

Isolation of a lymphadenopathy-associated virus from a patient with the acquired immune deficiency syndrome

M. L. B. BECKER, F. H. N. SPRACKLEN, W. B. BECKER

Summary

A virus similar to the lymphadenopathy-associated virus or human T-lymphotropic virus type III, which has been described in association with the acquired immune deficiency syndrome (AIDS) by several laboratories elsewhere in the world, was isolated from a Cape Town patient with lymphadenopathy and acquired immune deficiency. This virus has the characteristic morphogenesis and ultrastructure and its genome encodes the virus-specific p24 protein. It is T-lymphotropic and produces the characteristic cytopathic effect. It can be serially propagated in a human lymphocyte line of the T4⁺ phenotype. This isolate is being used in diagnostic immunofluorescence assays for virus-specific antibodies.

S Afr Med J 1985; **68**: 144-147.

Material and methods

Lymphocyte cultures

Peripheral blood and umbilical cord lymphocytes. Blood was collected into tubes containing lithium heparin (Teklab, Durham, England). The lymphocytes were separated by gradient centrifugation on Histopaque-1077 (Sigma, St Louis, Mo., USA). Aliquots of lymphocytes were then seeded into tissue culture flasks at a cell concentration of 10^6 /ml in RPMI-1640 medium (Gibco, Paisley, Scotland), supplemented with 0.2% Na(HCO₃)₂, 20% fetal calf serum (Gibco), 2% L-glutamine (Merck, Darmstadt, Federal Republic of Germany), penicillin 100 IU/ml, streptomycin 60 µg/ml, 10 - 20% Lymphocult-T (Biotest, Frankfurt, Federal Republic of Germany) and 1% phytohaemagglutinin (PHA 15; Wellcome, Beckenham, Kent, England), and cultured in an atmosphere of 5% CO₂ in air. The medium was replaced at intervals of 2 - 3 days with omission of the PHA. On day 8 the cultured peripheral lymphocytes were co-cultivated with an equal number of umbilical cord T-cell blasts. These had been stimulated for 3 days with PHA and were treated with Polybrene (Sigma) 2 µg/ml for 30 minutes before co-cultivation.

Cervical lymph node lymphocytes. The biopsied lymph node was diced with a scalpel and pressed through a fine-mesh stainless steel sieve into culture medium. The dispersed lymphocytes were then separated and cultured as described above.

Splenic lymphocytes. Spleens removed from trauma victims were diced and the cells dispersed into suspension. The lymphocytes in the suspension were separated by gradient centrifugation and cultured as described above.

Storage of lymphocytes. Cell suspensions were frozen at a density of $1 - 2 \times 10^6$ /ml in medium containing 10% dimethyl sulphoxide and stored in 2 ml ampoules in liquid nitrogen. When required, ampoules were thawed rapidly and the lymphocytes washed in culture medium and then resuspended and cultured as described above.

Immunofluorescence assays

Immunofluorescence assay was used either to demonstrate viral antigen in infected cells or to detect serum antibodies to these antigens. The cultured cells were washed in fluorescent antibody buffer (FA buffer; Difco, Detroit, Mich., USA) and resuspended to a concentration of 2.5×10^6 /ml. The cell suspension was spotted in amounts of 10 µl onto Multislide (Hendley-Essex, Loughton, Essex, England) and air-dried for 1 hour at 37°C. The slides were then fixed for 10 minutes in acetone at 4°C, air-dried at 37°C and stored at -20°C. For the assay 10 µl serum appropriately diluted in phosphate-buffered saline (PBS) was added to each spot and the slides were incubated in a humidified atmosphere at 37°C for 45 minutes. They were then washed three times for 5 minutes in PBS and rinsed in distilled water. Fluorescein isothiocyanate-conjugated sheep anti-human immunoglobulin (Wellcome) was diluted 1 in 40 in PBS and added to each spot

The acquired immune deficiency syndrome (AIDS) was recognized as a clinical entity in 1981 in the USA.¹ A retrovirus was isolated from a patient with acquired immune deficiency and generalized lymphadenopathy by a group at the Institut Pasteur, Paris, who named the virus lymphadenopathy-associated virus (LAV).²

Retrovirus strains subsequently isolated from AIDS patients in the USA were found to be distinct from human T-lymphotropic virus types I and II (HTLV-I and HTLV-II) and were named HTLV-III.³ These isolates were compared with LAV and found to be strains of the same retrovirus.⁴ HTLV-III is an important pathogen that has spread rapidly in high-risk groups, especially homosexual men. In the absence of specific treatment or a vaccine, urgent steps are required to interrupt transmission of the virus, especially via blood products. The accompanying paper⁵ reports the clinical features of two Cape Town patients, one with AIDS and the other with AIDS-related complex, who had antibodies to HTLV-III.⁵ We report here the isolation of HTLV-III from the patient with AIDS. The availability of this virus will facilitate the specific diagnosis of local cases, because the virus material supplied from overseas is restricted to research use only and commercial preparations are costly.

Department of Medical Virology, University of Stellenbosch and Tygerberg Hospital, Parowvallei, CP

M. L. B. BECKER, B.SC. (MED.), M.B. CH.B., PH.D.

W. B. BECKER, M.MED. (PATH.), M.D., F.R.C. (PATH.), F.C.M. (PATH.),
Professor and Head

Department of Medicine (West Block), Somerset Hospital and University of Cape Town

F. H. N. SPRACKLEN, M.D., F.R.C.P., F.A.C.C., Head of Department and Honorary Senior Lecturer

in amounts of 10 μ l. The slides were reincubated for 30 minutes and washed as described above before applying a coverslip mounted in fluorescent antibody mounting fluid (Difco). Appropriate control cells and sera were included in each assay. The slides were examined with a fluorescence microscope using incident light illumination.

Electron microscopy

Cultured lymphocytes were fixed and embedded and ultra-thin sections were prepared for transmission electron microscopy as previously described.⁶

Radio-immunoprecipitation assays (RIPAs)

These assays were carried out using reference sera in order to identify viral proteins by their immunoprecipitation from radiolabelled lysates of virus-infected lymphocytes. Alternatively, sera were tested for specific antibodies by their ability to immunoprecipitate radiolabelled viral antigens from lysates of infected lymphocytes. Appropriate control uninfected cell lysates and negative sera were included in assays.

Splenic lymphocytes were infected by adding stock virus-infected cells in a ratio of 1 to 10 and culturing for 18 hours. The cells were then centrifuged, resuspended in methionine-free Eagle's minimal essential medium (Gibco) at a cell concentration of 2×10^6 /ml, and incubated for 1 hour to deplete endogenous stores of methionine. After the addition of

³⁵S-methionine to 10 μ Ci/ml (Amersham, Bucks., England) incubation was continued overnight. The radiolabelled cells were centrifuged and resuspended in 100 μ l per 10^6 cells of lysis buffer (150 mM NaCl, 10 mM tris HCl, pH 7.4, 1 mM ethylenediamine tetra-acetic acid (EDTA), pH 8.0, 1 mM phenylmethylsulphonyl fluoride, 1% Nonidet P-40) and kept on ice for 15 minutes, after which they were centrifuged for 10 minutes at 2000 rpm to remove cell nuclei. The supernatant cytoplasmic extract was centrifuged for 60 minutes at 100 000 g to clear it of protein aggregates and diluted 1 in 5 with immunoprecipitation buffer A (IPBA) (150 mM NaCl, 25 mM tris HCl, pH 8.2, 1 mM EDTA, pH 8.0, 1% Nonidet P-40, 1% sodium dodecyl sulphate (SDS), 0.5% sodium azide, 1 mg/ml bovine serum albumin (BSA)). The cleared extract was mixed on ice for 30 minutes with Protein A-Sepharose beads (Pharmacia, Uppsala, Sweden) which had been precoated with normal human serum to adsorb substances reacting nonspecifically with human serum or Sepharose. The beads were removed by centrifugation at 10 000 g for 5 minutes. The adsorbed supernatant was mixed in aliquots of 100 μ l with 2 μ l of each test serum which had been precoated onto 20 μ l of Protein A-Sepharose beads, and the mixture was gently shaken on ice for 60 minutes. The beads were recovered from each test sample by centrifugation for 2 minutes at 2000 g and washed, essentially as described by Lee *et al.*⁷ In brief, the beads were washed once in 1 ml IPBA, resuspended in 100 μ l IPBA and layered onto 1 ml immunoprecipitation buffer B (IPBA plus 0.5M NaCl and 30% sucrose but without BSA). They were centrifuged for 2 minutes at 2000 g, washed once in 1 ml immunoprecipitation buffer C (125 mM tris HCl, pH 6.8, 2% Nonidet P-40, 0.1% SDS) and boiled in SDS-polyacrylamide gel electrophoresis sample buffer to elute the bound proteins. Samples were electrophoresed on 12% discontinuous SDS-polyacrylamide gels, essentially as described by Laemli.⁸ Gels were fluorographed with Amplify (Amersham), dried and exposed on Fuji RX film in X-ray cassettes.

Results

Virus culture

The peripheral blood lymphocytes (PBLs) of patient 2 were cultured with some difficulty, but giant-cell formation indicative of virus replication was noted on day 8. At this point lymphoblasts from 3-day-old cultures of umbilical cord lymphocytes were added and a cytopathic effect (CPE) with giant-cell formation was well developed 6 days later (Fig. 1). The first sign of the characteristic CPE was the appearance of large pseudopod-like protrusions from the edge of clusters of growing cells. Dispersion of the clusters revealed giant cells with multiple nuclei arranged in a horseshoe or ring with pseudopod formation most evident in the early stages. In older cultures the giant cells were dispersed and rounded. The virus was re-isolated from a second sample of blood taken after an interval of 5 months.

Viral CPE has been transferred serially over 20 times to date in cultures of PHA-stimulated PBLs or splenic lymphocytes, and may be evident as early as 18 hours after inoculation. In addition, infection could be serially transferred in two T4⁺ lymphoblastoid cell lines which we have established from co-cultures of the PBLs of vervet monkeys and human umbilical cord cells (unpublished data). These two cell lines are particularly suitable as a source of infected cells for the detection of serum antibodies by immunofluorescence assay.

Immunofluorescence assay

Cultured lymphocytes infected with the isolated virus were assayed for HTLV-III antigens using sera from patients with

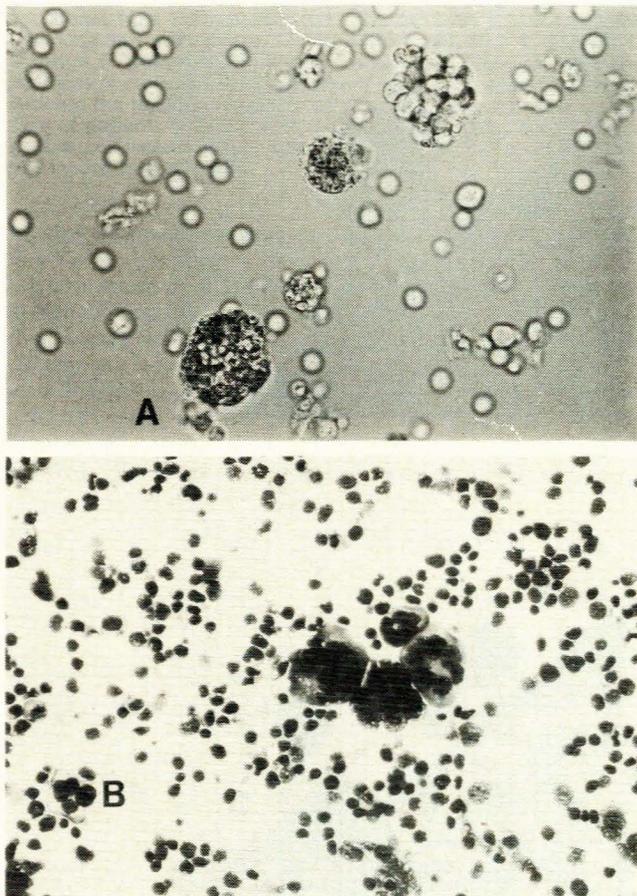


Fig. 1. Photomicrographs of infected lymphocyte cultures showing the cytopathic effect induced by the HTLV-III isolate. A feature is the formation of characteristic giant cells which tend to have their nuclei arranged in a ring as shown in a fresh preparation under phase contrast (A) ($\times 150$) and in a fixed, stained preparation (B) ($\times 60$).

TABLE I. TITRATION OF HTLV-III REFERENCE SERA BY IMMUNOFLUORESCENCE ASSAY AGAINST LYMPHOCYTES INFECTED WITH THE LAV ISOLATE

Sera	Titre
Patient 1	160
Patient 2	80
Reference sera*	
Patient 1	1 280
Patient 2	80
Patient 3	640

*Courtesy of Dr R. Sher, SAIMR, Johannesburg.

AIDS as reference sera (by courtesy of Dr R. Sher) which had been shown to contain HTLV-III antibodies by immunofluorescence assay with the HTLV-III-infected H9 cell line of Dr R. C. Gallo.³ The infected cells reacted specifically with all the reference sera to the titres shown in Table I.

Electron microscopy

The presence of a virus associated with the CPE in cultured lymphocytes was confirmed by transmission electron microscopy of ultrathin sections of the cells (Fig. 2). Virus was

absent in uninfected control cells. Viral particles were seen budding from the cytoplasmic membrane and lying free of the cells. The virion was approximately 100 nm in diameter and showed a characteristic core which appears cigar-shaped in the lengthwise orientation and rounded in cross-section. The core was significantly smaller and morphologically quite distinct from the core of the type C particles of HTLV-I and HTLV-II.⁹

RIPAs

The sera of patients 1 and 2 caused specific precipitation of viral peptides of molecular weight 24 kD, 110 kD and 130 kD from lysates of virus-infected lymphocytes biosynthetically labelled with ³⁵S-methionine (Fig. 3). Comparable amounts of p110 and p130 were precipitated by both sera, but the serum of patient 1 had considerably higher anti-p24 reactivity.

Patient 1 had serum immunoglobulin levels approximately three times normal, while patient 2 had levels within the normal range.⁵

Discussion

The virus reported here was isolated from the PBLs of a patient with AIDS. It is a lymphotropic cytopathic virus

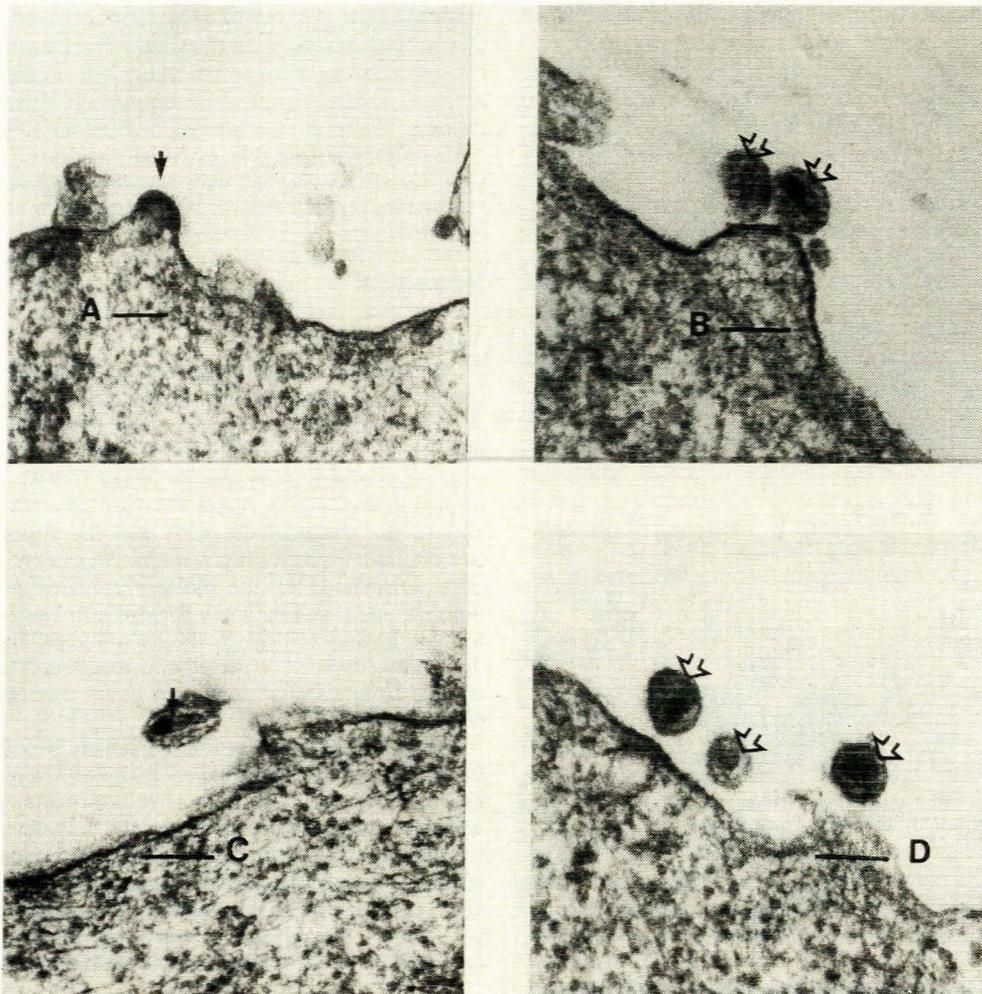


Fig. 2. Transmission electron micrographs of HTLV-III-infected lymphocyte cultures. Incipient budding of a virus from the cell surface can be seen (A) (arrow). The free-lying virus particles (B, C and D) show the characteristic dark-staining core which is elongated or rounded depending on the plane of section (arrows). The bars represent 100 nm.

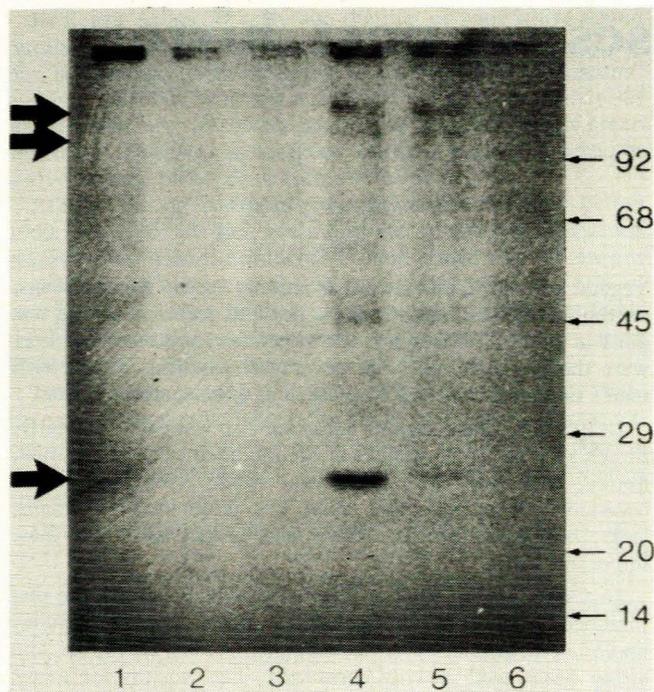


Fig. 3. SDS-PAGE analysis of proteins immunoprecipitated by sera from patients 1 and 2. The labelled cell lysate used in lanes 1 - 3 was from control cells and that in lanes 4 - 6 was from virus-infected cells (lanes 1, 4 — serum of patient 1; lanes 2, 5 — serum of patient 2; lanes 3, 6 — normal human serum). The positions of molecular-weight markers are shown. The bold arrows indicate the positions of p130, p110 and p24 precipitated by the sera of patients 1 and 2 from infected cell lysates but not from control cell lysates. There was no precipitation of p130, p110 or p24 by the control serum.

which was grown from the patient's PBLs and serially passaged in a human lymphocyte line of the T4⁺ phenotype. The CPE was characteristic of HTLV-III. AIDS reference sera reacted in high dilution with the virus-infected lymphocytes in immunofluorescence assays. The morphogenesis and morphology on electron microscopy of ultrathin sections of virus-infected cells were also typical of HTLV-III.

RIPAs using the AIDS reference sera confirmed specific immune precipitation of HTLV-III antigens from virus-infected cells. There was no reaction with negative control sera

and no reaction with goat anti-p24 HTLV-I antiserum (kindly supplied by Dr Gallo). A comparison of the precipitation of viral antigens by the sera of patients 1 and 2 on SDS-polyacrylamide gel electrophoresis indicates that patients may well show quantitative variation in their serum antibody response to individual viral antigens. We are investigating this variation with a larger sample of sera in an attempt to correlate the pattern of precipitating antibodies with the clinical findings. The RIPA test may prove diagnostically and prognostically useful.

We feel justified in concluding from our data that the virus isolate is a strain of HTLV-III virus. We have no extraneous HTLV-III or HTLV-infected cell lines in our laboratory. Our isolate is being used in an immunofluorescence assay for serum antibodies to HTLV-III with the prospect of developing an enzyme-linked immunosorbent assay. These tests are free of the limitation to research use which applies to virus strains or infected cell lines supplied from overseas.

We acknowledge the excellent technical assistance of Susan Engelbrecht, Antoinette Grobbelaar, Cecilia Piek and Brenda Robson.

Research grants from the University of Stellenbosch are gratefully acknowledged.

REFERENCES

- Centers for Disease Control. Kaposi's sarcoma and *Pneumocystis pneumonia* among homosexual men — New York City and California. *MMWR* 1981; **30**: 305-308.
- Barré-Sinoussi F, Chermann JC, Rey F *et al*. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* 1983; **220**: 868-871.
- Popovic M, Sarngadharan MG, Read E, Gallo RC. Detection, isolation and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 1984; **224**: 497-500.
- Ratner L, Gallo RC, Wong-Staal F. HTLV-III, LAV, ARV are variants of the same AIDS virus. *Nature* 1985; **313**: 636-637.
- Spracklen FHN, Whittaker RG, Becker WB, Becker MLB, Holmes CM, Potter PC. The acquired immune deficiency syndrome: a report of 2 confirmed cases in Cape Town and comments on human T-cell lymphotropic virus type III infections. *S Afr Med J* 1985; **68**: 139-143.
- Becker WB, Piek C, Robson B. Retroviruses in cultured peripheral lymphocytes from vervet monkeys seroreactive to HTLV-I. In: *Proceedings of the 23rd Conference of the Electron Microscopy Society of Southern Africa*. Johannesburg: Electron Microscopy Society of South Africa, 1984: 83-84.
- Lee JS, Trowsdale J, Bodmer WF. cDNA clones coding for the heavy chain of human HLA-DR antigen. *Proc Natl Acad Sci USA* 1982; **79**: 545-549.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680-685.
- Schüpbach J, Popovic M, Gilden RV, Gonda MA, Sarngadharan MG, Gallo RC. Serological analysis of a subgroup of human T-lymphotropic retroviruses (HTLV-III) associated with AIDS. *Science* 1984; **224**: 503-505.