Serological relationship between reverse transcriptases from human T-cell lymphotropic viruses defined by monoclonal antibodies

Evidence for two forms of reverse transcriptases in the AIDS-associated virus, HTLV-III/LAV

A. Chandra, T. Gerber, S. Kaul, C. Wolf, I. Demirhan and P. Chandra*

Laboratory of Molecular Biology, Center of Biological Chemistry, University Medical School, Theodor-Stern-Kai 7, D-6 Frankfurt/Main 70, FRG

Received 13 March 1986

The immunological relationship between reverse transcriptases purified from human T-cell lymphotropic viruses (HTLV-I, HTLV-II, HTLV-III) was defined using monoclonal antibodies specific for HTLV-III reverse transcriptase, secreted by a mouse/mouse hybridoma clone (4F8) developed in our laboratory. The viral proteins from HTLV-I and HTLV-II do not bear any cross-reactive epitope to antibodies secreted by this clone. These antibodies specifically cross-react with HTLV-III reverse transcriptase. The antibodies failed to neutralize the catalytic activity of reverse transcriptase; however, after immunoprecipitation with a magnetic conjugate of goat anti-mouse IgG, the residual activity was completely inhibited. This shows that the antibodies are not directed towards the catalytic active center of the enzyme. Using an immunoblotting technique (Western blotting), we have found two cross-reactive proteins with HTLV-III lysate with molecular masses of 53 and 66 kDa. This suggests that HTLV-III possesses two reverse transcriptase activities with a common determinant recognized by the same epitope.

Monoclonal antibody *Reverse transcriptase* *Human lymphotropic virus* *AIDS*

1. INTRODUCTION

Human T-cell lymphotropic retroviruses (HTLV) constitute a well characterized group of retroviruses, with tropism for OKT4+ T-helper cells. Three subgroups have been identified. HTLV-I, isolated in 1980 [1], is the etiological agent of adult T-cell leukemia; HTLV-II, first isolated from a patient with hairy cell leukemia [2], has not yet been linked to any human disease. More recently, a third member of this family, HTLV-III [3,4], has been shown to be the etiological agent of the acquired immune deficiency syndrome (AIDS). HTLV-III is similar to the lymphadenopathy-associated virus (LAV), previously described by Barré-Sinoussi et al. [5].

The subtype classification of the AIDS-associated virus as HTLV-III was implicated by the initial observations of Essex et al. [6,7] who reported antibodies to HTLV-I antigens in patients with AIDS, followed by a series of immunological data and homologies in nucleic acid sequences. Schupbach et al. [8] and Sarngadharan et al. [9] have reported that some of the antigens of HTLV-I and HTLV-III bear cross-reactive epitopes. The sequence studies using cloned HTLV-III and HTLV-I genomes have revealed some sequence homologies, especially in the *gag/pol* region, between both the viruses [10,11].

To study the cross-reactivity between antigens of

* To whom correspondence should be addressed

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

HTLV-III and other related viruses, we have recently generated hybridoma (mouse/mouse) clones which secrete monoclonal antibodies to p24, p31, gp41, gp120 and reverse transcriptase. Here, we will describe the serological analysis of reverse transcriptases purified from HTLV-I, HTLV-II and HTLV-III, using monoclonal antibodies to HTLV-III reverse transcriptase; the hybridoma clone used carries the designation 4F8.

2. EXPERIMENTAL

Human T-cell lymphotropic viruses I, II and III were obtained from Dr R.C. Gallo and Dr Prem Sarin, National Cancer Institute, Bethesda, MD, USA.

2.1. *Preparation of monoclonal antibodies*

BALB/c mice were immunized with successive intraperitoneal inoculations of detergent lysates (1% Triton X-100) of density-gradient purified HTLV-III produced in H9 cells. First inoculation was done with 150 μg viral protein emulsified in complete Freund's adjuvant, followed by 3 boosters given 2 weeks apart using 100 μg of the viral antigen in incomplete Freund's adjuvant. The last booster was given with a purified preparation of reverse transcriptase from HTLV-III (50 μg protein) in incomplete Freund's adjuvant. 84 h after the 5th inoculation, splenic lymphocytes (1×10^8 cells) were mixed with NS-1 mouse-myeloma cells (4×10^7 cells) and centrifuged ($1000 \times g$, 7 min). The fusion procedure, cell culturing and cloning of hybridomas were similar to the procedures described in [12].

Supernatant fluids from the hybridoma cultures were screened for antibodies to HTLV-III proteins by the enzyme-linked immunosorbent assay (ELISA) in which we used disrupted HTLV-III as antigen. Hybridoma cultures which reacted positive in this assay were further analyzed by Western blotting and by ELISA using purified reverse transcriptase as one of the antigens. One hybridoma clone, designated 4F8, was found to secrete antibodies reacting only with the purified reverse transcriptase.

For preparing ascites fluid containing monoclonal antibodies BALB/c mice were primed by injecting 0.5 ml pristane (2,6,10,14-tetramethylpentadecane) intraperitoneally. 10–14 days later 5×10^6–1×10^7 exponentially growing hybridoma cells were transplanted by the same route. After 2–3 weeks, 2–6 ml ascites were obtained.

2.2. *Purification of reverse transcriptases*

Reverse transcriptases from HTLV-I, HTLV-II and HTLV-III were purified as in [13,14]. However, we discovered that HTLV-III reverse transcriptase contains a glycoprotein as contaminant which cannot be removed by the usual purification procedures [15,16]. For this reason, HTLV-III reverse transcriptase was additionally purified by affinity chromatography on lectin-Sepharose 4B (Pharmacia, Freiburg) column, using α-methylmannoside as eluent to remove the glycoprotein.

2.3. *Immunoblotting (Western blotting)*

Lysates from HTLV-I, HTLV-II and HTLV-III were fractionated by SDS-polyacrylamide slab gel (PAGE) electrophoresis [14]. The proteins were transferred to nitrocellulose sheets using an electroblotting device (LKB Transphor, unit 2005) overnight at 35 V. The nitrocellulose sheets were incubated overnight at 4°C in phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). The sheets were cut into strips and kept at −80°C until used.

Each strip was incubated at 37°C for 2 h in PBS containing 2.5% BSA, 2.5% normal goat serum and 0.05% Tween 20. The strip was washed with PBS-Tween buffer and incubated with hybridoma medium (1:100) for 30 min at 37°C. The strip was washed again and incubated with biotinylated goat anti-mouse IgG (1:1000) for 30 min at 37°C. After washing in PBS-Tween buffer, the strip was reincubated in PBS containing horseradish peroxidase-avidin D conjugate for 30 min at 37°C. After final washing, the strip was incubated at room temperature in PBS containing 4-chloro-α-naphthol (0.75 mg/ml), methanol (25%) and 2% solution of hydrogen peroxide (60 μl/5 ml). The strip was washed twice and finally air-dried.

2.4. *Assay of reverse transcriptase activity*

Reverse transcriptase activity was measured by adding 20 μl of the enzyme fraction to a volume of 30 μl, which gave a final concentration of 50 mM Tris-HCl buffer (pH 8.0), 50 mM KCl, 15 mM $MgCl_2$, 1 mM dithiothreitol, 20 μM each of the

complementary dNTP, or tritium-labeled dGTP and 1.25 μg template primer $(rC)_n \cdot (dG)_{12}$. The reaction mixtures were incubated for 30 min at 37°C. The reaction was terminated by the addition of 0.36 mg BSA and 3 ml trichloroacetic acid (10%, w/v) containing 20 mM Na pyrophosphate. Acid-precipitable material was collected on Whatman glass-fibre discs (GF/C), washed and counted in a scintillation spectrometer. Each assay was carried out in triplicate.

3. RESULTS AND DISCUSSION

Biochemical characterization of the purified reverse transcriptase from HTLV-III has been reported from this laboratory [13,14]. In these studies, the enzyme was purified from disrupted virions by column chromatography on DEAE-cellulose columns (DE 23 and DE 52, Serva, Heidelberg), followed by a final purification on the phosphocellulose column. The step-wise isolation yielded an approx. 2000-fold purification of reverse transcriptase starting from the virus lysate [13,14]. The M_r of this enzyme which eluted as a single peak of activity from the phosphocellulose column was estimated to be 98000. Using this preparation in this study to characterize the hybridoma clones, we discovered a cross-reactivity for this antigen by hybridoma clones secreting antibodies to reverse transcriptase and gp41. This indicated to us that the reverse transcriptase used in our study was contaminated by a glycoprotein, probably gp41. Further purification of the phosphocellulose enzyme on a lectin-Sepharose 4B column removed the glycoprotein, and we were able to obtain a reverse transcriptase which showed no cross-reactivity with other hybridoma fluids. The hybridoma clone secreting monoclonal antibodies which reacted specifically to HTLV-III reverse transcriptase was designated 4F8, and used in our serological experiments.

The supernatant fluid obtained from the clone 4F8 was screened by ELISA using disrupted HTLV-III as antigen. Wells of a 96-well plastic tray were coated overnight with lysates of density-banded HTLV-I, HTLV-II and HTLV-III containing different amounts of antigen (table 1). The remaining protein-binding sites were saturated with 1% BSA in PBS. 50 μl of the hybridoma supernatant was then added to each well and incubated for 1 h at 37°C. The immuno-reactivity was measured by adding affinity purified goat anti-mouse IgG + M conjugated with β-galactosidase, using *p*-nitrophenyl-β-D-galactopyranoside as substrate. The activities were measured at 405 nm in an ELISA reader.

The results shown in table 1 demonstrate that viral proteins from HTLV-I and HTLV-II do not

Table 1

Serological relationship between human T-lymphotropic retroviruses defined by monoclonal antibodies

Experiment	$E_{405} \times 10^3$ at various virus (protein) concentrations							
	0(PBS/BSA)	15	30	60	125	250	500	1000
	(ng/well)							
HTLV-I	57	63	63	55	57	55	67	53
HTLV-II	64	69	64	51	63	77	60	50
HTLV-III	50	60	61	54	75	96	198	288

Supernatant fluid obtained from clone 4F8 was screened by ELISA using disrupted HTLV-III as antigen. Wells of a 96-well plastic tray were coated overnight with HTLV-I, HTLV-II and HTLV-III lysates containing different amounts of antigen. After saturation of the remaining protein-binding sites with 1% BSA, 50 μl of culture supernatant from 4F8 was added to each well. The cross-reactivity was measured by adding anti-mouse IgG + M conjugated with β-galactosidase, using *p*-nitrophenyl-β-D-galactopyranoside as substrate. The activities were measured in an ELISA reader at 405 nm

bear any cross-reactive epitope to antibodies secreted by the clone 4F8. As a control in these experiments, BSA was used. On the other hand, a concentration-dependent cross-reactivity is exhibited by the viral antigens from HTLV-III against antibodies secreted by 4F8 clone.

Following the procedure described above, wells of 96-well plastic trays were coated overnight with reverse transcriptases purified from HTLV-I, HTLV-II and HTLV-III. To document the specificity of 4F8 clone for reverse transcriptase, another hybridoma clone secreting monoclonal antibodies against the gp120 antigen of HTLV-III, designated 1A6, was included in this experiment. As follows from table 2, no cross-reaction between reverse transcriptases of HTLV-I and HTLV-II was observed towards antibodies to HTLV-III reverse transcriptase (clone 4F8). In the same experiment, the antibodies secreted by the clone 1A6 show no cross-reactivity towards the HTLV-III reverse transcriptase.

The specificity of antibodies secreted by the clone 4F8 for HTLV-III reverse transcriptase was further documented by immunoblotting. The electrophoretic separation of lysates from HTLV-I (lane 1), HTLV-II (lane 2) and HTLV-III (lane 3) are shown in fig.1A; lane 4 shows the separation of affinity-purified M_r markers (phosphorylase *b*, 94000; BSA, 67000; ovalbumin, 43000; carbonic anhydrase, 30000; soybean trypsin inhibitor, 20100 and α-lactalbumin, 14400). Fig.1B depicts the immunoblots of HTLV-I (lane 1), HTLV-II (lane 2) and HTLV-III (lane 3), developed against antibodies secreted by the clone 4F8. There is no cross-reacting band seen on HTLV-I and HTLV-II strips. The strip with HTLV-III shows two prominent bands in the region of M_r 53000 and 66000. To separate the two reactivities, the clone 4F8 was subjected to repeated cycling. Even after the 4th cycling of 4F8 clone, the two reactivities could not be separated. This indicates that both the reactive antigens, 53 and 66 kDa proteins, have a common determinant recognized by the same epitope.

To elucidate the biochemical nature of 4F8-antibody interaction to HTLV-III reverse transcriptase, we have measured the catalytic activity of reverse transcriptase in the presence of antibodies. The data in table 3 show that antibodies secreted by the clone are unable to neutralize the enzymatic activity. Measurement of residual activi-

Table 2

Serological relationship between reverse transcriptases from human T-lymphotropic viruses defined by monoclonal antibodies to HTLV-III-RT

Experiment	$E_{405} \times 10^3$ at various RT concentrations (protein)							
	0(PBS/BSA)	15	30	60	125	250	500	1000
	(ng/well)							
HTLV-I RT								
(4F8)	51	56	44	48	47	68	48	39
(1A6)	45	44	41	45	56	56	53	39
HTLV-II RT								
(4F8)	45	45	56	46	54	61	51	39
(1A6)	49	42	42	48	53	55	50	42
HTLV-III RT								
(4F8)	55	44	49	70	82	154	231	287
(1A6)	44	50	56	52	50	53	55	42

Following the procedure described in the text and table 1, wells were coated overnight with various amounts of reverse transcriptases purified from HTLV-I, HTLV-II and HTLV-III. The clone 1A6, serving as negative control, secretes antibodies against glycoprotein gp120

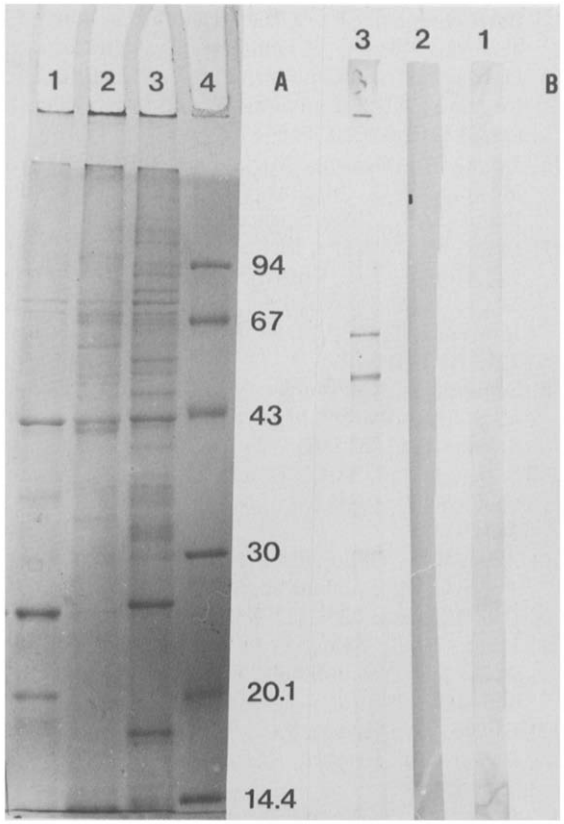

Fig.1. (A) SDS-polyacrylamide gel electrophoresis of virus lysates. 100 μl virus suspension containing 4 μl Tris-HCl (pH 6.8), 20 μl SDS and DTT (1%) was incubated in a boiling water bath for 5 min. To this were added 80 μl glycerine and 10 μl bromophenyl blue (10%); 15 μl of this suspension was used per slab. Other experimental details have been described [14]. Lanes: 1, HTLV-I; 2, HTLV-II; 3, HTLV-III; 4, protein markers: phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (14.4 kDa). (B) Immunoblot transfer analysis of antigenic cross-reactivities of HTLV-I, HTLV-II and HTLV-III towards antibodies secreted by hybridoma 4F8. Lanes: 1, HTLV-1; 2, HTLV-II; 3, HTLV-III. Experimental details are described in section 2.

ty by immunoprecipitation of the antigen-antibody complex however, shows a concentration-dependent inhibition of the enzyme activity. These data indicate that the antibodies secreted by 4F8 clone are not directed towards the active center of HTLV-III reverse transcriptase.

Table 3

Catalytic activity of HTLV-III reverse transcriptase in the presence of antibodies secreted by the hybridoma clone 4F8

Antibody concentration[a]	[^{3}H]dGMP incorporation (pmol/10 μl)	
	Neutralizing[b] activity	Residual[c] activity
Control[d]	110(100)[e]	120(100)
1:2000	ND	51.6(43)
1:200	99.3 (90.2)	22.8(18.9)
1:20	92.28(83.9)	ND

[a] Ascites prepared from clone 4F8 (see section 2) was used as the source of antibodies. The dilution factor depicts the concentration of antibodies in the ascites fluid

[b] Neutralizing activity was measured by preincubating 20 μl of the enzyme with 20 μl of the properly diluted ascites fluid overnight. 10 μl of this was used to assay the enzyme activity

[c] 10 μl of the enzyme was preincubated with 10 μl of the properly diluted ascites overnight at 4°C. To this was added 100 μl of magnetic conjugate of goat anti-mouse IgG (Biomag M4400, Sebak, Aidenbach, FRG), and the tubes were placed on a magnetic separation device (Sebak). After 1 h, 30 μl aliquots were pipetted from the supernatant and assayed for the residual enzyme activity. All estimations were done in triplicate

[d] In the control experiments, 1:100 dilution of ascites from another clone which showed no cross-reactivity to reverse transcriptase was used. Other procedures were identical

[e] Figures in parentheses represent the percent of control value

The biochemical and immunological data presented above suggest that there are two forms of reverse transcriptase activities having a common determinant for the antibodies secreted by clone 4F8. The antibodies secreted by clone 4F8 are non-neutralizing, and are specific for HTLV-III reverse transcriptase. Thus, the reverse transcriptase of HTLV-III is serologically different from those from HTLV-I and HTLV-II. Immunoblotting shows two forms of reverse transcriptase of M_r 53000 and 66000. There are two possible mechanisms to explain this heterogeneity of HTLV-III reverse transcriptase: one possibility is that the product of the *pol* gene is a polyprotein

which is processed at different sites producing two forms of the molecule; the other is that the parent enzyme of M_r 66000 is processed by a protease associated to the HTLV-III genome. Experimental evidence is in favor of the second possibility. We have recently shown [14] that HTLV-III reverse transcriptase can be separated by isoelectric focusing into two peaks with isoelectric points of 5.75 and 6.25. These differences are probably due to cleavage of roughly 100 amino acids from the carboxyl end by a specific protease. The characterization of two enzymes separated by isoelectric focusing should help in answering these questions.

ACKNOWLEDGEMENTS

We are grateful to Drs Robert C. Gallo and Prem S. Sarin for providing us with HTLV-I, HTLV-II and HTLV-III. It is a pleasure to acknowledge the skilled technical assistance of Mrs M. Dzwonkowski. This work was supported by a grant from Deutsche Krebshilfe eV.

REFERENCES

[1] Poiesz, B.J., Ruscetti, F.W., Gazdar, A.F., Bunn, P.A., Minna, J.D. and Gallo, R.C. (1980) Proc. Natl. Acad. Sci. USA 77, 7415–7419.

[2] Kalyanaraman, V.S., Sarngadharan, M.G., Robert-Guroff, M., Miyoshi, I., Blayney, D., Golde, D. and Gallo, R.C. (1982) Science 218, 571–573.

[3] Popović, M., Sarngadharan, M.G., Reed, E. and Gallo, R.C. (1984) Science 224, 497–500.

[4] Gallo, R.C., Salahuddin, S.Z., Popovic, M., Shearer, G.M., Kaplan, M., Haynes, B.F., Palker, T.J., Redfield, R., Oleska, J., Safai, B., White, F., Foster, P. and Markham, P.D. (1984) Science 224, 500–503.

[5] Barré-Sinoussi, F., Chermann, J.C., Rey, F., Nugeyne, M.T., Chamaret, S., Gruest, J., Dauguet, C., Axler-Blin, C., Vezinet-Brun, F., Rouzioux, C., Rozenbaum, W. and Montagnier, L. (1983) Science 220, 868–871.

[6] Essex, M., McLane, M.F., Lee, T.H., Falk, L., Howe, C.W.S., Mullins, J.I., Cabradilla, C. and Fracis, D.P. (1983) Science 220, 859–862.

[7] Essex, M., McLane, M.F., Lee, T.H., Tachibana, N., Mullins, J.I., Kreiss, J., Kasper, C.K., Poon, M.C., Landay, A., Stein, S.F., Cabradilla, C., Lawrence, D.N. and Evatt, B.L. (1983) Science 221, 1061–1064.

[8] Schupbach, J., Popovic, M., Gilden, R., Gonda, M., Sarngadharan, M.G. and Gallo, R.C. (1984) Science 224, 503–505.

[9] Sarngadharan, M.G., Bruch, L., Popovic, M. and Gallo, R.C. (1985) Proc. Natl. Acad. Sci. USA 82, 3481–3484.

[10] Arya, S.K., Gallo, R.C., Hahn, B., Shaw, G.M., Popovic, M., Salahuddin, S.Z. and Wong-Staal, F. (1984) Science 225, 927–930.

[11] Hahn, B.H., Shaw, G.M., Popovic, M., Gallo, R.C. and Wong-Staal, F. (1984) Nature 312, 166–169.

[12] Galfré, G., Howe, S.C., Milstein, C., Butcher, G.W. and Howard, J.C. (1977) Nature 266, 550–556.

[13] Chandra, P., Vogel, A. and Gerber, T. (1985) Cancer Res. (Suppl.) 45, 4677s–4684s.

[14] Chandra, A., Gerber, T. and Chandra, P. (1986) FEBS Lett., in press.

[15] Chandra, P., Steel, L.K., Laube, H., Balikcioglu, S., Mildner, B., Ebener, U., Welte, K. and Vogel, A. (1980) Cold Spring Harb. Conf. on Cell Proliferation 7, 775–791.

[16] Vogel, A. and Chandra, P. (1981) Biochem. J. 197, 553–563.