RNA DEPENDENT DNA SYNTHESIS IN CELL FREE PREPARATIONS OF HUMAN LEUKEMIA CELLS*

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SUMMARY. A cell free preparation of human leukemic cells, grown in tissue culture, incorporated H^3-thymidine phosphate into an acid insoluble product which was rendered acid soluble by the action of DNAase but not KOH or RNAase. RNAase, if added before incubation of the reaction mixture, prevented incorporation of the isotope into the product. For maximum incorporation Mg and all four deoxyribonucleotide triphosphates must be present. The enzyme has the properties of an RNA dependent DNA polymerase.

RNA primed DNA polymerase has been reported to be a component of seven virions which are tumorogenic for mice, monkeys, cats or fowl but has not been reported in normal cells of animals or man (1, 2, 3). If human leukemia has a viral etiology, this distinctive enzyme activity should be detectable in leukemia cells. To test this possibility, a continuous line of cells established from a case of human leukemia was selected and examined for RNA primed DNA polymerase activity. Evidence for the presence of this distinctive enzyme in these cells is presented in the following.

MATERIALS AND METHODS

The Z597 Cell is a continuous cell line established (in the laboratory of W. H. Murphy) from leukocytes of a patient with granulocytic leukemia.

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The cells have been characterized with regard to morphology, growth potential, chromosomal pattern, antigenicity and viral susceptibility. After 96 serial passages, they retain properties of human cells and appear to contain C type virus particles (unpublished results, W. H. Murphy). For these studies they were grown in Roux bottles in monolayers with Eagle's medium (4) supplemented with 20 percent fetal calf serum and passaged every four days.

The assay mixture contains the following components whose concentrations are recorded in molarity: $MgCl_2$ (12×10^{-3}); deoxyribonucleotide triphosphates, dATP, dCTP and dGTP (1.6×10^{-3}); dithiothreitol (4.3×10^{-3}); KCl (80×10^{-3}); H^3-methyl - TTP (1.80×10^{-6}), specific activity 17.4 C/m mole, 2399 counts/min/p mole.

NDT buffer used to pretreat some enzyme preparations is composed of a nonionic detergent, Nonidet-40 (.25%), Tris-HCl (.10M Tris-hydroxymethylaminomethane pH 8.3) and dithiothreitol (0.150M).

Assay Procedure - 0.1 ml of assay mixture was combined with enzyme which had been suspended in 0.1M Tris-HCl buffer pH 8.3 and sufficient additional buffer to give a final volume of 0.2 ml and incubated at 37°C. The reaction was stopped by adding 0.5 ml of 0.1M pyrophosphate, 0.05 ml of calf serum, and 0.5 ml of 25% trichloroacetic acid. The washed pellet was dissolved in formic acid and counted in scintillation fluid with an Ansitron scintillation counter (5).

Sources of Materials - DNAase cryst. (Worthington Biochem.Corp.), RNAase 5 x cryst. (Nutritional Biochem. Corp.), Micrococcal nuclease (Worthington Biochem. Corp.), trypsin (Seravic Lab.), lysozyme (Sigma), Nonidet-40 (Shell), deoxyribonucleotide triphosphates (Schwarz).

EXPERIMENTAL AND RESULTS

Preparations of enzyme which incorporate H^3-thymidine phosphate into acid insoluble material were made from Z597 cells. The cells were scraped from six Roux bottles into 15 ml of growth media. The combined cells (198×10^6) were centrifuged for 10 min. at 1000 g and resuspended in 10 ml of 0.01M KH_2-K_2HPO_4 buffer containing 0.002M $MgCl_2$ at pH 7.7. After being sedimented a second time (1000 g, 10 min), they were resuspended in 4 ml of the same buffer and held at 0°C for 5 minutes, then separated into nuclei and cytoplasm by 25 to 30 strokes of a Dounce homogenizer. Immediately the preparation was diluted with an equal volume of cold 0.12M Tris-HCl buffer containing 0.12M NaCl and 0.002M glucose at pH 8. Nuclear and cytoplasmic fractions were obtained by centrifugation (1000 g, 10 min). The supernatant fraction was centrifuged at 60,000 g, for one hour. The resulting pellet was resuspended in two ml of 0.1M Tris-HCl buffer at pH 8.3 and sonicated at full power, one minute, in a Raytheon sonic oscillator and used as a source of enzyme. Three different lots of enzyme were used in the various experiments described. All operations were at 4°C.

Incorporation of H^3-thymidine phosphate into acid insoluble material could be shown to be approximately a linear function of the concentration of the enzyme preparation (Lot III) present in the reaction mixture (Table 1). Appropriate dilutions of the enzyme were made and 0.075 ml of each combined with 0.025 ml of NDT buffer (Cf. materials). After 30 min., 0°C, 0.1 ml of assay mixture was added, the mixture was incubated one hour at 37°C and the incorporation of H^3 determined in the acid insoluble material. Isotopic incorporation was directly proportional to the enzyme concentration when dilutions greater than 30% were employed.

Table 1. Incorporation of H^3-Thymidine Phosphate into Acid Insoluble Material

Enzyme (ml)	Buffer (ml)	Incorporation cpm*	cpm*/.01 ml Enzyme
.0125	.0625	575	460
.0250	.0500	1500	632
.0375	.0375	2406	667
.0500	.0250	2806	572
.0750	.0000	2441	326

*Corrected by the value obtained for an unincubated control, 31 cpm.

Essential components of the reaction mixture were determined by omitting various components singly or in combinations and determining the effect on the incorporation of H^3-thymidine phosphate into acid insoluble material. The assay results in Table 2 indicate an absolute requirement for $MgCl_2$ and enzyme. All four nucleotide triphosphates must be present for maximum incorporation of thymidine phosphate. The reaction is partially inhibited by actinomycin (5 µg/ml).

Table 2. Essential Components of the Reaction Mixture

Component	Incorporation of H^3-thymidylate cpm
Complete*	3287
- $MgCl_2$	2
- dATP	532
- dCTP	444
- dGTP	233
- dATP-dCTP-dGTP	34
- Enzyme	0
+ Actinomycin (5 µg)	908

To 0.075 ml of a 1:2 dilution of enzyme, (Lot II), was added 0.025 ml of NDT buffer and 0.1 ml of assay mixture (cf. Materials and Methods)* from which the indicated components were omitted. After 60 min. incubation 37°C, the acid insoluble material was tested for H^3 incorporation.

RNA-dependence of the enzyme reaction was established by pretreating the enzyme preparation for 30 min. at 0°C with RNAase and then assaying the enzyme for its capacity to incorporate H^3-thymidine phosphate. RNAase was heated 100°C for 10 min. prior to use (to destroy possible

DNAase content). The effects of pretreatment of the enzyme with buffer, a nonionic detergent (Nonidet-40), Nonidet plus trypsin, and lysozyme were determined in like manner. From the results (Table 3), it is seen that incorporation of H^3-thymidine phosphate is reduced 78 to 83 percent by RNAase treatment. The enzyme activity is not markedly affected by Nonidet or lysozyme but almost eliminated by trypsin. The activity resistant to RNAase (25 μg/ml) was not destroyed when the concentration of RNAase was doubled and the temperature increased to 22°C. The lack of effect of lysozyme, a basic protein, suggests the effect of RNAase is due to its enzymatic activity rather than some other property of the protein.

Table 3. Effect of Pretreatment of Enzyme on Incorporation of H^3-thymidylate in Acid Insoluble Material

Pretreatment Condition +		Incorp. H^3-thymidylate cpm*
Temp	Reagent	
	Experiment 1	
0°C	Buffer	5669
0°C	Nonidet-40 (.25%)	5980
0°C	Nonidet-40 (.25%) + Trypsin (400 μg/ml)	160
0°C	Nonidet-40 (.25%) + RNAase (400 μg/ml)	1303
	Experiment 2	
0°C	Buffer	1053
22°C	Buffer	1214
0°C	RNAase (200 γ/ml)	242
0°C	RNAase (400 γ/ml)	240
22°C	RNAase (400 γ/ml)	215
0°C	Lysozyme (400 γ/ml)	1297

*All corrected by value of unincubated control 56 cpm (Exp. 1); 72 cpm (Exp. 2). 0.025 ml of detergent, buffer (0.1M Tris. HCl, pH 8.3 and .150M dithiothreitol), or hydrolytic enzymes at concentrations indicated were combined with 0.075 ml of polymerase enzyme (Lot II) Exp. 1 or (Lot IV) Exp. 2 and incubated for 30 min. at temperatures indicated. Then 0.1 ml of the assay mixture was added, incubated 37°C for 60 min. and the isotope incorporated into acid insoluble material determined.

Some properties of the acid insoluble product into which H^3-thymidine phosphate was incorporated were determined by the effects of

various hydrolytic agents upon it. The enzyme was incubated with the complete assay mixture, one hour, 37°C, to produce tritium labeled acid insoluble material. Aliquots (0.1 ml) of the reaction mixture were then combined with solutions of KOH, $MgCl_2$, DNAase plus $MgCl_2$ or RNAase plus $MgCl_2$. The first was incubated 18 hours at 37°C and the others for one hour at 37°C. The isotopic content of the acid insoluble material was then determined and compared with that of an aliquot of the reaction mixture which received no hydrolytic treatment. The data in Table 4 indicate the product containing H^3-thymidine is rendered completely acid soluble by DNAase, but not by RNAase or KOH. The acid insoluble product into which H^3-thymidine phosphate is incorporated has the properties of DNA.

Table 4. Properties of the Acid Insoluble Polymerase Product*

Reagent	Incubation time	Incubation temp.	H^3-thymidylate Incorp. cpm**
NONE	- - -	- - -	2139
+$MgCl_2$ (10 mM)	1 hr.	37°	1497
RNAase (40 µg/ml)	1 hr.	37°	1780
DNAase (40 µg/ml)	1 hr.	37°	17
KOH (0.6M)	18 hr.	37°	1651

*0.1 ml of reaction mixture which had been incubated at 37°C and contained the H^3-thymidine labeled acid insoluble product was combined with one ml of the listed hydrolytic agents, incubated and then the H^3 activity of the acid precipitable residue was determined.

+The RNAase and DNAase were prepared in 10 mM $MgCl_2$.

**The recorded radioactivity is corrected for the value obtained for an unincubated control.

DISCUSSION

The finding of a human cell line capable of continuous culture, which contains RNA dependent DNA synthetic activity, and from which reproducible preparations of enzyme activity can be obtained should facilitate continuing study of the enzyme leading to its characterization,

function and cell relationships.

The enzyme activity obtained from these cells is not distinctly different from that reported in tumorogenic virions, some of which require detergent activation while others do not. The activity seems to be in the form of a unit complex composed of an enzyme with a self contained RNA. Preliminary isopycnic centrifugation associated the major part of the activity with a structure of low density (1.105) suggesting some lipid content in addition to protein and RNA. However, there is no evidence that the active particle is a virion.

During the preparation of this manuscript comparable results with primary isolated human leukemic cells from several individual patients with lymphoblastic leukemia were reported in the literature (6).

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