

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 diag-

nosed 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

can be transmitted by intimate contact or by whole blood or separated blood components (2). As indicated by Popovic *et al.* (3), we and others have suggested that specific human T-lymphotropic retroviruses (HTLV) cause AIDS (4, 5). Many properties of HTLV are consistent with this idea (6).

An association of members of the HTLV family with T lymphocytes from some AIDS or pre-AIDS patients was reported previously. For example, the first subgroup of HTLV to be characterized, HTLV-I, was isolated recently from T cells from about 10 percent of AIDS patients, and a virus related to HTLV-II was isolated from one AIDS patient (4). Another HTLV isolate was obtained from the lymph nodes of a patient with lymphadenopathy and at risk for AIDS (7). This isolate has been difficult to grow in quantities sufficient to permit its characterization. HTLV proviral DNA was detected in T lymphocytes from two additional AIDS patients (8) and HTLV-related antigens were found in another two patients (4). Studies in which disrupted HTLV-I or the purified structural proteins (p24 or p19) were used to detect antibodies in serum samples from patients with AIDS and pre-AIDS indicated that 10 to 15 percent of the patients had been exposed to HTLV-I (9). Essex and his co-workers, using HTLV-infected T-lymphocyte cultures to detect antibody in serum samples, found that about 35 percent of patients with AIDS and pre-AIDS had been exposed to HTLV (5). Further studies suggested that at least some of the antigens detected in this system were products of the genome of a member of the HTLV family (10), but it was not known whether the antibodies were specifically against HTLV-I, HTLV-II, or a virus of a different subgroup.

With the availability of large quantities of HTLV-III (3), it became possible to develop specific immunological reagents that would facilitate its characterization. HTLV-III was found to share many properties with other HTLV isolates (6), but it was morphologically, biologically, and antigenically distinguishable (3, 11). Here we describe the detection and isolation of HTLV-III from a large number of patients with AIDS and pre-AIDS.

For these studies we used cell culture conditions previously developed in our laboratory for the establishment of T lymphocytes in culture and for the detection and isolation of HTLV-I and HTLV-II from leukemic donors (12). Evidence for the presence of HTLV-III included: (i) viral reverse transcriptase (RT) activi-

ty (12) in supernatant fluids; (ii) transmission of virus by coculturing T cells with irradiated donor cells or with cell-free fluids (3, 13); (iii) observation of virus by electron microscopy (12, 13); and (iv) the expression of viral antigens in indirect immune fluorescence assays using serum from a patient positive for antibodies to HTLV-III as described (5,

11), or antisera prepared against purified, whole disrupted HTLV-III (11). Cells and supernatant fluids were also monitored for the expression of HTLV-I and HTLV-II by using antibodies to the viral structural proteins p19 and p24 and by indirect immune fluorescence and radioimmunoprecipitation procedures (14). As summarized in Table 1, we found

Fig. 1. Reverse transcriptase activity from lymphocytes established in cell culture from a patient with pre-AIDS. Viable cell number and  $Mg^{2+}$ -dependent RT activity were determined by established procedures (13). Symbols:  $\circ$ , viable cell number in 1.5 ml of growth medium;  $\bullet$ , RT in 5  $\mu$ l of fivefold concentrated conditioned medium sampled at the indicated time. A sudden vertical drop in the dashed curve indicates the time of subculturing of cells to the indicated cell number. Arrow indicates the time of addition of rabbit antiserum to  $\alpha$ -interferon to a portion of the cultured cells (also see legend to Table 1).

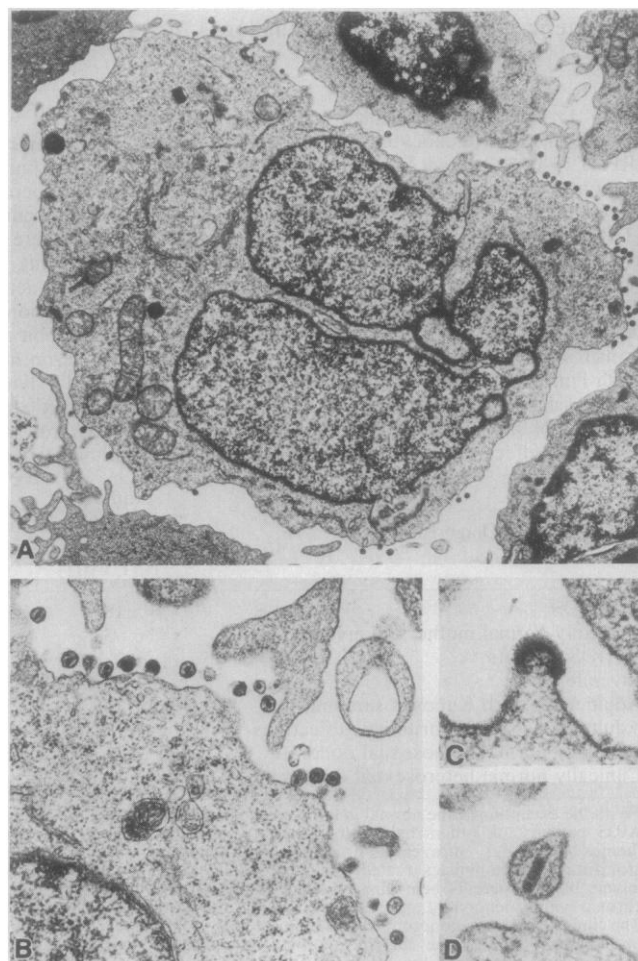
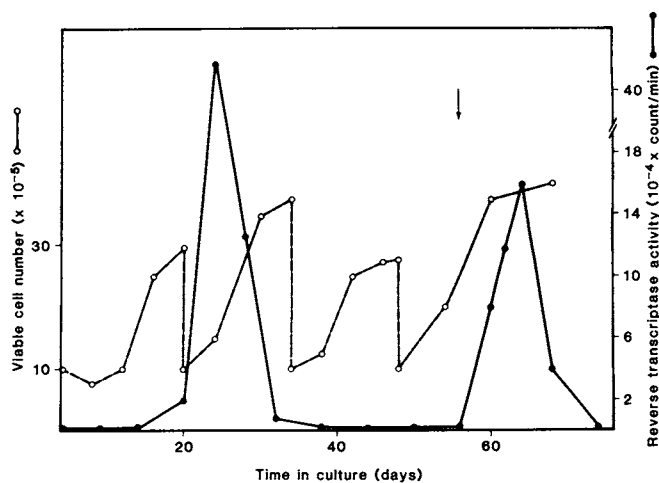


Fig. 2. Transmission electron micrographs of fixed, sectioned lymphocytes from a patient with pre-AIDS. (A)  $\times 10,000$ ; (B)  $\times 30,000$ ; (C and D)  $\times 100,000$ .

HTLV-III in 18 of 21 samples from patients with pre-AIDS, from three of four clinically normal mothers of juvenile AIDS patients, three of eight juvenile AIDS patients, 13 of 43 of adult AIDS patients with Kaposi's sarcoma, and 10 of 21 adult AIDS patients with opportunistic infections. Virus was detected in only one of 22 samples from clinically normal, nonpromiscuous homosexual males believed to be at only moderate risk for AIDS. It is interesting, however, that 6 months after these tests were conducted the one positive normal homosexual subject developed AIDS. In no instance, 0 of 115, was virus detected in or isolated from cells of the normal volunteers. Samples from 15 of these were tested under rigorously controlled conditions, which included addition of antibody to  $\alpha$ -interferon.

Primary cells from patients usually produce virus for 2 to 3 weeks (Fig. 1). After this time the production of virus declines even though the culture may contain actively replicating cells that can be maintained for long periods in the presence of added T-cell growth factor (TCGF). In some instances virus release can be reinitiated by the addition of antibody to  $\alpha$ -interferon (Fig. 1). The HTLV-III-producing cell cultures were characterized by established immunolog-

ical procedures (13). They were predominantly T lymphocytes (E rosette receptor-positive, OKT3<sup>+</sup> and Leu1<sup>+</sup>) with a helper-inducer phenotype (OKT4<sup>+</sup> and Leu3<sup>+</sup>).

The fairly uniform morphological appearance of HTLV-III is shown in Fig. 2. The diameter of the virus is 100 to 120 nm, and it is produced in high numbers from infected cells by budding from the cell membrane. A possibly unique feature of this virus is the cylindrical shaped core observed in many mature virions.

The incidence of virus isolation reported here probably underestimates its true incidence since many tissue specimens were not received or handled under what we now recognize as optimal conditions (15). This is particularly so for the samples received from late-stage AIDS patients. Such samples usually contain many dying cells and very few viable T4 lymphocytes. However, a high proportion of patients with AIDS and pre-AIDS have circulating antibody to HTLV-III (11).

The HTLV-III produced by cultured T cells from patients with AIDS and pre-AIDS is highly infectious and can be readily transmitted to fresh umbilical cord blood and adult peripheral blood or bone marrow lymphocytes. The production of HTLV-III by these cells is tran-

sient, often declining to undetectable levels by 2 to 3 weeks after infection (data not shown). The transmission of HTLV-III to an established T-cell line (3), however, now makes possible its production in large quantities for detailed analyses and for development of reagents for its detection (3, 11).

That the viruses we have named HTLV-III belong to the HTLV family is indicated by their T cell tropism, Mg<sup>2+</sup>-dependent RT of high molecular weight, antigenic cross-reactivity with HTLV-I and -II (11), cytopathic effects on T lymphocytes (3), and their morphological appearance in the electron micrograph. HTLV-III also contains some structural proteins similar in size to those of other members of the HTLV family (11).

These studies of HTLV-III isolates from patients with AIDS and pre-AIDS and from some healthy individuals at risk for AIDS provide strong evidence of a causative involvement of the virus in AIDS.

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Table 1. Detection and isolation of HTLV-III from patients with AIDS and pre-AIDS. Peripheral blood leukocytes were banded in Ficoll-Hypaque, incubated in growth medium (RPMI 1640, 20 percent fetal bovine serum, and 0.29 mg of glutamine per milliliter) containing phytohemagglutinin (PHA-P; 5  $\mu$ g/ml) for 48 hours at 37°C in a 5 percent CO<sub>2</sub> atmosphere. They were then reseeded with growth medium containing 10 percent purified T-cell growth factor (TCGF). Cells and conditioned media from these lymphocytes were assayed for the presence of HTLV-III. Samples exhibiting more than one of the following were considered positive: repeated detection of a Mg<sup>2+</sup>-dependent reverse transcriptase activity in supernatant fluids; virus observed by electron microscopy; intracellular expression of virus-related antigens detected with antibodies from seropositive donors or with rabbit antiserum to HTLV-III; or transmission of particles, detected by RT assays or by electron microscopic observation, to fresh human cord blood, bone marrow, or peripheral blood T lymphocytes. All isolates are distinguishable from HTLV-I or HTLV-II by several criteria and are classified as HTLV-III on the basis of similar morphological features observed by electron microscopy (Fig. 1); similar cytopathic effects (3); antigenic cross-reactivity (11); and nucleic acid analysis (16).

Diagnosis*	Number positive for HTLV-III	Number tested	Percent positive
Pre-AIDS	18	21	85.7
Clinically normal mothers of juvenile AIDS patients	3	4	75.0
Juvenile AIDS	3	8	37.5
Adult AIDS with Kaposi's sarcoma	13	43	30.2
Adult AIDS with opportunistic infections	10	21	47.6
Clinically normal homosexual donors	1	22	4.5
Clinically normal heterosexual donors	0	115	0

\*With the exception of the normal heterosexual donors and some of the clinically normal mothers of juvenile AIDS patients, all others belong to one of the groups of people identified as being at risk for AIDS (homosexual males, intravenous drug users, Haitian immigrants, heterosexual contacts of members of a group at risk, hemophiliacs treated with pooled blood products, recipients of multiple blood transfusions, and infants born of parents belonging to other groups at risk). Pre-AIDS includes patients with unexplained chronic lymphadenopathy and leukopenia, with an inverted T4 (helper)/T8 (suppressor) lymphocyte ratio. The clinically normal, nonpromiscuous, homosexual subjects are from Washington, D.C., and are believed to be at moderate risk. The clinically normal heterosexual donors include both male and female subjects believed not to be at risk for AIDS.

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15. For virus isolation, samples of freshly drawn, heparinized peripheral blood or bone marrow, yielding a minimum of  $10^7$  viable cells (greater than 90 percent), are needed. These samples must contain the cells of interest, namely, OKT4<sup>+</sup> T cells, which are frequently depleted in AIDS patients.
16. S. Arya *et al.*, in preparation.
17. We thank M. Gonda for electron microscopy and A. Patel, S. Roberson, A. Fladager, and E. Reid for technical assistance. We are also indebted to many clinical collaborators who provided patient materials.

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## Serological Analysis of a Subgroup of Human T-Lymphotropic Retroviruses (HTLV-III) Associated with AIDS

**Abstract.** *The two main subgroups of the family of human T-lymphotropic retroviruses (HTLV) that have previously been characterized are known as HTLV-I and HTLV-II. Both are associated with certain human leukemias and lymphomas. Cell surface antigens (p61 and p65) encoded by HTLV-I are frequently recognized, at low titers, by antibodies in the serum of patients with acquired immunodeficiency syndrome (AIDS) or with signs or symptoms that precede AIDS (pre-AIDS). This suggests an involvement of HTLV in these disorders. Another subgroup of HTLV, designated HTLV-III, has now been isolated from many patients with AIDS and pre-AIDS. In the studies described in this report, virus-associated antigens in T-cell clones permanently producing HTLV-III were subjected to biochemical and immunological analyses. Antigens of HTLV-III, specifically detected by antibodies in serum from AIDS or pre-AIDS patients and revealed by the Western blot technique, are similar in size to those found in other subgroups of HTLV. They include at least three serologically unrelated antigenic groups, one of which is associated with group-specific antigens (p55 and p24) and another with envelope-related (p65) proteins, while the antigens in the third group are of unknown affiliation. The data show that HTLV-III is clearly distinguishable from HTLV-I and HTLV-II but is also significantly related to both viruses. HTLV-III is thus a true member of the HTLV family.*

Members of the family of human lymphotropic retroviruses (HTLV) have the following features in common: a pronounced tropism for OKT4<sup>+</sup> lymphocytes (1), a reverse transcriptase (RT) with a high molecular weight (100,000) and a preference for Mg<sup>2+</sup> as the divalent cation for optimal enzymatic activity (2, 3), and the capacity to inhibit T cell function (4) or, in some cases, kill T cells (5). Many HTLV also have the capacity to transform infected T cells (1). The two major subgroups that have been characterized (6) are HTLV-I, which is causatively linked to certain adult T-cell malignancies (7), and HTLV-II, which was first identified in a patient with hairy cell leukemia (8).

Viruses of the HTLV family have been detected in some patients with the acquired immunodeficiency syndrome (AIDS) (9) or with pre-AIDS, a condition frequently progressing to AIDS (10). A high proportion of patients with AIDS or pre-AIDS, as well as a significant number of hemophiliacs, have antibodies in their serum that recognize a cell surface glycoprotein (gp61) that is present on certain human T cells infected with HTLV-I (11). Gp61 and p65, a slightly larger protein that is a homolog of gp61 and occurs in another cell line producing HTLV-I, were subsequently shown to be related to the HTLV viral glycoprotein (12, 13). Studies of blood transfusion recipients who later developed AIDS

and of their blood donors have revealed the presence, in the blood of the donors, of antibodies to a retrovirus of the HTLV family (14). These findings suggest an involvement of viruses of the HTLV family in the cause of AIDS and pre-AIDS. An involvement of HTLV-I alone appeared doubtful, however, because antibody titers to gp61 of HTLV-I in these patients are generally very low and antibodies to the structural proteins of HTLV, notably p24 and p19 (15), are not detectable in most AIDS patients (16). Instead, it seemed likely that another member of the HTLV family might be involved in the etiology of AIDS. Here we describe our studies of a group of cytopathic viruses (collectively designated HTLV-III) isolated from patients with AIDS or pre-AIDS. Isolation of these viruses was achieved by means of a novel system permitting the continuous growth of T-cell clones infected with the cytopathic types of HTLV found in these disorders (17). We show that antigens associated with human cells infected by HTLV-III are specifically recognized by antibodies in serum from AIDS and pre-AIDS patients, and present a preliminary biochemical and immunological analysis of these antigens.

Lysates of two immortalized and infected human T-cell clones, H4/HTLV-III and H17/HTLV-III (17), were tested with samples of human serum in a strip radioimmunoassay (RIA) based on the Western blot technique (18). The sera were from patients with AIDS or pre-AIDS, from contacts of such patients, and from homo- or heterosexual male controls. Sera from the same patients were also tested by the enzyme-linked immunosorbent assay (ELISA) with purified HTLV-III as part of a larger, systematic serologic study of the prevalence of antibodies to HTLV-III in AIDS and pre-AIDS patients (19).

Representative results are shown in Fig. 1. Sera from patients with AIDS or pre-AIDS, and from some homosexuals and heroin-addicts, recognized a number of specific antigens not detected by sera from heterosexual subjects. The most prominent reactions were with antigens of the following molecular weights: 65,000, 60,000, 55,000, 41,000, and 24,000. Antigens with molecular weights of approximately 88,000, 80,000, 39,000, 32,000, 28,000, and 21,000 gave less prominent reactions. The reaction with the antigen of 55,000 (p55) only occurred in sera that also recognized p24, suggesting a relationship between the two antigens.

The specificity of these reactions was

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