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DEOXYNUCLEOSIDE PHOSPHORYLATING ENZYMES IN MONKEY AND HUMAN TISSUES SHOW GREAT SIMILARITIES, WHILE MOUSE DEOXYCYTIDINE KINASE HAS A DIFFERENT SUBSTRATE SPECIFICITY

ASTER HABTEYESUS, ANNA NORDENSKJÖLD, CHRISTINA BOHMAN and STAFFAN ERIKSSON*

Medical Nobel Institute, Department of Biochemistry I, Karolinska Institutet, Stockholm, Sweden

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Abstract—Three key enzymes in the anabolic phosphorylation of deoxyribonucleosides and deoxyribonucleoside analogs were purified i.e. cytoplasmic thymidine kinase (TK1), mitochondrial thymidine kinase (TK2) and cytoplasmic deoxycytidine kinase (dCK) from human, mouse and monkey liver and spleen. Their subunit structure and substrate specificities were compared. Extensive purification of TK1 and dCK from mouse spleen and TK2 from mouse and monkey livers revealed major polypeptide bands of 25, 30 and 28 kD, respectively, on sodium dodecyl sulphate–polyacrylamide gel electrophoresis which are very similar to the subunit molecular weights of the corresponding human enzymes. Affinity purified polyclonal antibodies against human dCK also cross-reacted with 30 kD bands in extracts from both mouse and monkey spleen. Thus, the molecular weights of the subunits of these three enzymes appeared to be very similar in all three species. TK1 and TK2 from these different sources appeared to have similar substrate specificities against several deoxyribonucleoside analogs. However, mouse dCK differed significantly from monkey and human dCK in its capacity to phosphorylate dAdo and 2′,3′-dideoxycytidine (ddCyd) with a V_{max} approximately 10-fold lower than that of the two latter enzymes. The K_m and V_{max} values for dCyd and arabinocytosine appeared to be very similar with the enzymes from all three species. The fact that mouse dCK shows low activity with dAdo and ddCyd explains differences reported previously in the metabolism of dAdo and ddCyd in mouse compared to that in human lymphocytes. These results argue against the use of mice as model systems for human deoxynucleoside metabolism.

Mammalian cells have the capacity to salvage extracellular deoxyribonucleosides by phosphorylating them to deoxyribonucleotides which can subsequently serve as DNA precursors. The rate limiting enzymes in this pathway are usually deoxyribonucleoside kinases that produce deoxyribonucleoside monophosphates. Thymidine kinase 1 (TK1†, the cytoplasmic enzyme), thymidine kinase 2 (TK2, the mitochondrial enzyme) and deoxycytidine kinase (dCK) are key enzymes in this metabolism. Large differences have been found in the expression of these three enzymes in different tissues and in cells of different cell cycle phases [1–6].

The substrate specificities and tissue distribution of deoxyribonucleoside kinases are of great pharmacological interest since the activation of cytostatic and several antiviral nucleoside analogs is dependent on the activity of these enzymes. The introduction of 2′,3′-dideoxynucleosides as effective and clinically useful anti-HIV drugs has led to an intensive characterization of the biochemical pathways through which these compounds are activated [7–13]. Furthermore, the elucidation of the molecular mechanism of several combined immunodeficiency diseases associated with the lack of adenosine deaminase and purine nucleoside phosphorylase has illustrated the role of deoxynucleoside kinases in the accumulation of toxic deoxynucleotides in lymphoblastoid cells [14, 15].

There is a great need for the establishment of relevant animal model systems both for studies of the heritable immunodeficiency diseases and for the acquired immunodeficiency disease (AIDS) caused by HIV infection. Carson *et al.* [16] reported previously large differences in the metabolism of deoxyadenosine between mouse and human lymphocytes. Furthermore, important species differences were observed by Balzarini *et al.* [17] in the anti-retroviral activity and intracellular metabolism of 3′-azidothymidine and 2′,3′-dideoxycytidine, two of the most effective anti-HIV agents known today. They concluded that extrapolation from metabolic data obtained with murine (or other animal) cell lines to human cells may be misleading.

* To whom correspondence should be addressed.

† Abbreviations: TK1, cytoplasmic thymidine kinase; TK2, mitochondrial thymidine kinase; dCK, deoxycytidine kinase; AZT, 3′-azido-2′,3′-dideoxythymidine; FLT, 3′-fluoro-2′,3′-dideoxythymidine; dUrd, 2′-deoxyuridine; FdUrd, 5-fluoro-2′-deoxyuridine; ddCyd, 2′,3′-dideoxycytidine; araC, arabinosylcytosine; Ado, adenosine; dCyd, deoxycytidine; dAdo, 2′-deoxyadenosine; dGuo, 2′-deoxyguanosine; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; wt, wild type; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; CHAPS, 3[(3-chloramidopropyl)dimethylammonia]-1-propane-sulfonate.

Methods of completely purifying thymidine kinase 1 and thymidine kinase 2 [18, 19] as well as deoxycytidine kinase from human leukemic spleen [20] have been described recently. In the present investigation we used these methods to purify the corresponding enzymes from monkey and mouse liver and spleen in order to make a preliminary characterization of their properties, both with regard to subunit structure and enzyme kinetics. Our results demonstrate that while human and monkey deoxynucleoside kinases appear to be similar, mouse deoxycytidine kinase shows subtle but significant differences in substrate specificity which may explain the altered deoxynucleoside metabolism in mouse cells, described above. This data is of importance when choosing appropriate animal models for human diseases involving deoxynucleoside salvage.

MATERIALS AND METHODS

Materials. [^{3}H]dAdo, [^{3}H]dCyd, [^{3}H]dGuo and [^{3}H]Thd were purchased from Amersham International (Amersham, U.K.). dAdo, Thd and dCyd came from the Sigma Chemical Co. (St Louis, MO, U.S.A.) while dGuo came from P-L Biochemicals Inc (St Goar, Germany). Discs used in the enzymatic assays were Whatman DE-81 filter paper discs. ATP was bought from the Sigma Chemical Co. and 3[(3-chloramidopropyl)dimethylammonia] - 1 - propane - sulfonate (CHAPS) was purchased from Boehringer Mannheim (Mannheim, Germany). Monkey spleen and liver were from a 6-year-old male cynomolgus macaque (*Macaca fascicularis*) weighing 4 kg. Mouse livers and spleens were from female NMRI mice, approximately 3 months old. CEM wt and CEM AraC 8D cells were generously provided by B. Ullman, University of Oregon (Portland, OR, U.S.A.) and grown and harvested as described previously [15].

Preparation of extracts and DEAE-Sepharose chromatography. Frozen tissue was thawed and homogenized with a Waring blender in an isolation buffer containing 250 mM sucrose, 5 mM Hepes (pH 7.4) and 0.1 mM EGTA, and, after rinsing once with homogenization buffer (50 mM K_3PO_4, 250 mM sucrose, 5 mM DTT, 5 mM benzamide, 0.5 mM PMSF) was homogenized mannually with 10–15 strokes in a glass homogenizer followed by sonication for 20 sec. The suspension was centrifuged at 14,000 rpm for 30 min. The supernatant was treated with streptomycin sulphate and at a final concentration of 0.7% with continuous stirring for 25 min followed by centrifugation for 15 min at 14,000 rpm. The supernatant was collected and proteins were precipitated by addition of ammonium sulfate to 60%. Precipitated proteins were dissolved in buffer A with 50 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 20% glycerol and 5 mM mercaptoethanol, and dialysed against 20 volumes of the same buffer for 12 hr with two buffer changes. The resulting material was applied to a DEAE-Sepharose fast flow (Pharmacia LKB Biochemicals, Uppsala, Sweden) column (5 mg protein/mL), equilibrated with buffer A and eluted by a linear gradient of 0–0.5 M KCl in buffer A.

Affinity chromatography in Thd-Sepharose and TTP-Sepharose. The fractions from DEAE-chromatography containing only thymidine kinase (TK1) activity were pooled and directly applied to thymidine-Sepharose (*p*-aminophenyl-3′-TMP-Sepharose, prepared as described in Ref. 5), which was equilibrated with buffer C containing 50 mM potassium phosphate (pH 7.5), 10% glycerol, 5 mM $MgCl_2$, 5 mM dithiothreitol and 0.5 mM of the detergent CHAPS. The column was washed with 10 volumes of the same buffer and 5 volumes of the same buffer with 10 mM potassium phosphate buffer. TK1 was eluted with 2 column volumes of 100 μM Thd in the latter buffer.

Fractions containing both Thd kinase (TK1) and dCyd kinase activity (eluting with 0.05 M KCl) were pooled and then applied to and eluted from a Thd-Sepharose column, as described above for TK1.

Fractions containing dCK (eluting with 0.2 M KCl) were directly applied to a hydroxylapatite column and eluted with a phosphate gradient as described previously [21]. The active fractions were pooled and dialysed against 10 mM Tris–HCl (pH 7.6), 5 mM $MgCl_2$, 1 mM DTT and 10% glycerol. The dialysed protein was applied to a TTP-Sepharose [21] column which was washed with the same buffer followed by a wash with the same buffer containing 60 mM KCl. Bound dCK was eluted with 1 mM TTP added to the buffer and concentrated on a second small hydroxylapatite column equilibrated with buffer containing 10 mM potassium phosphate (pH 7.6), 20% glycerol, 5 mM Mg_2Cl and 1 mM DTT. dCK was eluted with the same buffer containing 0.2 M potassium phosphate and the protein fractions contained no detectable TTP, as determined spectrophotometrically.

Enzyme assays. Enzyme activity was measured by a radiochemical method as described essentially by Ives *et al.* [21]. Thymidine kinase activity was measured with 10 μM [^{3}H]Thd (or other Thd-dUrd analogs), 2.5 mM ATP, 2.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 15 mM NaF and 2.5 mM DTT. The cocktail for Ado or dAdo kinase activity determination contained 10 μM [^{3}H]Ado or 100 μM [^{3}H]dAdo, 50 μM EHNA (a gift from Burroughs Wellcome, Research Triangle Park, NC, U.S.A.), 100 mM KCl, 2.5 mM DTT, 50 mM Tris–HCl (pH 7.5), 15 mM NaF, 2.5 mM $MgCl_2$ and 2.5 mM ATP. The cocktail for dCK activity contained 10 μM [^{3}H]dCyd (or ddCyd and araC), 10 μM tetrahydrouridine (Calbiochem, San Diego, CA, U.S.A.), 2.5 mM ATP, 2.5 mM $MgCl_2$, 50 mM Tris–HCl (pH 7.5), 100 mM KCl, 15 mM NaF and 2.5 mM DTT. In all cases, the specific activity of the substrate was around 100 cpm/pmol. Aliquots of the assay mixtures were spotted on Whatman DE-81 filters which were washed, eluted and counted as described previously [20]. Proteins were determined by the method of Bradford [22] using bovine serum albumin as standard.

Analysis of substrate saturation kinetic experiments was done using the Michaelis–Menten equation and a non-linear regression data analysis program (Enzfitter by R. J. Leatherbarrow from Elsevier-Biosoft).

Preparation of antibodies against dCK. Immune serum against human dCK was produced as

described, essentially, by Sherley and Kelly [18], by inoculation of protein into the poplitea lymph nodes of a rabbit. Initially, four lymph nodes were injected with a total of 50 μg >90% pure human dCK, prepared as described [20], emulsified with Freunds Complete adjuvant. Approximately 40 μg of the same material, emulsified with Freunds incomplete adjuvant, was used to booster the rabbit by subcutaneous injection, four times on the back. Sera were collected 10 days after the last three boosters.

The sera were tested by immunoblotting methods as follows: crude extracts and pure dCK were electrophoresed in 12% SDS–polyacrylamide gels [20] and transferred to nitrocellulose (Transblot transfer medium, 0.2 μm, Biorad, Munich, F.R.G.) by electroblotting for 1 hr, as described by Towbin *et al.* [23]. The sheets were blocked for 1 hr with buffer containing 0.5% Tween 20 and 0.5 M NaCl in PBS followed by a 3–18 hr incubation with antiserum at different dilutions in the same buffer to allow antibody binding to occur. The nicrocellulose filters were then washed three times for 20 min with the same buffer, followed by incubation for 1 hr with 1:1000 diluted anti-rabbit alkaline phosphatase coupled sheep IgG (Sigma, Immunochemicals), washed and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazodium in 0.1 M ethanolamine–HCl buffer (pH 9.6) [24].

Several other protein bands besides dCK cross-reacted with the crude antisera and, therefore, an affinity purification of the anti dCK-antibodies was performed as described, essentially, by Olmsted [25]. In summary, 20 μg pure human dCK were applied to a SDS gel and electrophoresed and immunoblotted as described above. The dCK band was identified and the corresponding piece of nitrocellulose sheet was cut out. The filter pieces thus obtained were blocked, washed and incubated with 1 mL antiserum diluted with 9 mL PBS and 0.02% sodium azide for 18 hr at room temperature. The filters were then transferred to a large syringe and washed with 20 mL ice-cold PBS, followed by 5 mL 50 mM Tris–HCl (pH 7.5), 5 mM EDTA and 150 mM NaCl. Bound antibodies were eluted with 6 mL 0.2 M glycine–HCl buffer (pH 2.8) with 0.1% BSA for 2 min, followed immediately by neutralization with an equal volume 1 M sodium phosphate buffer. The filters were then washed with 6 mL cold PBS which was added to the eluate. This solution contained approximately 20 μg protein/mL and was designated purified antibody.

RESULTS

Purification and characterization of pyrimidine deoxynucleoside kinases from spleen and liver extracts

Thymidine kinase 1 as well as thymidine kinase 2 and deoxycytidine kinase can be extracted from normal and leukemic spleen and separated and partially purified by ammonium sulphate precipitation and DEAE-chromatography [19, 20]. dCyd and Thd kinase activities were determined in fractions obtained by ion exchange chromatography of proteins from both the spleen and liver of human, mouse and monkey. Figure 1 shows the results with mouse tissues but very similar results were obtained also with tissues from humans [19, 26] and monkeys. There were two thymidine kinase peaks in spleen extracts; one major activity that did not bind to the column and which corresponded to the basic enzyme TK1, and a second eluting with 0.05 M KCl showing both dCK and TK activity, corresponding to TK2. With approximately 0.2 M KCl cytoplasmic dCK eluted, this being the major dCyd phosphorylating activity (Fig. 1A). Two major peaks of dAdo phosphorylating activity were also observed; one that eluted with low salt, corresponding to adenosine kinase (see below), and a secoond peak coinciding with cytoplasmic dCK; but the relative levels of these activities varied in spleen extracts from mouse as compared to those of human and monkey (Table 1) [3, 26].

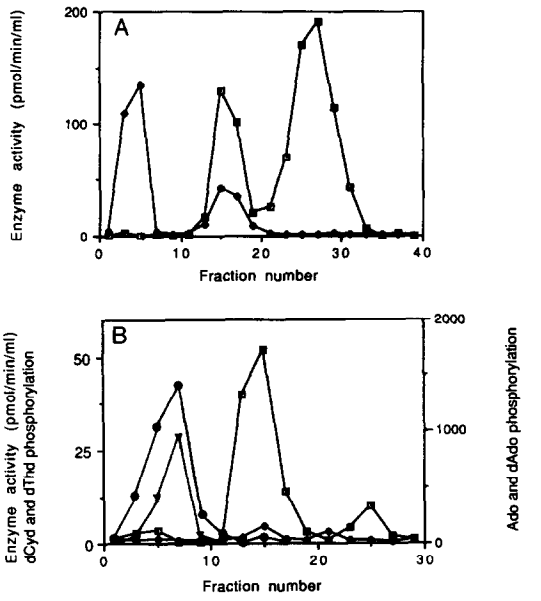

Fig. 1. DEAE-Sepharose chromatography of extracts from mouse spleen (A) and liver (B). Proteins were applied to the columns which were eluted with linear KCl-gradient from 0–0.5 M. Ado (●), dAdo (▲), dCyd (□) and dThd (■) phosphorylating activities were determined in the fractions as described in Materials and Methods.

Table 1. Substrate specificity of human, mouse and monkey dCK

	Human dCK		Mouse dCK		Monkey dCK	
Substrates	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)	K_m (μM)	V_{max} (%)
dCyd	1.5	100*	1.8	100†	2	100‡
AraC	20	200	30	180	8	145
ddCyd	60	75	30	4	30	25
dAdo	80	420	21	50	200	600

The 100% values for dCyd correspond to the following V_{max} values (nmol/min/mg): *200, pure human dCK; †0.2, DEAE purified mouse dCK; ‡0.5, DEAE purified monkey dCK. The kinetic constants were determined as described in Materials and Methods. Values presented are from one typical saturation curve, repeated at least twice with very similar results.

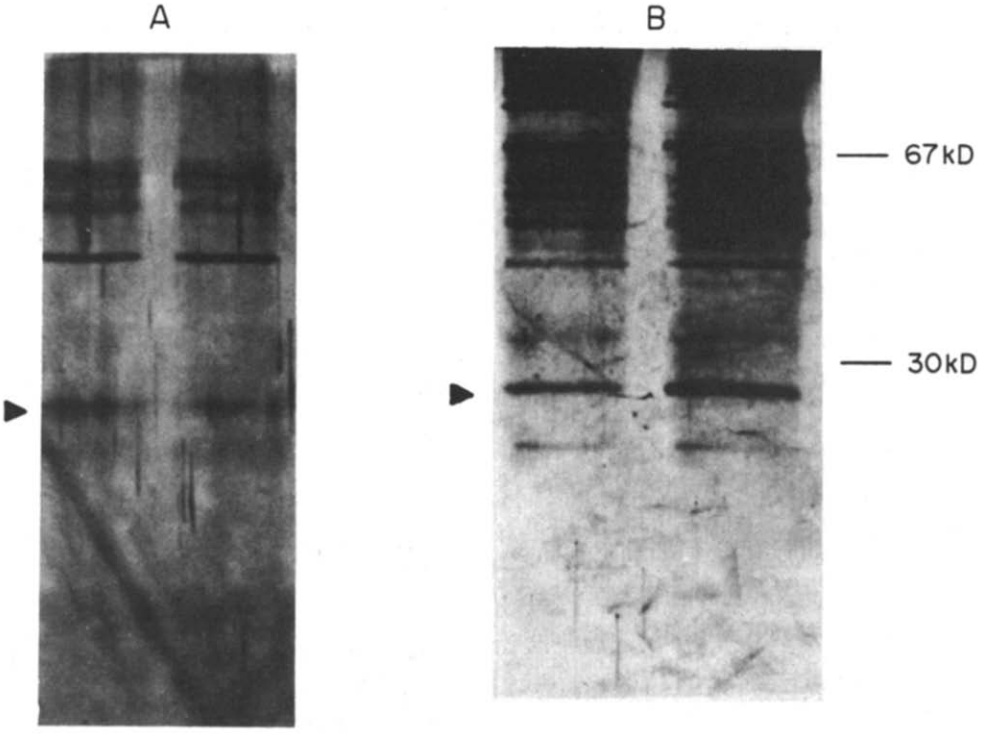

Fig. 2. SDS gel electrophoresis of fractions from thymidine-Sepharose containing maximal mouse TK1 (A) and TK2 (B) activity. The polypeptide bands were developed by silver staining and the arrows indicate the position of the presumed TK1 and TK2 bands. The bovine serum albumin (67 kD) and carbonic anhydrase (30 kD) were used as molecular weight markers.

Liver extracts gave a different elution profile with only one peak of dCyd and low Thd kinase activity corresponding to TK2 (Fig. 1B). Ado kinase as well as dAdo kinase activity coeluted in fractions eluting with low salt (Fig. 1B), indicating that it was Ado kinase activity that was the only dAdo phosphorylating activity in liver extracts. A minor dCyd phosphorylating activity was also observed which may correspond to cytoplasmic dCK. Total TK2 activity per gram tissue was roughly the same in the three species tested. Thus, DEAE-chromatography resulted in separation of the deoxynucleoside kinases and an overall purification of 10–20-fold compared to the crude extracts. These results are in accordance with recent results obtained with extracts from duck spleen and the liver [27].

As discussed below, in some cases, due to the lack of sufficient amounts of starting material, the partially purified enzyme fractions after DEAE-chromatography were tested for their substrate specificity but in the case of mouse spleen TK1, TK2 and dCK, as well as monkey liver TK2, further purification was possible. The procedures followed closely those developed for leukemic human spleen enzymes [19, 20] i.e. using thymidine-Sepharose for TK1 and hydroxylapatite and thymidine-Sepharose for TK2, as described in Materials and Methods. In the case of mouse TK1, SDS gel electrophoresis and silverstaining of fractions from thymidine-Sepharose revealed a band at 25–26 kD whose intensity coincided with peak enzyme activity (Fig. 2A). Affinity chromatography of mouse TK2 showed a band around 28 kD coeluting with the activity (Fig. 2B). These results indicate that the subunit molecular weights of mouse TK1 and 2 are very similar to those of the corresponding human enzymes [18, 19].

In the case of thymidine-Sepharose-purified monkey liver TK2, we could not detect any protein bands but photoaffinity labeling of the fraction after Thd-Sepharose revealed a 28 kD band that was photolabeled specifically. This was very similar to that which has been observed with human TK2 (Jansson O, Bohman C, Munch-Petersen B and Eriksson S, in preparation).

Antibodies to human dCK were prepared using relatively low amounts of pure enzyme as antigen which were injected into rabbit poplitea lymph nodes. However, the crude antiserum cross-reacted with several other bands in Western blot analysis and we, therefore, performed an affinity purification on nitrocellulose bound 30 kD dCK by the method of Olmsted [25]. With this purified antibody the 30 kD dCK band was the predominant one stained in crude extracts and in fractions from DEAE-chromatography. We therefore tested the dCK fractions isolated from human, mouse and monkey spleen and found that a 30 kD band was stained in all cases (Fig. 3). Immunostaining of these bands could be blocked by the addition of excess pure human dCK (data not shown). No 30 kD band was observed in the TK2-containing fractions from monkey liver.

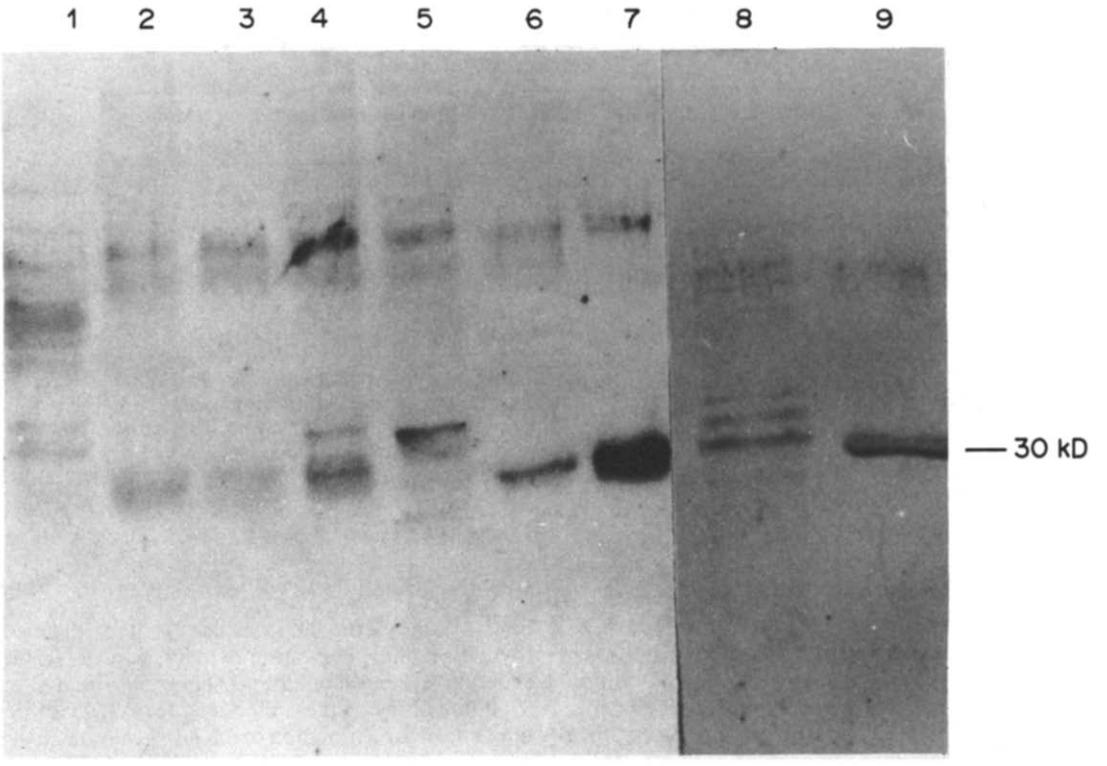

Fig. 3. Immunoblots of protein extracts and purified dCK using affinity purified rabbit polyclonal antibodies against human dCK. Cell extracts and 12% SDS–polyacrylamide gels were prepared and electroblotted to nitrocellulose, the antibodies were affinity purified and the blots were developed as described in Materials and Methods. Lanes contained the following: (1) 60 μg proteins from monkey liver DEAE-fractions with maximum TK2 activity; (2) 60 μg proteins from monkey spleen DEAE-fractions with maximal dCK activity; (3) 60 μg proteins from the corresponding human fractions; (4) and (5) 40 μg proteins from DEAE-fractions of wild type and dCK negative (araC 8D) CEM cells, respectively; (6) and (9) 5 μg hydroxylapatite purified human dCK (approximately 1% pure); (7) 40 ng pure human dCK; (8) 10 μg hydroxylapatit purified mouse dCK.

To demonstrate the identify of the 30 kD band, DEAE-fractions from CEM T-lymphoma wild type cells and a mutant (araC-8D) lacking dCK activity [15] were also tested on the same blot. The mutant cells did not show any band at 30 kD, either in the crude extracts or in the corresponding DEAE-fractions, while several cross-reacting bands were observed in both mutant and wild type fractions (Fig. 3). Thus, we may conclude that antibodies against the human enzyme cross-react with mouse and monkey dCK, and they all appear to have the same subunit molecular weight.

Substrate specificity of mouse and monkey TK1 and TK2

Due to limitations in the amount of starting material we had to use only partially purified (approximately 20-fold higher specific activity than crude extracts, see legend to Table 2) but completely separated enzymes. The capacity of both TK1 and TK2 to phosphorylate several different pharmacologically important pyrimidine analogs e.g. AZT [7, 8], FLT [12] and araC was determined and compared to that of the pure human enzymes [19, 28].

TK1 from all sources showed high activity with dUrd and AZT, somewhat lower activity with FLT and no activity with cytosine containing deoxynucleosides (Table 2). TK2, on the other hand, showed high activity with Thd, dUrd, FdUrd and dCyd, while AZT and araC were much less effective and no activity was found with FLT. The fact that mouse TK2 showed decreased activity with Thd as compared with dCyd is probably related to the fact that this fraction contained some Thd degrading enzyme(s). Highly purified TK2 from monkey liver appeared to have the same substrate specificity as pure human spleen TK2, which is in line with recent data from pure human liver TK2 (Jansson O, Bohman C, Munch-Petersen B and Eriksson S, in preparation). In conclusion, we found from this limited characterization of thymidine kinase of mouse and monkey, no significant difference to the pure human enzymes [19, 28].

Substrate specificity of dCK from monkey and mouse spleen

Table 1 shows K_m and V_{max} values for dCK from three different sources with some of the known substrates. Two dCyd analogs of pharmacological

Table 2. Substrate specificity of human, mouse and monkey TK1 and TK2

Substrates	Relative activity at 20 μM (%)					
	Human TK1	Mouse TK1	Monkey TK1	Human TK2	Mouse TK2	Monkey TK2
Thd	100*	100†	100‡	100§	100‖	100¶
dCyd	≤6	≤1	≤1	85	180	89
dUrd	95	53	ND	120	57	54
FdUrd	58	ND	ND	110	ND	43
AraC	ND	ND	ND	≤5	≤3	≤7
AZT	49	64	40	5	16	8
FLT	17	30	10	≤1	≤10	≤4

The 100% values correspond to the following specific activities (nmol/min/mg): *9000, pure lymphocyte enzyme; †0.4, spleen DEAE-fraction; ‡0.3, spleen DEAE-fraction; §200, pure spleen enzyme; ‖0.1 spleen, DEAE-fraction; ¶100, purified monkey liver enzyme. The values represent means of the least two determinations. The difference in detection levels is due to the difference in specific activity of the radioactive substrates used.

ND, not determined.

importance were tested i.e. araC, a much used cytostatic compound and ddCyd, one of the most efficient anti-HIV agents known [7, 9], in addition to dCyd and dAdo. In the case of monkey enzyme, we did not have sufficient amounts of spleen for a complete purification of the enzyme but with mouse dCK we performed several additional steps in the purification, leading to a highly purified enzyme preparation with a major peptide of 30 kD (data not shown). However, the overall yield of enzyme activity was low and this enzyme was apparently much less stable during purification than the human enzyme. We therefore used DEAE-purified dCK from monkey and mouse (approximately 10-fold purified compared to crude extracts; see legend to Table 1) for the basic characterization of the substrate specificity of the enzyme.

The apparent K_m and V_{max} values for dCyd and araC were similar for the pure human enzyme [20] and both mouse and monkey dCK. However, there were distinct and reproducible differences in the cases of ddCyd and dAdo. While monkey and human dCK showed very similar values for the different enzymes, mouse dCK had a several fold lower V_{max} (and a somewhat lower K_m) for ddCyd and dAdo as compared to that for dCyd (Table 1). Addition of dCyd caused competitive inhibition of the dAdo phosphorylation of mouse dCK with an apparent K_i of 0.33 μM. We also tested the capacity of mouse dCK to phosphorylate dGuo and the activity in this case was similar to that with dAdo i.e. several fold lower than that which has been shown for the human enzyme [20]. With the highly purified mouse dCK we found activity with dCyd as substrate only; no activity with 100 μM ddCyd or dAdo was seen.

DISCUSSION

In the present investigation, three deoxynucleoside kinases were separated and (partially) purified from mouse and macaque monkey tissues in order to make a biochemical characterization of their subunit structure and substrate specificities. The human and mouse genes for TK1, which is the principal thymidine phosphorylating activity in proliferating cells, have been cloned and sequenced, and show 87% homology at the amino acid level [29, 30]. To our knowledge no monkey TK1 gene has been sequenced but it is unlikely that primate TK1 genes differ substantially. It is, therefore, not surprising that we do not observe any significant differences in the biochemical properties of the three TK1s, nor were there substantial differences in the substrate specificities towards natural pyrimide deoxyribonucleosides and the 2′,3′-dideoxy analogs tested here. However, it has been shown previously [17] that in human lymphoid cells AZT accumulates as AZTMP whereas in murine cells it is more efficiently converted to AZTDP and AZTTP. It appears that mouse thymidylate kinase has a higher capacity to phosphorylate AZTMP than the corresponding human enzyme which has been shown to have a very low V_{max} for AZTMP [31]. This fact has important biological consequences and it emphasizes the danger of extrapolating conclusions obtained with mouse cells to human cells.

The mitochondrial deoxypyridine kinase (TK2) has not been studied in great detail and the gene has not been cloned. We found here that the subunit molecular weight and substrate specificity of TK2 from mouse and monkey spleen and liver appear to be similar to those found for the pure human leukemic spleen enzyme [19, 28]. The pharmacological implications of the deoxynucleoside substrate specificity of TK2 have been discussed elsewhere [19]. It is likely that this enzyme is also highly conserved in other species but further studies are needed to clarify this point.

The unexpected finding from this study was the fact that although mouse dCK appeared to have the same subunit molecular weight as the human and monkey enzymes and showed immunological cross-reactivity with them, it had a different substrate specificity. Purine deoxynucleosides and the analog ddCyd appeared to be used as substrate several fold less efficiently by the mouse enzyme, as compared

to human and monkey dCK. For instance, at 10 μM concentration of dAdo or ddCyd, mouse dCK phosphorylated these nucleosides at approximately 10 and 5% of the rate of dCyd phosphorylation, while the responding values for human dCK were 100 and 50%, respectively.

Murine dCK was purified extensively by Meyers and Kreis [32], and they found no activity with dAdo or dGuo as substrate. Here, we have shown that partially purified mouse dCK does phosphorylate dAdo (and dGuo) and that dCyd is a competitive inhibitor of this reaction, excluding that our partially purified mouse preparation contained other dAdo kinases(s). However, also in this study further purification resulted in a dCK preparation that showed no significant dAdo activity and we suspect that this might have been due to a selective inactivation process analogous to that which has been shown to occur with human dCK [33]. In the case of human dCK, it was observed that the temperature optimum and half life of the enzyme differed depending on which substrate was used and it was suggested that the purine phosphorylating form of dCK was less stable than its dCyd phosphorylating form [33]. A similar inactivation of mouse dCK appears likely to occur and may explain why purified mouse dCK lacks purine deoxynucleoside kinase activity.

Carson *et al.* [16] showed previously that mouse tissues have a different distribution of dAdo kinase(s) to that of human tissues; in the former case it corresponded to that of adenosine kinase while in the latter it followed that of dCK. Furthermore, dAdo phosphorylation by mouse lymphocytes at low concentrations could be inhibited by adenosine but not dCyd, while in the case of human lymphocytes the reverse was true. Their conclusion was that dCK was more important in phosphorylating dAdo in human than in mouse lymphocytes. The results presented here provide an explanation for this difference; mouse dCK showed a reduced capacity to phosphorylate dAdo compared to human dCK. A comparison of the primary sequences of mouse and human dCK may provide information on the molecular background to this diverging substrate specificity.

Balzarini *et al.* [17] have demonstrated that mouse L1210 cells accumulate 10–100-fold less of the products of the phosphorylation of ddCyd as compared to human Molt-4 cells. Inefficient phosphorylation of ddCyd by mouse P388 leukemia cells was also reported recently by Plagemann and Woffendin [34]. A pronounced difference in the cytotoxicity of ddCyd towards mouse S49 and human CEM cells has also been observed by Ullman *et al.* [13] i.e. human cells were growth inhibited at 5 μM ddCyd, whereas no growth inhibition of S49 cells occurred at 400 μM ddCyd. We demonstrate here that the explanation for these results is most likely to be the difference in the capacities of mouse and human dCK to use this analog as substrate. This fact may also explain why in mouse but not in human lymphoblasts, ddCyd phosphorylation appears to be the rate limiting step in the intracellular accumulation of ddCTP [35].

Data presented here show clearly that human and monkey dCK appear less specific with regard to their phosphate acceptors as compared to mouse dCK and that this fact is of great importance when considering the capacity of mouse cells to phosphorylate certain deoxynucleoside analogs. Therefore, the mouse model may be less valid than the monkey model for the development of deoxynucleoside analogs intended for therapeutic use in humans.

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