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TIGHT BINDING INHIBITORS—VI

INTERACTIONS OF DEOXYCOFORMYCIN AND ADENOSINE DEAMINASE IN INTACT HUMAN ERYTHROCYTES AND SARCOMA 180 CELLS*

TIMOTHY ROGLER-BROWN, RAM P. AGARWAL† and ROBERT E. PARKS, Jr.
Section of Biochemical Pharmacology, Division of Biology and Medicine, Brown University, Providence, RI 02912, U.S.A.

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Abstract—The inactivation and reactivation of adenosine deaminase (ADA) by deoxycoformycin was studied in intact human erythrocytes and murine Sarcoma 180 cells *in vitro*. The second-order rate constant (k_1) for the association reaction between deoxycoformycin and intraerythrocytic ADA was calculated to be $5.1 \times 10^3\ M^{-1}\ sec^{-1}$. This is about 300 to 500-fold lower than the k_1 values determined either with hemolyzed human erythrocytes ($k_1 = 1.4 \times 10^6\ M^{-1}\ sec^{-1}$) or with partially purified human erythrocytic ADA ($k_1 = 2.6 \times 10^6\ M^{-1}\ sec^{-1}$). In intact erythrocytes only slight reactivation (<10 per cent) of the inhibited ADA (*EI* complex) was detectable over 24 hr, whereas with hemolysates about 50 per cent reactivation of the inhibited ADA was observed in about 25 hr ($k_2 = 7.7 \times 10^{-6}\ sec^{-1}$). The k_1 values with intact and supernatant fractions from homogenized Sarcoma 180 cells were determined to be $1.1 \times 10^4\ M^{-1}\ sec^{-1}$ and $4.2 \times 10^6\ M^{-1}\ sec^{-1}$ respectively. With intact Sarcoma 180 cells, negligible reactivation of ADA was seen during a 48-hr period. Preliminary studies indicate an important role for the erythrocytic nucleoside transport system on the apparent k_1 values and the rate of inactivation of ADA by deoxycoformycin in intact cells.

Recently there has been a marked upsurge of interest in adenosine deaminase (ADA).‡ This enzyme plays a key role in the regulation of adenine nucleotide metabolism as well as in the biological and biochemical behavior of a number of analog purine nucleosides, including compounds of chemotherapeutic potential, e.g. arabinosyl adenine[1], cordycepin[2], etc. The importance of ADA in the regulation of purine metabolism has been emphasized by the discovery that a genetic deficiency in ADA may be associated with a severe combined immunodeficiency disease (SCID-ADA deficient), in which children lack both T and B lymphocytic functions[3]. Other notable recent developments have been the discovery of two exceptionally potent tight-binding inhibitors of ADA, conformycin[4–6] and deoxycoformycin[7, 8]. These inhibitors display many of the characteristics expected of "transition-state" analogs as originally postulated by Pauling in 1948[9].

Earlier reports from this laboratory[10–14] that utilized certain new experimental approaches developed by Cha[10, 12] for the study of tight binding enzyme inhibitors revealed that coformycin and dCF have K_i values with human erythrocytic ADA in the range of 1×10^{-10} to 2.5×10^{-12} M. In those studies, it was possible to measure directly the velocity constants of the association reaction (k_1) between the inhibitor (I) and ADA (E) and of the dissociation rate constant (k_2) of the enzyme–inhibitor (EI) complex, e.g.

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EI \qquad (1)$$

In studies with partially purified human erythrocytic ADA and dCF, the k_1 and k_2 values were determined as $2.6 \times 10^6\ M^{-1}\ sec^{-1}$ and $6.6 \times 10^{-6}\ sec^{-1}$ respectively[13].

Recent reports from several laboratories have demonstrated that dCF, in combination with various adenosine analogs, markedly increases their chemotherapeutic effectiveness and/or their incorporation into the intracellular nucleotide pools[1, 2, 14–19]. Deoxycoformycin and other adenosine deaminase inhibitors, e.g. erythro-9-(2-hydroxy-3-nonyl)adenine[20], in combination with adenosine analogs are under consideration for pharmacologic testing which may lead to clinical trials. Therefore, it is important to examine the factors that influence the inactivation and reactivation of ADA in the presence of inhibitors such as dCF in intact cells as compared with the isolated enzyme. It seems likely that transfer of the inhibitor through the cellular membrane, either by diffusion or by a specific transport system, will be found to play a significant role. This paper describes studies of this type with intact human erythrocytes and murine Sarcoma 180 cells. A

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†Present address: Section of Medical Oncology, Departments of Pharmacology and Medicine, Boston University School of Medicine, Boston, MA 02118.

‡Abbreviations used: ADA, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4); coformycin, (3-β-D ribofuranosyl 6,7,8-trihydroimidazo [4,5-*d*] [1–3] diazepin-8-(R)-ol); dCF, deoxycoformycin, *d*-coformycin or [3-(2′-deoxy-β-D-erythro pentofuranosyl) 6,7,8 trihydroimidazo [4,5-*d*] [1, 3] diazepin-8-(R)-ol] or Covidarabine: HNBTGR, 2-amino-6-[(2-hydroxy-5-nitrobenzyl) thio]-9-β-D-ribofuranosyl purine; PNPase, purine nucleoside phosphorylase (purine nucleoside: orthophosphate ribosyltransferase, EC 2.4.2.1).

preliminary report of these findings has been presented[21].

MATERIALS AND METHODS

Deoxycoformycin (dCF, Covidarabine) samples were provided by Dr. H. W. Dion of Parke Davis & Co., Detroit, MI[7] and Dr. John Douros of the Drug Development Branch of the National Cancer Institute, Bethesda, MD. A nucleoside transport inhibitor, HNBTGR, was a gift from Dr. A. R. P. Paterson of the University of Alberta, Edmonton, Alberta. The concentrations of solutions of dCF were determined spectrophotometrically ($\epsilon_{282\,nm}$ in $H_2O = 8.0 \times 10^3$)[7]. Human erythrocytes were obtained through the Division of Hematological Research, Memorial Hospital, Pawtucket, RI. The hemoglobin-coated charcoal used in this study was prepared as described by Waxman *et al.*[22].

Preparation of cells

Human erythrocytes were separated from plasma and the buffy coat by washing twice in 0.9% NaCl and were suspended in about 1 vol. of a standard medium (potassium phosphate buffer, 50 mM, pH 7.4; NaCl, 75 mM; $MgCl_2$, 2 mM; glucose, 10 mM; penicillin, 10,000 units/l and streptomycin, 10,000 μg/l). After determination of the hematocrit of these suspensions, the cells were diluted to a final concentration of 20% (v/v), by addition of appropriate amounts of the standard medium.

Sarcoma 180 cells were maintained by serial transplantation of about 2.5×10^6 cells into the peritoneal cavities of female CDl mice. The cells were harvested 5–6 days after inoculation, washed twice in 0.9% NaCl solution and suspended in the standard medium to a final concentration of 10–20% (v/v). Homogenization of the Sarcoma 180 cells (10–20%, v/v, in standard medium) was performed by grinding the cells for 10 min using a mortar and pestle. The homogenate was centrifuged at 3000 rev/min for 5 min in a clinical centrifuge and the supernatant fluids were collected and utilized.

Determination of adenosine deaminase activity in intact cells

The activity of ADA in intact erythrocytes and Sarcoma 180 cells was determined by measuring ammonia liberation due to adenosine deamination. The measurement of ammonia was determined by a minor modification of the microdiffusion method of Seligson and Seligson[23], and the colorimetric procedure of Chaney and Marbach[24]. Suspensions of erythrocytes (4%, v/v) or Sarcoma 180 cells (4%, v/v) in standard medium were incubated with 1 mM adenosine. Aliquots (1 ml) were withdrawn immediately upon addition of adenosine (1 mM) and after 10-min incubation periods in a shaking water bath at 30°. The activity was stopped by adding aliquots to flasks containing 1 ml of saturated K_2CO_3 (100 g/100 ml) that were equipped with rubber stoppers holding glass rods with flared ground tips coated with 1 M citric acid. The flasks were placed on a rotary mixer (Multi-Purpose Rotator, Scientific Industries, Inc. Bohemia, NY) for at least 30 min and the ammonia was allowed to diffuse and to be trapped on the citric acid-coated rods. The ammonia collected was washed into a 1 ml solution containing phenol (0.53 M) and nitroprusside (1 mM). The color was developed by the addition of 1 ml sodium hydroxide (0.63 M) containing sodium hypochlorite (0.03 M). The indophenol formed was measured spectrophotometrically at 625 nm.

Earlier studies with washed human erythrocytes that employed this method[15, 25] demonstrated that ammonia liberation is linear with time for at least 30 min under these conditions and is essentially identical with both intact erythrocytes and hemolysates. The enzymic activities measured agreed closely with values obtained by standard spectrophotometric procedures[13]. Also, in control studies with Sarcoma 180 cells, no liberation of ammonia occurred in the absence of adenosine, and in the presence of adenosine, the rates of ammonia liberation were linear for at least 10 min. The adenosine deaminase activity determined by this method with Sarcoma 180 cells agreed well with that of supernatant fractions measured by spectrophotometric methods. Before attempting to apply this procedure to other cell types, however, it is essential that control studies be performed to establish that there is no spurious liberation of ammonia by reactions other than adenosine deaminase, e.g. amino acid oxidases, and that no consumption of ammonia occurs as in isolated hepatocytes that have an active urea cycle, or enzymes such as glutamate dehydrogenase, that might consume ammonia in the presence of α-ketoglutarate.

Determination of the association velocity constant (k_1) of deoxycoformycin and adenosine deaminase in intact erythrocytes

Twenty per cent (v/v) suspensions of human erythrocytes were incubated with dCF at 30° with thorough mixing on a shaking water bath. At timed intervals, the aliquots (0.5 ml) were withdrawn and centrifuged at 3000 rev/min for about 30 sec. The supernatant fluids were carefully removed from the sedimented erythrocytes. The erythrocytes were resuspended immediately to a final volume of 2.5 ml by addition of standard medium containing adenosine (final concentration 1 mM). The ADA activity was determined at 30° for 10 min by the microdiffusion method described above.

From the $T_{1/2}$ values (time required for 50 per cent inhibition) of the remaining ADA activities, the pseudo-first-order rate constants, k_λ, were determined[10, 12] as follows:

$$k_\lambda = 0.693/T_{1/2}. \quad (2)$$

The k_λ values were used to determine the k_1 values according to the following relationship:

$$k_\lambda = k_1[I] \quad (3)$$

where $[I]$ is the dCF concentration.

In experiments where uridine or HNBTGR was employed, 20% (v/v) erythrocytic suspensions in standard medium were preincubated with uridine

(1 mM) or HNBTGR (50 μM) for at least 1 hr at 30°; then dCF was added at the concentrations indicated. After incubation at 30° for various time intervals, 0.5-ml samples were withdrawn, centrifuged immediately and the supernatant fluids were removed. In experiments with uridine, the cell pellets were suspended in 2.4 ml of standard medium containing 1 mM adenosine and the residual ADA activity was determined by the microdiffusion method. In HNBTGR experiments, the pellet was washed once with 10 ml of standard medium. The cells were hemolyzed quickly by adding 2.15 ml of distilled water containing adenosine (final concentration 1 mM). After addition of 0.25 ml of 10-fold concentrated standard medium, the enzymic activity was determined. In studies with potent nucleoside transport inhibitors, e.g. HNBTGR, it is necessary to measure residual ADA activity in hemolysates rather than intact cells since compounds such as HNBTGR interfere with the measurement of ADA activity in intact erythrocytes[25]. From the remaining ADA activity, k_i values were determined as described above.

Determination of k_i *in human erythrocytic hemolysate*

Erythrocytes that had been washed twice in 0.9% NaCl and suspended in equal volumes of standard medium were hemolyzed by the addition of about 4–5 vol. of distilled water, producing a final hemolysate of about 20% erythrocytes by volume. To adjust the pH and salt concentration of the mixture to conditions identical with those used in studies with intact erythrocytes, 1 vol. of 10-fold concentrated standard medium was added to 9 vol. of hemolysate. The experiments were performed by adding appropriate concentrations of dCF to aliquots of these hemolysates with incubation at 30°. At appropriate times, the reaction was terminated by withdrawing 1-ml aliquots and diluting with 4 ml of standard medium, containing adenosine to a final concentration of 1 mM. The ADA activity was assayed and the k_i values were determined as described above.

Determination of k_i *in Sarcoma* 180 *cells*

Aliquots (0.5 ml) of a Sarcoma 180 cell suspension (10–20 per cent, v/v, in standard medium) were incubated at 30° with dCF at appropriate concentrations. It was necessary to perform the assay on individual samples because of the cellular aggregration that might occur during the incubation. At timed intervals the reaction was terminated by dilution with 4 vol. of standard medium containing adenosine (1 mM final concentration). The remaining ADA activity was determined then by the microdiffusion assay.

Determination of k_i *in supernatant fluids from homogenized Sarcoma* 180 *cells*

Supernatant fluids from Sarcoma 180 homogenates were diluted to a final concentration of 10% (v/v), based on the original volume of packed Sarcoma 180 cells, in standard medium. Aliquots (0.5 ml) were incubated with desired concentrations of dCF. The reactions were stopped at intervals by a 5-fold dilution of aliquots of the reaction mixture with standard medium containing adenosine (1 mM). The ADA activity was assayed by the microdiffusion technique described above with the use of 20-min incubation periods at 30°.

Attempts to determine the dissociation of the adenosine deaminase–deoxycoformycin complex in intact human erythrocytes and hemolysates

The ADA in intact cells was inhibited by incubation of erythrocytic suspensions (20 per cent, v/v) in standard medium with dCF (4×10^{-7} M) for about 1 hr at 30°. After this preincubation period the cells were sedimented by centrifugation for 5 min at 3000 rev/min and the supernatant fluids were discarded. Attempts to reactivate the inhibited ADA in intact erythrocytes and hemolysates were carried out by the three approaches as described below.

Dialysis of intact cells against hemoglobin-coated charcoal suspensions. Cells (ADA inhibited) were diluted with standard medium to a 4% suspension (v/v) and placed into dialysis tubing. Dialysis was carried out at room temperature with constant stirring, against 500 ml of standard medium, containing hemoglobin-coated charcoal (15 g) to absorb any free and/or dissociated dCF. At appropriate intervals 2.25-ml aliquots of the cell suspension were removed from the dialysis bag and the ADA activity was determined after addition of 0.25 ml of 10 mM adenosine (final concentration of 1 mM) to the cell suspension. Cells treated identically in the absence of dCF served as controls.

Direct treatment of ADA-inhibited intact erythrocytes with hemoglobin-coated charcoal. ADA-inhibited cells were adjusted to 20% (v/v) by addition of a hemoglobin-coated charcoal suspension (16 g/l) in standard medium. The reaction mixture was stirred gently at room temperature. At timed intervals 1.0-ml aliquots were withdrawn and the cells were hemolyzed by addition of 3.5 ml of distilled water with rapid vortexing for about 1 min. To restore the pH and salt concentration, 0.5 ml of 10-fold concentrated standard medium was added to the mixture. The mixture was centrifuged to remove the charcoal after 1 min of stirring. Aliquots of the supernatant fluid were withdrawn and assayed for ADA activity after addition of 1 mM adenosine. Similarly treated controls were performed simultaneously. In most experiments hemolysis was seen after more than 24 hr of stirring of intact erythrocytes with charcoal.

Direct treatment of ADA-inhibited erythrocytic hemolysates with hemoglobin-coated charcoal. ADA-inhibited cells were hemolyzed by addition of 3.5 vol. of distilled water to 1 vol. of the packed erythrocytes followed by rapid stirring on a vortex mixer. Samples were centrifuged at 3000 rev/min for 5 min to remove the erythrocytic ghosts. Salt concentration and pH were adjusted by addition of 0.5 ml of a 10-fold concentration of standard medium. One-vol. aliquots of this hemolysate were treated with 4 vol. of a charcoal suspension (16 g/l) in standard medium. At timed intervals aliquots were centrifuged to remove the charcoal and the

ADA activities in the supernatant fluids were measured.

Attempts to demonstrate dissociation of the adenosine deaminase–deoxycoformycin complex in intact murine Sarcoma 180 cells

The dissociation of the ADA–dCF complex of intact Sarcoma 180 cells was determined by following the ADA activity of 1.0-ml aliquots of 20% cell suspensions which had been inhibited with dCF (4×10^{-7} M). Aliquots removed after incubation at different intervals were washed twice with 10 ml of standard medium, suspended to 4% (v/v), and the ADA activity was assayed.

RESULTS

The rate constant (k_1) of the association reaction between deoxycoformycin and adenosine deaminase in intact human erythrocytes

Figure 1 presents semilogarithmic plots of the ADA activity in intact human erythrocytes incubated with various concentrations of dCF. The mean k_1 values for the association reaction between dCF and ADA in intact erythrocytes were estimated to be about 5.1×10^3 M^{-1} sec^{-1}. When similar experiments were performed with hemolyzed erythrocytes, the mean k_1 values were 1.4×10^6 M^{-1} sec^{-1} (Table 1). These apparent k_1 values in intact erythrocytes are 300 to 500-fold lower than the values obtained either with hemolysates or partially purified human erythrocytic ADA preparations[13, 21]. This strikingly slower association rate of inhibition of ADA in intact erythrocytes indicates that the erythrocytic membrane plays a key role in this process.

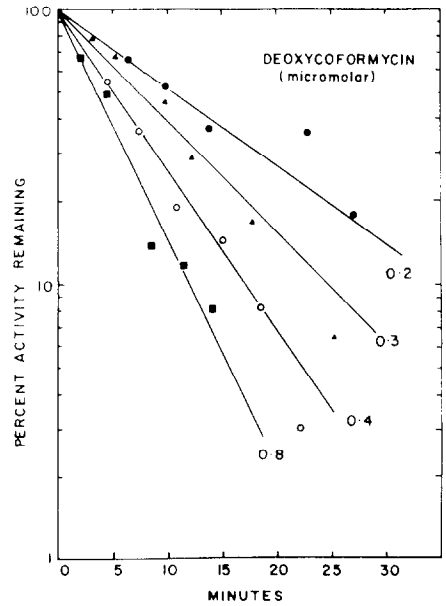

Fig. 1. Determination of the association velocity constant (k_1) of deoxycoformycin and adenosine deaminase in intact human erythrocytes. The appropriate concentration of deoxycoformycin was added to a 20% suspension of erythrocytes and incubated in a Dubnoff metabolic shaking incubator at 30°. Reactions were terminated at appropriate times by centrifugation of the sample in a clinical centrifuge at 3000 rev/min. The supernatant fluid was then removed from the pelleted erythrocytes; 2.4 ml of standard medium containing adenosine at a final concentration of 1 mM was added to the erythrocytes. The ADA activity was assayed by measuring ammonia evolution in a Dubnoff metabolic shaking incubator at 30°, according to the procedure described in Materials and Methods.

Table 1. Association rate constant (k_1) of deoxycoformycin and adenosine deaminase

Source	k_1 (M^{-1} sec^{-1})
Human erythrocytes	
Purified enzyme	2.6×10^6*
Hemolysate	1.4×10^6
Intact cells	5.1×10^3
Uridine (1 mM)	
Intact cells	1.1×10^3
Hemolysate	2.0×10^6
HNBTGR (50 μM)	
Intact cells	5.1×10^2
Hemolysate	4.1×10^6
Sarcoma 180	
Homogenate	4.2×10^6
Intact cells	1.1×10^4

*See Ref. 13.

The k_1 values of partially purified erythrocytic ADA and that of hemolysates are similar within a magnitude of 2-fold. This strongly suggests that there are no factors in hemolysates that interfere significantly with the interaction between ADA and dCF, e.g. human erythrocytes have unusually high activity of PNPase[26] and dCF might be considered as an analog of deoxyinosine, an excellent substrate for this enzyme. However when dCF (0.1 μmole) was incubated with several units of homogeneous human erythrocytic PNPase under optimal conditions for a period of 24 hr, no disappearance of the ADA-inhibitory activity of dCF could be detected. This indicates that, even under these rigorous conditions, dCF has no demonstrable substrate activity with human erythrocytic PNPase. Comparable quantities of deoxyinosine would have undergone phosphorolysis within seconds under these conditions.

Role of the erythrocytic nucleoside transport system on the rate of inhibition of intraerythrocytic adenosine deaminase by deoxycoformycin

The above observations strongly suggest that the erythrocytic nucleoside transport system studied extensively by Paterson *et al.*[27–30] may play a key role in the rate of inactivation of intraerythrocytic ADA by dCF. Initial studies of this question involved two types of experiments: (a) examination of competition between nucleosides and dCF for the nucleoside transporter; and (b) examination of the effect of the potent nucleoside transport inhibitor, HNBTGR on the rate of ADA inactivation in intact erythrocytes.

Uridine, a non-metabolizable ribonucleoside in mature human erythrocytes, interacts with the nucleoside transport system. Radiolabeled uridine has been one of the major biochemical tools employed in prior studies of this system[27, 31, 32]. Therefore, experiments were

performed to test whether uridine affects the apparent k_i values of the inactivation of intraerythrocytic ADA by dCF. As shown in Table 1, in the presence of 1 mM uridine, an apparent k_i of $1.1 \times 10^3\ M^{-1}\ sec^{-1}$ was determined, i.e. a value about 5-fold lower than in the absence of uridine. Further studies are in progres in attempts to evaluate more precisely the possible competitive relationship of the interaction between uridine and dCF. As shown in Table 1, the addition of 1 mM uridine to hemolysates did not alter the k_i value determined in the absence of uridine.

As shown in Fig. 2 and Table 1, the nucleoside transport inhibitor, HNBTGR, has a profound effect on the apparent k_i value of the inhibition of intraerythrocytic ADA by dCF. The addition of HNBTGR (50 μM) to intact erythrocytes decreased the apparent k_i value to $5.1 \times 10^2\ M^{-1}\ sec^{-1}$, i.e. about 10-fold, but did not decrease the k_i value determined with hemolysates. Also, as shown in Fig. 2, when the concentration of dCF was increased about 20-fold (0.4 to 8.0 μM) in the presence of HNBTGR (50 μM), the inhibitory effect of HNBTGR was partially overcome. A detailed study of this interaction is currently in progress and will be the subject of a future communication. All data obtained to date, in agreement with the above findings with uridine, are consistent with the hypothesis that the nucleoside transport system plays a key role in the rate of inactivation of intraerythrocytic ADA by dCF.

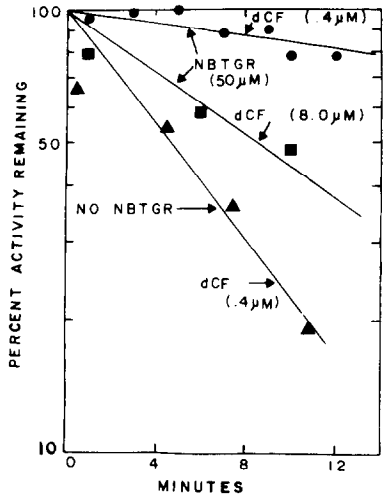

Fig. 2. Studies employing the nucleoside transport inhibitor. Erythrocytes (a 20% suspension in standard medium) were treated with or without 50 μM HNBTGR for about 1 hr at 30° in a Dubnoff metabolic shaking incubator. The association velocity constant was determined by adding deoxycoformycin (at a concentration of 0.4 to 8.0 μM) to 0.5 ml of the erythrocytic mixture. The association reaction was stopped by centrifuging the 0.5-ml mixture and removing the supernatant fluid from the pelleted erythrocytes. The pelleted erythrocytes were washed once in 10.0 ml of standard medium. The cells were resuspended in 2.4 ml of distilled water containing adenosine to a final concentration of 1 mM and mixed well in order to hemolyze the cells. The ADA activity was then assayed by the procedure described in Materials and Methods. The abbreviations HNBTGR and NBTGR (in the figure) represent the same compound.

Rate of inactivation of intracellular adenosine deaminase by deoxycoformycin in Sarcoma 180 cells

Studies similar to those described above for intact human erythrocytes and hemolysates have been performed with intact and homogenized murine Sarcoma 180 cells. As shown in Table 1, the results with Sarcoma 180 cells were similar to those obtained with human erythrocytes. The apparent k_i value for the inactivation of ADA by dCF in Sarcoma 180 cells was about 400-fold lower than the value measured with supernatant fractions of homogenized Sarcoma 180 cells. Interestingly, the k_i value determined with Sarcoma 180 supernatant fractions was close to the k_i value measured with purified erythrocytic ADA and the hemolysates. These studies with Sarcoma 180 cells indicate that, as with human erythrocytes, the cell membrane plays a key role in the rate of inactivation of ADA by dCF. However, to date we have not explored the important question of the possible role of a nucleoside transport system in Sarcoma 180 cells and other intact nucleated cells.

Attempts to dissociate the adenosine deaminase–deoxycoformycin complex in intact human erythrocytes and hemolysates

Studies with intact erythrocytes. The determination of the dissociation velocity constant (k_2) in erythrocytes was attempted by two procedures described in Materials and Methods. The first set of data in Table 2 was obtained by dialyzing intact erythrocytes (ADA inhibited) against a suspension of hemoglobin-coated charcoal. As can be seen in Table 2, negligible ADA activity was recovered even after 49 hr of incubation. Similarly, the addition of hemoglobin-coated charcoal directly to erythrocytic suspensions did not promote the dissociation of the ADA–dCF complex, as evidenced by the very low recovery of ADA activity after 24 hr of incubation (see Table 2). Considerable loss of ADA activity occurred in this experiment in control samples and on prolonged stirring progressive hemolysis was evident.

Studies with hemolysate. Figure 3 demonstrates the dissociation of the ADA–dCF complex in hemolysates. By extrapolation of the activity of the ADA reactivated during the first 12 hr the $T_{1/2}$ value for the dissociation of the ADA–dCF complex was estimated at about 25 hr. This value is similar to that obtained with partially purified ADA (29 hr)[13]. From this $T_{1/2}$ value the dissociation rate constant (k_2) was calculated to be $7.6 \times 10^{-6}\ sec^{-1}$. When intact erythrocytes were hemolyzed 24 hr after inactivation of the intracellular ADA by dCF (4×10^{-7} M), the rate of reactivation of enzymic activity was essentially identical to that observed in erythrocytes which were hemolyzed after 1 hr of preincubation with dCF (4×10^{-7} M, Fig. 3). This indicates that ADA does not undergo permanent inactivation when inhibited by dCF for prolonged periods in the erythrocyte. This finding also emphasizes further the importance of the intact erythrocytic membrane not only in the rate of inactivation of ADA in the cell, but also in the failure of ADA to recover activity in the intact cell.

Table 2. Dissociation of deoxycoformycin–adenosine deaminase (EI) complex in intact human erythrocytes

Time (hr)	Control (% of initial ADA activity)	dCF-treated* (% ADA activity recovered relative to initial control activity)
	Intact cells using dialysis bag	
0	100	0
3	91	3
13	81	7
29	90	2
38	96	8
49	118	5
	Intact cells with charcoal	
0	100	0
1	75	2
6	81	10
16	64	6
24	51	2

*dCF: 4×10^{-7} M.

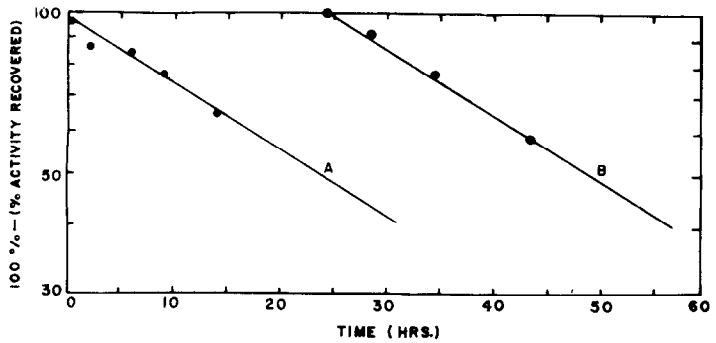

Fig. 3. Dissociation of adenosine deaminase–deoxycoformycin in human erythrocytic hemolysate. A 20% suspension of intact human erythrocytes was inhibited with 4×10^{-7} M deoxycoformycin at 30° in a Dubnoff metabolic shaking incubator. After preincubation with the inhibitor for 1 hr (A) or 24 hr (B), swelling and breakage of the cells were induced by the addition of 5 vol. of distilled water. Hemoglobin-coated charcoal was added then to the hemolysate mixture at a concentration of 0.016 g/ml and the adjustment of salt concentrations and pH was made by the addition of 10-fold concentrated standard medium to the mixture. At appropriate times aliquots were taken from this mixture and centrifuged to remove the charcoal. A portion of the hemolysate was separated from the charcoal and assayed for ADA activity by ammonia evolution as described previously in Materials and Methods.

Attempts to dissociate the adenosine deaminase–deoxycoformycin complex in intact Sarcoma 180 cells

When the dissociation of the ADA–dCF complex was examined with intact Sarcoma 180 cells *in vitro*, no appreciable recovery of ADA activity was observed over a 48-hr period, as shown in Table 3.

DISCUSSION

These investigations emphasize the crucial importance of the cell membrane, both with human

Table 3. Dissociation of deoxycoformycin–adenosine deaminase (EI) complex in intact Sarcoma 180 cells

Time (hr)	Control (% of initial ADA activity)	dCF-treated* (% ADA activity recovered relative to initial control activity)
0	100	1
3	99	4
6	118	3
9	105	4
12	75	1
48	81	1

*dCF: 4×10^{-7} M.

erythrocytes and Sarcoma 180 cells, in the biochemical behavior of the tight-binding ADA inhibitor, deoxycoformycin. In both cell types, the rate of inactivation of ADA by dCF was several hundred times slower than observed with the partially purified ADA, hemolysates or supernatant fractions of Sarcoma 180 homogenates. Furthermore, the reactivation of dCF-inhibited ADA in intact cells was found to be extremely slow (about 10 per cent or less in 48 hr). In contrast, the reactivation of ADA (partially purified or in hemolysates) followed first-order kinetics with $T_{1/2}$ values in the range of 25–30 hr. Although these observations indicate a key effect of the cellular membrane on the rate of reactivation of dCF-inhibited ADA, the possibility that ADA exists in an altered conformational state in the intact cells may not be ruled out on the basis of current evidence. It should be noted that the physical association of the enzyme with erythrocytic cell membrane has been suggested earlier from this laboratory[25]. However, the finding that the ADA inhibited in intact erythrocyte for 24 hr recovered activity with a $T_{1/2}$ of about 25 hr (see Fig. 3) indicates that irreversible damage to the enzyme did not occur during this prolonged period of inactivation. Since dCF and other potent ADA inhibitors have considerable clinical potential as possible immunosuppressive agents[33, 34] or potentiators of the action of adenosine analogs[1, 2, 14, 17], it is obvious that phenomena involving cellular membranes will have a profound influence on the pharmacologic behavior of this class of compound.

Recent evidence indicates that significant restoration of ADA activity can occur in tumor cells in intact animals over a period of about 6–12 hr following treatment with dCF[17, 35]. Mature adult human erythrocytes lack the metabolic machinery for protein synthesis or, in other words, resynthesis of adenosine deaminase and the present studies with Sarcoma 180 cells employed conditions that also did not favor protein synthesis. Therefore, it is possible that the inactivation of ADA by dCF will be much longer in duration with anucleated cells, e.g. erythrocytes, than in nucleated cells where ADA activity may be restored by synthesis of new enzymic protein. Of course, confirmation of these speculations must await detailed investigations with intact animals.

The evidence presented in Table 1 strongly suggests that the nucleoside transport system found in the cell membranes of erythrocytes and other tissues plays a significant role in the delivery of dCF into the erythrocyte. Uridine and HNBTGR, both of which have been demonstrated conclusively to interact with the human erythrocytic nucleoside transport system[27–32], significantly decrease the rate of inactivation of ADA by dCF in intact cells. In studies to be documented in detail elsewhere we have found that both of these compounds behave as competitive inhibitors of the interaction between dCF and the erythrocytic nucleoside transporter. It has been possible to determine K_d values for uridine and HNBTGR that are similar to those reported earlier for uridine and an analog of HNBTGR[22, 25, 26]. Recent reports by Paterson *et al.*[32, 36] indicate that differences occur in the nucleoside transport system among species and cell types, e.g. the nucleoside transport system of HeLa cells grown in culture does not interact significantly with dCF. Therefore, an area of interest for future study is the behavior of the nucleoside transport mechanisms of various mammalian cells (including tumors) with the ADA inhibitor dCF. Significant differences from one tissue to another could profoundly affect the pharmacologic and chemotherapeutic behavior of this type of compound and might indicate a need for appropriate structural modifications in the molecule to alter its physiologic disposition.

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