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CHARACTERIZATION OF THE RNA DEPENDENT DNA POLYMERASE OF A NEW HUMAN T LYMPHOTROPIC RETROVIRUS (LYMPHADENOPATHY ASSOCIATED VIRUS)*

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We described here the characteristics of the Reverse Transcriptase activity associated with the Lymphadenopathy Associated Virus (LAV). A critical concentration of non ionic detergent, all four deoxyribonucleosides triphosphates and the divalent cation Mg^{2+} are required for optimal endogenous enzyme activity. The endogenous reaction product is digested by DNase and not by RNase and its synthesis is only slightly inhibited by actinomycin D. Exogenous reactions are optimal using poly A oligo dT_{12-18} or poly Cm oligo dG_{12-18} as template primer and Mg^{2+} as divalent cation.

This enzyme can be distinguished from other cellular DNA polymerases activities and from Terminal deoxynucleotidyl Transferase (TdT) by purification from LAV infected T lymphocytes using phosphocellulose column.

Lymphadenopathy Associated Virus (LAV) has been isolated (1) (2) from a lymph node biopsy of an homosexual patient with lymphadenopathy, a syndrom which is often considered as an early clinical sign of acquired immunodeficiency syndrome (AIDS) (3). More recently, similar viruses have been obtained from patients presenting with AIDS (4). This disease is characterized by the development of opportunistic infections and/or Kaposi's sarcoma and seems to be due to a T cell dysfunction (5). The replication of LAV or LAV related viruses occurrs exclusively in an helper

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Abbreviations : RT, reverse transcriptase ; poly A oligo dT_{12-18}, poly(adenylic-acid)-oligo(deoxythymidilic-acid$_{12-18}$) ; poly dA oligo dT, poly(deoxyadenilic-acid)-oligo(deoxythymidilic-acid$_{12-18}$) ; poly C oligo dG, poly(cytidylic-acid)-oligo(deoxy-guanylic-acid) ; poly C_m oligo dG_{12-18}, poly(2'-O-methylcytidylic-acid)-oligo(deoxy-guanylic-acid$_{12-18}$).

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(OKT_4+) subpopulation of T lymphocytes (4). A prevalence of antibodies to LAV has been found in the sera of patients presenting with lymphadenopathy syndrom as well as with AIDS (4).The role of such viruses in the disease is not yet clear . Several characteristics indicate that LAV or LAV related viruses belong to the retroviruses family (1) (4). Budding particles at the plasma membrane have been observed in electron microscopy. The density of the virus in sucrose gradient is 1.16. A Mg^{2+} dependent reverse transcriptase activity has been found to be associated with RNA containing virions. In the present paper, we report some properties of the enzymes associated with purified virions or existing in extracts of infected cells.

MATERIALS AND METHODS

. Chemicals

^3H thymidine triphosphate and ^3H uridine were obtained from Amersham, Radiochemical Center. Unlabelled deoxyribonucleosides triphosphates were purchased from P.L Biochemicals. Synthetic template primers were supplied either by Boehringer or by P.L. Biochemicals. Purified bovine pancreatic DNase and activated DNA were obtained from Worthington Biochemicals Co. Actinomycin D was purchased from Calbiochem. Nycodenz was from Nyegaard, Oslo.

Cells

T lymphocytes from a normal blood donor were cultured and infected with LAV as previously described (1).

. Viruses

Studies on exogenous reactions were performed in the presence of synthetic template primers using a crude viral pellet from cell free supernatant obtained as indicated elsewhere (6).

For endogenous reactions using only the viral RNA as template primer, the virus was concentrated (7) and purified on sucrose gradient using isopycnic centrifugation.(8) In some experiments purified virions were prepared using isotonic gradients of Nycodenz by a procedure previously published (4).

. Enzyme purification

10^8 normal human T lymphocytes and 10^8 LAV infected human T lymphocytes (15 days after infection) were washed 2 times in P.B.S., then resuspended in Tris 50 mM (pH 7.5), KCl 500 mM, EDTA 0.1 mM, glycerol 10 %, dithictreitol (DTT) 1 mM, and frozen in liquid nitrogen and thawed three times. Cell homogenates were incubated 15 mn at 37°C with 0.5 % Triton and centrifuged at 100,000 g for 1 hour. The supernatants were removed, dialysed 6 hours (Tris 50 mM, EDTA 0.1 mM, KCl 100 mM, Glycerol 10 %).

RT, DNA polymerases and Terminal deoxynucleotidyl transferase (TdT) were prepared and assayed as previously described (9).

. Enzyme assays

The composition of reaction mixtures are indicated in the text. The reactions were terminated by adding 0.1 M sodium pyrophosphate, carrier nucleic acid and 20 % trichloroacetic acid. The acid precipitable radioactivity of each sample was determined as previously described. (1).

RESULTS

1) - Characterization of the enzyme reaction

Table I summarized the conditions required for the endogenous enzyme activity associated with purified LAV particles. Like other DNA polymerases, the enzyme needed for

Table I

Requirements of the endogenous LAV RNA dependent DNA polymerase reaction

Reaction mixture	^{3}HTMP incorporated during 45 mn (cpm)
- Complete	3994
- Minus Mg Cl_2	971
- Minus Mg Cl_2 plus $Mn(CH_3\ COO)_2$	1583
- Minus dATP	1447
- Minus dCTP	1595
- Minus dGTP	1630
- Minus Triton	871
- Minus virus	490
- Complete	4771
plus Actinomycin D	2544
- Complete	3725
- RNase pretreatment	740
	^{3}HTMP incorporated during 75 mn (cpm)
- Control	2784
- plus RNase	3561
- plus DNase	642

Approximatively 10 microgramms of viral proteins were incubated in a complete reaction mixture (100 microlitres) containing 50 mM Tris pH 7.8, 6 mM Mg Cl_2, 10 mM dithiothreitol, 0.05 % Triton X 100, 0.1 mM of each dXTP and 10 µCi of ^{3}HTTP (specific activity 30 Ci/mmol). Actinomycin D (50 µg/ml) or RNase A 100 µg/ml were added to Triton disrupted virions.
RNase treatement was performed for 30 mn at 37°C in the presence of 0.2 M NaCl prior addition of complete reaction mixture.
Endogenous reactions were carried out for 45 min à 37°C in the reaction mixture described above.
Then, RNase A (1 mg/ml) or DNase I (40 µg/ml) were added and the samples were incubated for 30 mn at 37°C. Control reaction was identical except for the omission of nucleases.
At zero-time incubation the background level was 863 cpm.

Table II

Response of viral enzyme to exogenous template-primers

Template primer	^{3}HTMP or ^{3}HGMP incorporated in 60 min. (cpm)
- Poly A oligo dT_{12-18}	62 758
- Poly C oligo dG_{12-18}	10 239
- Poly dA oligo dT_{12-18}	4 337

The ability of the enzyme to use a synthetic template primer was tested by adding each one at a final concentration of 0.05 absorbancy unit at 260 nm per ml in a reaction mixture (50 l) containing 50 mM Tris pH 7.8, 20 mM KCl, 1 mM dithiothreitol, 5 mM Mg Cl_2 and 10µCi of ^{3}HTTP (30 Ci/mmol) or ^{3}HdGTP (20 Ci/mmol). Reactions were initiated by adding the reaction mixture to a viral pellet previously disrupted with 0.1 % Triton. In this conditions of reaction, where only one dXTP was added, exogenous enzyme activity was only detected.

optimal activity all four deoxyribonucleosides triphosphates. The divalent cation Mg^{2+} was found to be necessary for the expression of enzyme activity as well as detergent treatment. The addition of actinomycin D to the reaction mixture reduced the synthesis of the product by approximatively 50 %. Incubation of disrupted virus with RNase reduced the ^{3}HTMP incorporation, providing evidence that the enzyme activity of LAV is dependent on RNA used as template.

As shown in table I, the nature of the endogenous reaction product was DNA since this product was sensitive to DNase treatment but was resistant to RNase. Alkali treatment was also without effect on this reaction product (data not shown). The stimulative effect of some exogenous template primers on the LAV DNA polymerase activity is illustrated on table II.

Both poly A oligo dT_{12-18} and poly C oligo dG_{12-18} which are known to be effective template primers for RNA tumor virus DNA polymerases (10, 11, 12, 13, 14), stimulated the enzyme activity. Only a weak reaction was observed when the reaction mixture contained poly dA oligo dT_{12-18}, an efficient template primer for cellular and bacterial DNA polymerases (10, 11, 12).

In the exogenous reaction (9, 10, 11), the reverse transcriptase activity was activated preferentially by Mg^{2+}. The optimal concentration was found to be 5 mM (data not shown). The divalent cation Mn^{2+} was approximatively 10 times less effective at 0.5 mM (concentration generally used for the reverse transcriptase of murine type C RNA viruses). The optimal pH for exogenous reaction was about 7.8 and the optimal concentration of detergent was between 0,05 % to 1 % (data not shown).

2) - Association of the enzyme with the LAV virion

In order to demonstrate that the RT activity is associated with the virus particles, ^{3}H-Uridine labelled LAV was centrifuged at 55000 rpm for 90 min. in a 5 to 35 per cent linear gradient of Nycodenz. The fractions were then tested for both ^{3}H-Uridine labelled acidoprecipitable material and polymerase activity. As shown on figure 1, the viral RNA and the exogenous polymerase

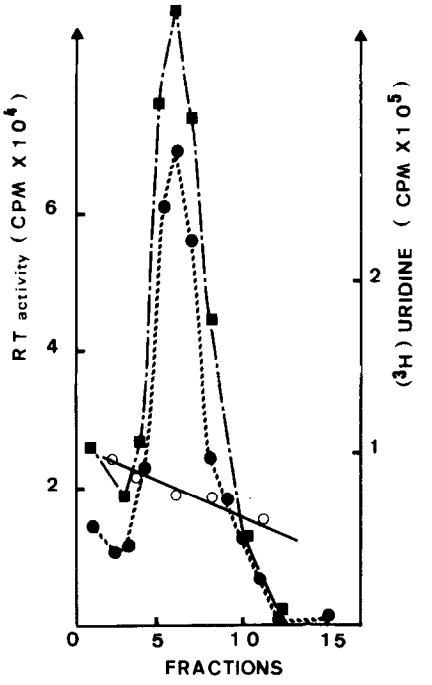

Figure 1 Virus producing cells were labelled for 12 hours with ^{3}H-Uridine (30 Ci/mmol ; 20 μCi/ml). Cell free supernatant was then harvested and the virus was concentrated by polyethyleneglycol precipitation before purification on Nycodenz gradient as described elsewhere (4). In control experiment Murine Leukemia Virus banded at the same density (data not shown). 200 μl fractions were collected and 5μl sample of each fraction were assayed for exogenous RT activity (■—■); 20μl of each fraction were precipitated with 20 percent trichloroacetic and the ^{3}H labelled acidoprecipitable material (●·····●) was collected on 0.45 μ Millipore filter and the radioactivity was measured in a Packard scintillation counter.

activity were found in coincident peaks at a density of about 1.10 g/ml. In similar experiment using ^{35}S-Methionine labelled virus, an exact coincidence was observed between the peak of RT activity and the fractions containing the proteins of LAV (data not shown).

3) - Purification of RT from LAV infected lymphocytes by phosphocellulose chromatography

As shown in fig. 2 using activated DNA as template primer, 3 peaks could be seen corresponding to α , γ and β polymerases at KCl 0.2, 0.4, 0.6 M respectively. With poly A oligo dT_{12-18}, 3 peaks were also observed : 0.2 - 0.4 and 0.6 M KCl corresponding respectively to RT activity, γ and α DNA polymerase activities. Using poly Cm dG_{12-18} as template primer, we identified the 0.2 M KCl peak as the only RT peak.

With non infected human lymphocytes, we noted only 2 peaks using poly A oligo dT_{12-18} (0.4 - 0.6 KCl), 3 peaks using activated DNA and no activity with poly Cm oligo dG. Comparing both results a LAV related R.T. activity could be detected only in LAV infected cells.

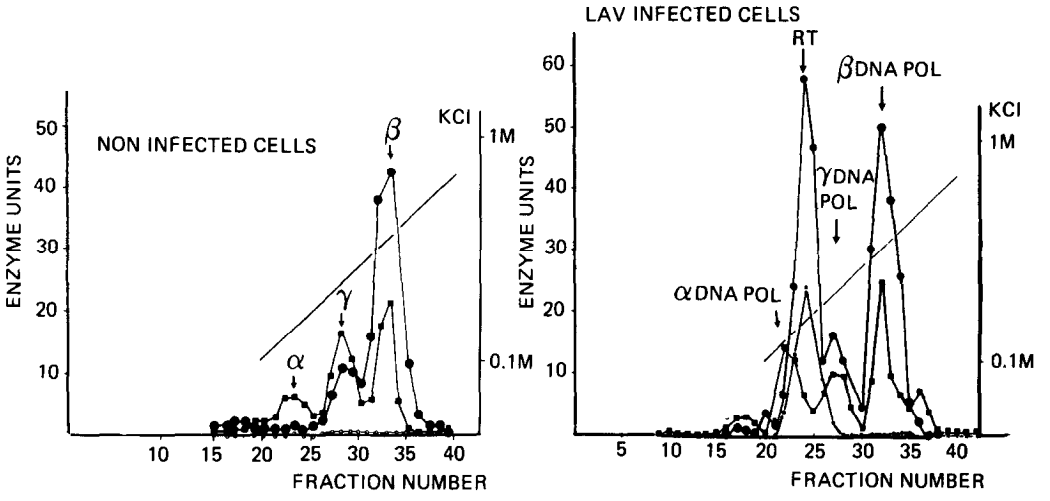

Figure 2 The figure shows the RT and DNA polymerase activity patterns obtained after a phosphocellulose chromatography and a 0.1 M to 1 M KCl elution. Enzyme conditions are identical to those described in legend of table II. One enzyme unit is defined as the amount which catalyses the incorporation of one nM of deoxynucleoside monophosphate into the product DNA during one hour at 37° C for 10^9 cells. (1 g equivalent).

○——○ poly Cm oligo dG_{12-18}
●——● poly A oligo dT_{12-18}
■——■ Activated DNA

A Terminal deoxynucleotidyl transferase (TdT) assay was also performed, showing a TdT activity peak, at 0.5 M KCl (data not shown) different from that of RT (0.2 M KCl).

DISCUSSION

Treatment of the virus with a non ionic detergent enhanced the endogenous activity indicating that disruption of the viral envelope promotes the enzymatic reaction. This requirement for detergent and the observation that, during isopycnic centrifugation purification, the enzyme activity remains associated with ^{3}H-Uridine labelled RNA and with ^{35}S-Methionine labelled proteins are consistent with the fact that the enzyme is a component of LAV. The omission of one deoxynucleoside triphosphate precursor decreases the endogenous reaction as expected for a DNA polymerase activity. Furthermore the endogenous reaction product is digested by DNase treatement. Preincubation experiment with RNase shows that the enzyme activity associated with LAV is an RNA directed DNA polymerase. Moreover,the endogenous reaction is partially inhibited by Actinomycin D.

LAV enzyme activity is strongly enhanced by synthetic template primers such as poly A oligo dT_{12-18} and poly C oligo dG_{12-18} which are known (10) to be preferentially used by reverse transcriptases of RNA tumor viruses.

Ionic requirement for optimal activity is obtained with the divalent cation Mg^{2+} . This characteristic is similar to that of Human T cell leukemia virus (HTLV) (15) although HTLV is not antigenically and morphologically related to LAV.(4). Thus, the enzymes of known human T lymphotropic viruses seem to be Mg^{2+} dependent.

LAV DNA polymerase can be purified from LAV infected human T lymphocytes by phosphocellulose chromatography and KCl elution (16). One of the three peaks (KCl 0.2 M) observed using poly A oligo dT_{12-18} as template primer is also obtained with poly C_m oligo dG, a reverse transcriptase specific template primer (17). No TdT activity was detected in this peak. Both results, the position in elution gradient and the template primer specificity indicate that the eluted enzyme is a reverse transcriptase.

These characteristics of the enzyme associated to LAV together with its morphology in electron microscopy (1)(2) - which is similar to other RNA tumor viruses such as Equine Infectious Anemia Virus, EIAV, (4) - allow to classify LAV in the Retrovirus family.

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