Assay method for monitoring the inhibitory effects of antimetabolites on the activity of inosinate dehydrogenase in intact human CEM lymphocytes

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A rapid and convenient method has been developed to monitor the inhibition of inosinate (IMP) dehydrogenase by antimetabolites in intact human CEM lymphocytes. This method is based on the determination of ^3H release from [2,8- ^3H]hypoxanthine ([2,8- ^3H]Hx) or [2,8- ^3H]inosine ([2,8- ^3H]Ino). The validity of this procedure was assessed by evaluating IMP dehydrogenase inhibition in intact CEM cells by the well-known IMP dehydrogenase inhibitors ribavirin, mycophenolic acid and tiazofurin. As reference materials, several compounds that are targeted at other enzymes in *de novo* purine nucleotide anabolism (i.e. hadacidine, acivicin) or catabolism (i.e. 8-aminoguanosine, allopurinol) were evaluated. There was a strong correlation between the inhibitory effects of the IMP dehydrogenase inhibitors (ribavirin, mycophenolic acid, tiazofurin) on ^3H release from [2,8- ^3H]Hx and [2,8- ^3H]Ino in intact CEM cells and their ability to decrease intracellular GTP pool levels. The other compounds (hadacidine, acivicin, 8-aminoguanosine, allopurinol) had no marked effect on ^3H release from [2,8- ^3H]Hx. Using this method, we demonstrated that the novel ribavirin analogue, 5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide, is a potent inhibitor of IMP dehydrogenase in intact cells.

INTRODUCTION

Inosinate (IMP) dehydrogenase (EC 1.1.1.205) catalyses the oxidation of IMP to xanthylate (XMP), an intermediate metabolite in the synthesis of GTP. During the reaction, the hydrogen atom located on the C-2 of the hypoxanthine ring is replaced by an hydroxy group. NAD^+ serves as the electron acceptor, and is reduced to $\text{NADH} + \text{H}^+$:

IMP dehydrogenase should be considered as a key enzyme in the *de novo* biosynthesis of purine nucleotides, in particular GMP. Its activity seems to be elevated in all neoplastic cells that have been examined so far [1,2]. Therefore IMP dehydrogenase may be considered as a useful target enzyme in cancer chemotherapy.

An example of a compound targeted at IMP dehydrogenase is tiazofurin, a C-nucleoside with marked cytotoxicity *in vitro* that has proved effective in inducing clinical responses in leukaemic patients [3]. The active metabolite of tiazofurin is TAD (tiazole carboxamide adenine dinucleotide), which is thought to interact with the NADH site of the enzyme. Ribavirin, a broad-spectrum antiviral agent that has proved effective against a number of DNA and RNA viruses *in vitro* [4], and has been investigated clinically in the treatment of influenza A and B viruses, respiratory syncytial virus, parainfluenza virus and a variety of exotic virus infections [5,6], also inhibits IMP dehydrogenase after it has been converted to its 5'-monophosphate by adenosine kinase. The decrease in the intracellular GTP and dGTP pools following ribavirin treatment is assumed to be the primary reason for its broad-spectrum antiviral activity and cytotoxicity. Thus compounds that are active as inhibitors of IMP dehydrogenase may be of potential utility as either antiviral or cytostatic compounds, or both.

We have now developed a useful, convenient and sensitive strategy for measuring IMP dehydrogenase activity, and the

inhibitory effects of antimetabolites thereupon, in intact human lymphocyte (CEM) cells.

EXPERIMENTAL

Cells

Human lymphocyte CEM cells and the hypoxanthine/guanine phosphoribosyltransferase (HGPRT)-deficient CEM/HGPRT⁻ cells (kindly provided by Dr. S. Erikson and Dr. A. Karlsson, Karolinska Institute, Stockholm, Sweden) were grown in 75 cm² tissue culture flasks (Falcon 3024F; Becton-Dickinson France S.A., Grenoble, France) in RPMI-1640 medium supplemented with 10% (v/v) inactivated fetal calf serum (Gibco Bio-Cult, Glasgow, Scotland, U.K.), 2 mM-L-glutamine (Flow Laboratories, Irvine, Scotland, U.K.) and 0.075% NaHCO_3 (Flow Laboratories).

Chemicals

Ribavirin (Virazole) was obtained from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). EICAR (5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide) was synthesized by A. Matsuda (Hokkaido University, Sapporo, Japan). 8-Aminoguanosine, mycophenolic acid, tiazofurin and allopurinol were kindly provided by Dr. D. Cooney and Dr. D. G. Johns (National Cancer Institute, NIH, Bethesda, MD, U.S.A.). Hadacidin was kindly provided by Dr. B. Ullman (Oregon Health Sciences University, Portland, OR, U.S.A.) and Dr. J. S. Driscoll (National Cancer Institute), and acivicin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Dr. R. A. De Abreu (Academisch Ziekenhuis, Nijmegen, The Netherlands). Hypoxanthine and inosine were obtained from Sigma Chemical Co.

Radiochemicals

The radiolabelled precursors [2,8- ^3H]hypoxanthine ([2,8- ^3H]Hx) and [2,8- ^3H]inosine ([2,8- ^3H]Ino) (specific radioactivity

Abbreviations used: TAD, tiazole carboxamide adenine dinucleotide; HGPRT, hypoxanthine/guanine phosphoribosyltransferase; EICAR, 5-ethynyl-1- β -D-ribofuranosylimidazole-4-carboxamide; Hx, hypoxanthine; Ino, inosine; PRPP, phosphoribosyl pyrophosphate.

22 and 25 Ci/mmol respectively) were obtained from Moravex Pharmaceuticals, Brea, CA, U.S.A. [$G-^3H$]Hx (3.4 Ci/mmol) was from Amersham Belgium, Gent, Belgium.

3H release from [2,8- 3H]Hx and [2,8- 3H]Ino

The activity of IMP dehydrogenase in the intact CEM/0 and CEM/HGPRT⁻ cells was measured by estimation of 3H release from [2,8- 3H]IMP that had been formed in the cells from [2,8- 3H]Hx or [2,8- 3H]Ino in the reaction catalysed by IMP dehydrogenase. Cells were collected by centrifugation at 300 *g* for 6 min, washed and resuspended in fresh RPMI-1640 culture medium without serum at a cell density of 2.5×10^6 cells/ml; 300 μ l of this cell suspension was taken into a plastic 13 ml tube (Falcon) together with 60 μ l of RPMI-1640 medium and 40 μ l (4 μ Ci) of radiolabelled Hx or Ino. At various times (i.e. 0, 5, 10, 20 and 30 min) (as indicated in the Figures), 100 μ l of the reaction mixture was withdrawn and mixed with 250 μ l of a freshly prepared cold suspension of Carbon Black (UCB, Leuven, Belgium) (100 mg/ml) in 5% trichloroacetic acid. After centrifugation at 1100 *g* for 10 min, 200 μ l samples of the supernatants were analysed for radioactivity. The amount of free 3H in the reaction mixture (before incubation with the cells) was less than 10% and 2% of the amount of labelled Hx and Ino added respectively. Since this amount did not change during incubation in the absence of cells, it was subtracted from the values obtained at different times of incubation, so as to obtain net values for the amount of 3H released enzymically by IMP dehydrogenase.

Inhibition of IMP dehydrogenase by test compounds in intact CEM cells

To evaluate the inhibitory effects of the test compounds on IMP dehydrogenase activity in intact CEM cells, essentially the same procedure was followed as described above. After preparation of the cells (2.5×10^6 /ml) in serum-free RPMI-1640 medium, 300 μ l of the cell suspension was taken into plastic 13 ml tubes together with 20 μ l of RPMI-1640 medium and 40 μ l of an appropriate amount of test compound (as indicated in the Figures), and the incubation with radiolabelled precursor (40 μ l) was started immediately after addition of the test compounds. At 10, 20 or 30 min, 100 μ l of the reaction mixture was withdrawn and the amount of released 3H was determined as described above.

Some test compounds were also evaluated for their inhibitory effects on 3H release by IMP dehydrogenase for longer incubation times with the CEM/0 cells. Briefly, 5 ml cell cultures (5×10^5 cells/ml) were incubated in the presence of appropriate concentrations of the test compounds. Immediately after addition of the test compounds, 0.5 ml of the cell culture was withdrawn, added to test tubes containing 1 μ Ci of [2,8- 3H]Hx (final Hx concentration 0.4 μ M), and further incubated at 37 °C. After a 30 min incubation period, a cold suspension of Carbon Black was added to precipitate cells and unreacted [2,8- 3H]Hx. After centrifugation, the amount of released 3H was determined. At 2, 4 and 6 h after initiation of the incubation of the CEM cells with the test compounds, the amount of released 3H was determined as described above.

Preparation of CEM cell extracts and determination of GTP pools by h.p.l.c.

The procedure for determining intracellular GTP pools has been described previously [7,8]. Briefly, 5×10^5 CEM cells/ml were incubated with various concentrations of ribavirin, mycophenolic acid, tiazofurin or EICAR for 2 h or 6 h in cell culture bottles containing 5 ml of cell suspension. Then the cells were centrifuged, washed with RPMI-1640 medium without serum

and L-glutamine, and precipitated with ice-cold methanol (60%). After standing for 10 min on ice, the precipitate was removed by centrifugation at 3000 *g* and the supernatants were analysed by h.p.l.c. using a Partisphere SAX-10 column. A linear gradient of 7 mM-(NH₄)H₂PO₄ (pH 3.8) (buffer B) to 250 mM-(NH₄)H₂PO₄ + 50 mM-(KCl) (pH 4.5) (buffer C) was used to separate GTP from the other nucleotides. The different successive chromatography steps were as follows: 10 min of 100% buffer B; a 10 min linear gradient from 100% buffer B to 60% buffer B + 40% buffer C; a 5 min linear gradient from 60% buffer B + 40% buffer C to 100% buffer C; 15 min of 100% buffer C; a 3 min linear gradient from 100% buffer C to 100% buffer B; and 5 min of buffer B to equilibrate the column. The retention time of GTP was 37.1 min.

Incorporation of [G- 3H]Hx in purine nucleotide pools of CEM cells

CEM cell cultures (5×10^6 cells/ml; 5 ml) were incubated for 5 h in the presence of 0.75 μ M-[G- 3H]Hx and various concentrations of EICAR (i.e. 0, 0.2, 0.5, 2.0 and 5.0 μ M). Then cell extracts were prepared as described above and radiolabelled IMP, GTP, AMP, ADP and ATP were separated on a Partisphere SAX-10 column using a linear gradient of buffer B to buffer C. The different chromatographic steps were as follows: 10 min of 80% water + 20% buffer B; a 5 min linear gradient to 100% buffer B; 15 min of 100% buffer B; a 20 min linear gradient to 60% buffer B + 40% buffer C; a 5 min linear gradient to 100% buffer C; 18 min of 100% buffer C; a 5 min linear gradient to 80% water + 20% buffer B; 5 min of 80% water + 20% buffer B. The retention times of IMP, GTP, AMP, ADP and ATP were 21.6, 70.8, 3.9, 50.5 and 63.7 min respectively. The radiolabelled fractions were assessed for radioactivity in the presence of HIONIC-FLUOR (Packard, Groningen, The Netherlands).

RESULTS

3H release from [2,8- 3H]Hx and [2,8- 3H]Ino in intact CEM/0 and CEM/HGPRT⁻ cells

3H release from [2,8- 3H]Hx was proportional to the time of incubation of 0.4 μ M-[2,8- 3H]Hx with CEM/0 cells (Fig. 1). When 10^6 CEM cells/ml were used, 3H was released linearly for up to 20 min, after which 3H release quickly levelled off. With 2×10^6 or 5×10^6 CEM cells/ml, the reaction proceeded linearly for up to 30 min before levelling off. However, 3H release was not proportional to cell density. With 5×10^6 cells/ml, only twice as much 3H was released as with 10^6 cells/ml after 30 min of incubation (Fig. 1). There was no marked difference between the amount of 3H released from [2,8- 3H]Hx and [2,8- 3H]Ino after a 20 min incubation period with CEM/0 cells, while [2,8- 3H]Ino released a slightly greater amount of 3H after 30 min incubation than did [2,8- 3H]Hx (Fig. 2). The amount of 3H released from [2,8- 3H]Hx and [2,8- 3H]Ino in CEM/HGPRT⁻ cells (CEM cells deficient in hypoxanthine/guanine phosphoribosyltransferase) was less than 5% or 1% of that released from [2,8- 3H]Hx or [2,8- 3H]Ino in the wild-type CEM/0 cells respectively.

Effect of the initial Hx and Ino concentration on 3H release from [2,8- 3H]Hx and [2,8- 3H]Ino

3H release from [2,8- 3H]Hx and [2,8- 3H]Ino in CEM/0 cells was examined as a function of different input concentrations of the compounds (i.e. 0.4, 2.4, 5.4, 10.4, 20.4 and 50.4 μ M), keeping the initial radiolabelled input constant. When only radiolabelled precursor was used, the initial Hx or Ino concentration was 0.4 μ M. The higher the input doses of unlabelled Hx and Ino, the lower the amounts of 3H released from [2,8- 3H]Hx and [2,8-

Fig. 1. ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ as a function of incubation time of CEM/0 cells with $0.4\text{ }\mu\text{M}$ - $[2,8\text{-}^3\text{H}]\text{Hx}$

CEM/0 cell density: 10^6 cells/ml ($\blacktriangledown$); 2×10^6 cells/ml ($\blacktriangle$); 5×10^6 cells ($\bullet$). Data are means of two independent experiments.

Fig. 2. ^3H release from $0.4\text{ }\mu\text{M}$ - $[2,8\text{-}^3\text{H}]\text{Hx}$ and $0.4\text{ }\mu\text{M}$ - $[2,8\text{-}^3\text{H}]\text{Ino}$ as a function of incubation time

CEM/0 ($\circ$, $\triangle$) or CEM/HGPRT $^-$ ($\bullet$, $\blacktriangle$) cells were incubated with either $[2,8\text{-}^3\text{H}]\text{Hx}$ ($\circ$, $\bullet$) or $[2,8\text{-}^3\text{H}]\text{Ino}$ ($\triangle$, $\blacktriangle$). Data are means of two independent experiments.

$^3\text{H}]\text{Ino}$ (results not shown). The results obtained for ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ were very similar to those obtained with $[2,8\text{-}^3\text{H}]\text{Ino}$. When the amounts of ^3H released from Hx or Ino were plotted as a function of substrate concentration, Michaelis-Menten kinetics were noted. Irrespective of the time point taken, i.e. 10, 20 or 30 min (results not shown), the K_m value for the conversion of Hx and Ino to XMP was between 1 and $2\text{ }\mu\text{M}$. This value is ~ 10 -fold lower than the K_m value of IMP with IMP dehydrogenase ($K_m\text{ }20\text{ }\mu\text{M}$) under cell-free experimental conditions [9].

Effect of antimetabolites on IMP dehydrogenase activity in CEM/0 cells

A number of antimetabolites, i.e. ribavirin, mycophenolic acid, tiazofurin, EICAR, hadacidin, acivicin, allopurinol and 8-aminoguanosine, were evaluated for their inhibitory effects on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ and $[2,8\text{-}^3\text{H}]\text{Ino}$ in CEM/0 cells (Fig. 3; Tables 1 and 2). Mycophenolic acid and ribavirin proved to be potent inhibitors of IMP dehydrogenase in intact CEM

Fig. 3. ^3H release from $0.4\text{ }\mu\text{M}$ - $[2,8\text{-}^3\text{H}]\text{Hx}$ in CEM/0 cells in the presence of different concentrations of ribavirin (a) and mycophenolic acid (b)

Ribavirin concentrations were $50\text{ }\mu\text{M}$ ($\bullet$), $20\text{ }\mu\text{M}$ ($\ast$), $10\text{ }\mu\text{M}$ ($\blacktriangle$), $5\text{ }\mu\text{M}$ ($\blacktriangledown$), $2\text{ }\mu\text{M}$ ($\square$) and $0\text{ }\mu\text{M}$ ($\circ$). Mycophenolic acid concentrations were $20\text{ }\mu\text{g/ml}$ ($\bullet$), $5\text{ }\mu\text{g/ml}$ ($\ast$), $2\text{ }\mu\text{g/ml}$ ($\blacktriangle$), $0.5\text{ }\mu\text{g/ml}$ ($\blacktriangledown$) and $0\text{ }\mu\text{g/ml}$ ($\circ$). Data are means of three independent experiments.

cells when their inhibitory effects were measured immediately after their addition to the cells. The inhibition was clearly concentration-dependent (Fig. 3). Mycophenolic acid at $20\text{ }\mu\text{g/ml}$ and ribavirin at $50\text{ }\mu\text{M}$ inhibited ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ by 80 and 95% respectively. The 50% inhibitory concentrations (IC_{50}) were $9.6\text{ }\mu\text{M}$ for ribavirin and $1.8\text{ }\mu\text{M}$ for mycophenolic acid (Fig. 3). In contrast, hadacidin (an inhibitor of adenylosuccinate synthetase) and acivicin (an inhibitor of GMP synthetase) (Scheme 1) had no marked effect on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ or $[2,8\text{-}^3\text{H}]\text{Ino}$ (Table 1), even when tested at concentrations (50 and $5\text{ }\mu\text{g/ml}$ respectively) that had strong cytostatic effects in CEM cells (results not shown). Interestingly, 8-aminoguanosine did not cause much inhibition of ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$, but strongly inhibited release from $[2,8\text{-}^3\text{H}]\text{Ino}$, the latter effect being nicely concentration-dependent within the concentration range 2– $100\text{ }\mu\text{g/ml}$ (Table 1). Whereas tiazofurin had no inhibitory effect on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ at a concentration as high as $100\text{ }\mu\text{M}$, EICAR inhibited ^3H release by 50% at a concentration of $18.5\text{ }\mu\text{M}$ (Table 2).

When the test compounds were preincubated with CEM cells before the addition of $[2,8\text{-}^3\text{H}]\text{Hx}$, their inhibitory effects on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ became more pronounced (Table 2). The

Scheme 1. Metabolic pathways of purine nucleotide metabolism

Abbreviations used: XO, xanthine oxidase; 5'-Nu, 5'-nucleotidase; PNP, purine nucleoside phosphorylase; Xa, xanthine; Xo, xanthosine; S-AMP-L, S-AMP lyase; S-AMP-S, S-AMP synthetase; IMP-D, IMP dehydrogenase; GMP-S, GMP synthetase.

Table 1. Inhibitory effect of various antimetabolites on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ and $[2,8\text{-}^3\text{H}]\text{Ino}$ in CEM/0 cells

The initial concentrations of $[2,8\text{-}^3\text{H}]\text{Hx}$ and $[2,8\text{-}^3\text{H}]\text{Ino}$ were $0.4\text{ }\mu\text{M}$. ^3H release was measured after a 10 min incubation period. The cells were not preincubated with the test compounds

Compound	Concentration	^3H release (c.p.m.) from	
		$[2,8\text{-}^3\text{H}]\text{Hx}$	$[2,8\text{-}^3\text{H}]\text{Ino}$
None (control)	—	26280	23500
Ribavirin	$50\text{ }\mu\text{M}$	5039	3500
	$20\text{ }\mu\text{M}$	9133	8000
	$10\text{ }\mu\text{M}$	14512	11510
	$5\text{ }\mu\text{M}$	18678	16602
	$2\text{ }\mu\text{M}$	22691	20401
Mycophenolic acid	$20\text{ }\mu\text{g/ml}$	1291	1185
	$5\text{ }\mu\text{g/ml}$	5092	2818
	$2\text{ }\mu\text{g/ml}$	11035	5341
	$0.5\text{ }\mu\text{g/ml}$	16638	18127
Hadacidin	$50\text{ }\mu\text{g/ml}$	30037	25392
	$20\text{ }\mu\text{g/ml}$	31867	25544
Acivicin	$5\text{ }\mu\text{g/ml}$	28942	23234
	$2.5\text{ }\mu\text{g/ml}$	29751	20774
8-Aminoguanosine	$100\text{ }\mu\text{g/ml}$	17080	785
	$20\text{ }\mu\text{g/ml}$	23745	3810
	$5\text{ }\mu\text{g/ml}$	—	8416
	$2\text{ }\mu\text{g/ml}$	—	12749

inhibitory effect of mycophenolic acid increased by 4–5-fold if preincubated with the CEM/0 cells for 4–6 h, whereas for ribavirin such increase in activity was already apparent after a 2 h preincubation period. Also, the inhibitory effect of tiazofurin on ^3H release increased by more than 10-fold after a 2 h preincubation period, and did not increase further upon longer preincubation periods with the cells. EICAR showed a 17-fold increase in inhibitory activity if preincubated with the cells for 2 h, and a 33-fold increase in activity when this preincubation was extended to 4–6 h (Table 2). Under the experimental conditions used, EICAR proved to be a 20-fold more potent

Table 2. Effects of test compounds on ^3H release from $[2,8\text{-}^3\text{H}]\text{Hx}$ in CEM/0 cells following various preincubation times

Except for the 6 h preincubation period (data taken from a single experiment), data represent means from three independent experiments. The concentration of $[2,8\text{-}^3\text{H}]\text{Hx}$ was $0.4\text{ }\mu\text{M}$. $[2,8\text{-}^3\text{H}]\text{Hx}$ was exposed to the drug-treated CEM cells for 30 min.

Compound	Preincubation time (h)...	IC_{50} (μM)			
		0	2	4	6
Mycophenolic acid		1.8	1.3	0.46	0.43
Ribavirin		9.6	1.0	0.73	1.1
Tiazofurin	≥ 100	10	8.2	10	
EICAR		18.5	1.1	0.56	0.56

Fig. 4. Correlation between intracellular GTP levels and the amount of ^3H released from $0.74\text{ }\mu\text{M}$ - $[\text{G-}^3\text{H}]\text{Hx}$

CEM/0 cells were incubated for 2 h in the presence of various concentrations of ribavirin (20, 5, 1 and $0.2\text{ }\mu\text{M}$) (●), EICAR (20, 5, 1, 0.2 and $0.05\text{ }\mu\text{M}$) (▼), tiazofurin (100, 20, 5 and $1\text{ }\mu\text{M}$) (■), and mycophenolic acid (5, 1, 0.2 and $0.05\text{ }\mu\text{g/ml}$) (▲). The control value is indicated by the symbol *. Data are means of two independent experiments.

Table 3. Incorporation of [G-³H]Hx into the IMP, GTP, AMP, ADP and ATP pools of EICAR-treated CEM cells

EICAR was incubated with CEM cells together with 0.74 μ M-[G-³H]Hx for 5 h.

EICAR (μ M)	³ H in pool (d.p.m.)				
	IMP	GTP	AMP	ADP	ATP
0	301	58221	15085	89597	943488
0.2	468	63254	12899	66694	996850
0.5	5909	37427	11573	60403	962912
2.0	30090	15937	14696	62420	1021883
5.0	39419	8826	14768	63574	1040450

inhibitor of IMP dehydrogenase than tiazofurin, and about as potent as ribavirin.

Effect of IMP dehydrogenase inhibitors on the GTP pools of CEM/0 cells

The effects of ribavirin, tiazofurin, mycophenolic acid and EICAR on the intracellular GTP pools were examined. Control CEM cell cultures contained 640 nmol of GTP/10⁹ cells. When CEM cells were exposed to ribavirin (20 μ M), mycophenolic acid (5 μ g/ml), EICAR (5 μ M) or tiazofurin (100 μ M) for 2 h, the GTP pools fell to 310, 190, 280 and 220 nmol/10⁹ cells respectively. The decrease in GTP levels was less pronounced at lower compound concentrations, irrespective of the nature of the compound (Fig. 4).

Effect of EICAR on the incorporation of [2,8-³H]Hx into IMP and adenine nucleotides in CEM cells

When CEM cells were exposed to EICAR at 5, 2, 0.5 and 0.2 μ M for 5 h, together with 0.4 μ M-[2,8-³H]Hx, the radiolabel was efficiently incorporated into adenine nucleotide pools (Table 3). Irrespective of the concentration of EICAR, the total amount of radiolabelled AMP, ADP and ATP ranged between 11573 and 14768 d.p.m., 60604 and 66694 d.p.m., and 962912 and 1040450 d.p.m. respectively (Table 3). In contrast, the radiolabel found in GTP decreased progressively from 63254 d.p.m. (0.2 μ M-EICAR) to 8826 d.p.m. (5 μ M-EICAR). Incorporation of [2,8-³H]Hx into the IMP pools markedly increased as a function of the EICAR concentration (Table 3).

DISCUSSION

The suitability, sensitivity and reliability of the ³H release assay from [2,8-³H]Hx as a tool for monitoring IMP dehydrogenase inhibition in intact (CEM) cells is demonstrated by the close correlation between the inhibitory effect of the test compounds on ³H release from [G-³H]Hx and their effect on the decrease in the GTP pools (as long as the inhibitory effect on ³H release is greater than 30%). At lower inhibitory effects on IMP dehydrogenase, however, GTP pools seem not to be affected, and this may be due to salvage synthesis of GMP and subsequent conversion of GMP into GDP and GTP. These data suggest that estimation of ³H release from [G-³H]Hx is more accurate than measuring intracellular GTP levels as a parameter of IMP dehydrogenase activity.

No extensive investigations on the measurement of IMP dehydrogenase activity in intact cells have been reported. Only Kalman & Hsiao [10] briefly reported on the effect of several compounds on ³H release from [2-³H]Ino in murine L1210 cells, and their preliminary observations on the inhibitory effects of mycophenolic acid, ribavirin and tiazofurin on ³H release from

[2-³H]Ino in L1210 cells were in good agreement with our findings in CEM/0 cells.

When radiolabelled [2,8-³H]Hx is used as the precursor for XMP in CEM cells, ~83% of Hx is metabolized to AMP, ADP and ATP, and only 17% to XMP, GMP, GDP and GTP [7]. Moreover, only 50% of the radiolabel of Hx (i.e. ³H on C-2) can be released, since the ³H present on C-8 of the purine base is not subject to release by IMP dehydrogenase. Taking into account that only 1 μ Ci of [2,8-³H]Hx was given to 2.5×10^5 cells, we could calculate that more than 50% of the radiolabel of C-2 of Hx was released within a 10 min incubation period. Therefore the levelling off of the ³H release curves as shown in Figs. 1–3 most likely reflects exhaustion of the radioactive precursor.

Our investigations indicate that in intact CEM cells, the following apply: (i) the conversion of Hx into IMP requires the action of HGPRT; (ii) Ino conversion into IMP also needs the action of HGPRT, which implies that the direct conversion of Ino to IMP by 5'-nucleotidase represents only a very minor pathway from Ino to IMP; (iii) Ino must be first converted to Hx by purine nucleoside phosphorylase before proceeding into IMP via HGPRT; and (iv) other enzyme reactions that may possibly result in ³H release at the C-2 and/or C-8 position of the Hx ring do not intervene (Scheme 1). One such enzyme is xanthine oxidase, which converts Hx into xanthine, and xanthine into uric acid. However, from our findings that (i) in CEM/HGPRT⁻ cells almost no ³H is released from either [2,8-³H]Hx or [2,8-³H]Ino, and (ii) ³H release from [2,8-³H]Hx is fully inhibited in the presence of high concentrations of IMP dehydrogenase inhibitors, it appears that xanthine oxidase does not play a role under our experimental conditions. This may be due to the well-known lack of xanthine oxidase activity in human lymphocytes (i.e. CEM cells). However, it should be mentioned that the examination of IMP dehydrogenase activity in those cell lines that contain marked amounts of xanthine oxidase (i.e. human liver and intestinal cells) should be performed in the presence of a xanthine oxidase inhibitor (i.e. allopurinol).

When several inhibitors of IMP dehydrogenase (i.e. ribavirin, mycophenolic acid, tiazofurin), adenylosuccinate synthetase (i.e. hadacidin), GMP synthetase (i.e. acivicin), purine nucleoside phosphorylase (i.e. 8-aminoguanosine) and xanthine oxidase (i.e. allopurinol) were added to the CEM cells, inhibition of ³H release from [2,8-³H]Hx only occurred in the presence of IMP dehydrogenase inhibitors. None of the other antimetabolites influenced ³H release from [2,8-³H]Hx. These observations point to the usefulness of the assay for detecting IMP dehydrogenase inhibitors and quantifying their inhibitory effects on IMP dehydrogenase in intact cells. Moreover, by comparing the inhibitory effects on ³H release from [2,8-³H]Hx and [2,8-³H]Ino, it should be possible to detect specific inhibitors of purine nucleoside phosphorylase and also quantify the inhibitory effect of such compounds on this enzyme in intact cells. Since the ³H release assay will also detect inhibition of HGPRT, it should be ascertained that the test compounds do not affect guanine incorporation into GTP pools.

When the CEM cells were preincubated for 2–6 h with the test compounds prior to the addition of [2,8-³H]Hx, the inhibitory effect of the IMP dehydrogenase inhibitors became much more pronounced. Mycophenolic acid, which does not need further metabolism to be inhibitory to IMP dehydrogenase, showed a 4–5-fold increase in its inhibitory potential upon 2–6 h preincubation with the cells. Ribavirin, which needs to be converted by adenosine kinase to ribavirin 5'-monophosphate [11,12] before it can inhibit IMP dehydrogenase [13], became about 10-fold more potent upon preincubation with the cells. Tiazofurin, which had no marked inhibitory effect on ³H release when added at 100 μ M together with [2,8-³H]Hx, became inhibitory at 10 μ M

if preincubated with the cells for 2–6 h. Tiazofurin has proven to be inhibitory to IMP dehydrogenase as its tiazole carboxamide adenine dinucleotide (TAD), which means that tiazofurin has to undergo at least two different metabolic conversions before it may become fully inhibitory to IMP dehydrogenase [14–16]. Thus increased activity of the test compounds upon preincubation with the cells may reflect, at least in part, intracellular metabolism to their corresponding active metabolites. Also, the time required for penetration of the test compounds into the cells may account for the increased inhibitory effects of the test compounds upon preincubation. Another interesting explanation for the increased inhibitory effects of the test compounds (i.e. mycophenolic acid) upon longer preincubation times with the cells may be the nature of the kinetic interaction of these compounds with IMP dehydrogenase. Indeed, Hupe and co-workers [17] recently provided evidence for an enzyme–XMP–mycophenolic-acid complex as a mechanism for the inhibition of the enzyme by the test compound. Our findings that XMP pools are progressively enhanced as a function of incubation time after seeding CEM cells (J. Balzarini & E. De Clercq, unpublished work) may therefore explain, at least in part, the increased inhibitory effect of mycophenolic acid on IMP dehydrogenase upon longer preincubation times of the cells with the test compound.

EICAR, a novel analogue of 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide that is endowed with broad-spectrum antiviral [18] and antileukaemic activity [19], proved to be one of the most potent inhibitors of IMP dehydrogenase described thus far. It was ascertained that EICAR does not markedly influence the 5-phosphoribosyl-1-pyrophosphate (PRPP) pools and the HGPRT activity of the cells, since incorporation of [2,8- 3 H]Hx into adenine nucleotide pools was unaffected (Table 3). Incorporation of [2,8- 3 H]Hx into IMP was markedly enhanced due to the intracellular accumulation of IMP in EICAR-treated cells (Table 3). These data argue against an inhibitory effect of EICAR on HGPRT activity or PRPP pools, since adequate PRPP and HGPRT levels are required to incorporate [2,8- 3 H]Hx-derived radiolabel into IMP and adenine nucleotide pools. The 33-fold increase in the inhibitory effect of EICAR on IMP dehydrogenase following a 4 h preincubation period indicates that EICAR may need to be thoroughly metabolized before being able to inhibit the enzyme or, alternatively, is poorly phosphorylated by its activating enzyme(s), thereby slowly accumulating the active metabolite as a function of time. The metabolic pathway(s) that are involved in the conversion of EICAR to its active metabolite(s) are not yet known. However, EICAR is not markedly cleaved by purified calf spleen purine nucleoside phosphorylase (from Boehringer) to its corresponding base and ribose 1-phosphate under conditions where inosine is completely hydrolysed within 10 min (results not shown). Also, EICAR is equally cytostatic to CEM/0 and CEM/HGPRT⁺ cells (i.e. IC_{50} s of 1.12 μ M and 0.78 μ M respectively). These observations argue against the possibility that EICAR is first subject to phosphorylation by purine nucleoside phosphorylase

before the base is metabolized via HGPRT to EICAR 5'-monophosphate.

In conclusion, a rapid, sensitive and reliable assay has been developed to measure IMP dehydrogenase activity in intact cells; this assay appears useful for the detection and quantification of the inhibitory effects of IMP dehydrogenase inhibitors on this enzyme activity in intact cells.

This investigation was supported by grants from the Belgian Fonds voor Geneeskundig Wetenschappelijk Onderzoek (Project no. 3.0097.87), the Belgian Geconcerteerde Onderzoeksacties (Project No. 90/94-2), and the Nationaal Fonds voor Wetenschappelijk Onderzoek (Project no. 7.0040.90). We thank Ria Van Berwaer and Miette Stuyck for excellent technical assistance, and Christiane Callebaut for dedicated editorial assistance.

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