Deoxyribonucleic Acid Polymerase Activity Associated with a Plasma Particulate Fraction from Patients with Chronic Lymphocytic Leukemia¹

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Plasma from two cases of chronic lymphocytic leukemia have been examined for deoxyribonucleic acid (DNA) polymerase activity. In both cases, detectable enzyme activity was present. In the plasma from a patient known to have chronic lymphocytic leukemia for 10 years, the enzyme activity was sufficiently high to study product formation, buoyant density of the enzyme activity, deoxyribonucleoside triphosphate requirements, and kinetics of DNA synthesis. These studies are presented in this report.

It was possible to detect very low levels of virus by measuring deoxyribonucleic acid (DNA) polymerase from culture medium of cells shortly after infection with strain MC29 virus, when the noninfected control cultures showed no enzyme activity (11). These studies and earlier work on DNA polymerase activity from other laboratories (2, 4-10) encouraged us to investigate the possibility that a similar enzyme activity could be detected in the leukemia of man.

This is a report on the initial studies of the DNA polymerase activity in plasma from two cases of chronic lymphocytic leukemia (CLL). Results in this preliminary report encourage further research in this direction.

MATERIALS AND METHODS

Preparation of enzyme. Blood from the patients was collected by using ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The final EDTA concentration in the blood approximated 1 mg of EDTA per ml of blood. The blood sample was centrifuged at 3,000 to 4,000 $\times g$ for 10 min. Plasma was removed and frozen immediately at -40°C and later transferred to -70°C for storage.

Plasmas were thawed and centrifuged again at 3,000 to 4,000 $\times g$ for 10 min before enzyme preparation. The particulate fraction from the clarified plasma was collected by centrifuging at 50,000 rev/min in a Spinco SW50 rotor for 60 min. The pellets were resuspended in 0.1 ml of TKGE buffer [0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.3, 0.1 M KCl, 0.004 M EDTA, and 10%

glycerol] and mixed with an equal volume of 2 $\times$ Nonidet solution [0.5% Nonidet (Shell P40) plus 0.13 M dithiothreitol]. The detergent-treated enzyme fraction was incubated for 5 hr in an ice bath before assay.

Determination of buoyant density of fraction containing enzyme activity. Plasmas from patients with CLL, from chicks infected with avian myeloblastosis virus (AMV), or from tissue culture fluid containing MC29 virus were centrifuged at 5,000 rev/min for 10 min to clarify the fluids. The particulate material was centrifuged onto a 95% glycerol pad for 1 hr at 50,000 rev/min in a Spinco SW50 rotor. Supernatant plasma was removed, care being taken not to disturb the pellet on top of the 95% glycerol. A 1.5-ml glycerol gradient ranging from 35 to 85% glycerol was applied directly on top of the pellet fraction, and the gradient was overlaid with mineral oil to the top of the centrifuge tube. All glycerol solutions were prepared in 0.01 M Tris-hydrochloride buffer (pH 8.3) containing 0.1 M NaCl, 0.01 M MgCl_2 , 0.002 M EDTA, and 5×10^{-4} M dithiothreitol. The gradients were centrifuged at 50,000 rev/min in a Spinco SW50 for 2 hr, and 0.1-ml fractions were collected from the centrifuge tube. Densities along the gradient were estimated from refractive indexes of alternate fractions. Each fraction was treated with 0.1 ml of 0.5% Nonidet containing 0.3 M dithiothreitol and allowed to stand in ice for 12 hr before assay for DNA polymerase activity. Fractions were assayed for enzyme by using the standard procedure. Incubation conditions are given in the figure legends.

Enzyme assay. A standard reaction mixture contained, in a volume of 0.1 ml: Tris-hydrochloride (pH 8.3), 4 μmoles ; MgCl_2 , 0.8 μmole ; NaCl, 6 μmoles ; reduced glutathione, 0.37 μmole ; unlabeled

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deoxyribonucleoside triphosphates, 0.02 μ mole; 3 H-labeled deoxyribonucleoside triphosphate, 1 μ Ci (counts per minute per picomole are reported in legends). DNA (native) and enzyme were added in amounts indicated in each experiment. All assays were performed at 37 C, and the reactions were terminated and counted as previously described (11).

RESULTS

We have observed DNA polymerase activity in the pellet fraction from plasmas of patients having CLL. The results in Table 1 show the enzyme activity from the plasma from an 87-year-old male (CLL-JV) diagnosed as having had CLL for 10 years who had not received chemotherapy. Control of this condition was mainly through transfusion, and, at the time of plasma collection, he had a white cell count of 500,000 cells/mm³. Over a 7-day period after this first collection, he received five 60 Co therapy treatments, and his blood cell count had fallen to 250,000 cells/mm³ when a second plasma collection was made 8 days after the first collection. Plasmas from both collection periods were found to contain enzyme activity. However, a third collection made immediately after infusion of 1 unit of red cells contained no detectable enzyme activity. The reason for this loss of DNA polymerase from the plasma is not known. A fourth collection was made after cobalt therapy had reduced the white cell count to 6,000 cells/mm³, and the plasma had no DNA polymerase activity.

The other patient (CLL-DD) was newly diagnosed as having CLL and was under transfusion therapy during the period of plasma collection. The patient was a 70-year-old female with a white cell count of 30,000 cells/mm³. Over 90% of the white cells in both patients were lymphocytes.

In both cases shown in Table 1, enzyme activity in the plasma pellets was increased by the detergent treatment, as was found with animal tumor viruses (8, 10). Inaccessibility of the DNA polymerase activity implied that the enzyme was bound in a particulate and not available to the substrates in the reaction mixture until released by the detergent treatment. Enzyme activity was stimulated by the addition of *Micrococcus lysodeikticus* DNA to the assay. There was little evidence that rI:rC homopolymer duplex stimulated synthesis of DNA polymer. This template stimulates synthesis by enzymes in some animal tumor viruses but has little template activity with other tumor virus enzymes (G. Weber, unpublished data).

DNA polymerase activity associated with the pellet fraction from plasma was found to meet the criterion of having a requirement for all four

TABLE 1. DNA polymerase activity in plasma from chronic lymphocytic leukemia (CLL) patients

Plasma	En- zyme ^a (ml)	Template	³ H-dGTP ^b incorporation (counts/min)	
			Without deter- gent	With deter- gent ^c
Expt 1				
CLL-JV	.01	ML-DNA ^d	81	201
CLL-JV	.05	ML-DNA	125	570
CLL-JV	.01	rI:rC ^e	72	47
CLL-DD	.01	ML-DNA	82	91
CLL-DD	.05	ML-DNA	50	170
CLL-DD	.01	rI:rC	84	89
Expt 2				
CLL-JV	.04			76
CLL-JV	.04	ML-DNA		525
CLL-DD	.03			48
CLL-DD	.03	ML-DNA		134

^a In the first experiment, 0.01 ml of enzyme preparation corresponds to particulates from 0.25 ml of patients' plasma, and reactions were for 1 hr at 37 C. In the second experiment, 0.02 ml of enzyme corresponds to particulates from 0.25 ml of plasma and the reactions were incubated for 2 hr at 37 C.

^b One picomole of 3 H-deoxyguanosine triphosphate (dGTP) = 2,000 counts/min.

^c Detergent treatment of pellet was done as described.

^d ML-DNA was obtained from *M. lysodeikticus*, and 4 μ g was added to assay.

^e We prepared rI:rC by mixing 300 μ g of poly I with 100 μ g of poly C in 1 ml of 0.2 M NaCl. A 0.01-ml amount of this mixture was added to the assay.

deoxyribonucleoside triphosphates. Results in Table 2 show that the enzyme from CLL-JV plasma was greatly depressed by leaving out only one of the deoxyribonucleoside triphosphates. The activity in the other plasma from CLL did not show as strong dependency, but synthesis by the complete system was very low.

These first two experiments provided assurance that the plasmas contained a DNA polymerase that was stimulated by DNA template and synthesized a DNA heteropolymer. It was apparent that the activity in CLL-JV plasma was sufficient to examine the product of the DNA-primed reaction on a CsCl buoyant density gradient. The amount of 3 H-labeled product was enhanced by doubling the reaction mixture and incubating for 2 hr. The reaction was terminated as previously described (11), and the product and DNA template were banded on an isopycnic CsCl

TABLE 2. Effect of omission of deoxyribonucleoside triphosphate^a

Condition ^b	Plasma	³ H-TTP incorporation (counts/min)
Complete	CLL-JV	672
Without dATP	CLL-JV	113
Without dCTP	CLL-JV	148
Without dGTP	CLL-JV	103
Complete	CLL-DD	117
Without dATP	CLL-DD	94
Without dCTP	CLL-DD	66
Without dGTP	CLL-DD	46

^a *M. lysodeikticus* DNA was added at a level of 4 μ g per assay. The reaction mixtures were incubated at 37 C for 2 hr. A 0.04-ml amount of enzyme fraction (same preparation as in Table 1, experiment 2) was used, and 1 pmole of ³H-thymidine triphosphate (TTP) was equivalent to 2,700 counts/min. Background counts were subtracted.

^b Abbreviations: dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate.

FIG. 1. Isopycnic centrifugation of the ³H-labeled DNA product in CsCl. ³H-labeled product from a two-fold assay (0.4 ml) and 50 μ g of *Micrococcus lysodeikticus* DNA were mixed with CsCl to give an approximate density of 1.70 and centrifuged for 44 hr at 40,000 rev/min in a SW50 Spinco rotor at 20 C. The open circles represent the marker DNA (density = 1.72 g/cm³), and the closed circles represent the ³H-product.

gradient. Results of this experiment are reported in Fig. 1. The product of the reaction had a major band at a density of 1.72 which was the density of the DNA template.

There was considerable radioactivity located in the dense regions of the CsCl gradients. It was not

possible to examine this product further since the supply of enzyme was limited. If this represents DNA product, then one might speculate that it has complexed with a more dense molecule.

Kinetics of product synthesis. The rate of ³H-deoxyguanosine triphosphate (dGTP) incorporation into trichloroacetic acid-insoluble polymer was shown to be linear for 2 hr (Fig. 2). The enzyme from CLL-JV had the greater rate of synthesis of the two preparations. It appears from this data that the sensitivity for detecting enzyme activity in leukemic plasma can be increased by extending the reaction time since the reaction rate was linear. In a separate experiment, plasmas from individuals with no known blood problems (normal white cell number) were examined for a particulate enzyme in the blood plasma. The procedures for preparation and assay were identical to those used for the CLL plasmas except twice as much plasma was used for the enzyme preparation. The enzyme activity was measured over a 2-hr period, and the results are given in Fig. 3. The data show that there is no synthesis of polymer by either preparation over the 2-hr

FIG. 2. Kinetics of ³H-deoxyguanosine triphosphate (dGTP) incorporation into DNA by polymerase. Incorporation of ³H-labeled substrate into DNA product was assayed at the intervals indicated in the figure by using 0.2-ml reaction mixtures, with each assay containing particulates from 0.5 ml of plasma and 4 μ g of DNA primer per ml. The closed triangles indicate the results from CLL-JV plasma, and the open triangles were assays from CLL-DD plasma. One picomole of ³H-dGTP equalled 2,000 counts/min.

FIG. 3. Absence of DNA polymerase activity in nonleukemic plasmas. Plasmas from two healthy individuals were tested for DNA polymerase activity by using the standard assay. Enzyme activity from blood donors is indicated in the figure by open and closed triangles at the various time periods. The assay mixtures for this experiment contained particulates from twice the volume of plasma as was used in Fig. 2. One picomole of ^3H -deoxyguanosine triphosphate equalled 2,000 counts/min.

period. Absence of particulate enzyme activity in plasma from healthy individuals demonstrated that the activity from the leukemic plasmas was not a measure of low enzyme activity normally found in plasma. Obviously, from this experiment, we could not exclude the possibility that the appearance of DNA polymerase in the plasma particulate fraction from CLL was not a secondary effect from the stress of the disease.

Buoyant density of enzyme fraction. The second collection of 14 ml of plasma from CLL-JV, 8 days after the initial collection, was used for studies on the buoyant density of the enzyme activity from the leukemic plasma. The white blood cell count at the collection time was 252,000 cells/mm³. The procedures for pelleting and for floating the enzyme in a glycerol density gradient were given above. Tumor virus from AMV plasma was treated in the same manner to compare banding positions in the glycerol gradient. Strain MC29 from tissue culture was used also to compare the density of the virus enzyme in the same density gradient. This gradient was centrifuged at a separate time.

The AMV preparation had been frozen and thawed a number of times and consisted mainly of damaged virus particles. This AMV preparation on the first thawing had nearly all intact virus particles with a buoyant density of 1.16 g/cm³. Generally, intact RNA tumor virus bands in a density gradient at 1.16 g/cm³, and a shift in buoyant density from 1.24 to 1.27 indicates removal of the outer virus envelope (1, 3).

Ríman has observed a shift in density of intact AMV virion to 1.23 to 1.25 after forming viral cores by detergent treatment (*personal communication*).

The procedure for floating the enzyme in a glycerol density gradient produced a broad band of polymerase activity in all gradients and was chosen to avoid transfer of small quantities of particulate material. Figure 4 illustrates the distribution of enzyme activity from CLL-JV plasma in a density gradient. The enzyme activity floated in the gradient to produce broad bands with peak enzyme activity in the density range of 1.20 to 1.23 g/cm³ and a minor band at the density of 1.15 g/cm³. This demonstrates that the enzyme activity is associated with a particulate fraction having a density range that is similar to that found for core type particles. Figures 5 and 6 provide a comparison of distribution of the enzyme activity by preparations of AMV (Fig. 5) and MC29 virus (Fig. 6). The density range observed for these virus preparations indicates

FIG. 4. Isopycnic centrifugation of particulate fraction from plasma of CLL-JV in a preformed glycerol density gradient. The pellet fraction from the plasma was elevated in a glycerol gradient by the same procedure as used for avian myeloblastosis virus in Fig. 5. The volume of each reaction mixture was 0.2 ml, and the assays were incubated 4 hr at 37 C. *Micrococcus lysodeikticus* DNA (6 μg) was used as the template, and 0.02 ml of each gradient fraction (0.2 ml) was assayed. ^3H -deoxyguanosine triphosphate (dGTP) was used as labeled substrate (1 pmole = 2,000 counts/min).

FIG. 5. Isopycnic centrifugation of avian myeloblastosis virus in a preformed glycerol density gradient. The virus particles were pelleted against a 95% glycerol pad and elevated to their density in a glycerol gradient by using the procedures described. Reaction mixture volume was 0.2 ml, and the assays were incubated for 60 min at 37 C. Each fraction was assayed for enzyme activity by using *Micrococcus lysodeikticus* DNA template (4 μ g) and 0.025 ml of the gradient fractions (0.1 ml). ^3H -deoxyguanosine triphosphate (dGTP) was used as labeled substrate (1 pmole = 2,000 counts/min).

that most of the virions are present as core particles or nucleocapsids (1, 3). Minor peaks at 1.16 indicate the presence of some intact virus particles.

DISCUSSION

The results reported in this paper were derived from two cases of CLL. The DNA polymerase activity obtained in these experiments was low, which we fully expected. Nevertheless, the kinetic data and the experiments on the omission of triphosphates from the incubation mixture were convincing evidence that the enzyme activity measured in plasmas of CLL was indeed valid.

Evidence that the enzyme activity was contained in a particle came from the observations that detergent was able, in both cases, to increase DNA polymerase activity and that the enzyme floated in a glycerol gradient to a density which was characteristic of core particles.

There seems little doubt that a particulate enzyme did exist in the CLL-JV plasma. It remains to be seen if this enzyme activity can be detected frequently and under what conditions of therapy the enzyme activity will persist. We

FIG. 6. Isopycnic centrifugation of strain MC29 tumor virus in a preformed glycerol density gradient. The virus particles were pelleted and banded in a glycerol gradient the same as the avian myeloblastosis particles in Fig. 5. Volume of each reaction mixture was 0.2 ml, and the assays were incubated for 60 min at 37 C. *Micrococcus lysodeikticus* DNA (4 μ g) was used as template, and 0.025 ml of each gradient fraction (0.1 ml) was assayed. ^3H -deoxyguanosine triphosphate (dGTP) was used as labeled substrate (1 pmole = 2,000 counts/min).

have indications that certain therapy will eliminate detectable enzyme activity. Obviously more studies need to be done to determine if the enzyme activity is directly related to the disease or whether the appearance of the enzyme activity in the plasma is due to a functional failure by a diseased system, permitting an accumulation of normal enzyme from fragmented cells of the blood.

These studies suggest, however, that a new opportunity for study of human neoplasms may be available.

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