SELECTIVE INHIBITION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT) BY ADENOSINE RIBONUCLEOSIDE TRIPHOSPHATE (ATP) AND ITS APPLICATION IN THE DETECTION OF TdT IN HUMAN LEUKEMIA

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SUMMARY:

TdT is strongly inhibited in the presence of ATP, while other DNA polymerases, i.e., α, β, γ and reverse transcriptase are resistant to ATP inhibition. This selective inhibition of TdT in the presence of ATP has been used as a diagnostic aid to identify TdT in extracts of leukocytes obtained from acute lymphocytic leukemia (ALL) and chronic myelogenous leukemia (CML) patients.

INTRODUCTION:

Identification and monitoring of TdT in human leukemia has great diagnostic value, since it appears to be a marker for the presence of immature (blast) T cells (1-7). This is particularly important when one deals with an undifferentiated leukemia where classical cell surface marker studies do not permit their classification into individual B or T cell type. In certain cases of CML in blast crisis TdT determinations have helped to clarify that the observed crisis is indeed due to an upsurge of T lymphoblast concentration as a secondary response to the existing B cell myelogenous leukemia. Obviously, an unequivocal determination of TdT is of prime importance in the diagnosis and classification of leukemia. In our biochemical studies on the active site of TdT we found that ribonucleoside triphosphates stongly inhibit TdT, ATP being the most potent of all the triphosphates. Details of the mechanistic aspect of ATP inhibition will appear elsewhere (Modak, in preparation). A practical application of this inhibitory effect in terms of unequivocal identification of TdT in leukemic cell extracts was investigated and is the subject of the present communication.

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MATERIALS AND METHODS:

Rat thymus was provided by Dr. Morris S. Zedeck, while ALL and CML cells were generously given by Dr. Roland Mertelsmann. Radioactive chemicals were purchased from Amersham Searle, Inc. Various template primers and unlabelled triphosphates were bought from P.L. Biochemicals. All other chemicals were of reagent grade. DNA polymerases α and γ from HeLa cells were kindly donated by Dr. A. Weissbach of Roche Institute for Molecular Biology; while DNA polymerase β from calf thymus was prepared according to a procedure of Chang (8). Rauscher leukemia viral (RLV) reverse transcriptase was purified using affinity chromatography of polycytidylate-agarose columns (9). Calf thymus TdT was purified according to the procedure of Chang and Bollum (10).

All procedures were carried out at 0-4°C. Crude extracts from thymus, ALL or CML cells were obtained by detergent treatment of 3x freeze-thawed samples according to the procedure of McCaffrey et al. (1). Extracts were centrifuged at 150,000g for 1 hr at 4°C and the supernatants applied at a flow rate of 8 mls/hr to a 0.5 x 12cm phosphocellulose column which had been previously equilibrated with TEDG buffer [50 mM Tris-HCl pH 7.8, 1 mM EDTA, 1 mM DTT, 0.1% (w/v) bovine serum albumin and 20% glycerol]. The columns were washed with 50 ml of the same buffer containing 100 mM KCl. Enzyme activity was eluted with a 0.1-0.65M KCl gradient in TEDG buffer. Fractions of approximately 2 mls were collected and 20 µl of each fraction was assayed for various enzyme activities as described below.

Enzyme assays: Enzyme reactions were carried out in a final volume of 100 µl and contained the following components: 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 50 µg bovine serum albumin, 20 µM appropriate tritiated nucleoside triphosphate, adjusted to a final specific activity of 1,000 cpm/pmole, and 0.5 µg of template primer. The type and concentration of divalent cation (Mg^{2+} or Mn^{2+}) together with additional components, whenever required for various template primers and enzymes are as follows:

(i) poly (rA) (dT)12-18 directed synthesis: Reactions were carried out in the presence of 0.5 mM $MnCl_2$ and 50 mM KCl.

(ii) poly (dC) (dG)12-18 directed synthesis: For this template-primer reaction Mg^{2+} (2.5 - 5 mM) was used as a divalent cation.

(iii) Terminal deoxyribonucleotidyl transferase was assayed as described by Marcus et al. (7) using oligo (dA)12-18 as a primer in the presence of 0.5 mM $MnCl_2$ and [^{3}H]dGTP as a substrate.

Incubations were carried out at 37°C/1 hr and were terminated by the addition of 5% (w/v) trichloroacetic acid (TCA) containing 0.01M sodium pyrophosphate. The acid-insoluble activity was collected on Whatman GF/B filters, washed extensively with TCA containing sodium pyrophosphate and subsequently with water, and ethanol. The filters were then dried and counted in a toluene based scintillation fluid (11).

RESULTS AND DISCUSSION:

In the human leukemic cell at least 5 distinct DNA polymerases have been identified (12). All of these enzymes, i.e. DNA polymerases α, β, γ and viral related reverse transcriptase, with the exception of TdT may be considered replicative polymerases. Identification of TdT (which is normally restricted

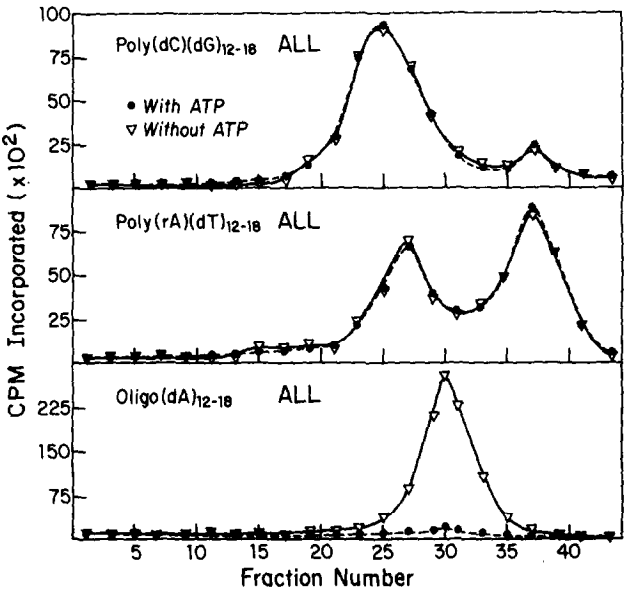

Figure 1.

Phosphocellulose chromatography of acute lymphocytic leukemia (ALL) extract. Assays were carried out in the presence (●——●) and absence (▽——▽) of 50 μM ATP using different template primers as described in materials and methods.

to thymus and bone marrow only), in these cells, implies their lineage to thymus and the leukemia in question may be diagnosed as of T cell origin (4). In a study of childhood ALL patients, monitoring of TdT levels in response to chemotherapy, has been shown to be of great diagnostic value, in terms of initiation and/or termination of treatment and monitoring patients' progress (12 & 13). Because of the presence of other DNA polymerases, and the possibility of non-specific low level incorporation of substrate under TdT assay conditions by them, it is important to unequivocally identify TdT, particularly, when it is present in low levels. We have observed that ATP is a strong inhibitor of TdT but has no action on any of the other replicative DNA polymerases (Table 2). This selective inhibition of TdT, then, becomes a diagnos-

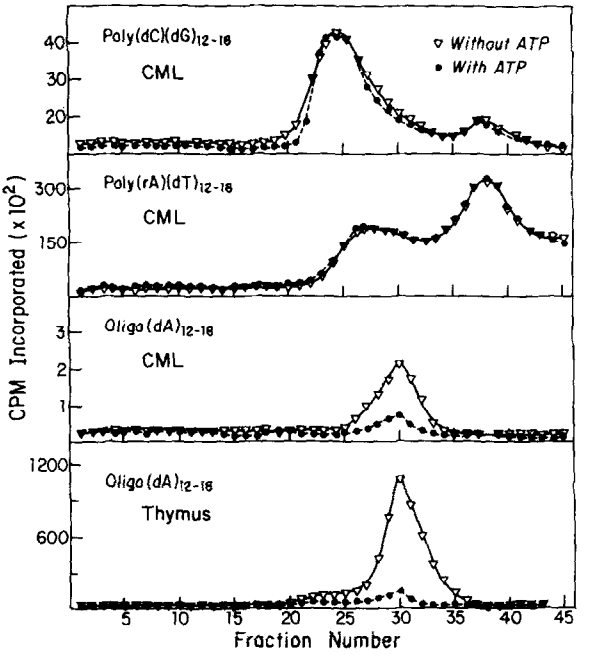

Figure 2

Phosphocellulose chromatography of chronic myelogenous leukocyte (CML) extract and thymus extract. Assays were carried out with (•——•) and without (▽——▽) 50 μM ATP as described in materials and methods. Data for phosphocellulose fractions obtained from thymus extracts with poly (dC) $(dG)_{12-18}$ and poly (rA) $(dT)_{12-18}$ directed synthesis are not shown as there was no inhibition of these activities with ATP.

tic test for identification and confirmation of TdT in the fractions obtained from leukemic cell extracts. We first successfully applied this test to a known population of TdT positive cells i.e., leukocytes from an ALL patient, as well as thymus tissue. The profile of DNA polymerases from these extracts and their susceptibility to ATP is shown in Fig. 1. When the cell extract from CML in blast crisis, was similarly examined, low levels of oligo dA-

Table 1

Effect of ATP on Terminal Deoxynucleotidyl Transferase Activity

Amount of Enzyme	dGTP Incorporation (CPM)	
	Control	+ ATP
2 µl	221	57
5 µl	598	65
10 µl	1,500	120
20 µl	3,355	155
30 µl	4,175	159

Fraction #30 off phosphocellulose column from CML extracts (Fig. 2) was concentrated and different quantities ranging from 2 - 30 µl were used for determining the effect of ATP on TdT activity. Assay conditions are described in materials and methods.

Table 2

Response of purified DNA polymerases α, β, γ, TdT and RLV reverse transcriptase to different template-primers and the effect of ATP

Name of Enzyme	Template-primers					
	Oligo$(dA)_{12-18}$		Poly$(rA)\cdot(dT)_{12-18}$		Poly$(dC)\cdot(dG)_{12-18}$	
	Substrate Incorporation (CPM)					
	Control	+ATP	Control	+ATP	Control	+ATP
HeLa DNA polymerase α	40	60	490	472	76,395	74,861
Calf thymus DNA polymerase β	50	51	6,860	7,051	3320	3350
HeLa DNA polymerase γ	110	103	1,320	1,303	7,074	6,840
Calf thymus TdT	16,820	269	267	297	88	92
RLV reverse transcriptase	105	107	14,510	13,880	20,790	20,973

Conditions for Oligo $(dA)_{12-18}$, poly $(rA)\cdot(dT)_{12-18}$ and poly $(dC)\cdot(dG)_{12-18}$ directed synthesis by various polmerases are given in materials and methods.

dependent activity were found, which were also susceptibile to ATP inhibition (Fig. 2), indicating that the activity under examination, although low, is true TdT. This enzyme fraction was further concentrated and tested for susceptibility to ATP and the results are presented in Table 1. The activity is proportional to enzyme concentration even at 10 fold dilution, but the percent inhibition by ATP remains constant. This observation together with the fact that ATP has no effect on mammalian DNA polymerases α, β and γ as well as mammalian viral reverse transcriptase (Table 2) makes this test a valuable tool in diagnostic identification of TdT.

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REFERENCES:

1. McCaffrey, R., Smoler, D.F. and Baltimore, D. (1973) Proc. Nat. Acad. Sci. USA 70:521.
2. Sarin, P.S. and Gallo, R.C. (1974) J. Biol. Chem. 249:8051.
3. Bhattacharya, J.R. (1974) Biochem. Biophys. Res. Comm. 62:367.
4. Gallo, R.C. (1975) N. Engl. J. Med. 292:804.
5. McCaffrey, R., Harrison, T.A., Parkman, R. and Baltimore, D. (1975) N. Engl. J. Med. 292:775.
6. Coleman, M.S., Hutton, J.J., de Simone, P. and Bollum, F.J. (1974) Proc. Nat. Acad. Sci. USA 71:4404.
7. Marcus, S.L., Smith, S.W., Jarowski, C.I. and Modak, M.J. (1976) Biochem. Biophys. Res. Commun. 70:37.
8. Chang, L.M.S. (1973) J. Biol. Chem. 248:3789.
9. Modak, M.J. and Marcus, S.L. (1977) J. Biol. Chem. 252:11.
10. Chang, L.M.S. and Bollum, F.J. (1971) J. Biol. Chem. 246:909.
11. Modak, M.J. (1976) Anal. Biochem. 75:340.
12. Coleman, M.S., Greenwood, M.F., Hutton, J.J., Bollum, F.J., Lampkin, B. and Holland, P. (1976) Cancer Res. 36:120.
13. Hutton, J.J. and Coleman, M.S. (1976) Br. J. Haematol. 34:447.