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Purification of Deoxycytidine Kinase from Various Human Leukemic Cells by End-product Analog Affinity Chromatography

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Abstract: Homogeneous human deoxycytidine kinase was purified in one step from a variety of spontaneous human leukemic cells (T-ALL, B-ALL, B-CLL, AML, CML), and from cultured T-lymphoblast cells (MOLT-4) using the newly developed affinity medium, dCp_4-Sepharose. Starting with an ammonium sulfate fraction, purification was achieved in one step with the kinase being eluted from a column by the end product inhibitor, dCTP. The purified deoxycytidine kinase from T-ALL cells phosphorylated deoxyadenosine and deoxyguanosine, as well as deoxycytidine. The enzyme purified from T-ALL and B-CLL cells yielded one major band with a molecular weight of 52 kDa determined by SDS-polyacrylamide gel electrophoresis. AML and CML cells yielded one 52 kDa band and an extra band of 30 kDa molecular weight. On the other hand, B-ALL and MOLT-4 cells showed a low molecular weight band of 30 kDa only. However, the electrophoretic mobilities of enzymatic activity in 12% non-denaturing gels were identical for the dCyd kinase from all different kinds of leukemic cell lines, except that the B-ALL, B-CLL, and MOLT-4 cell preparations had an extra minor peak, all at the same position. dAdo and dCyd phosphorylating activities co-migrated indicating that these activities are all associated with the same protein. Two new methods, a disk implantation method and a nitrocellulose powder method were used with a small amount of enzyme protein to raise polyclonal antibodies against dCyd kinase purified from T-ALL cells.

Key words: affinity media, deoxycytidine kinase, leukemic cells, purification.

Deoxycytidine kinase (dCyd kinase; NTP : deoxycytidine 5′-phosphotransferase, EC 2.7.1.74) is the pyrimidine salvage pathway enzyme which catalyzes the phosphorylation of 2′-deoxycytidine to 2′-deoxycytidine 5′-monophosphate in the presence of a nucleoside triphosphate as a phosphate donor.

The dCyd kinase has been purified from the cytosol of human cells which phosphorylates more than one deoxynucleoside substrate, but in varying ratios, and with various K_m values (Durham and Ives, 1970; Yamada *et al.*, 1983; Hurley *et al.*, 1983; Sarup and Fridland, 1987; Datta *et al.*, 1989). The same enzyme also phosphorylates purine deoxynucleosides including several nucleoside analogues (Eriksson *et al.*, 1991; Kierdaszuk *et al.*, 1992). However it is not yet clear how many mutispecific kinases are expressed in human cytosol.

Mutant cells lacking deoxynucleoside kinase activities showed resistance to deoxynucleoside analogs used as chemotherapeutic agents, which indicates that one or more of these activities plays an important practical role in the activation of these drugs used as anticancer or antiviral agents (Verhoef *et al.*, 1981; Bhalla *et al.*, 1984). It has been shown that dCyd kinase contributes to the effectiveness of important chemotherapeutic nucleosides by phosphorylation, including Cytosine arabinoside (cytarabine), Adenine arabinoside, 2-fluoroadenine arabinoside, Guanine arabinoside, 5-fluoro deoxycytidine, 2′,3′-dideoxy cytidine, and 6-Thioguanine deoxyriboside (Plagemann *et al.*, 1978; Ullman *et al.*, 1988; Cooney *et al.*, 1986; Heinemann *et al.*, 1988; Kierdaszuk *et al.*, 1992).

Metabolic phosphorylation of these nucleosides to their nucleoside triphosphate derivatives is required for biological action *in vivo* and dCyd kinase is responsible for activating them to monophosphates (Chu *et al.*, 1968; Schrecker, 1970). Because dCyd kinase is the rate-limiting enzyme in the activation of many important anticancer and antiretroviral drugs, it has attracted attention in studies of drug resistance and sensitivity.

The purification and characterization of dCyd kinase in human cells is a challenging problem since this enzyme is unstable and comprises only a small fraction

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of total cellular proteins. The synthesis of suitable affinity chromatography media was critical to the study of this enzyme. Various kinds of multisubstrate-type inhibitors of deoxynucleoside kinases have been synthesized for affinity chromatography media. dNp_4A (deoxynucleoside 5′-adenosine 5″-p^1,p^4-tetraphosphate) was synthesized and coupled to Sepharose, and proved to be useful as an affinity ligand for resolving and purifying closely related kinase species (Ikeda and Ives, 1985). Homogeneous dCyd kinase was purified from human T-lymphoblast cells using a dCp_4A-Sepharose column (Kim *et al.*, 1988) and a kinetic mechanism and end-product regulation have been proposed (Kim and Ives, 1989).

Previous studies have shown that dCyd kinase is subject to strong feedback inhibition by its distal product, dCTP, in several systems (Durham and Ives, 1971; Cooper and Greer, 1973). dCTP inhibited the dCyd kinase more potently than dCp_4A, and exhibited kinetic effects quite similar to the multisubstrate analog, dCp_4A (Ikeda *et al.*, 1988).

A new affinity ligand dCp_4 has been synthesized hoping to find a means of fractionating a putative more-weakly-binding dCyd kinase isoenzyme, and also to increase the affinity column capacity. This study shows that using the ideal affinity medium, dCp_4-Sepharose, dCyd kinase can be purified from a variety of human leukemic cells to isolate isoenzymes of dCyd kinase. With a small amount of purified dCyd kinase protein, two methods using nitrocellulose membrane were developed to raise polyclonal antibodies. Western immunoassay was also performed.

Materials and Methods

Materials

Nucleosides and nucleotides were obtained from Sigma, Boehringer Mannheim, P-L Biochemicals, or Calbiochem. Tritiated nucleosides were supplied by ICN. The affinity ligand dCp_4 was synthesized according to the method of Ikeda and Ives (1985). Adipic acid dihydrazide-Agarose purchased from Sigma was coupled to a periodate-oxidized affinity ligand to construct an affinity column.

Chemicals for polyacrylamide gel electrophoresis and Bradford reagent were supplied by Bio-Rad Laboratories. Nitrocellulose membrane was purchased from Schleicher & Schuell. An immunoassay kit was obtained from Vector Laboratories. All other reagents and chemicals used were of reagent grade. Human leukemic lymphocytes, obtained by leukapheresis, were provided by the Tumor Procurement Service of the Ohio State University Comprehensive Cancer Center.

Enzyme preparation and assay

Human leukemic cells were centrifuged at $4,000\times g$ for 15 min and the supernatant was discarded. Red cells were removed by hypotonic lysis.

White cells were collected and disrupted by brief sonication in Seligmann balanced salt solution containing 0.05% EDTA and 2 mM dithioerythritol. Particulate fractions were sedimented at $20,000\times g$ for 20 min, and the supernatant fraction was stored at -80℃.

Preliminary to purification of the enzyme, nucleic acids were precipitated using streptomycin sulfate (1 g/g protein, pH 7.0). The supernatant solution was fractionated with ammonium sulfate. The protein which precipitated between 30% and 63% saturation was dissolved in equilibration buffer (EB, 20 mM Tris buffer, pH 8.0 at 4℃) containing 20% glycerol, 2 mM Dithioerythritol, and 2 mM EDTA.

Deoxynucleoside kinase activities were assayed using the disk anion-exchange method as described by Ives (1984). The dCyd kinase assay mixture contained 10 mM ATP, 12 mM $MgCl_2$, 0.1 M Tris-HCl (pH 7.5 at 37℃), 20 μM dCyd, [^{3}H]dCyd (0.5 μCi/assay), 5% glycerol, and 2 mM dithioerythritol. One enzyme activity unit was defined as the amount of enzyme which catalyzed the formation of 1 nmole of deoxynucleotide per minute. The protein concentration of the enzyme was measured by the method of Bradford (1978).

Affinity chromatography

A 1.8 ml dCp_4-Sepharose column was equilibrated with EB. Several protease inhibitors were added to the sample before it was passed through the affinity column, but the column itself was not equilibrated with these inhibitors. Final protease inhibitor concentrations in the sample were: 1,10-phenanthroline (5 mM), benzamidine (1 mM), and diphenylcarbamyl chloride (0.1 mM). After adding the sample at 4℃, the column was washed with 10 vol of EB, followed by 10 volumes of the same buffer plus 0.3 mM ATP and 0.5 M NaCl to remove any nonspecifically-bound proteins. Then, the ATP and salt were removed by washing again with 5 volumes of EB. The dCyd kinase was eluted by adding 0.3 mM dCTP in EB to the column and allowing it to stand overnight before resuming flow and collecting the eluate. Mg^{2+} ion was not required either for retention or elution of the enzyme.

Polyacrylamide gel electrophoresis

Sodium Dodecyl Sulfate (SDS) polyacrylamide gel electrophoresis was run by the method of Laemmli (1970) with a 4% stacking gel and a 12% separating gel. Discontinuous non-denaturing polyacrylamide gel electrophoresis, in 8 to 15% acrylamide gels, was run

according to the procedure of Laemmli, but without SDS, using a BIO-RAD model 360 mini vertical slab-cell at 4℃. To identify which protein band contained enzyme activity in non-denaturing gels, an unstained parallel channel was cut into 2 mm slices, soaked in assay mixture overnight, then assayed for enzyme activity (Gower *et al.*, 1979).

Molecular weight determinations

The relative molecular mass (M_r) of the native enzyme was determined by sedimentation equilibrium in a Beckman Airfuge by the method of Pollet *et al.* (1979), modified as described by Chakravarty *et al.* (1984). Either bovine serum albumin or Dextran T-40 was added (5 mg/ml) to the dCyd kinase samples to stabilize the density gradients, and glycerol was added to standard tubes to equal its concentration (6.7%) in the diluted kinase samples. Ten successive 10 μl samples were withdrawn from the meniscus of each tube and assayed for enzyme activity and protein content. Molecular weights were also estimated by observing the effect of polyacrylamide gel concentrations (8, 10, 12, and 15%) on electrophoretic mobilities of native proteins (Hedrick and Smith, 1968).

Immunization of rabbits

Disk implantation method: Prior to immunization New Zealand white rabbits (3~4 kg) were bled for preimmune serum. Nitrocellulose disks prepared with a 1-hole punch were sterilized under UV light, and each disk was moistened with 15 μl of a solution containing 0.2~0.3 μg of antigen protein. The rabbit was anesthetized and five longitudinal 1 cm incisions were made in the thigh muscle of one leg. To each disk, 25 μl of Freund's complete adjuvant was applied and two disks were implanted in each incision in the muscle. A total of 2 μg of antigen protein was used for the first immunization of each rabbit. The muscle was then closed by suturing with sterile gut followed by stitches in the skin. After three weeks Freund's incomplete adjuvant (25 μl) was applied to each disk and the two disks were surgically placed in each incision. These disks carried a total of 1 μg of antigen protein. A second booster immunization was performed with 1 μg of antigen protein, and the animal was bled via cardiac puncture.

Nitrocellulose powder method: An aqueous milky suspension of nitrocellulose powder was prepared in a blender and collected by centrifugation. Approximately 20 μg of enzyme protein was allowed to adsorb overnight onto the nitrocellulose powder equilibrated with 15 mM potassium phosphate (pH 8.0), containing 20% glycerol, then the nitrocellulose was collected by centrifugation. The pellet was emulsified in Freund's complete adjuvant and injected along the back muscle of a New Zealand rabbit. After three weeks, a booster dose (10 μg of protein on 50 μl of nitrocellulose), emulsified in Freund's incomplete adjuvant was injected intramuscularly. Four weeks later a second booster was administered subcutaneously at various sites on the back.

ELISA(Enzyme-Linked-Immunosorbent-Assay)

One hundred μl of each solution containing purified dCyd kinase (antigen) in a sodium carbonate coating buffer (pH 9.6) was added to the wells of an Immulon II microtiter plate. The plate was washed with 0.01 M Phosphate buffer (PB). A blocking solution containing 1% BSA in PB was added and the plate was incubated for 1 h. The plate was washed three times with 0.01 M PBS-Tween (0.05%). One hundred μl samples of diluted serum and a set of controls including preimmune serum were added to the appropriate wells. The plate was incubated and washed three times with PBS-Tween. One hundred μl of a 1/1000 dilution of peroxidase conjugated goat anti-rabbit IgG in PBS-Tween was added and incubated. The plate was washed three times and 100 μl of the peroxidase substrate solution was added. The substrate solution contained 4 mg of o-phenylene diamine, and 10 μl of 30% H_2O_2 in 10 ml of Phosphate citrate buffer (pH 5). The reaction was stopped by adding 50 μl of 1.25 M H_2SO_4 to each well. The abserbance at 490 nm for each well was read on the ELISA plate reader.

Results

Purification of the enzyme from T-ALL

The new dCp_4-Sepharose affinity column was tested first with the human T-ALL cell ammonium sulfate fraction to compare with a dCp_4A-Sepharose column. The result is shown in Fig. 1. The 30~63% ammonium sulfate fraction (37 U, 339 mg protein) of human T-ALL cells was applied to the dCp_4-Sepharose column with protease inhibitors (1,10-phenanthroline, benzamidine, and diphenylcarbamyl chloride), and dCyd kinase activity was eluted biospecifically by the inhibitory end-product, dCTP. A near-saturating mixture of both substrates, dCyd and ATP was considerably less effective than dCTP as an eluent. Although the dCp_4-Sepharose column contained only 1.8 ml of gel, and the sample was the same size as was normally applied to a 4 ml dCp_4A-Sepharose column, the run-through activity was cut nearly in half and a relatively good recovery of activity was obtained. Little activity was eluted with 0.3 mM dCTP immediately after applying the sample

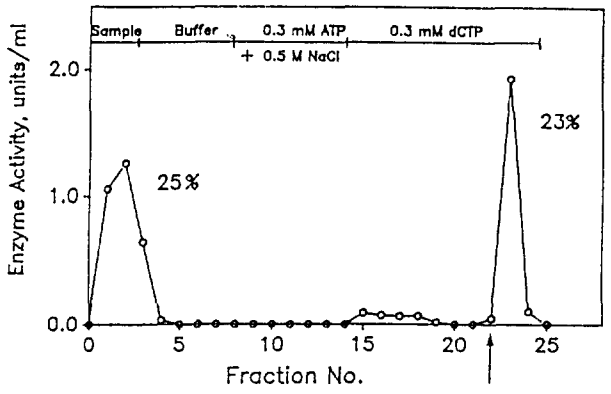

Fig. 1. Purification of dCyd kinase from T-ALL cell by dCp_4-Sepharose affinity column. Arrow denotes an overnight pause in the elution process.

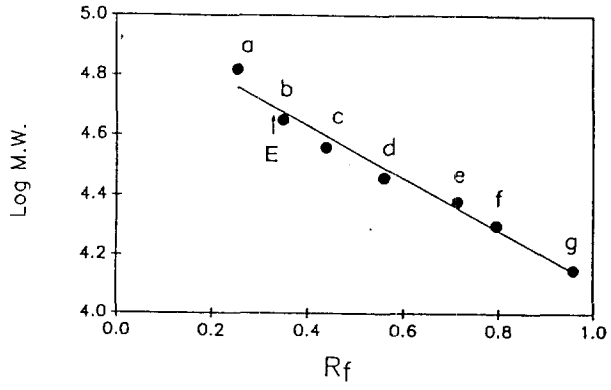

Fig. 2. Determination of the molecular weight by SDS gel electrophoresis. Standard proteins are as follow: a, bovine albumin; b, egg albumin; c, glyceraldehyde-3-phosphate dehydrogenase; d, carbonic anhydrase; e, trypsinogen; f, trypsin inhibitor; g, a-lactalbumin. E indicates the R_f of the purified dCyd kinase from T-ALL.

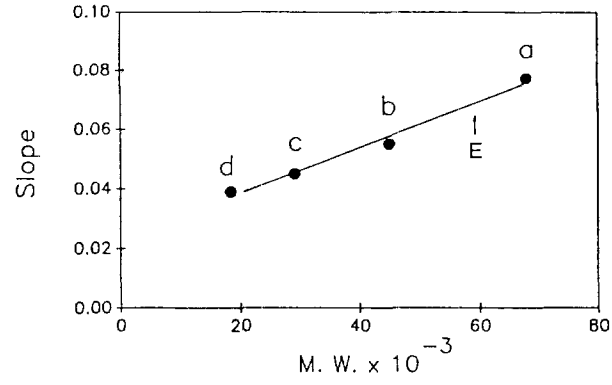

Fig. 3. Estimation of the molecular weight of the native dCyd kinase by the "Ferguson" relationship. The slopes of plots of mobility *vs.* total acrylamide concentration were determined for a series of standard proteins. a, bovine serume albumin; b, ovalbumin; c, carbonic anhydrase; d, b-lactoglobulin monomer. E indicates the purified dCyd kinase from T-ALL.

and most of the activity emerged after the column stood overnight with protease inhibitors in the sample. Thus, the slow release of kinase was even more pronounced than in experiments using the dCp_4A-Sepharose column. The amount of activity in the run-through fraction depended on the load of protein applied, and the specific activity of the enzyme eluted was approximately 21 nmole/min/mg. Columns were used repeatedly over a period of a year or more with only a gradual deterioration of performance.

Molecular weight of the native enzyme from T-ALL

Judging from the single band obtained with the affinity-purified enzyme from T-ALL in SDS-polyacrylamide gel electrophoresis, the preparation was virtually homogeneous. Only one band of protein was observed upon electrophoresis in nondenaturing gels, as well. Assurance that the stained band was, in fact, the enzyme was provided by activity assays in gel slices from a parallel channel. A series of such assays on gels containing several concentrations of acrylamide monomer revealed congruent migration of both the activity and the protein band. Had the protein bearing the activity and the protein yielding the stained band been different molecular species, they should be diverged as the gel concentration was changed (Hedrick and Smith, 1968). Since they did not, and since only one band was detected by both procedures, it can be concluded that the enzyme was practically homogeneous. A molecular weight of 52,000± 2000 daltons was determined for the silver-stained protein band in SDS-polyacrylamide gel electrophoresis (Fig. 2). Silver-staining gave a sharper band with a slightly higher relative mobility than did larger amounts of protein stained with Coomassie Blue.

Sedimentation equilibrium determinations were carried out with different batches of dCyd kinase from T-ALL cells prepared in the presence of protease inhibitors. An average molecular weight of 59,300± 3700 daltons was obtained for the native enzyme, assuming a partial specific volume of 0.725. An identical value was derived from mobility analysis of the enzyme activity after electrophoresis in non-denaturing polyacrylamide gels of various concentrations (Fig. 3), even though the latter determination was affected by the shape, as well as the mass, of the molecule. Although the molecular weight estimated for the native enzyme was somewhat larger than the 52 kDa value determined for the denatured polypeptide, it seems clear that this enzyme consists of a single polypeptide chain.

Substrate specificity

dCyd kinase purified from T-ALL cells retained the ability to phosphorylate dAdo and dGuo, but dCyd is

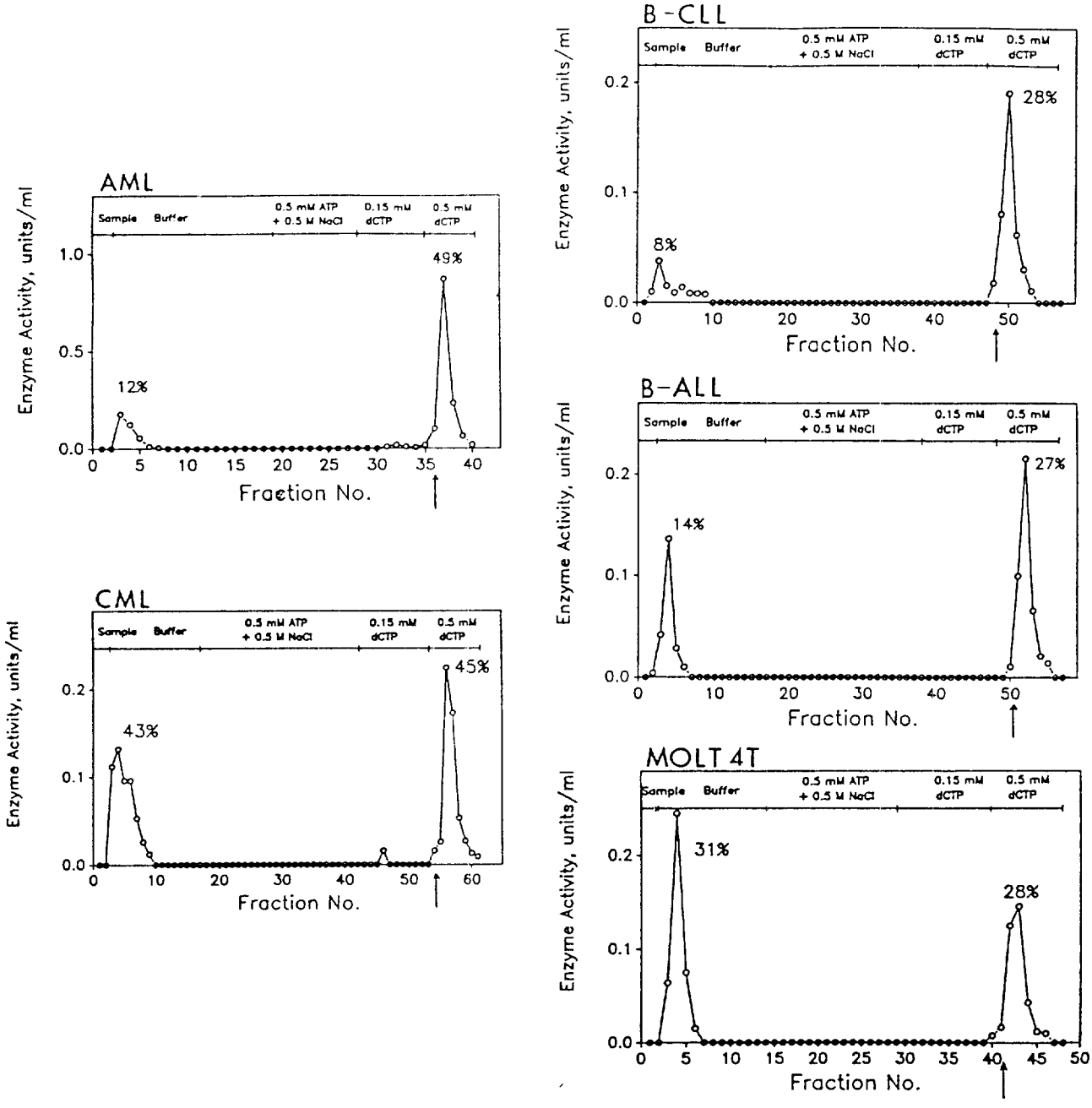

Fig. 4. Purification profiles of dCyd kinase from different kinds of human leukemic cells and cultured human T-lymphoblasts by dCp_4-Sepharose affinity column.

clearly the preferred substrate.

Possible isoenzymes from a variety of other leukemic cells

In an attempt to find a putative more-weakly-binding isoenzymes using the new affinity column, dCyd kinase was purified from a variety of other leukemic cells. Crude extracts from a variety of human leukemic cells with similar dCyd kinase activities were applied to the column. As shown in Fig. 4, the dCp_4-Sepharose column retained much more dCyd kinase from all of the different human leukemic cell lines and MOLT 4T cells (cultured human T-lymphoblasts) than the dCp_4A-Sepharose column. The enzyme was eluted effectively at 0.5 mM dCTP and high salt and ATP concentrations did not displace the enzyme from the column. Therefore, enzyme interaction with the column was probably not due to either ion-exchange or broadly-specific nucleotide interactions with the phosphate-donor site of the enzyme.

The recovery of activity was higher with myeloblastic leukemia cells (AML and CML) than with lymphoblastic

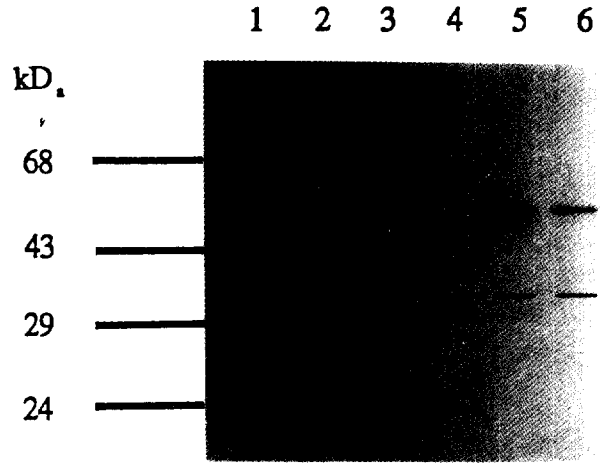

Fig. 5. SDS-polyacrylamide gel electrophoresis of dCyd kinase purified from different human leukemic cells. Lane 1 : T-ALL; Lane 2 : B-ALL; Lane 3 : B-CLL; Lane 4 : MOLT-4; Lane 5 : AML; Lane 6 : CML

leukemia cells (B-CLL, B-ALL, T-ALL and MOLT 4). When a MOLT 4 cell extract was applied to the dCp_4 A-Sepharose column, all of the activity ran through the column. This indicates that the dCp_4-Sepharose column which retained MOLT 4 kinase activity, is much more powerful for purifying dCyd kinase, and for finding isozymic forms of the deoxynucleoside kinases, if more than one type of enzyme is present.

Purity analysis

Proteins purified by the dCp_4-Sepharose column from various human leukemic cells and MOLT 4 cells were analyzed for purity. The peak fraction of each dCp_4-Sepharose chromatography was concentrated by Centricon 10 micro-concentrator, and applied to 12% SDS-polyacrylamide electrophoresis gel. For comparison, the enzyme purified from T-ALL was also applied. As seen in Fig. 5, enzymes purified from B-CLL cells showed one major band that had the same R_f value as the enzyme purified from T-ALL cells (52 kDa). The enzymes from AML and CML cells showed two bands of molecular weight 52 and 30 kDa. On the other hand, enzyme proteins from B-ALL and MOLT 4 cells showed one lower molecular weight band (30 kDa).

Electrophoretic mobility of activity

To compare the electrophoretic mobilities of enzyme activities purified from different cells on the dCp_4-Sepharose column, 12% non-denaturing polyacrylamide gel electrophoresis was performed. The enzymes purified from CML and AML cells were applied to 12% non-denaturing electrophoresis gel, and for comparison, with the T-ALL enzyme (Fig. 6A). Fig. 6B shows the results obtained when the MOLT-4, B-CLL, and B-ALL enzymes were applied to one gel, along with the T-ALL enzyme. dCyd kinase purified from lymphoid cells (T-ALL, B-ALL, B-CLL and MOLT 4 T) and myeloid cells (AML and CML) showed the same electrophoretic mobility in non-denaturing polyacrylamide gel. However, B-ALL, B-CLL, and MOLT 4 T cells exhibited an extra minor peak (all at the same position) in addition to the major peak.

dAdo kinase activity was also assayed from the lane containing the B-ALL cell protein. Both dAdo and dCyd phosphorylating activities co-migrated, indicating that these activities are associated with the same protein (also in Fig. 6A and 6B).

ELISA

The relatively small amounts of enzyme protein available made conventional antibody production impractical. Instead, a new technique, the disk implantation method was used. A total of 4 μg of enzyme purified from T-ALL cells was used as an antigen for one rabbit to raise polyclonal antibodies. To determine the titer of the antiserum, ELISA was performed. As shown in Fig. 7, the titer was low with the crude antiserum. The nitrocellulose powder method of immunization was attempted as a variation designed to increase the titer. However, the titer was not increased (data not shown). It seems likely that human dCyd kinase was not strongly immunogenic since the same procedures gave high titer with bacterial dCyd kinase (personal communication from Dr. Seiichiro Ikeda, The Ohio State Univ.)

Western immunodetection

Western immunoassays of the purified enzyme from T-ALL cells were performed with antiserum raised by the disk implantation method. Immunodetection revealed a single sharp band of immunoreactive material at 52 kDa in the affinity-purified T-ALL cell enzyme fraction (data not shown). Further studies should be carried out to determine whether the 30 kDa band from other leukemic cells might represent a cross-reactive dCyd kinase isoenzyme, or a proteolyzed product.

Determination of the N-terminal amino acid sequence

It appeared likely that the N-terminus of human dCyd kinase purified from T-ALL cells was blocked. An 80 pmol sample subjected to gas-phase sequence analysis after extensive filtration dialysis on a centricon unit yielded a maximum of only about 8 pmol of Edman-reactive material (data not shown).

Discussion

This study showed that a highly biospecific affinity

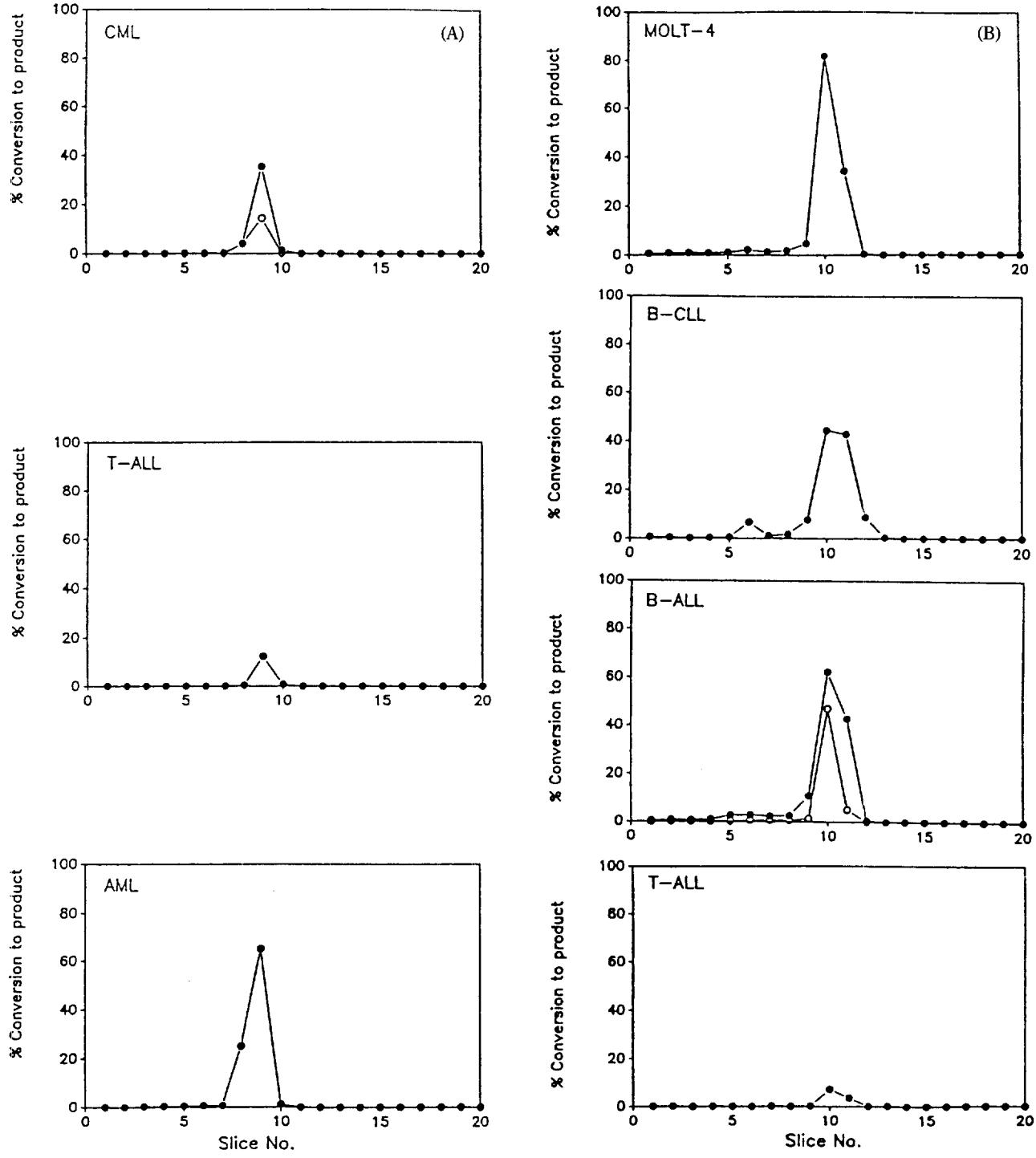

Fig. 6. Electrophoretic mobility profiles of enzyme activity from AML, CML cells and comparison with the enzyme from T-ALL cell in one gel (A) and from MOLT-4, B-CLL, B-ALL and T-ALL in one gel (B). (●–●) dCyd kinase; (○–○) dAdo kinase.

system provided a simple and efficient method for isolating apparently homogeneous dCyd kinase and its isoenzyme. Using the newly developed affinity media, dCp_4-Sepharose, dCyd kinase was purified to apparent homogeneity from a variety of human leukemic cells (T-ALL, B-ALL, CLL, AML, and CML), and from cultured T-lymphoblasts (MOLT 4).

The purification of human dCyd kinase from leukemic spleen (Bohman and Eriksson, 1988), leukemic lymphoblasts and myeloblasts (Sarup and Fridland, 1987), myeloid leukemia cell mitochondria (Wang *et al.*, 1993), and cultured T-lymphoblasts (Datta *et al.*, 1989) involved several purification steps, such as ion-exchange chromatography, hydrophobic chromatography, and general specificity affinity chromatography.

Datta *et al.* (1989) used a dCTP-Sepharose 4B col-

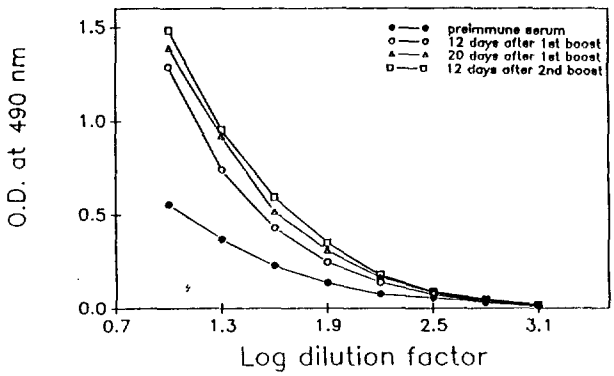

Fig. 7. Determination of the titer, by ELISA, of crude antiserum raised by the disk implantation method.

umn in the last purification step for human dCyd kinase from MOLT-4 cells. The enzyme activity was eluted even with 2 mM ATP and 2.4 mM $MgCl_2$, and final enzyme elution was performed with 0.1 mM dCyd, 2 mM ATP, 2.4 mM $MgCl_2$, and 2 mM dTTP. Apparently, the triphosphate group alone was not sufficient for the efficient binding of dCyd kinase. Comparing the binding efficiencies of the enzyme to dCp_4-Sepharose with that of dCTP-Sepharose, the extra phosphate might considerably strengthen the interaction of a derivatized dCTP with the enzyme. With the dCp_4-Sepharose column the binding was so tight that enzyme activity was not eluted continuously in one day even with dCTP.

Regardless of the sample load or the eluent concentration, not all of the activity could be eluted immediately on addition of dCTP, and pronounced tailing was seen. An additional peak always emerged if the column flow was resumed after standing overnight in the presence of dCTP. Extensive comparisons were made in establishing the common identity of the two peaks; the apparent K_m values, native and SDS-gel electrophoretic patterns, isoelectric pH values, and molecular weights were virtually identical (Kim *et al.*, 1988). It seems that enzyme dissociation is a slow step. The fact that all of the recoverable enzyme emerges in a very small volume, if the column sets overnight with dCTP, suggests that it is a slow off rate rather than an unfavorable dissociation equilibrium which causes initial tailing of the activity. Therefore, the column was allowed to stand overnight with dCTP before the enzyme was collected as a single peak.

The dCp_4-Sepharose purified dCyd kinase from T-ALL cells had a broad substrate specificity. The apparent K_m values, which were small for dCyd (3.3 μM) but much larger for dAdo (890 μM) and dGuo (640 μM), were determined from dCp_4A-Sepharose purified dCyd kinase (Kim *et al.*, 1988). dCyd kinase purified from human leukemic spleen (Bohman and Eriksson, 1988), MOLT-4 (Datta *et al.*, 1989), and human leukemic-lymphoblasts and myeloblasts (Sarup and Fridland, 1987) also contained major phosphorylating activity for dAdo and dGuo.

dCyd kinase was purified to apparent homogeneity from human T-ALL cells using the multisubstrate analog dCp_4A (Kim *et al.*, 1988). While the dCp_4A-Sepharose medium used for purification of T-ALL dCyd kinase was highly specific, its capacity was limited by apparent steric interference with binding, causing a substantial portion of the activity to run through the column. The run-through fraction might conceivably consist of a different isoenzyme. Therefore, a medium which retains all of the dCyd kinase activity, and which yields nearly all the enzyme activity as pure protein, is the ideal.

The new dCp_4-Sepharose affinity column retained dCyd kinase from all different kinds of human leukemic cells tested with relatively good recovery rates (27~49 %). SDS-gel electrophoresis revealed that the protein purified from B-CLL cells had the same molecular weight band (52 kDa) as the protein from T-ALL cells, but the enzyme from AML and CML cells, which had higher enzyme recovery rates than other leukemic cells, showed two bands of 52 kDa and 30 kDa. B-ALL and MOLT-4 dCyd kinases showed only one low molecular weight band (30 kDa). This result coincides with other observations. Datta *et al.* (1989) found a 30.5 kDa protein band derived from dCyd kinase purified from MOLT 4 cells and suggested that the enzyme consisted of two subunits. A similar subunit molecular weight was reported for the enzyme purified from B-cell lymphomas of the human leukemic spleen (Bohman and Eriksson, 1988). Only one form of dCyd kinase activity was found, and the subunit molecular weight was 30 kDa. The enzyme phosphorylated dCyd, dAdo, and dGuo. The apparent molecular weight of the active enzyme was 60 kDa. The cDNA coding for human dCyd kinase of MOLT 4 cells has been cloned by Chottiner *et al.* (1991), confirming that the enzyme exists as a dimer with a subunit molecular mass of 30.5 kDa. However, no subunit molecular weight was determined for the enzyme purified from T-ALL cells or cultured CCRF-CEM (human T-lymphoblasts cells) (Sarup and Fridland, 1987).

Therefore, it is possible that at least two isoenzymes of human dCyd kinase exist in different kinds of human leukemic cells as the dCp_4-Sepharose column retained two different isoenzymes of dCyd kinase. The M_r values of native isoenzymes were similar since they showed the same electrophoretic mobility of enzyme activity in non-denaturing gels. The extra minor activity

peak exhibited by B-lymphoblast and MOLT-4 cell enzymes may be due to aggregation of the enzymes. Previous studies have shown that proteolysis can occur on the column during purification (Kim *et al.*, 1988). Further studies should be carried out to determine if the 30 kDa band of AML and CML cells is an isoenzyme or a proteolyzed product of dCyd kinase.

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