trimodule System from Waters Associates (Cheshire, UK). 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 limited (Glasgow, Scotland).

Results and Discussion

Cell lines $APRT_1$, $HGPRT_1$, PNP and PPRPS were relatively uniform in size and contained from 0.14 to 0.18 mg protein/10^6 cells (mean of 7 determinations per cell type). However, $HGPRT_2$ cells were microscopically much larger, with 0.244 mg protein/10^6 cells. Thus, in order to eliminate any difference resulting from cell size, all results in the metabolic experiments (table I) were expressed on a protein basis.

The results of duplicate incubations with 8-^{14}C dAR in short-term experiments in 5 different enzyme deficient cell lines are compared in table I. In the absence of the ADA inhibitor EHNA, extracellular radioactivity was found mostly as hypoxanthine (H) and to a lesser degree as deoxyinosine (dHR), indicating complete deamination of the dAR. The only exception was the PNP deficient cell line where the majority of counts were found as dHR, due to its inability to cleavage the deoxynucleoside (the small number of counts in H were due to the lability of the dHR in the acid extraction step). Within the cells with pathways for the catabolism and salvage of dAR (PPRPS, $HGPRT_2$ and $APRT_1$) the counts were predominantly found as ATP, ADP and GTP, with minor incorporations into NAD^+ and IMP pools.

Addition of 10 μM EHNA to the incubation medium introduced some changes in the pattern of radiolabelled distribution (table I). The extracellular mediums contained variable amounts of dAR but the most important observation was that still up to 50 % of the counts were as dHR and H. In addition, in the cell lines with intact PNP and HGPRT activities, radiolabelled ATP, ADP, NAD^+, IMP and GTP were also detected. This confirms the fact that the deamination of dAR was only partially inhibited by 10 μM EHNA.

The results obtained with the $HGPRT_1$ cell line, which lacks the ability to recycle the purine base hypoxanthine derived from the catabolism of dAR, demonstrates unequivocally the ineffectiveness of ADA inhibition. Accumulation of 40 % of the counts in dHR in the PNP deficient cell line, which could not metabolize dHR any further, also supports the incomplete inhibition by 10 μM EHNA.

It is equally noteworthy that, despite the ineffectiveness of ADA inhibition, some dATP (in some instances significant amounts) was formed by all these EB virus derived B cell lines. This was also noted in separate studies using the effective ADA inhibitor, 2′-deoxycoformycin, and is discussed elsewere (11), as is the unexpected finding of some counts in ATP in both the PNP and completely HGPRT deficient cell lines.

The concentrations of dAR (25 μM) and EHNA (10 μM) used in this report were chosen because they are at the lower and higher limits, respectively, of the concentrations used in other published

Table I. *Metabolism of 8-^{14}C deoxyadenosine with (+) and without (—) 10 μM EHNA.*
Metabolism of radiolabelled dAR (25 μM) by five different cell lines (1×10^6 cells) incubated for 2 h at 37 °C with and without 10 μM EHNA. Counts found in the different (d)nucleotide pools in the soluble fraction within the cell are expressed in pmoles/mg protein/2 h, as compared with counts found as (d)nucleosides and bases in the medium, expressed in nmoles/mg protein/2 h. Results are the mean (± SD) for the number of experiments (n) indicated. dAR = deoxyadenosine; dHR = deoxyadenosine; H = hypoxanthine; — = < 1 pmol/mg protein/10^6 cells.

			Soluble fraction (pmoles/mg protein/2 h)						Medium (nmoles/mg protein/2 h)		
Cell line	EHNA	n	ATP	ADP	dATP	NAD+	IMP	GTP	dAR	dHR	H
PRPPS	—	6	485 ± 73	132 ± 32	—	33 ± 14	49 ± 19	103 ± 23	—	5.9 ± 0.5	7.4 ± 0.1
	+	4	562 ± 74	64 ± 27	64 ± 4.3	30 ± 19	49 ± 13	122 ± 24	6.3 ± 0.8	3.1 ± 0.3	3.0 ± 0.5
$HGPRT_1$	—	4	—	—	—	—	—	—	—	1.4 ± 0.3	10.9 ± 0.2
	+	4	30 ± 10	—	29 ± 7.0	—	—	—	5.1 ± 0.2	2.5 ± 0.2	3.9 ± 0.1
$HGPRT_2$	—	5	116 ± 9.0	6 ± 1.4	—	—	—	14 ± 4.0	—	3.6 ± 0.6	8.2 ± 0.7
	+	4	73 ± 22	3 ± 1.8	93 ± 38	—	—	3 ± 1.9	3.4 ± 0.2	2.5 ± 0.2	3.1 ± 0.5
PNP	—	5	9 ± 3.0	—	—	—	—	—	—	11.6 ± 1.1	1.1 ± 0.2
	+	6	60 ± 14	—	6 ± 3.0	—	—	—	8.1 ± 0.2	5.5 ± 0.2	0.2 ± 0.1
$APRT_1$	—	7	832 ± 78	119 ± 35	—	14 ± 5.0	44 ± 18	159 ± 32	—	2.7 ± 0.4	7.8 ± 0.2
	+	4	412 ± 86	44 ± 12	27 ± 9.0	10 ± 3.0	—	71 ± 16	6.2 ± 0.4	1.7 ± 0.2	3.1 ± 0.3

studies. Higher concentrations of the deoxynucleoside have been extensively used in dAR toxicity experiments together with low concentrations of EHNA (5-10 μM) to simulate *in vitro* ADA deficiency (5, 8, 14, 15).

Other workers using extracts from cultured lymphocytes, not intact cells, have also noted that EHNA was a poor inhibitor compared to 2'-deoxycoformycin (3). EHNA did not inhibit ADA completely even at the limits of its solubility (687 μM). The use of EHNA is based on early reports which indicated that it was an effective ADA inhibitor at concentrations as low as 5 μM (13, 15). Consequently many workers have used EHNA at this low concentration to inhibit ADA, despite the fact that at least one report has suggested that the concentration of EHNA needed to inhibit ADA in intact lymphocytes may be ten fold that for homogenates (7).

This is of particular relevance to recent studies such as those demonstrating that micromolar concentrations of dAR with 5 μM EHNA were toxic to an earlier phase (G_1) of the cell cycle in cultured leukaemic T-cells (5), besides the fact that this combination was toxic to non-dividing lymphoid cells, including peripheral blood lymphocytes (8). Such findings are of importance since they question the concept that toxicity to the immune system in inherited ADA deficiency relates exclusively do dATP accumulation, with subsequent inhibition of ribonucleotide reductase and hence cell division.

It has been previously noted in a system using intact erythrocytes (9) that EHNA was a much more effective inhibitor of ADA when using adenosine compared with dAR as substrate. The present studies show that it is vital to check the effectiveness of any inhibitor with the appropriate substrate in the system to be used, particularly when using intact cells. The results confirm the finding that any study relating to deoxynucleoside toxicity in cultured lymphocytes using EHNA as ADA inhibitor is not an effective model for the *in vivo* situation in ADA deficiency. Consequently they may well be monitoring an effect resulting from the metabolism and accumulation of the substrate in other purine, rather than deoxypurine pools (11).

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Resumen

Los inhibidores enzimáticos eritro-9(2-hidroxi-3-nonil) adenina (EHNA) y 2'-desoxicoformicina han sido utilizados por diversos autores para la reproducción *in vitro* de la deficiencia congénita de adenosín desaminasa (ADA). En el presente estudio sigue el metabolismo de la 8-C^{14} desoxiadenosina (dAR) en ausencia y presencia de EHNA, 10 μM, en linfocitos en cultivo continuo procedentes de pacientes deficientes en enzimas implicados en el catabolismo y reciclaje de la dAR. Los resultados obtenidos muestran que el EHNA, 10 μM, no impide el catabolismo de la dAR, no siendo, por lo tanto, un modelo apropiado para el estudio de la toxicidad presentada por el sistema inmune en la deficiencia de ADA.

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