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17. There are five coal-fired power plants in the Oak Ridge area. Kingston burns 4.0×10^9 kg of coal per year; Bull Run, 2.3×10^9 kg/year; Y-12, 1.1×10^8 kg/year; K-25, 3.2×10^7 kg/year; and the ORNL power plant, 2.5×10^7 kg/year; the total in the Oak Ridge area is approximately 6.5×10^9 kg/year.
18. Iron is most available to plants in the ferrous form: uptake into blades of *Macrocystis* and *Phaseolus* roots and shoots has been shown to be dependent on the prereduction of Fe(III) to Fe(II); S. L. Manley, *Plant Physiol.* **68**, 914 (1981). Iron uptake is also enhanced by reduction of rhizosphere *p*H [A. N. Sarkar and R. G. Wyn Jones, *Plant Soil* **66**, 361 (1982)].
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Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and Pre-AIDS

Abstract. *A cell system was developed for the reproducible detection of human T-lymphotropic retroviruses (HTLV family) from patients with the acquired immunodeficiency syndrome (AIDS) or with signs or symptoms that frequently precede AIDS (pre-AIDS). The cells are specific clones from a permissive human neoplastic T-cell line. Some of the clones permanently grow and continuously produce large amounts of virus after infection with cytopathic (HTLV-III) variants of these viruses. One cytopathic effect of HTLV-III in this system is the arrangement of multiple nuclei in a characteristic ring formation in giant cells of the infected T-cell population. These structures can be used as an indicator to detect HTLV-III in clinical specimens. This system opens the way to the routine detection of HTLV-III and related cytopathic variants of HTLV in patients with AIDS or pre-AIDS and in healthy carriers, and it provides large amounts of virus for detailed molecular and immunological analyses.*

Epidemiologic data suggest that the acquired immunodeficiency syndrome (AIDS) is caused by an infectious agent that is horizontally transmitted by intimate contact or blood products (*1–3*). Though the disease is manifested by opportunistic infections, predominantly *Pneumocystis carinii* pneumonia (*4*), and by Kaposi's sarcoma (*5*), the underlying disorder affects the patient's cell-mediated immunity (*6*), resulting in absolute lymphopenia and reduced subpopulations of helper T lymphocytes ($OKT4^+$). Moreover, before a complete clinical manifestation of the disease occurs, its prodrome, pre-AIDS, is frequently characterized by unexplained chronic lymphadenopathy or leukopenia involving helper T lymphocytes (*5, 6*). This leads to the severe immune deficiency of the patient and suggests that a specific subset of T cells could be a primary target for an infectious agent. Although patients with AIDS or pre-AIDS are often chronically infected with cytomegalovirus (*7*) or hepatitis B virus (*8*), for various reasons these appear to be opportunistic or coincidental infections. We have proposed that AIDS may be caused by a virus from the family of human T-cell lymphotropic retroviruses (HTLV) (*9*) that includes two major, well-characterized subgroups of human retroviruses, called human T-cell leukemia-lymphoma viruses, HTLV-I (*9–12*) and HTLV-II (*9, 11, 13*). The most common isolate, HTLV-I, is obtained mainly from patients with mature T-cell malignancies (*9, 12*). Seroepidemiological studies, the biological effects of the virus in vitro, and nucleic acid hybridization data indicate that HTLV-I is etiologically associated with the T-cell malignancy of adults that is endemic in certain areas of the south of Japan (*14*), the Caribbean (*15*), and Africa (*16*). HTLV-II was first isolated from a patient with a T-cell variant of hairy cell leukemia (*13*). To date, this is the only reported isolate of HTLV-II from a patient with a neoplastic disease. Virus isolation and seroepidemiological data show that both HTLV-I and HTLV-II can sometimes be found in patients with AIDS (*17*).

That a retrovirus of the HTLV family might be an etiological agent of AIDS was suggested by the findings (i) that another retrovirus, feline leukemia virus, causes immune deficiency in cats (*18*); and that (ii) retroviruses of the HTLV family are T-cell tropic (*12, 19*); (iii) preferentially infect helper T cells ($OKT4^+$) (*12, 19*); (iv) have cytopathic effects on various human and mammalian cells, as demonstrated by their induction of cell syncytia formation (*20*); (v) can alter some T-cell functions (*21*); (vi) can in some cases selectively kill T cells (*22*); and (viii) may be transmitted by intimate contact and blood products (*9*). Also consistent with an HTLV etiology were the results of Essex and Lee and their colleagues showing the presence of antibodies to cell membrane antigens of HTLV-infected cells in serum samples from more than 40 percent of patients with AIDS (*23*). This antigen has since been defined as part of the envelope of HTLV (*24*). The more frequent detection in AIDS patients of antibodies to a membrane protein rather than to HTLV-I internal structural core proteins (*25*), together with the low incidence of isolations of HTLV-I or HTLV-II from AIDS patients, also suggested that a new variant of HTLV might be present.

The original detection and isolation of HTLV-I were made possible by the discovery of T-cell growth factor (TCGF) (*26*), also called interleukin 2 (IL-2), which stimulates the growth of different subsets of normal and neoplastic mature T cells (*27*), and by the development of sensitive assays for reverse transcriptase (RT), an enzyme characteristic of retroviruses (*28*). The procedures used previously for the transmission and continuous production of HTLV-I and -II were first worked out in mammalian cells transformed by avian sarcoma virus (*29*). These methods involved cocultivation of the transformed cells with cells permissive for the particular virus strain. Normal human T cells in cocultivation experiments preferentially yielded HTLV of both subgroups. Some of these viruses showed an immortalizing (transforming) capability for certain target T cells (*9, 12*). We thought that HTLV variants that have cytopathic effects on their target cells but do not immortalize them might be more important in the cause of AIDS. In fact, such variants were frequently but only transiently detected when normal T cells were used as targets in cocultivation or cell-free transmission experiments. This transience was our main obstacle to the isolation of these cytopathic variants of HTLV from patients with AIDS or pre-AIDS. We subsequently found a cell line that is highly susceptible to and permissive for cytopathic variants of HTLV. This cell line can grow permanently after infection with the virus. We report here the estab-

lishment and characterization of this new immortalized T-cell population and its use in the isolation and continuous high-level production of HTLV variants from patients with AIDS and pre-AIDS.

Several neoplastic human cell lines established in vitro were assayed for susceptibility to infection with HTLV-I and -II and with many of the more cytopathic retroviruses isolated from AIDS patients (*30*). One neoplastic aneuploid T-cell line, derived from an adult with lymphoid leukemia, was found to be susceptible to infection with the new cytopathic virus isolates. This cell line, termed HT, has produced HTLV-variants in sufficient quantities to permit the development of specific immunologic reagents and nucleic acid probes that can be used to characterize new isolates and compare them with HTLV-I and HTLV-II (*30*). These cytopathic variants differ from HTLV-I and -II not only in their biological effects but also in several immunological assays and in their morphology (*31*). They nevertheless have many properties similar to HTLV-I and -II. For example, they are T4 lymphotropic, they have a similar RT (*30*), they cross-react with several structural proteins in heterologous radioimmune assays with serum from AIDS patients and with antisera to the virus raised in animals (*31*), and they induce syncytia. These new HTLV isolates are collectively designated HTLV-III, although it is not yet proved that they are identical.

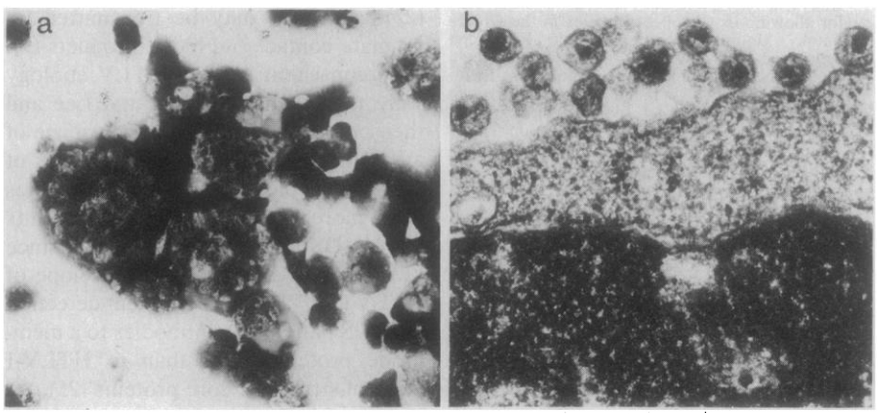

Fig. 1. Light and electron microscopic examination of clone H4/HTLV-III. (a) H4/HTLV-III cells were characterized by the presence of large multinucleated cells that showed, with Giemsa-Wright staining, a characteristic arrangement of their nuclei (×350). (b) Electron micrograph of the cells showing the presence of extracellular viral particles (×60,000).

Table 1. Response of cloned T-cell populations to infection with HTLV-III. Single-cell clones were isolated as described (*34*, *35*) from a long-term cultured aneuploid HT cell line exhibiting mature T-cell phenotype [$OKT3^+$ (62 percent), $OKT4^+$ (39 percent), and $OKT8^-$] as determined by cytofluorometry with a fluorescence-activated cell sorter. The cultures are routinely maintained in RPMI 1640 medium containing 20 percent fetal calf serum (FCS) and antibiotics. The terminal cell density of the parental cell culture, seeded at a concentration of 2×10^5 cells per milliliter of culture media, was in the range of 10^6 to 1.5×10^6 cells per milliliter after 5 days of culture.

Characteristics after infection	Clones*							
	H3	H4	H6	H9	H17	H31	H35	H38
Total cell number ($\times 10^6$)								
At 6 days	1	1.5	1.5	0.3	0.4	0.3	0.5	1.8
At 14 days	2.2	7.3	7.5	10	4.7	5.0	4.5	3.2
Multinucleated cells (%)*								
At 6 days	24	42	32	7	13	14	30	45
At 14 days	45	48	45	30	22	45	60	60
Immunofluorescence positive cells (%)†								
At 6 days								
Rabbit antiserum to HTLV-III	55	56	32	32	39	21	10	87
Patient serum (E.T.)	56	29	21	ND	ND	ND	ND	73
At 14 days								
Rabbit antiserum to HTLV-III	50	74	60	97	71	40	20	80
Patient serum	45	47	56	78	61	43	22	89
Reverse transcriptase activity ($\times 10^4$ cpm/ml)‡								
At 6 days	2.4	1.8	2.1	4.1	2.6	1.4	1.7	2.5
At 14 days	16.2	18.1	16.1	20.2	17.1	13.4	15.1	18.2

*Cell smears were prepared from cultures 6 and 14 days after infection and stained with Wright-Giemsa. Cells with more than five nuclei were considered to be multinucleated. Cloned cells from uninfected cultures also contained some multinucleated giant cells; however, the arrangement of the multiple nuclei in a characteristic ring formation (see Fig. 1a) was lacking and the number of these cells was much less (0.7 to 10 percent). †Cells were washed with phosphate-buffered saline (PBS) and resuspended in the same buffer at concentration 10^6 cells per milliliter. Approximately 50 μl of cell suspension was spotted on a slide, air dried, and fixed in acetone for 10 minutes at room temperature. Slides were stored at −20°C until use. Twenty microliters of either rabbit antiserum to HTLV-III (diluted 1:2000 in PBS) or serum from the patient (E.T.) diluted 1:8 in PBS was applied to cells and incubated for 50 minutes at 37°C. The fluorescein-conjugated antiserum to rabbit or human immunoglobulin G was diluted and applied to the fixed cells for 30 minutes at room temperature. Slides were then washed extensively before microscopic examinations. The uninfected parental cell line as well as the clones were consistently negative in these assays. ND, not done. ‡Virus particles were precipitated from cell-free supernatant as follows: 0.3 ml of 4*M* NaCl and 3.6 ml of 30 percent (weight to volume) polyethylene glycol (Carbowax 6000) were added to 8 ml of harvested culture fluids and the suspension was placed on ice overnight. The suspension was centrifuged in a Sorvall RC-3 centrifuge at 2000 rev/min at 4°C for 30 minutes. The precipitate was resuspended in 300 μl of 50 percent (by volume) glycerol (25 m*M* tris-HCl, *p*H 7.5, 5 m*M* dithiothreitol, 150 m*M* KCl, and 0.025 percent Triton X-100). Virus particles were disrupted by addition of 100 μl of 0.9 percent Triton X-100 to 1.5*M* KCl. Reverse transcriptase assays were performed as previously described (*10*, *28*) (see comments to Fig. 2b) and expressed in counts per minute per milliliter of culture medium.

The cell line HT was tested for HTLV before being infected in vitro and was negative by all criteria including lack of proviral sequences (*32*). Continuous production of HTLV-III was obtained after repeated exposure of parental HT cells (3×10^6 cells pretreated with polybrene) to concentrated culture fluids harvested from short-term cultures of T cells (grown with TCGF) obtained from patients with AIDS or pre-AIDS. The concentrated fluids were first shown to contain particle-associated RT. When cell proliferation declined, usually 10 to 20 days after exposure to the culture fluids, the fresh (uninfected) HT cells were added to the cultures. Culture fluids from the infected parental cell line were positive for particulate RT activity, and about 20 percent of the infected cell population was positive in an indirect immune fluorescence assay in which we used serum from a hemophilia patient with pre-AIDS (patient E.T.). Serum from E.T. also contained antibodies to proteins of disrupted HTLV-III (*33*) but did not react with cells infected with HTLV-I or HTLV-II.

The parental T-cell population was extensively cloned in order to select the most permissive clones that would preserve high rates of growth and virus production (for example, see clones 4 and 9 in Table 1). A total of 51 single-cell clones were obtained by both capillary (*34*) and limited dilution (*35*) techniques using irradiated mononuclear cells from peripheral blood of a healthy donor as a feeder. The clones were infected with HTLV-III by exposure to concentrated

virus (2×10^6 cells of each clone and 0.1 ml of virus). Then cell growth and morphology, expression of cellular viral antigens, and RT activity in culture fluids were assessed 6 and 14 days after infection. Results for eight of these clones are shown in Table 1. Although all of these clones were susceptible to and permissive for the virus, there were considerable differences in their ability to proliferate after infection. For example, the cell number decreased by 10 to 90 percent from the initial cell count within 6 days after infection. The percentage of T cells positive for viral antigens ranged from 10 to 80 percent, as determined by immune fluorescence assays with serum from patient E.T. and with antiserum from rabbits infected repeatedly with disrupted HTLV-III. At 14 days after infection, the total cell number and the proportion of HTLV-III positive cells had increased in all eight clones. The virus positive cultures consistently showed a high proportion of round giant cells containing numerous nuclei (Fig. 1a). These cells resemble those induced by HTLV-I and -II (*9*) except that the nuclei exhibit a characteristic ring formation. Electron microscopic examinations showed that the cells released considerable amounts of virus (Fig. 1b).

Both virus production and cell viability of the infected clone H4 (H4/HTLV-III) were monitored for several months. Although virus production fluctuated (Fig. 2a), culture fluids harvested and assayed at approximately 14-day intervals consistently showed particulate RT activity which has been followed for over 5 months. The viability of the cells ranged from 65 to 85 percent and the doubling time of the cell population was approximately 30 to 40 hours (data not shown). Thus the data show that this permanently growing T-cell population can continuously produce HTLV-III.

The yield of virus from H4/HTLV-III cells was assessed by purification of concentrated culture fluids through a sucrose density gradient and assays of particulate RT activity in each fraction collected from the gradient. As shown in Fig. 2b, the highest RT activity was found at a density of 1.16 g/ml, which is similar to other retroviruses. The highest RT activity was found in the fractions with the largest amount of virus, as determined by electron microscopy. The actual number of viral particles determined by this method was estimated (*36*) to be about 10^{11} per liter of culture fluid.

We have used clones H4 and H9 for the long-term propagation of HTLV-III from patients with AIDS and pre-AIDS.

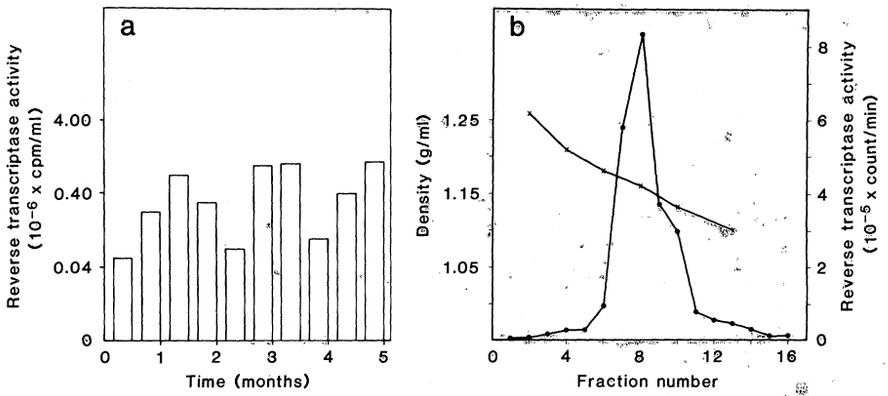

Fig. 2. (a) Continuous HTLV-III production from H4/HTLV-III in long-term culture was characterized by fluctuation in the amount of released virus as assessed by RT activity in the culture fluid (for details, see Table 1 and Fig. 2b). Viability of the infected cells was in the range of 60 to 90 percent. (b) Sucrose density gradient banding of HTLV-III showed the highest particulate RT activity at a density of 1.16 g/ml. A cell-free virus concentrate from a culture of H4/HTLV-III was layered on a 20 to 60 percent (by weight) sucrose gradient in 10 m*M* tris-HCl (*p*H 7.4) containing 0.1*M* NaCl and 1 m*M* EDTA and centrifuged overnight at 35,000 rev/min in a Spinco SW47 rotor. Fractions of 0.7 ml were collected from the bottom of the gradient and portions were assayed for RT (●) with $(dT)_{15} \cdot (A)_n$ being used as the primer template and Mg^{2+} as the divalent cation according to the methods described earlier (*10, 28*). Density of sucrose (X) was determined by refractive index measurements.

HTLV-III was isolated from four patients by the cocultivation method and from one patient by cell-free infection of these T-cell clones (Table 2). The transmission was monitored by RT activity, electron microscopic examinations, and expression of viral protein. When the H4 cells thus infected were fixed with acetone and tested with rabbit antiserum to HTLV-III and with serum from patients E.T., the percentage of positive cells was between 5 and 80 percent. HTLV-III has also been isolated in our laboratory from a total of 48 patients by the more conventional methods for isolation of HTLV (*30*). Some of these isolates have now successfully been transmitted to the HT clones for production and detailed analyses.

A few T-lymphocyte retroviruses that differed from HTLV-I and -II but were associated with lymphadenopathy syndrome were detected earlier (*37, 38*). One such virus, called LAV, was reported to be unrelated to HTLV-I or -II (*38*). Moreover, serum samples from 37.5 per-

Table 2. Isolation of HTLV-III from patients with AIDS and pre-AIDS.

Patient*	Diagnosis	Origin	Virus expression†			
			RT Activity ($\times 10^4$ cpm)	Percent positive cells in immune fluorescence assay		Electron microscopy
				Rabbit antiserum	Serum from E.T.	
R.F.	AIDS (heterosexual)	Haiti	0.25	80	33	ND
S.N.	Hemophiliac (lymphadenopathy)	United States	6.3	10	ND	+
B.K.	AIDS (homosexual)	United States	0.24	44	5	+
L.S.	AIDS (homosexual)	United States	0.13	64	19	+
W.T.	Hemophiliac (lymphadenopathy)	United States	3.2	69	ND	ND

*Cocultivation with H4 recipient T-cell clone was performed with fresh mononuclear cells from peripheral blood of patients R.F. and S.N., respectively. For patients B.K. and L.S. cocultivation was performed with T cells grown in the presence of exogenous TCGF (10 percent by volume) for 10 days. The ratio of recipient to donor (patients') cells was 1:5. The mixed cultures were maintained in RPMI 1640 medium (containing 20 percent FCS and antibiotics) in the absence of exogenous TCGF. H9 cells were also infected by exposing the cells to concentrated culture fluids harvested from T-cell cultures of patient W.T. The cultures were grown in the presence of exogenous TCGF for 2 weeks before the culture fluids were harvested and concentrated. Cells of H9 clones were treated with polybrene (2 μg/ml) for 20 minutes and 2×10^6 cells were exposed for 1 hour to 0.5 ml of 100-fold concentrated culture fluids positive for particulate RT activity. †HTLV-III virus expression in cells infected by the coculture and cell-free methods was assayed approximately 1 month after cultivation in vitro. Note a considerable fluctuation in HTLV-III expression. For details of the RT and indirect immune fluorescence assays see Table 1.

cent of patients with AIDS were found to react with it (*38*). In contrast, HTLV-III is related to HTLV-I and -II (*31, 39*) and, by all criteria, this new virus belongs to the HTLV family of retroviruses. In addition, more than 85 percent of serum samples from AIDS patients are reactive with proteins of HTLV-III (*33*). These findings suggest that HTLV-III and LAV may be different. However, it is possible that this is due to insufficient characterization of LAV because the virus has not yet been transmitted to a permanently growing cell line for true isolation and therefore has been difficult to obtain in quantity.

The transient expression of cytopathic variants of HTLV in cells from AIDS patients and the previous lack of a cell system that could maintain growth and still be susceptible to and permissive for the virus represented a major obstacle in detection, isolation, and elucidation of the precise causative agent of AIDS. The establishment of T-cell populations that continuously grow and produce virus after infection opens the way to the routine detection of cytopathic variants of HTLV in AIDS patients and provides the first opportunity for detailed immunological (*31, 33*) and molecular analyses of these viruses.

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Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and at Risk for AIDS

Abstract. *Peripheral blood lymphocytes from patients with the acquired immunodeficiency syndrome (AIDS) or with signs or symptoms that frequently precede AIDS (pre-AIDS) were grown in vitro with added T-cell growth factor and assayed for the expression and release of human T-lymphotropic retroviruses (HTLV). Retroviruses belonging to the HTLV family and collectively designated HTLV-III were isolated from a total of 48 subjects including 18 of 21 patients with pre-AIDS, three of four clinically normal mothers of juveniles with AIDS, 26 of 72 adult and juvenile patients with AIDS, and from one of 22 normal male homosexual subjects. No HTLV-III was detected in or isolated from 115 normal heterosexual subjects. The number of HTLV-III isolates reported here underestimates the true prevalence of the virus since many specimens were received in unsatisfactory condition. Other data show that serum samples from a high proportion of AIDS patients contain antibodies to HTLV-III. That these new isolates are members of the HTLV family but differ from the previous isolates known as HTLV-I and HTLV-II is indicated by their morphological, biological, and immunological characteristics. These results and those reported elsewhere in this issue suggest that HTLV-III may be the primary cause of AIDS.*

The acquired immunodeficiency syndrome known as AIDS was initially recognized as a separate disease entity in 1981 (*1*). Groups reported to be at risk for AIDS include homosexual or bisexual males (about 70 percent of reported cases), intravenous drug users (about 17 percent of cases), and Haitian immigrants to the United States (about 5 percent of cases). Also at risk are heterosexual contacts of members of the highest risk group, hemophiliacs treated with blood products pooled from donors, recipients of multiple blood transfusions, and infants born of parents belonging to the high-risk groups (*2*). AIDS is diagnosed as a severe, unexplained, immune deficiency that usually involves a reduction in the number of helper T lymphocytes and is accompanied by multiple opportunistic infections or malignancies. A number of other clinical manifestations, when occurring in members of a group at risk for AIDS, are identified as its prodrome (pre-AIDS). These include unexplained chronic lymphadenopathy or leukopenia involving a reduction in the number of helper T lymphocytes (*1, 2*). The increasing incidence of this disease, the types of patients affected, and other epidemiological data suggest the existence of an infectious etiologic agent that