Antibodies Reactive with Human T-Lymphotropic Retroviruses (HTLV-III) in the Serum of Patients with AIDS

Abstract. *In cats, infection with T-lymphotropic retroviruses can cause T-cell proliferation and leukemia or T-cell depletion and immunosuppression. In humans, some highly T4 tropic retroviruses called HTLV-I can cause T-cell proliferation and leukemia. The subgroup HTLV-II also induces T-cell proliferation in vitro, but its role in disease is unclear. Viruses of a third subgroup of human T-lymphotropic retroviruses, collectively designated HTLV-III, have been isolated from cultured cells of 48 patients with acquired immunodeficiency syndrome (AIDS). The biological properties of HTLV-III and immunological analyses of its proteins show that this virus is a member of the HTLV family, and that it is more closely related to HTLV-II than to HTLV-I. Serum samples from 88 percent of patients with AIDS and from 79 percent of homosexual men with signs and symptoms that frequently precede AIDS, but from less than 1 percent of heterosexual subjects, have antibodies reactive against antigens of HTLV-III. The major immune reactivity appears to be directed against p41, the presumed envelope antigen of the virus.*

The incidence of the acquired immunodeficiency syndrome (AIDS) in homosexual men with multiple sexual partners, intravenous drug abusers, hemophiliacs, blood transfusion recipients, and close heterosexual contacts of members of these high-risk groups (*1–7*) strongly suggests that the disease spreads by the transmission of an infectious agent (*8, 9*). The agent's primary targets within the body appear to be specific subpopulations of T cells. The severe immune deficiency of AIDS patients results from an unusually low proportion of helper T lymphocytes ($OKT4^+$) and a resulting lack of many helper functions, including production of antibodies by B cells (*1, 3*).

Retrovirus infections are known to lead to depressed immune functions in animal systems. For example, in cats, a major result of infection with feline leukemia virus (FeLV) is loss of normal immune function. More FeLV-infected cats die from consequences of this immune dysfunction than from the leukemia itself (*10*). FeLV provides an example of a single T-cell tropic retrovirus that causes both target cell proliferation (leukemia) and depletion (immunosuppression). By analogy, a human retrovirus with a tropism for T cells should be considered a serious candidate in the etiology of human AIDS. Two subgroups of a family of human T-lymphotropic retroviruses (HTLV) have been isolated and characterized (*11*). The first, HTLV-I, was isolated from a black American with an aggressive form of T-cell lymphoma (*12*) and has been etiologically linked to the pathogenesis of adult T-cell leukemia-lymphoma (ATL) (*13–15*). Infection with HTLV-I in vitro can alter T-cell function (*16*) and, in some cases, lead to T-cell death (*17*). HTLV-II was isolated from a patient with a T-cell variant of hairy cell leukemia (*18*).

Although there are distinct differences between HTLV-I and HTLV-II, they have the following common features: a tropism for $OKT4^+$ lymphocytes (*19*); a Mg^{2+}-dependent reverse transcriptase (RT) of high molecular weight (100,000) (*20*); some antigenic cross-reactivity in their proteins (*18*); a novel set of nucleotide sequences called pX at the 3′ end of the viral genome; a limited amount of nucleic acid homology in their genomes (*21*); and similar morphology. Both HTLV-I and HTLV-II have been isolated from cultured T cells of patients with AIDS (*22, 23*). Another retrovirus was isolated from a homosexual patient with chronic generalized lymphadenopathy (*24*), a syndrome that often precedes AIDS and is therefore referred to as pre-AIDS. Proviral DNA of HTLV-I was detected in the cellular DNA of two AIDS patients (*25*), and serum samples from some patients were shown to react with antigens of HTLV-I (*26*). A larger proportion of the sera reacted with a cell membrane antigen specific to HTLV-I–infected cells (*27*). This antigen has since been identified as a precursor of the envelope glycoprotein, gp46, of HTLV-I (*28, 29*). However, the correlation between AIDS and serum antibodies to HTLV-I protein (including the cell membrane antigen, p61) is weak.

These results are consistent with the idea that the primary cause of AIDS is another member of the HTLV family with limited cross-reactivities with the known HTLV subgroups. Sera with high titers of antibodies to the AIDS-specific virus might show a detectable reaction with antigens of HTLV-I and HTLV-II, whereas the reaction of sera with low titers might be too weak to recognize in such a cross-reactive system. Our attempts to isolate other retroviruses from AIDS patients resulted in the identification of a number of HTLV isolates that are similar to each other but are distinguishable from HTLV-I and HTLV-II. These new isolates are designated HTLV-III and are described in the ac-

Table 1. Antibodies to HTLV-III in serum samples from patients with AIDS and pre-AIDS and from control subjects. Wells of 96-well Immulon plates were coated overnight with a lysate of density-banded HTLV-III (*30*) at 0.5 μg protein per well in 100 μl 50 m*M* sodium bicarbonate buffer, *p*H 9.6. The wells were washed with water and incubated for 20 minutes with 100 μl of 5 percent bovine serum albumin in phosphate buffered saline (PBS). The wells were washed again in water, and then 100 μl of 20 percent normal goat serum in PBS were added to each well, followed by 5 or 10 μl of the test sera. These were allowed to react for 2 hours at room temperature. The wells were washed three times with 0.05 percent Tween-20 in PBS and incubated for 1 hour at room temperature with peroxidase-labeled goat antiserum to human immunoglobulin G at a dilution of 1:2000 in 1 percent normal goat serum in PBS. The wells were successively washed four times with 0.05 percent Tween-20 in PBS and four times with PBS and reacted with 100 μl of the substrate mixture containing 0.05 percent orthophenylene diamine and 0.005 percent hydrogen peroxide in phosphate-citrate buffer, *p*H 5.0. The reactions were stopped by the addition of 50 μl of 4*N* H_2SO_4, and the color yield was measured with a Dynatech ELISA reader. Assays were done in duplicate and absorbance reading greater than three times the average of four normal negative control readings was taken as positive.

Subject	Number positive for antibodies to HTLV-III	Number tested	Percent positive
Patients with AIDS	43	49	87.8
Patients with pre-AIDS	11	14	78.6
Intravenous drug users	3	5	60
Homosexual men	6	17	
Sexual contact of AIDS patient	1	1	
Persistent fatigue	1	1	
Other	4	15	26.6
Other controls	1	186	0.5
Normal subjects	1	164	0.6
Patients with hepatitis B virus infection	0	3	
Patient with rheumatoid arthritis	0	1	
Patients with systemic lupus erythematosus	0	6	
Patients with acute mononucleosis	0	4	
Patients with lymphatic leukemias	0	8	

companying reports (*30–32*). Here we describe the use of HTLV-III in an immunological screening of serum samples from patients with AIDS and pre-AIDS and from individuals at increased risk for AIDS.

The virus was purified from supernatants of cell cultures supporting the continuous production of HTLV-III (*30*). The virus showed a difference in the makeup of its protein components as revealed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of a sucrose density banded preparation (Fig. 1, lane 2). Like HTLV-I (lane 1), and unlike common mammalian retroviruses (for example, Rauscher murine leukemia virus, lane 3), HTLV-III (lane 2) has a major group-specific antigen (*gag* protein) with a molecular weight of 24,000 (p24). It has a reverse transcriptase with a molecular weight of about 100,000, another protein with a molecular weight of 41,000 (presumably the envelope glycoprotein), and shows a tropism for $OKT4^+$ lymphocytes. However, it lacks the band separating at a molecular weight of 19,000 (p19). Instead, it has a smaller band that is missing in HTLV-I. Immunological studies presented in an accompanying report (*32*) also indicate that HTLV-III is antigenically different from HTLV-I and -II, but that it also shares a variety of antigenic determinants with them, especially with HTLV-II. This relatedness has also been confirmed by comparison of nucleotide sequences of the three types of HTLV (*33*).

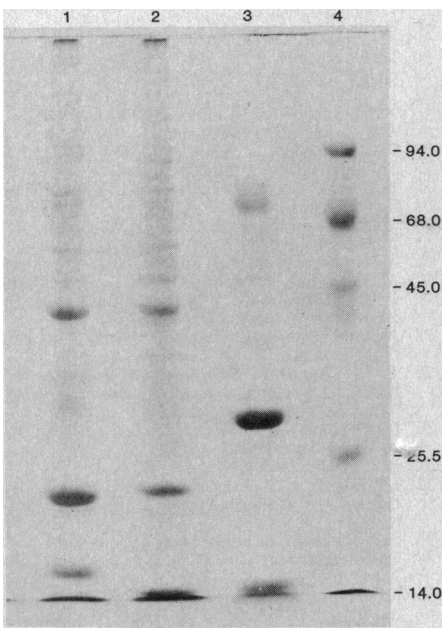

Fig. 1. Comparison of the SDS–polyacrylamide gel profile of HTLV-III with profiles of HTLV-I and Rauscher murine leukemia virus (R-MuLV). Lane 1, HTLV-I; lane 2, HTLV-III; lane 3, R-MuLV; lane 4, molecular weight standards: phosphorylase b (94,000), bovine serum albumin (68,000), ovalbumin (45,000), chymotrypsinogen (25,500), and lysozyme (14,000).

Serum samples were obtained from patients with clinically documented AIDS, Kaposi's sarcoma, sexual contacts of AIDS patients, intravenous drug abusers, homosexual men, and heterosexual subjects. These sera were tested for their reactivity to HTLV-III by means of the enzyme-linked immunosorbent assay (ELISA) (*34*). Lysates of sucrose density banded HTLV-III were coated on 96-well microtiter plates. The test sera were diluted with normal goat serum, added to the wells, and allowed to react for 2 hours or overnight at room temperature. The primary immune complex formed with the antibodies in the human sera was detected by adding peroxidase-labeled goat antiserum to human immunoglobulins and assaying for a colored peroxidase reaction product (*34*). The results are presented in Table 1. Of 49 clinically diagnosed AIDS patients, 43 (88 percent) showed serum reactivity in this assay. Two of the subjects whose serum reacted positively with the HTLV preparation had developed AIDS after receiving blood transfusions, one in Haiti and the other in Aruba. Of 14 homosexual men with pre-AIDS, 11 (79 percent) were positive. Of 17 homosexual men with no clinical symptoms of AIDS, seven were positive. At least one of these was known to be a long-time sexual partner of a patient with clinically diagnosed AIDS. Another had persistent fatigue and possibly other early symptoms of AIDS. Because these 17 men had been seeking medical assistance, they are not a representative sample of the homosexual population, and the high incidence of HTLV-III–specific antibodies in their sera may not reflect the true incidence in the homosexual population. One of the three intravenous drug abusers that were positive for serum antibodies to HTLV-III was also a homosexual. Serum samples from only one of 186 control subjects reacted positively in this test. These control subjects included three with hepatitis B virus infection, one with rheumatoid arthritis, six with systemic lupus erythematosus, four with acute mononucleosis, and eight with various forms of lymphatic leukemias and lymphomas, some of whom were positive for HTLV-I. The rest were normal donors of unknown sexual preference including laboratory workers ranging in age from 22 to 50.

To understand the molecular nature of the antigens recognized by ELISA, we conducted the following experiment. A lysate of HTLV-III was fractionated by SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose sheet by the electrophoretic blotting (Western) technique of Towbin *et al.* (*35*). The nitrocellulose sheet was cut into 0.5-cm strips and reacted with samples of the human sera. Antigen-antibody complexes formed were detected by autoradiog-

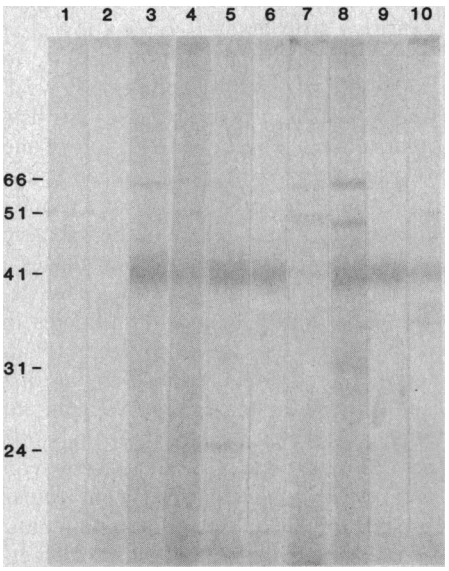

Fig. 2. Identification of HTLV-III antigens recognized by sera of AIDS patients. HTLV-III was lysed and fractionated by electrophoresis on a 12 percent polyacrylamide slab gel in the presence of SDS. The protein bands on the gel were electrophoretically transferred to a nitrocellulose sheet according to the procedure of Towbin *et al.* (*35*). Strip solid-phase radioimmunoassays were then performed as described (*36*). The sheet was incubated at 37°C for 2 hours with 5 percent bovine serum albumin in 10 m*M* tris-HCl, *p*H 7.5 containing 0.9 percent NaCl and cut into 0.5-cm strips. Each strip was incubated for 2 hours at 37°C and 2 hours at room temperature in a screw cap tube containing 2.5 ml of buffer-1 (20 m*M* tris-HCl, *p*H 7.5, 1 m*M* EDTA, 0.2*M* NaCl, 0.3 percent Triton X-100, and 2 mg of bovine serum albumin and 0.2 mg of human Fab per milliliter). Test sera (25 μl) were then added to individual tubes containing the strips and incubation was continued for 1 hour at room temperature and overnight in the cold. The strips were washed three times with a solution containing 0.5 percent sodium deoxycholate, 0.1*M* NaCl, 0.5 percent Triton X-100, 1 m*M* phenylmethylsulfonyl fluoride, and 10 m*M* sodium phosphate, *p*H 7.5. The strips were incubated for 1 hour at room temperature with 2.4 ml of buffer-1 and 0.1 ml of normal goat serum. Affinity-purified and ^{125}I-labeled goat antiserum to human immunoglobulin (μ chain and Fc fragment) (1.25×10^6 count/min) were added to the reaction mixture and the incubation was continued for 30 minutes at room temperature. The strips were washed as described, dried, mounted, and exposed to x-ray film. Strip 1, adult T-cell leukemia; strip 2, normal donor; strip 3, mother of a child with AIDS; strips 4 and 6 to 10, AIDS patients; and strip 5, patient with pre-AIDS.

raphy after incubation of the strips with ^{125}I-labeled goat antibody to human immunoglobulin. Figure 2 shows that the antigen most prominently and commonly detected among all of the sera from AIDS patient had a molecular weight of 41,000 (p41). This corresponds to one of the major proteins of the virus (Fig. 1) and is presumably the envelope protein. Strip 7 shows the result obtained with serum from an AIDS patient that reacted negatively in the ELISA but in this more sensitive strip assay it gave a low, but definitely positive, result. Reactivity to p24 of the virus was generally very weak and was clear only in two cases (strips 4 and 5). This may be a reflection of the relative titer toward different antigens. One would expect the highest antibody titer against the envelope of the infecting agent, especially if the infection causes a pronounced immune deficiency and decreased capacity to make antibodies in response to subsequent antigenic challenge. Additional reactivities against antigens with molecular weights of 66,000 and 51,000 were seen in some sera. In strip 8 the serum reacted with an additional antigen that has a molecular weight of 31,000. These additional antigens appear to be related to those detected by sera from the same patients in HTLV-III–producing cells (*33*). Strips 1 and 2 show that sera from a patient with ATL who was positive for HTLV-I and from a normal subject do not react with the antigens of HTLV-III.

Of particular interest is the finding that among the serum samples that reacted positively with HTLV-III two were from young children (ages 7 months and 2 years). These children were free of known opportunistic infections including cytomegalovirus, Epstein-Barr virus, *Pneumocystis carinii*, and fungus. The mother of one of them was positive in both tests described here. The children presumably acquired the infection in utero, by their mother's milk, or by another route.

Among the positive serum samples from AIDS patients there appears to be a wide variation in antibody titer to HTLV-III. Generally, the titers in sera from patients with advanced AIDS are significantly lower than those in sera from newly diagnosed patients and patients with pre-AIDS. This is consistent with the idea that HTLV-III infection causes an initial lymphoid proliferation but eventually causes death of the target lymphocytes (OKT4$^+$) leading to the abnormal T4$^+$/T8$^+$ ratios and loss of helper T-cell functions including antibody production by B cells. Therefore, the low or negative result in the ELISA of sera from some cases of advanced AIDS may be a consequence of the natural course of the disease. To prove this it will be necessary to study antibody titers in sera obtained at intervals from subjects at risk for the disease. The serum of one AIDS patient showed a low positive titer, but serum from his homosexual partner with no symptoms of AIDS had a significantly higher antibody titer. It is interesting that the serum of one AIDS patient that was negative in the ELISA did show a definite but low positive reaction with p41 in the more sensitive Western blot assay (Fig. 2, strip 7). The ELISA with purified p41 might prove to be even more sensitive. It is significant that although HTLV proviral sequences were clearly detected in DNA from cell samples obtained from two AIDS patients early in the course of their disease, these sequences could not be detected in cells obtained after 1 year in one case and 2 months in the second case (*25*). It is conceivable that the subset of T lymphocytes that forms the target of the provirus had been depleted before the second samples were obtained in each case.

In conclusion, we have shown a high incidence of specific antibodies to HTLV-III in patients with AIDS and pre-AIDS. Among the antibody-positive cases reported here a few are of particular importance with respect to the transmission of the disease. For example, the mother of the baby with AIDS was positive for HTLV-III as was a long-term sexual partner of a homosexual with AIDS. Recipients of blood products originating from individuals at risk for AIDS were also positive for HTLV-III and, as described in an accompanying report (*31*), the virus has been isolated from several children with AIDS as well as from their mothers. The data presented here and in the accompanying reports (*30–32*) suggest that HTLV-III is the primary cause of AIDS.

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