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Continuous Production of a Cytopathic Human T-Lymphotropic Virus in a Permissive Neoplastic T-Cell Line

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We developed cloned populations from the commonly available, well-characterized cell line HUT-78. These cloned cells grow permanently after infection with isolates of human T-lymphotropic virus type III, also called lymphadenopathy virus (HTLV-III/LAV), from patients with acquired immune deficiency syndrome and related syndromes. In contrast, activated human T cells are lysed after HTLV-III/LAV infection. The infected cloned cells have been in culture continuously for 6 months and have produced high levels of extracellular reverse transcriptase (400,000 cpm/ml). This level is comparable to that of similarly infected normal human T cells. Three weeks after infection with HTLV-III/LAV, more than 90% of the cloned HUT-78 cells lysed; the remaining cells continued to grow. Approximately 80% of these cells expressed HTLV-III/LAV antigens by immunofluorescence. The extracellular virus of the chronically infected cell line was shown to be similar to other HTLV-III/LAV isolates by competition radioimmunoassay, by reactivity with human serum, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. This HTLV-III/LAV-infected immortalized cell line enables the continuous production of large amounts of virus.

The etiologic agent of acquired immune deficiency syndrome (AIDS) is the retrovirus, human T-lymphotropic virus type III (HTLV-III), also called lymphadenopathy-associated virus (LAV; herein referred to as HTLV-III/LAV). HTLV-III/LAV has a cytopathic effect on its target cells, T4-positive lymphocytes (9). Infected T cells are destroyed by the virus, which makes it difficult to produce large amounts of virus for molecular and immunologic analysis. Popovic et al. (14) reported the development of a cell line resistant to the cytopathic effect of the virus and yet permissive for virus replication (H9). By cocultivating H9 cells with patient's mononuclear cells, Popovic demonstrated the use of this cell line for virus isolation. Most published procedures for virus isolation require cocultivation of patient material with activated human T lymphocytes (2, 4, 7, 9). These lymphocytes are expensive and time consuming to process, and cocultures must be frequently monitored for virus production by using an assay for viral reverse transcriptase.

HUT-78 is a well-characterized T4-positive continuous lymphocyte cell line derived from a patient with Sezary syndrome (3). Ordinarily, T-cell growth factor (TCGF) (15) is required to grow human T cells in continuous culture. However, some cultured T cells infected with HTLV-I and -II become independent of exogenous TCGF (11, 13); the HUT-78 cell line does not require exogenous TCGF. HUT-78 cells, however, show no evidence of HTLV-I, -II, or -III proviral sequences or expression (5). Levy et al. (7) first reported the successful infection of HUT-78 with an AIDS-associated retrovirus and subsequently used the infected cells to examine the serum of AIDS patients for antibodies to AIDS-associated retrovirus by immunofluorescence. Daniel et al. (1) reported using HUT-78 cells to isolate simian T-lymphotropic virus type III from macaques. They found no difference in the sensitivity of HUT-78 cells and human T cells growing in the presence of TCGF for isolation or growth of simian T-lymphotropic virus type III.

We have now developed a continuously producing, permanently growing cell line derived from the cloned populations of the HUT-78 cell line. We compared virus prepared from infected normal adult lymphocytes with virus prepared from infected cloned cells and demonstrated the reliability of both preparations for serologic investigations. We also examined the potential of our HUT-78 cloned cell lines for use in isolating virus from patient material.

MATERIALS AND METHODS

Cloning. The HUT-78 cell line was cloned in 24-well tissue culture dishes by the limiting dilution technique (0.3 cells per well) with irradiated normal peripheral blood lymphocytes as feeder cells. After incubation for 7 days at 35°C in an atmosphere of 5% carbon dioxide, 1.0 ml of medium (RPMI 1640 containing 20% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml) was removed and replaced with fresh medium. After 2 to 3 weeks, the resulting clones were transferred to 25-cm^2 tissue culture flasks and gradually expanded.

Infection. The cloned cells were infected with HTLV-III/LAV (CDC 451), an isolate obtained at the Centers for Disease Control from a hemophilia patient with AIDS (4). Reverse transcriptase (5,000 cpm/10^6 cells) was added in the presence of Polybrene (1 μg/ml). The cells continued to proliferate and were split and fed every 3 to 4 days.

Reverse transcriptase assay. Culture supernatants were assayed for particle-associated reverse transcriptase activity by using poly(A)$(dT)_{12-18}$ as the template primer and Mg^{2+} as the divalent cation (2).

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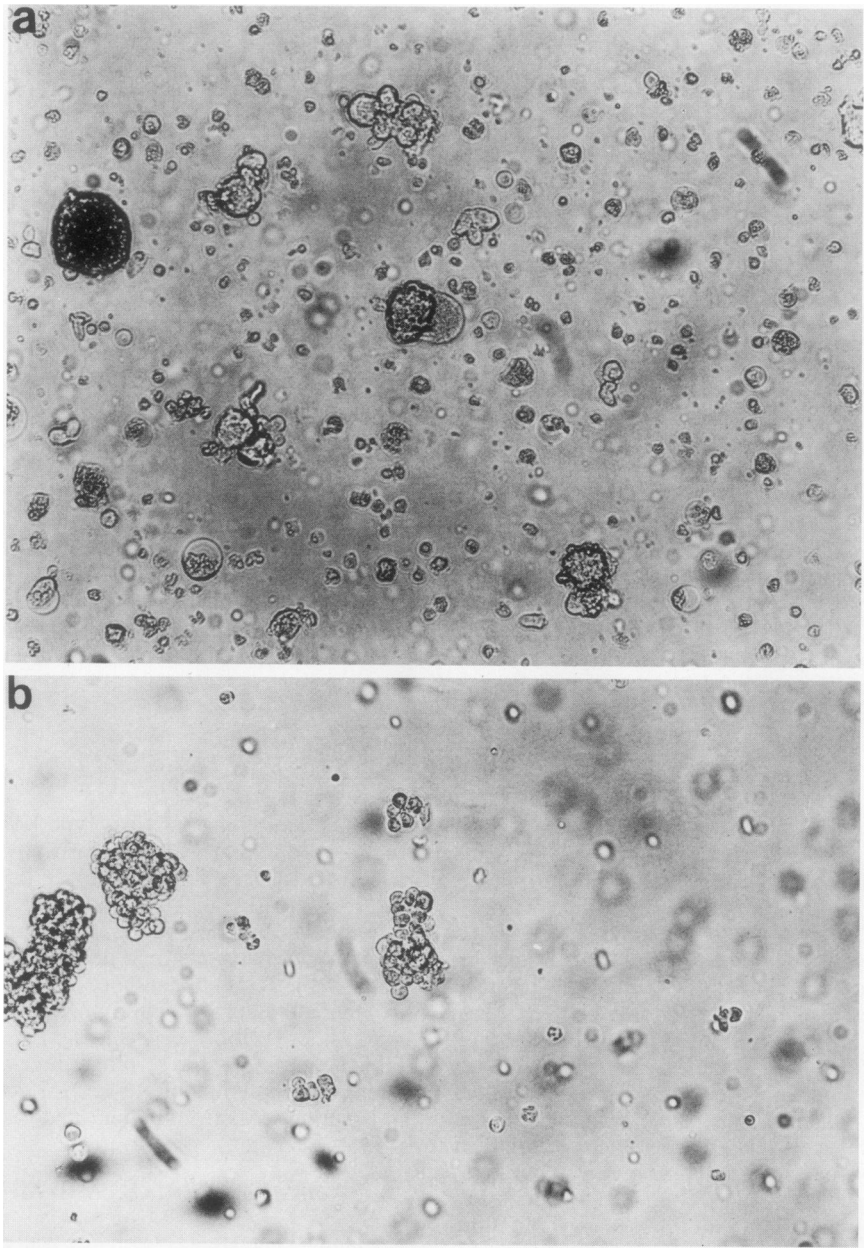

FIG. 1. Appearance of 6D5 cells infected with HTLV-III/LAV (CDC 451). (a) Characteristic cytopathic effect seen 2 to 3 weeks after infection; (b) normal appearance of cells seen 6 weeks after infection.

Direct fluorescent antibody technique. The cells were fixed in acetone and then assayed by standard indirect immunofluorescence procedures using 1:500 diluted fluorescein isothiocyanate-conjugated immunoglobulin G (IgG) from a patient with a high antibody titer to LAV (9).

SDS-PAGE. Sucrose-gradient-banded HTLV-III/LAV (CDC 451) from the cloned cell line and from adult T lymphocytes was prepared and lysed (5). Lysates were fractionated by polyacrylamide gel electrophoresis (PAGE) on a 12% polyacrylamide slab gel in the presence of sodium dodecyl sulfate (SDS) (6). The resulting protein bands were stained with Coomassie blue and photographed.

Radioimmunoassay. A competition radioimmunoassay was carried out with ^{125}I-labeled HTLV-III/LAV (CDC 451) p24 and a limiting dilution (1:3,000) of hyperimmune rabbit antibody to HTLV-III/LAV (CDC 451) (4). Serial dilutions (100 μl) of solubilized virus (initial concentration, 10 μg) in buffer 1 (20 mM Na_2HPO_4 [pH 7.6], 200 mM NaCl, 1 mM EDTA, 0.3% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, and bovine serum albumin [2 mg/ml]) were incubated with the immune serum for 1 h at 37°C. Labeled HTLV-III/LAV (CDC 451) p24 (8,000 cpm in 100 μl of buffer 1) was added; the mixture was further incubated at 37°C for 2 h and then overnight at 4°C. A 20-fold excess of goat antiserum to rabbit IgG was then added, and the volume was made up to 1 ml in buffer 1. The samples were further incubated at 37°C for 2 h and at 4°C for 1 h and then centrifuged at 3,000 × *g* for 20 min. The supernatants were

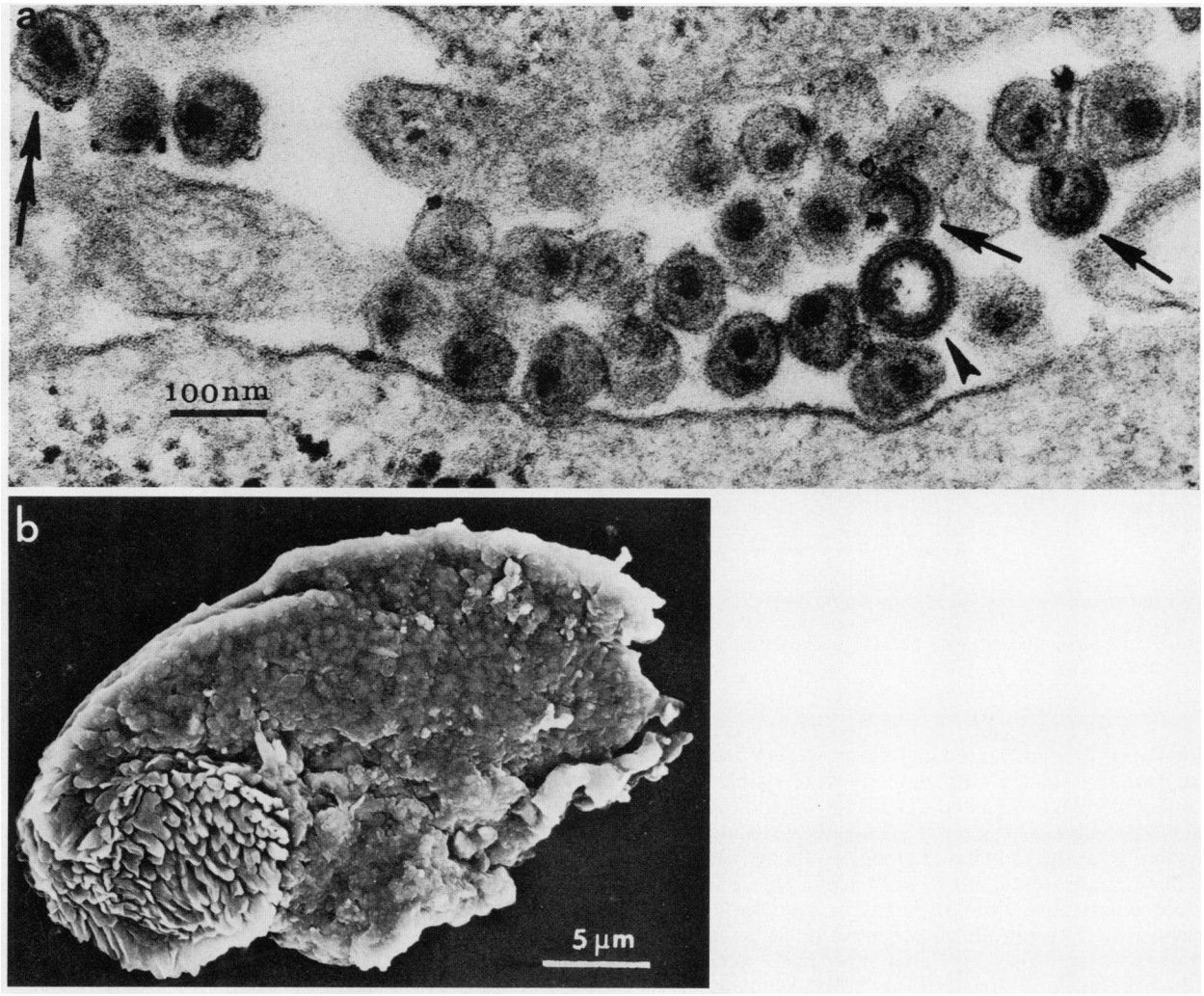

FIG. 2. Electron micrographs of 6D5 cells infected with HTLV-III/LAV (CDC 451). (a) Thin-section electron micrograph showing virus particles with a diameter of 100 nm and an eccentric nucleoid in an electron-dense core. Arrows, Budding profiles; double-headed arrow, bar-shaped nucleoid; arrowhead, empty ring. (b) Scanning electron micrograph of balloon cell. The cell measures 32 by 26 μm, as opposed to the normal small lymphocyte with a diameter of 5 μm.

aspirated, and the radioactivity in the sediment was determined with a gamma counter.

Enzyme-linked immunosorbent assay. Sucrose gradient-banded virus was solubilized in 0.5% Triton X-100, diluted in carbonate buffer (pH 9.6), and coated on the surface of 96-well microtiter plates (Immulon II; Dynatech Laboratories, Inc., Alexandria, Va.) at a concentration of 200 ng per well. After overnight refrigeration the wells were blocked with 100 μl of phosphate-buffered saline (pH 7.2) containing 0.05% Tween 20 and 5.0% bovine serum albumin. After 1 h, the wells were washed three times with phosphate-buffered saline containing 0.05% Tween 20, and then 100 μl of human serum diluted 1:50 in blocking solution was added to each well. After overnight refrigeration, the wells were washed three times and incubated for 90 min with 100 μl of a 1:2,000 dilution of peroxidase-conjugated goat anti-human IgG (Cappel, Cochranville, Pa.) per well. The plates were then washed three times, and the color was developed by using a substrate solution of 0.01% *o*-phenylenediamine dihydrochloride (Sigma Chemical Co., St. Louis, Mo.) and 0.01% hydrogen peroxide in 50 mM citrate buffer (pH 4.6). The reaction was stopped by adding 100 μl of 2 N sulfuric acid. The optical density was determined by using a Dynatech enzyme-linked immunosorbent assay reader at 490 nm. A positive test was one that gave an optical density of 4 times the average optical density of a panel of normal control sera.

RESULTS

Establishment of continuously producing permanent cell line. Twenty-two separate clones of the HUT-78 cell line were established and infected with CDC 451 virus. Only two of these clones could not be productively infected as determined by testing for reverse transcriptase and the appearance of cytopathic effects. On day 15 after infection, the cells exhibited cytoplasmic ballooning (Fig. 1a). By day 20, reverse transcriptase levels had peaked between 200,000 and 800,000 cpm/ml. Nuclear enlargement and cell lysis accompanied the rise in reverse transcriptase levels. The cultures, containing a few single cells, much cellular debris, and some

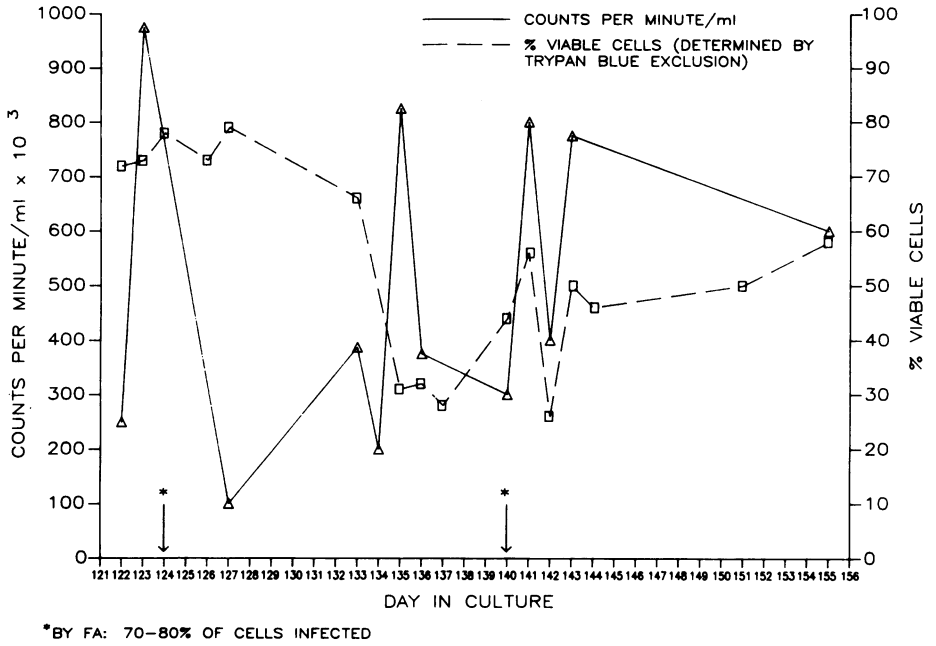

FIG. 3. Reverse transcriptase activity and percent viable cells of 6D5 clone continuously producing HTLV-III/LAV (CDC 451). FA, Fluorescent antibody.

ballooned or multinucleated giant cells were left stationary. After 2 weeks, small clumps of cells were visible in two of the infected clones, 6D5 and 7D6. By 3 weeks, cell division in these two clones was rapid. The cells were morphologically similar to the uninfected clone, no longer demonstrating the dramatic cytopathic effect (Fig. 1b). Thin-section electron microscopy showed that virus particles were 70 to 100 nm in diameter, with an eccentric nucleoid in an electron-dense core (Fig. 2a). The bar-shaped nucleoid and budding profiles are typical of this virus. Scanning electron microscopy showed that a typical balloon cell measured 32 by 26 μm, as opposed to the normal small lymphocyte with a diameter of 5 to 8 μm (Fig. 2b).

Reverse transcriptase levels fluctuated as the culture was expanded and fed (Fig. 3). The percentage of viable cells also fluctuated over time. No correlation was observed between cell viability and level of reverse transcriptase activity (Fig. 3). By immunofluorescence, 70 to 80% of the 6D5 cells were infected.

The cloned cell line was characterized by established immunologic procedures (10). Cells were exclusively T lymphocytes ($OKT3^+$, $T10^+$, and $Leul^+$) with a helper-inducer phenotype ($OKT4^+$). Cells were negative for E rosette receptors ($T11^-$) and TCGF receptors (Tac^-).

Comparison of CDC 451 grown in 6D5 and in adult lymphocytes. By measuring the concentration of purified disrupted viral protein (8), the yield of virus from 6D5 cells was 620 μg/ml, and the yield from adult lymphocytes was 750 μg/ml. Comparison of the SDS-PAGE profiles of virus produced from 6D5 cells and from adult lymphocytes shows an identical makeup of the protein components, with major bands at molecular weights 18,000, 24,000, and 45,000 (Fig. 4). A competition radioimmunoassay was used to compare the antigenic characteristics of the major core protein (p24) of CDC 451 purified from 6D5 cells and from adult lymphocytes. The virus preparations were used as competitive antigens in a homologous radioimmunoassay for HTLV-III/LAV ^{125}I-labeled p24. Virus from both 6D5 cells and from adult lymphocytes competed successfully for the rabbit antibody to HTLV-III/LAV (Fig. 5). The identical curve given by both viral preparations indicates that the major core proteins of virus grown in 6D5 cells and in adult lymphocytes are identical.

Enzyme-linked immunosorbent assay. With antigen prepared from CDC 451-infected 6D5 cells an enzyme-linked immunosorbent assay was developed and compared with our standard enzyme-linked immunosorbent assay using an antigen prepared from CDC 451-infected adult lymphocytes (4). With two minor exceptions, results with both antigens were identical. Both antigens detected antibody in 43 of 44 AIDS patients and in 43 of 46 with lymphadenopathy syndrome (as did antigen from HTLV-III-infected H9 cells). Antibody was not detected by any antigen in 20 of 20 rheumatoid arthritis patients, 10 of 10 lupus erythematosus patients, and 50 of 50 CDC employees (none worked in a laboratory). Antibody was detected in 1 of 40 randomly selected blood donors when CDC 451 from adult lymphocytes or HTLV-III from H9 cells was used as the antigen, whereas antibody was detected in two (including 1 equivocal result) of 40 random donors when 6D5-CDC 451 was used as the antigen.

DISCUSSION

Few reported mature neoplastic T-cell lines have been HTLV negative and independent of exogenous TCGF (12). Since there was a chance that our cloned cells infected with HTLV-III/LAV might yield other transforming viruses, we wanted to verify that the HTLV-III/LAV (CDC 451) virus with which we inoculated the 6D5 cloned cells was identical to the virus produced by those cells. We demonstrated identity by competition radioimmunoassay of the major core proteins, by SDS-PAGE of the viral proteins, and by serologic comparison.

We determined that virus purified from infected 6D5 cells

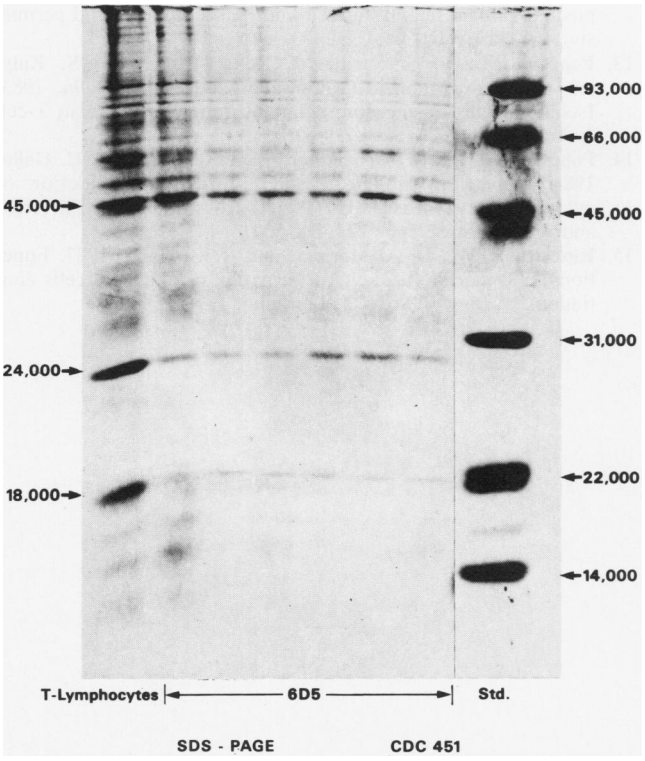

FIG. 4. SDS-PAGE of HTLV-III/LAV (CDC 451) purified from stimulated adult lymphocytes (left lane) and from 6D5 cells (six different preparations in inner lanes). Right lane shows the molecular weights of the marker proteins.

was an acceptable substitute for virus purified from adult T cells for use in serologic assays. The sensitivity and specificity of the ELISA with CDC 451 virus produced in 6D5 cells were identical to the sensitivity and specificity of the ELISA with CDC 451 produced in adult lymphocytes: 95.5 and 99.1%, respectively. The actual yield of virus from 6D5 cells was somewhat less than the yield of virus from adult lymphocytes (620 and 750 μg/ml, respectively). However, using the cloned cell line is a better method of virus production because the 6D5 cells are easier to manipulate and the purity of the virus recovered is equivalent to that produced by using adult lymphocytes.

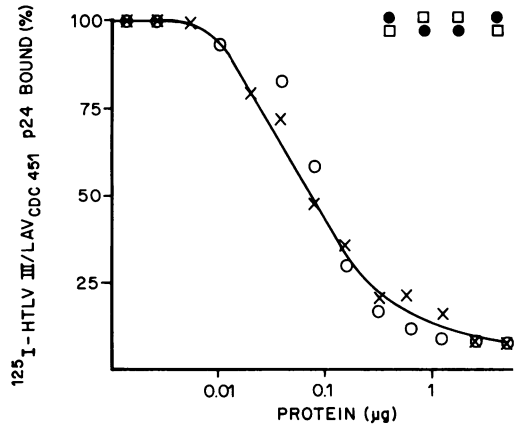

FIG. 5. Competition radioimmunoassay of HTLV-III/LAV (CDC 451) from 6D5 cells (×) and from adult lymphocytes (○), HTLV-I (●), and HTLV-II (□).

A striking feature observed by light microscopy was the appearance of balloonlike cells. By monitoring cultures for this characteristic appearance, the need for frequent reverse transcriptase assays may be minimized. The use of these cloned HUT-78 cells to cocultivate lymphocytes and other material from AIDS patients may be a practical and reliable alternative to the use of activated human lymphocytes for virus isolation. In preliminary studies with five different virus isolates, we found equal recovery of virus from both 6D5 cells and stimulated adult lymphocytes. Daniel et al. reported similar findings (1). By immunofluorescence, we found 70 to 80% of 6D5 cells positive for HTLV-III/LAV antigens. Levy et al. (7), on the other hand, using a similar assay found that only 40% of HUT-78 cells were infected with AIDS-associated retrovirus type 2. Likewise, Popovic et al. (12), using monoclonal antibodies in performing an immunofluorescence technique, found 21% of HTLV-III infected HUT-78 cells positive for p15 antigens and 36% positive for p24 antigens. The differences observed among laboratories in the percentage of cells infected may be due to differences in methodology or differences in viral strains. However, by cloning the HUT-78 cell line we may have selected a more susceptible cell population.

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