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Nucleoside kinases in T and B lymphoblasts distinguished by autoradiography

(immunodeficiency/adenosine deaminase/purine nucleoside phosphorylase)

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ABSTRACT **Nucleoside kinases catalyze the initial step leading to the accumulation of deoxypurine nucleotides that occurs in patients with inherited deficiencies of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) and purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1). This accumulation is thought to interfere with DNA synthesis in lymphocytes and, thus, to cause the immune defects associated with these enzymopathies. However, there is controversy about the identity of the nucleoside kinases that are responsible for intracellular phosphorylation of deoxyadenosine in adenosine deaminase deficiency and deoxyguanosine in purine nucleoside phosphorylase deficiency. To distinguish the nucleoside kinases present in T and B lymphoblastoid cells, we have coupled discontinuous PAGE with autoradiography. This procedure showed that deoxycytidine kinase (NTP:deoxycytidine 5′-phototransferase, EC 2.7.1.74), deoxyadenosine kinase (ATP:deoxyadenosine 5′-phosphotransferase, EC 2.7.1.76), and adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) are all present in both T and B lymphoblasts. While adenosine kinase is expressed at nearly equal levels in B and T cells, the deoxynucleoside kinases are expressed at much lower levels in B cells than in T cells. The autoradiographic data agreed with assays of the nucleoside kinase activities. Molecular weights were determined by using 5–10% polyacrylamide gels. M_r values were 29,000 for adenosine kinase, 41,000 for deoxyadenosine kinase, and 53,000 for deoxycytidine kinase and its isozyme. The reduced expression of deoxycytidine and deoxyadenosine kinases in B lymphoblasts may account for the lower accumulation of deoxypurine nucleotides in B cells as compared with T cells.**

Deficiencies of two metabolically related enzymes, adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) (1–3) and purine-nucleoside phosphorylase (purine-nucleoside:orthophosphate ribosyltransferase, EC 2.4.2.1) (2–4), are associated with severe combined immune deficiency and defective T-cell function, respectively. The major underlying mechanism causing the immune defects in both diseases is thought to be accumulation of dATP (2, 3, 5, 6) and dGTP (2, 3, 5, 7), respectively, both of which interfere with the action in lymphocytes of ribonucleotide reductase, an enzyme essential for DNA synthesis. This accumulation of deoxyribonucleotides in both deficiencies is initiated by the action of nucleoside kinases. While it is known that lymphoid cells possess high phosphorylative activities towards both deoxyadenosine and deoxyguanosine (7–10), it has not been determined which specific nucleoside kinases are involved, and their substrate specificities have also been debated (8, 11–17). Some investigators have reported that mammalian cells possess both adenosine kinase (ATP:adenosine 5′-phosphotransferase, EC 2.7.1.20) and deoxyadenosine kinase (ATP:deoxyadenosine 5′-phosphotransferase, EC 2.7.1.76) as separate enzymes (8, 15, 16, 18, 19). However, others have suggested that mammalian cells contain only adenosine kinase (12, 20). There is general agreement on the presence of deoxycytidine kinase (NTP:deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) in lymphoid tissues (7–10, 17, 19), but no evidence has been presented for a separate deoxyguanosine kinase enzyme.

The purpose of this study was to distinguish the kinases present in T and B lymphoblastoid cells and to investigate their substrate specificities. For this purpose we used polyacrylamide gel electrophoresis (PAGE) following by autoradiography. This procedure also permitted determination of M_r values for the kinases.

EXPERIMENTAL PROCEDURES

Materials. 2′-Deoxy[2,8-^{3}H]adenosine (25 Ci/mmol; 1 Ci = 37 GBq) and 2′-deoxy[5-^{3}H]cytidine (22 Ci/mmol) and deoxy[8-^{3}H]guanosine (5 Ci/mmol) were from ICN. The other reagents were from Sigma.

Cell Lines. The human T-cell lines CCRF-CEM, CCRF-HSB, and 8402, and the human B-cell lines 8392 and RPMI 7666 were obtained and grown as described (21, 22). The B-cell line SL-BM was established from peripheral blood lymphocytes of a normal individual by infection with Epstein–Barr virus (22). The CCRF-CEM mutant T cell lacking adenosine kinase and deoxycytidine kinase (13) was a generous gift from D. A. Carson (Scripps Clinic, LaJolla, CA).

Kinase Assays. Deoxyguanosine kinase and deoxycytidine kinase activities were measured in cell extracts as described (7), and deoxyadenosine kinase and adenosine kinase were measured by the method of Ives *et al.* (23) but with the addition to the reaction mixture of 10 μM *erythro*-[9-(2-hydroxy-3-nonyl)] adenosine as an adenosine deaminase inhibitor. The substrate concentrations were 300 μM for deoxyguanosine, deoxycytidine, and deoxyadenosine, and 20 μM for adenosine—each dissolved in 0.1 M Tris·HCl (pH 7.9) containing 10 mM ATP, 12 mM $MgCl_2$, and 10 mM dithiothreitol. The deoxyguanosine kinase assay contained purified rabbit anti-human purine nucleoside phosphorylase IgG (7).

PAGE and Molecular Weight Determinations. Discontinuous tube (7-mm i.d.) PAGE at pH 8.9 was performed as described (24). Molecular weights were determined by retardation coefficients obtained from polyacrylamide concentrations of 5–10% by the method of Bryan (25). A calibration curve was made with lactalbumin, bovine carbonic anhydrase, chicken egg albumin, and the monomer and dimer of bovine albumin. Linear regression was used to establish the slopes of the Ferguson plots of 100 log (relative mobility × 100) against polyacrylamide percentage (26) and the calibration plot of log K_r (retardation coefficient) against log M_r (25).

Autoradiography. The radiolabeled nucleotide products of the kinase reactions were immobilized by lanthanum precipitation by the method of Tischfield *et al.* (27). Tube gels of 7

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or 5 mm i.d. were run with equivalent protein loads. After electrophoresis, the gels were cut in half and incubated for 60 min at 37°C with radiolabeled nucleoside in 2.5 ml of 5 mM ATP/6 mM $MgCl_2$/10 mM dithiothreitol/0.1 M Tris·HCl, pH 7.9. The radioactivity used gave substrate levels of less than 1 μM: 10 μl of deoxycytidine (1 mCi/ml; 22 Ci/mmol); 10 μl of adenosine (1 mCi/ml; 32 Ci/mmol); 20 μl of deoxyadenosine (1 mCi/ml; 5 Ci/mmol). In the competitive substrate experiments, the incubation solution contained 1 mM unlabeled nucleoside. After incubation, the gels were washed and treated with 0.1 M lanthanum chloride (27), then washed and treated with Amplify (Amersham), and dried under vacuum at 60°C. Dry gels were exposed to Kodak X-Omat film at −70°C for 3–5 days.

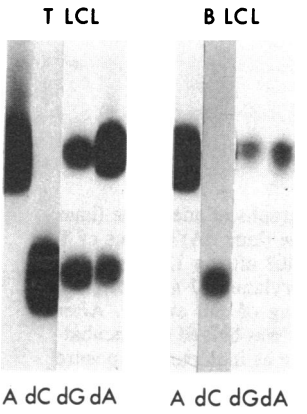

FIG. 1. Autoradiographs of nucleoside kinases of T lymphoblastoid cell CCRF-CEM (T LCL) and B lymphoblastoid cell SL-BM (B LCL). Tritiated substrates are indicated at the bottom of the gels. For both cell types, the samples were 50 μg in lanes A (adenosine) and dC (deoxycytidine) and 300 μg in lanes dG (deoxyguanosine) and dA (deoxyadenosine). The origin was at the top, with downward migration in 7% polyacrylamide 7-mm i.d. gels to the anode. Autoradiographic exposure was for 3 days at −70°C.

RESULTS

Kinase Assays. Kinase activities towards deoxynucleosides were significantly higher in T cells than B cells, whereas adenosine phosphorylation was similar in both cell types (Table 1). The kinase assays also were done on T-cell extracts in the presence of each of the three unlabeled alternative nucleosides at 0.4 mM concentration. The only assay showing a major change associated with the presence of unlabeled nucleoside was that of deoxyguanosine kinase with added deoxycytidine, where the activity was reduced from 80.6 to 25.8 nmol/hr per mg of protein. For assays of adenosine, deoxyadenosine, and deoxycytidine phosphorylation in the presence of unlabeled nucleosides, no significant decreases were observed (data not shown). In these assays the ratio of unlabeled to tritiated nucleoside was 20:1 for adenosine kinase and 1.3:1 for deoxyadenosine, deoxyguanosine, and deoxycytidine kinases.

Autoradiographic Detection of Kinases. Cell extracts from T and B cells were analyzed to distinguish their kinase activities towards deoxyadenosine, deoxycytidine, deoxyguanosine, and adenosine as shown in Fig. 1. There were marked differences between T and B cells in the radioactive zones produced from the deoxynucleosides. The electrophoresed T-cell extracts showed two distinct strong zones on the gels incubated with deoxyadenosine and deoxyguanosine. The analogous gels with the same amount of protein extract from B cells gave single weaker bands that had the slower migration rate. The gels incubated with deoxycytidine showed a strong band for T cells and a weak band of similar electrophoretic mobility for B cells. Both T and B cell extracts gave similar strongly radioactive single bands with adenosine, apparently corresponding in migration rate to the slower-moving bands with deoxyguanosine and deoxyadenosine activity. Similar results were obtained from T-cell lines 8402 and CCRF-HSB and B-cell line RPMI 7666. The fact that both T- and B-cell extracts produced intense zones of activity against adenosine, but that the B-cell activities against deoxyadenosine were distinctly less than those of T cells, strongly suggested the presence of separate kinases against adenosine and deoxyadenosine having similar electrophoretic mobilities. To detect a difference in the two migration rates, T-cell extracts were run in 9% polyacrylamide for 12 hr, and the complementary gel halves were incubated with adenosine and deoxyadenosine (Fig. 2). The gels, which were dried in the same alignment, show that the adenosine kinase activity and the slower-moving deoxyadenosine kinase activity do represent two different proteins. Similar results were obtained with 10% polyacrylamide (data not shown). In Fig. 2 the faster migrating band developed with deoxyadenosine corresponds to the faster migrating bands obtained with deoxycytidine, deoxyguanosine, and deoxyadenosine in Fig. 1.

The presence of two separate kinases phosphorylating adenosine and deoxyadenosine was further investigated by analyzing extracts of a mutant CCRF-CEM T cell that lacked both adenosine kinase and deoxycytidine kinase activity (13). As shown in Fig. 3 the gels incubated with radiolabeled adenosine and deoxycytidine did not develop the intensely staining single bands that were produced by extracts of the parent cell line (Fig. 1). The gels incubated with radiolabeled deoxyadenosine and deoxyguanosine both showed a single intense band, analogous to the slower-migrating zone shown in Fig. 1, and the complete absence of the faster-migrating band that was visualized with deoxyadenosine, deoxyguanosine, and deoxycytidine with extracts from the parent cell line (Fig. 1). These autoradiographs provide further support for distinct adenosine and deoxyadenosine kinases.

Autoradiography with Competitive Substrates. To characterize the substrate specificities of the kinases, gels containing electrophoresed T-cell extracts were incubated with the radiolabeled substrates without and with the addition of the three alternate unlabeled nucleosides (Fig. 4). For the set of gels incubated with radioactive deoxyguanosine, the addition of deoxycytidine prevented formation of the faster-migrating band, whereas coincubation with deoxyadenosine precluded development of the slower-migrating zone. Unlabeled adenosine did not visibly change the intensity of either band. In these autoradiographs the unlabeled nucleosides exceeded the radiolabeled deoxyguanosine by 625:1. These results show that the slower-migrating protein visualized with deoxyguanosine is unlikely to be adenosine kinase and is probably deoxyadenosine kinase as demonstrated in Figs. 2 and 3.

From the set of gels incubated with deoxyadenosine, the addition of unlabeled adenosine did not prevent development of the two radioactive zones. However, the addition of deoxyguanosine eliminated the slower migrating band and left unchanged the intensity of the faster-migrating zone. The

Table 1. Nucleoside kinase activities in T and B cells

	Phosphorylating activity, nmol/hr per mg of protein			
	dGuo	dCyd	dAdo	Ado
T cells	83.5 ± 22.9	65.8 ± 16.9	79.8 ± 10.6	277.6 ± 99.1
B cells	15.9 ± 6.7	8.4 ± 2.6	16.2 ± 7.2	350.8 ± 32.8

Values are means ± SD for determinations on T-cell lines CCRF-CEM, CCRF-HSB, and 8402 and B-cell lines 8392, RPMI 7666, and SL-BM.

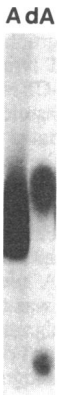

FIG. 2. Autoradiographs of adenosine (lane A) and deoxyadenosine (lane dA) kinases of T lymphoblastoid cell 8402 after a 12-hr electrophoresis in 9% polyacrylamide 7-mm i.d. gels. The sample was 200 μg of cell extract. After electrophoresis the gel was halved and incubated in tritiated substrate as indicated. Exposure was as in Fig. 1.

converse was seen with the addition of deoxycytidine—i.e., the faster-migrating band was eliminated, and the slower-migrating band was relatively unaltered. In these gels the unlabeled nucleoside was in excess of the radioactive deoxyadenosine by 3125:1. These results suggest that the slower-migrating band is a deoxyadenosine kinase with deoxyguanosine kinase activity and the faster-migrating band is deoxycytidine kinase with deoxyadenosine- and deoxyguanosine-phosphorylating activity. As expected, we demonstrated no enzyme with exclusively deoxyguanosine kinase activity.

In the gel sets incubated with tritiated adenosine and deoxycytidine, the radioactive zones were not greatly altered by any of the unlabeled substrate additions. The ratio of unlabeled to radiolabeled nucleoside was 8300:1 for the adenosine incubations and 5600:1 for the deoxycytidine incubations. These results indicate that the single intense bands seen with radiolabeled adenosine and deoxycytidine are due to adenosine kinase and deoxycytidine kinase, respectively. A weak band of intermediate mobility was seen in the gels incubated with deoxyadenosine and deoxycytidine. When the gels were run with more protein or exposed to film for a longer time, these bands were well developed and could be visualized with deoxycytidine, deoxyadenosine, and deoxyguanosine but not with adenosine (data not shown).

Molecular Weights from PAGE. Molecular weights were estimated for the two T-cell bands produced in the presence of radioactive deoxyguanosine and deoxyadenosine, the single intense bands associated with radiolabeled deoxycytidine and adenosine respectively, and the weak band of intermediate mobility obtained from gels incubated with deoxyadenosine and deoxycytidine (Table 2). Similar molecular weight values (≈53,000) were obtained for the intense band developed with deoxycytidine and the faster-migrating band developed with deoxyguanosine and deoxyadenosine.

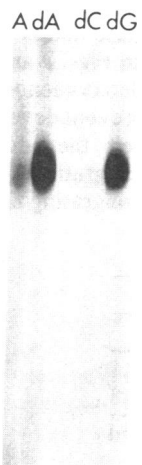

FIG. 3. Autoradiographs of nucleoside kinases of mutant T lymphoblastoid cell CCRF-CEM that lacked adenosine and deoxycytidine kinases (13). The 7% polyacrylamide 5-mm i.d. gels were exposed as in Fig. 1. Sample sizes were 150 μg in gels with radiolabeled deoxyadenosine (lane dA) and deoxyguanosine (lane dG) and 25 μg in gels with radiolabeled adenosine (lane A) and deoxycytidine (lane dC).

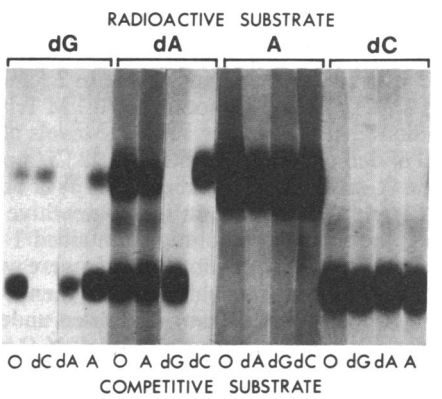

FIG. 4. Autoradiographs of nucleoside kinases of T lymphoblastoid cell CCRF-CEM. The tritiated nucleosides indicated at the top of the gel were coincubated with 1 mM unlabeled substrate indicated at the bottom. Sample sizes were 50 μg in gels with radioactive adenosine (A) and deoxycytidine (dC) and 300 μg in gels with radioactive deoxyguanosine (dG) and deoxyadenosine (dA). The 7% polyacrylamide 7-mm i.d. gels were exposed as in Fig. 1.

These are designated in Table 2 as deoxycytidine and deoxyguanosine/deoxyadenosine. The slower-migrating band observed with deoxyadenosine has a M_r of 41,000 and the single band obtained with adenosine had a M_r of 29,000. From the weak band seen with deoxyadenosine and deoxycytidine (Fig. 3), the same M_r of 51,000 was obtained, suggesting an isozyme of deoxycytidine kinase, designated $dCyd_i$ in Table 2.

DISCUSSION

Marked differences were obtained from the kinase assays of B- and T-cell extracts, with T cells possessing considerably higher phosphorylating activity than B cells towards deoxycytidine, deoxyguanosine, and deoxyadenosine. In contrast, adenosine kinase activities were similar for T and B cells. It has been shown that deoxycytidine kinase activity is high in thymus cells (8–10, 12, 15), that deoxyadenosine kinase activity is higher in thymus than spleen cells (15), and that deoxyguanosine phosphorylation is elevated in T lymphoblastoid cells (7). However, similar activities of deoxyadenosine kinase in T and B lymphoblasts have also been reported (19, 30).

The kinase assays were in agreement with the autoradiography, in which bands of reduced intensity were obtained with B cells after incubation with deoxycytidine, deoxyguanosine, and deoxyadenosine. The radioactive zone visualized

Table 2. Molecular weights by gel electrophoresis (5–10% polyacrylamide) on T-cell extracts

Bands with kinase activity	$M_r \times 10^{-3}$ This work*	Reported elsewhere	Refs.
dCyd†	53 ± 4	49–60	8, 17, 20
$dCyd_i$‡	51 ± 3		
dGuo/dAdo§	53 ± 2		
dAdo¶	41 ± 3	41–63	18, 28
Ado	29 ± 3	25–40	8, 20, 29

*Values are means ± SD from three gels.
†Fastest migrating band in deoxycytidine lanes.
‡Slower-migrating band in deoxycytidine lanes and an intermediate band in deoxyadenosine lanes (Fig. 4).
§Faster-migrating band in deoxyguanosine and deoxyadenosine lanes (Figs. 1 and 4).
¶Slower-migrating band in deoxyadenosine lanes.

with adenosine was similar with extracts of both B and T cells. If, as has been suggested (12–14), adenosine kinase were the major source of deoxyadenosine phosphorylation in T and B cells, autoradiographs developed with adenosine and deoxyadenosine should be similar for both cell types. The fact that such a similarity was not observed strongly suggested that two distinct enzymes, phosphorylating adenosine and deoxyadenosine, respectively, are present in T and B cells. This suggestion was confirmed by autoradiography, which showed the presence of deoxyadenosine kinase in CCRF-CEM T-cell mutant lacking both adenosine kinase and deoxycytidine kinase, and by autoradiography following prolonged electrophoresis, which demonstrated that adenosine kinase could be separated from deoxyadenosine kinase.

The autoradiographs made after addition of unlabeled nucleosides showed that there are two enzymes with phosphorylating activities toward both deoxyguanosine and deoxyadenosine—i.e., deoxyadenosine kinase and deoxycytidine kinase. The activity of deoxyadenosine kinase was not inhibited by an 8000-fold excess of adenosine or deoxycytidine. Also, the mutant T-cell line that expressed deoxyadenosine kinase, although lacking adenosine kinase and deoxycytidine kinase, did not show significant autoradiographic activity with adenosine or deoxycytidine. These findings are in agreement with previous studies of deoxyadenosine kinase that showed adenosine and deoxycytidine were very poor substrates (8, 18). Adenosine kinase activity, visualized by autoradiography, was not inhibited by a nearly 6000-fold excess of deoxyadenosine, deoxyguanosine, or deoxycytidine. Previous studies showed that mammalian adenosine kinase had poor activity towards deoxyadenosine (31, 32).

Assays performed after addition of competitive substrates showed significant reductions only with deoxycytidine added to the deoxyguanosine kinase assay. This lack of correlation with the autoradiography, where unlabeled deoxycytidine and deoxyadenosine changed the patterns obtained with deoxyguanosine, and unlabeled deoxyguanosine and deoxycytidine altered the bands given by deoxyadenosine, undoubtedly reflects the considerably higher ratios of unlabeled to labeled nucleoside possible with the autoradiographic procedure.

The determination of molecular weight by PAGE confirmed the presence of adenosine, deoxyadenosine, and deoxycytidine kinase in lymphoblastoid cells. The almost identical electrophoretic mobility and similar molecular weights of adenosine and deoxyadenosine kinases probably explains previous failure to separate the two enzymes by ion-exchange or gel-filtration chromatography. It also may have contributed to the lack of agreement regarding the presence (15–19) or absence (11–14, 20) of deoxyadenosine kinase in mammalian cells. An isozyme of deoxycytidine kinase with activity towards deoxycytidine, deoxyadenosine, and deoxyguanosine was observed in T lymphoblasts. This isozyme probably contributes to the multiple peaks obtained when tissue extracts are analyzed by ion-exchange column chromatography (19, 20).

Gower *et al.* (10) have demonstrated a specific mitochondrial kinase from calf thymus with activity toward guanosine as well as deoxynucleosides. As the T- and B-cell extracts did not show any guanosine kinase activity by autoradiography (data not shown), it is unlikely that the cytosolic deoxyadenosine kinase we have demonstrated resulted from contamination with mitochondria. A unique deoxyguanosine kinase with an acid pH optimum has been reported in neonatal mouse skin (33). Incubation of gels with radiolabeled substrates at pH 5.5 did not produce any extra zones of kinase activity (data not shown), suggesting that this deoxyguanosine kinase is not present in human lymphocytes.

It is reasonable to suggest that the differences shown between T and B cells in expression of deoxycytidine and deoxyadenosine kinases may contribute to the biochemical mechanisms involved in the immune defects related to deficiencies of adenosine deaminase and purine nucleoside phosphorylase. Furthermore, knowledge of the distribution and expression of nucleoside kinases in lymphoid cells is important to the design and application of nucleoside analogs for use in immunosuppression and chemotherapy.

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