Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 5530–5534, August 1985
Medical Sciences

Isolation of infectious human T-cell leukemia/lymphotropic virus type III (HTLV-III) from patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related complex (ARC) and from healthy carriers: A study of risk groups and tissue sources

(lymphadenopathy-associated virus/cytopathic virus/T lymphocytes)

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Communicated by Ludwik Gross, March 25, 1985

ABSTRACT **Acquired immunodeficiency syndrome (AIDS) and AIDS-related complex (ARC) are thought to be caused by human T-cell leukemia/lymphotropic virus type III (HTLV-III). Since the fall of 1982, independent isolates of HTLV-III have been obtained in this laboratory, in collaboration with several clinical groups, from 101 AIDS and ARC patients and healthy donors at risk for AIDS. Most isolates were from peripheral blood T lymphocytes established in cell culture, but some were obtained from bone marrow, lymph node, brain tissue, and cell-free plasma and from cells associated with saliva, cerebrospinal fluid, and semen. Virus was isolated from approximately 50% of AIDS patients, 85% of ARC patients, and 30% of healthy individuals at risk for AIDS. The risk groups included homosexuals, promiscuous heterosexuals, i.v. drug users, recipients of blood or blood products, and spouses and offspring of AIDS patients and others at risk for AIDS. A high correlation was seen between persistent levels of serum antibody and the ability to isolate virus from patient or donor leukocytes. Immunologic and nucleic acid analysis demonstrated that the virus isolates were highly related, although substantial diversity was observed in the restriction enzyme cleavage patterns of those studied in detail. Biological analysis of cells from infected patients and donors as well as from normal peripheral blood mononuclear cells exposed to virus *in vitro* demonstrated that OKT4/Leu3a+ (helper/inducer) lymphocytes were preferentially infected and were subjected to a characteristic cytopathic effect. The availability of multiple isolates of virus from a number of different patients and donors will greatly facilitate the characterization of HTLV-III and the study of possible biological and/or biochemical variants of the virus responsible for the development of AIDS, ARC, and related diseases.**

Human T-cell leukemia/lymphotropic virus type III (HTLV-III) (1–4), also named lymphadenopathy-associated virus (LAV) (5, 6), is thought to be the primary cause of a spectrum of immunological disorders, with acquired immunodeficiency syndrome (AIDS) as the most severe clinical manifestation. The implication of HTLV-III as the causative agent was based primarily on the high correlation between development of disease and prior exposure to virus, evidenced by the isolation of virus (1, 2) and the detection of antibodies to viral proteins in patient sera (3, 4, 7). Furthermore, the *in vitro* effect of HTLV-III on immunologically active normal lymphocytes was found to be consistent with the hematological status of patients observed during the course of disease (1, 2, 8, 9). Here we describe the isolation of infectious virus from 47 AIDS patients, 35 AIDS-related-complex (ARC) patients, and 19 clinically healthy donors at risk for AIDS. Virus was isolated from peripheral blood lymphocytes and other tissues.

It was recently observed that, although all isolates of HTLV-III are highly related, many differ from others in their restriction endonuclease cleavage pattern, suggesting some genomic diversity (10). The availability of many isolates of HTLV-III from patients and donors with such a wide variety of clinical manifestations and geographical and social backgrounds will be useful in determining the extent, biological consequences, and the significance, if any, of these differences. They may also be helpful in defining any regional strain variations.

MATERIALS AND METHODS

Preparation of Cells and Tissues. Heparinized peripheral blood and bone marrow mononuclear cells were collected and processed as described (1, 2). Cells were prepared from lymph nodes and brain biopsy specimens by mincing tissues and banding in Ficoll-Hypaque before introduction into cell culture. Cell-free plasma was filtered and used, either directly or after removal of particulate material by centrifugation, to infect normal peripheral blood mononuclear cells. Saliva samples were processed as described (11). Cells were established in culture with interleukin 2 (1, 2) following incubation (48 hr, 37°C, 5% CO_2) in growth medium (RPMI 1640, 20% fetal calf serum) supplemented with phytohemagglutinin (PHA-P, 5 μg/ml), as described (1, 2, 8, 11).

Isolation of Virus. Supernatant fluids from cell cultures were monitored for particulate reverse transcriptase activity (1, 2, 8, 11). Selected cells were also examined by electron microscopy for viral particles. The presence of infectious virus was determined by transmission of cell-free virus to mitogen-stimulated normal peripheral blood mononuclear cells pretreated with Polybrene (2–24 hr, 2 μg/ml) or DEAE-dextran (30 min, 25 μg/ml) (1, 2, 8).

Characterization of Cells. The phenotype of cells was determined in a live-cell indirect immunofluorescence assay using OKT4/Leu3a (helper/inducer phenotype), OKT8/Leu-2a (cytotoxic/suppressor phenotype), and other cell-specific monoclonal antibodies as previously described (1, 2, 8).

Characterization of HTLV-III. Infected cells were fixed and monitored for expression of viral proteins by indirect immunofluorescence procedures (1,2) using rabbit anti-

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Abbreviations: HTLV-III, human T-cell leukemia/lymphotropic virus type III; AIDS, acquired immunodeficiency syndrome; ARC, AIDS-related complex.

HTLV-III (2, 12) or monoclonal antibodies to HTLV-III p24 (13). Selected cell cultures were tested for the presence of HTLV-III proteins by competition radioimmunoassays using ^{125}I-labeled HTLV-III p24 and specific antisera (12).

HTLV-III proviral DNA was detected in selected cells by Southern blot procedures using cloned HTLV-III DNA as described elsewhere (10). High molecular weight DNA was prepared by standard methods. DNA (5–20 μg) was digested with *Sst* I according to the supplier's recommendations, and DNA was electrophoresed in 0.8 cm thick 0.7% agarose slab gels. Gels were then blotted in 10× NaCl/Cit (1× = 0.15 M NaCl/15 mM sodium citrate, pH 7) onto 0.1-μm nitrocellulose filters (Schleicher & Schuell). Hybridizations were at 37°C for 18 hr in 2.4× NaCl/Cit/40% (vol/vol) formamide/10% (wt/vol) dextran sulfate/0.1% bovine serum albumin/0.1% polyvinylpyrrolidone/0.1% Ficoll/tRNA (20 μg/ml). Filters were washed for 2 hr at 65°C in 1× NaCl/Cit. The *Sst* I–*Sst* I insert from clone λBH-10 (14) was used as probe at 3 × 10^6 dpm/ml (specific activity ≈ 2 × 10^8 dpm/μg). Blots were exposed to Kodak XAR-5 film for 1–3 days.

RESULTS

The description and clinical status of the 101 patients and donors from whom HTLV-III was isolated are summarized in Table 1. The minimum criteria used to identify new HTLV-III isolates were (*i*) release of particulate, Mg^{2+}-requiring, viral reverse transcriptase into cell culture supernatant fluids (1, 2); (*ii*) transmission of virus to cultures of normal human peripheral blood mononuclear cells or to permissive T-cell lines with resulting characteristic cytopathic effects and release of virus (1, 2); and (*iii*) detection of HTLV-III proteins by indirect immunofluorescence assays using virus-specific monoclonal antibody (13) or hyperimmune sera (12).

Virus was isolated from individuals with a wide variety of risk factors and at different stages of disease. In addition to samples from patients with clinically defined AIDS (15, 16) or ARC, samples from clinically healthy individuals considered to be at risk for AIDS were also examined. Peripheral blood and bone marrow mononuclear cells, lymph nodes and brain, saliva, or plasma were processed as described in *Materials and Methods* and were established in cell culture. Virus was isolated from body fluids [e.g., saliva, plasma, or cerebrospinal fluid (data not shown)] by direct transmission or cocultivation of leukocytes with stimulated normal human lymphocytes or permissive T-cell lines (ref. 11 and unpublished work). The ability to isolate virus varied depending on the condition and number of cells received from each patient or donor. However, the overall prevalence (number of isolates/number of patients tested) of virus isolation was approximately 50% for AIDS patients, 85% for ARC patients, and 30% for healthy donors at risk for AIDS. In contrast, HTLV-III was not detected in, nor isolated from, 150 randomly selected normal donors. The ability to isolate virus from several tissues and body fluids from the different patients and donors is summarized in Table 2. The isolation of virus from leukocytes found in the semen of a healthy patient at risk for AIDS (17) and from AIDS patients (18), previously reported by our collaborators, is also included in Table 2.

Examples of the production of HTLV-III by leukocytes established in culture from a HTLV-III-seropositive patient and by normal peripheral blood cells infected *in vitro* are shown in Fig. 1. Following their establishment in cell culture the peripheral blood mononuclear cells, in this instance from a healthy homosexual male, released virus within 2 weeks. It was apparent that the loss of helper/inducer T cells, which requires many months or even years in infected individuals, is greatly accelerated when these cells are cultured *in vitro*. Coincident with the release of virus, a reduction in the number of viable cells remaining in culture was seen. An examination of different cell types demonstrated that the cells chiefly affected were T cells with the helper/inducer pheno-

Table 1. Summary of the isolation of HTLV-III from AIDS and ARC patients and from clinically normal donors at risk for AIDS

Patients or donors	Clinical diagnosis	HTLV-III isolates, no./no. attempted
Homosexual males*	AIDS	36/74
	ARC	21/23
	Normal†	14/56
Nonhomosexual males and females		
Intravenous drug users	ARC	2/3
Hemophiliacs	AIDS	4/6
	ARC	1/2
Transfusion recipients	AIDS	1/2
	ARC	1/1
Juveniles	AIDS	3/6
	ARC	1/1
Mothers of juveniles with AIDS‡	AIDS	1/3
	ARC	1/2
	Normal	2/4
Promiscuous males§	AIDS	2/2
	ARC	6/6
Spouses of AIDS and ARC patients	ARC	1/2
	Normal	3/7
Prostitute	ARC	1/1
Random donors	Normal	0/150

Peripheral blood leukocytes, bone marrow, or lymph nodes were collected, processed, and introduced into *in vitro* culture as described (1, 2). Saliva (11) and plasma (unpublished method) were processed and used to transmit virus directly to fresh or established cell lines in some instances. All but 5 of the AIDS/ARC patients and donors at risk for AIDS reported here were seropositive for antibody to HTLV-III (3, 4, 7). Isolation of virus was described previously (1, 2) and in *Materials and Methods*.

*These include 4 male Haitian immigrants who may or may not be homosexuals.

†These include 1 clinically normal homosexual donor who donated blood to at least three juveniles who subsequently developed immune deficiency.

‡Additional risk factors for AIDS identified in 2 mothers were heterosexual promiscuity (prostitution) and i.v. drug abuse.

§These men were identified in collaboration with R. Redfield, Walter Reed Army Institute of Research, as part of a study of U.S. military personnel.

Table 2. Prevalence and sources of HTLV-III isolates

Tissue source	HTLV-III isolates, no./no. attempted		
	Healthy individuals	ARC patients	AIDS patients
Peripheral blood	16/50	31/38	43/88
Bone marrow	NT	1/6	NT
Lymph nodes	NT	4/4	NT
Brain abscess	NT	NT	2/3
Plasma	NT	3/6	NT
Saliva*	4/6	4/10	0/4
Semen†	1/1	NT	2/2

All donors and patients belong to the risk groups described in Table 1. NT, not tested.

*Most saliva samples were provided by J. E. Groopman (New England Deaconess Hospital, Boston) and a portion of these results were reported previously (11).

†Isolation of HTLV-III from lymphocytes found in semen of patients or high-risk donors was performed in collaboration with D. Ho and M. Hirsch (Massachusetts General Hospital, Boston) (17) and D. Zagury (Univ. of Paris, Paris, France) (18).

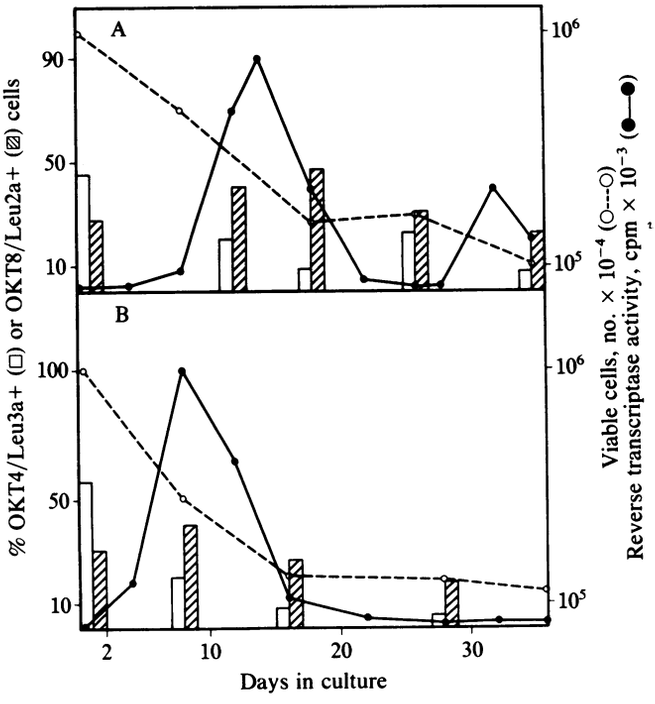

FIG. 1. Release of HTLV-III by cultured cells from a seropositive, healthy, homosexual male (*A*) and by infected fresh normal peripheral blood mononuclear cells (*B*). Patient cells were processed, stimulated, and cultured and virus from an ARC patient was used to infect normal human peripheral blood mononuclear cells as described previously (1, 2).

type (OKT4/Leu3+). Following the initial "burst" of virus production a low number of surviving OKT4/Leu3+ cells were sometimes observed; in these instances, a second release of detectable levels of virus with concomitant cell death was usually seen (Fig. 1).

Likewise, when virus collected from primary patient cells was used to infect cells from fresh peripheral blood, bone marrow, or cord blood, a similar pattern of virus expression with accompanying cell death was seen (Fig. 1). However, the amount of virus released was usually much higher and the destruction of involved cells more rapid and complete than with the primary cell cultures. In addition to demonstrating the presence of infectious virus in patients' cells, the transmission of virus to secondary cell lines enabled the collection of large amounts of virus and infected cells to be used for detailed characterizations and comparisons. In many instances, characteristic HTLV-III retroviruses were visualized by electron microscopic examination of cultured primary and secondarily-infected cells. An example of an infected leukocyte and budding virus is shown in Fig. 2.

The viruses isolated from cells established in cell culture were all found to be antigenically related. As determined by use of HTLV-III-specific monoclonal antibody (13) or hyperimmune antisera (1, 2, 12) and indirect, fixed-cell, immunofluorescence procedures, the percentage of cells in primary cell cultures and in infected fresh peripheral blood cells reacting with these specific antisera usually ranged from 5 to 20% (data not shown). Selected infected cells were further analyzed by more-sensitive, homologous competition radioimmunoassays. A comparison of representative isolates of HTLV-III is shown in Fig. 3. In these experiments, proteins prepared from cells infected with different HTLV-III isolates were used to compete with the precipitation of purified HTLV-III p24 by homologous antibody. The similar slopes of the competition curves obtained for cell lysates from HTLV-III-infected T-cell line H9 (2) and from normal

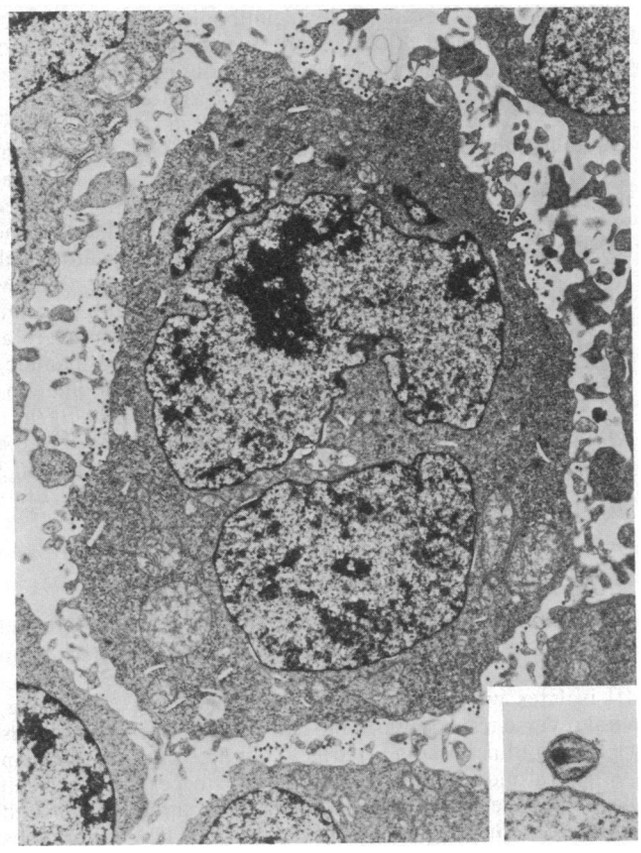

FIG. 2. Transmission electron micrograph of a cell from an ARC patient. Cells were fixed, sectioned, and observed as described (1, 3). (×5400.) (*Inset*) Mature viral particle. (×81,000.)

peripheral blood lymphocytes, infected by virus from AIDS and ARC patients and from a healthy homosexual male, indicate the relatedness of the various isolates. It is apparent from the displacement of the competition curves obtained with some samples that the infected cells contain various amounts of viral antigens.

The relatedness of several virus isolates was also demonstrated by Southern blot hybridization analysis. As shown in Fig. 4, the HTLV-III-specific probe hybridized with DNA from cells infected with virus (*i*) from cells pooled from AIDS and ARC patients (2); (*ii*) from a single AIDS patient; (*iii*) from a juvenile ARC patient; and (*iv*) from a healthy homosexual male. The heterogeneity in the sizes of HTLV-III-related DNA fragments has been reported previously (10) and indicates that minor genome differences exist among some of the viral isolates, although closely related isolates have also been found (unpublished observation in collaboration with G. Shaw and B. Hahn).

DISCUSSION

It is clear from the data presented here that HTLV-III can be isolated from the cells of patients and donors at all stages of the AIDS-disease spectrum. As noted previously (1), isolation of virus is more efficient from ARC patients than from AIDS patients. This likely reflects the relative number and viability of cells carrying the virus in ARC and AIDS patients—e.g., the number of OKT4/Leu3a+ cells is usually lower in AIDS patients. The prevalence of detectable virus in healthy individuals at risk for AIDS also varied depending on the degree of risk. Virus was isolated from 50% (13 of 26) of highly promiscuous homosexual men, whereas it was obtained from only 15% (6 of 41) of donors with a limited

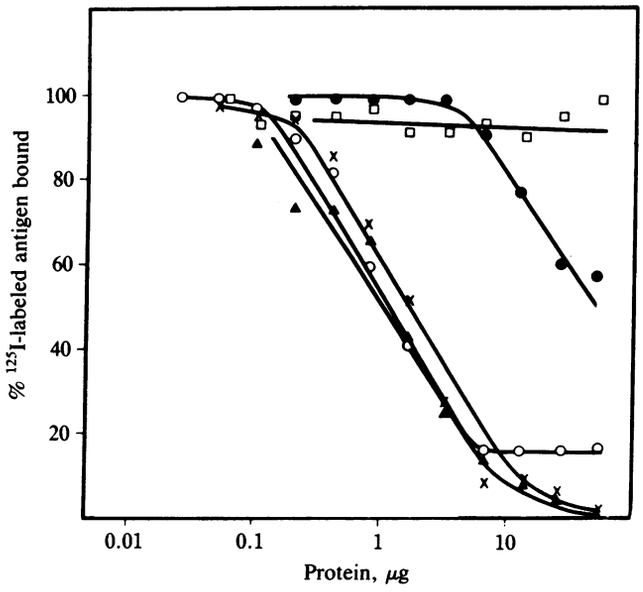

FIG. 3. Homologous competition radioimmunoassay for HTLV-III p24. Precipitation of HTLV-III p24 (^{125}I-labeled) by homologous antisera was assayed in the presence of various amounts of extracts of infected and control cells. Assays were performed as described (3, 4, 7). Extracts tested were from HTLV-III-infected T-cell line H9 (×), uninfected H9 cells (□), normal peripheral blood cells infected by virus from an AIDS patient (○), normal peripheral blood cells infected by virus from a juvenile with lymphadenopathy (●), and normal peripheral blood cells infected by virus from a healthy homosexual male (▲).

contact, such as nonpromiscuous or monogamous homosexual men and other healthy at-risk donors. In both categories, the frequency of isolation was similar to the seropositivity, which is ≈50% for healthy promiscuous homosexual males and ≈30% for healthy nonpromiscuous homosexual males (1, 7, 11).

The number of individuals infected by HTLV-III as indicated by virus isolation (summarized in Table 1) is clearly an underestimate of the true number of these individuals,

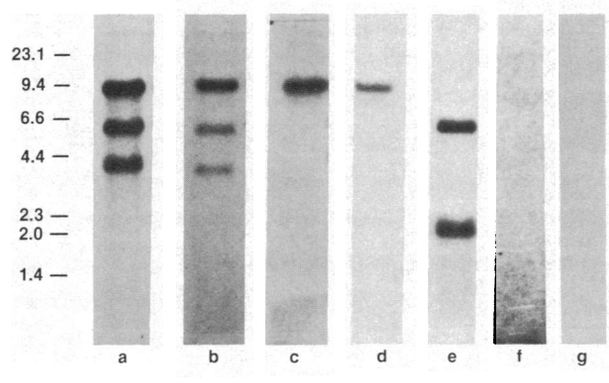

FIG. 4. Southern blot hybridization analysis of DNA from HTLV-III-infected cells. High molecular weight DNA was prepared, digested with *Sst* I, electrophoresed in agarose, blotted onto nitrocellulose, and hybridized with molecularly cloned, ^{32}P-labeled HTLV-III DNA (10, 14). Lanes: a, T-cell line H9, infected by pooled virus from AIDS and ARC patients; b, T-cell line H4, infected by pooled virus from AIDS and ARC patients; c, T-cell line H4, infected by virus from an AIDS patient; d, T-cell line N65, established from a clinically normal homosexual male (donated blood to individuals who subsequently developed AIDS); e, T-cell line JM, infected by virus from a juvenile pre-AIDS patient; f, uninfected T-cell line H9; g, uninfected T-cell line JM. Markers at left (in kilobases) show positions corresponding to fragments of *Hin*dIII-digested phage λ DNA.

particularly those with AIDS, since seroepidemiological studies have demonstrated an exposure to HTLV-III in >90% of AIDS and ARC patients (3, 4, 7). In fact, by using freshly collected specimens and by testing cells from individuals more than once, virus can also be isolated from a larger proportion of patients (data not shown). These observations suggest that individuals with a persistent antibody titer to viral proteins usually harbor cells from which infectious HTLV-III can be isolated. However, it is also possible that in rare instances, an individual could be immunized to viral antigens as a result of frequent exposure to viral antigens. For example, this could conceivably happen with some hemophiliacs who receive repeated treatment with processed blood products. In view of the importance of identifying potential virus carriers, it should be noted that the presence of serum antibodies to HTLV-III is not an absolute determining factor in identifying virus-infected individuals since, in infrequent instances, virus has been isolated from clinically normal, seronegative donors (19).

Even though the majority of virus isolates were from peripheral blood leukocytes, it is clear that other body tissues and fluids contain cells that can harbor infectious virus (Table 2). Although the number of other tissues tested are low relative to the number of peripheral blood samples tested, there is an indication that some (e.g., lymph nodes) may be an even richer source of infected cells. The finding of virus-positive cells in patient semen and of virus in cell-free plasma is also consistent with likely routes of virus transmission in certain high-risk groups, such as homosexuals, heterosexual contacts of high-risk patients and donors, and recipients of blood or blood products (15, 16). However, the presence of virus or virus-positive cells in patient or donor saliva (11) also suggests the possibility of its transmission by nonvenereal mechanisms or without the use of blood or blood products. However, no evidence has been found suggesting an efficient transmission of virus by casual contact only.

All 101 virus isolates were classified as members of the type-III subgroup of HTLV based on their immunological reactivity with specific monoclonal antibody or hyperimmune antisera and by their cytopathic effect on normal peripheral blood mononuclear cells *in vitro*. Six individual virus isolates further characterized by detailed immunological and nucleic acid analyses also showed relatedness to each other. Details of the protein and nucleic acid comparisons of virus isolates of HTLV-III have been presented elsewhere (3, 4, 10, 12, 14).

The frequent isolation of virus from AIDS patients and not from the normal population and the *in vitro* effect of virus on freshly isolated T-cells demonstrate its link to this disease process and support the observation that many of the immune-deficiency syndromes known by other names (ARC, persistent generalized lymphadenopathy, etc.) are likely different manifestations of the same disease process.

Even though it is clear that HTLV-III is causatively involved in the immune disorder AIDS and its prodromes (see ref. 20 for review), the actual mechanism by which it exerts its pathologic effect is not known. Recent observations suggest that the apparent tropism for T4+ lymphocytes by HTLV-III is due to a similarity, if not identity, between the virus receptor and T4 antigens on the surface of helper/inducer T lymphocytes. These antigens are also recognized by OKT4, Leu3a, and other related antibodies (21–23). It is interesting to note that HTLV-III has several properties very similar to other pathogenic viruses, including visna virus (24). Based on the presence of (*i*) genes coding for *trans*-activating proteins (25–27), (*ii*) similar numbers and sizes of structural proteins, (*iii*) similar size and divalent-cation requirements of viral reverse transcriptase, and/or (*iv*) limited protein and nucleic acid homology, in addition to varying degrees of pathogenic effects on infected cells, it is

likely that members of the HTLV group, bovine leukemia virus (BLV), and members of the lentivirus group (e.g., visna virus) form a group of related retroviruses (reviewed in ref. 24).

Genomic diversity among HTLV-III isolates has been reported (10) and was also apparent in the viruses analyzed here, although some very closely related HTLV-III isolates have also been observed (unpublished work in collaboration with G. Shaw and B. Hahn). The reasons for and significance of the genomic differences observed could be important in understanding the etiology and pathogenicity of these viruses and in the control and management of resulting diseases. For example, it would be interesting to see whether specific genomic changes are reflected as differences in clinical status or, alternatively, represent a regional difference in the source of virus. Any of several factors could contribute to the genomic variations observed. For example, HTLV-III is an efficiently replicating virus and the generation of changes in viral genes resulting from rapid replication was noted in other systems (28). Also, differences in life-style or exposure to other agents, including other members of the HTLV group, or in the genetic background of the infected individuals could be involved. The availability of multiple isolates of HTLV-III obtained from such a wide variety of patients and donors from different geographical areas will undoubtedly facilitate the detailed characterization needed to resolve these and other questions concerning the involvement and control of HTLV-III in AIDS and related syndromes.

We gratefully acknowledge the cooperation of many clinical investigators throughout the world in providing patient material, Dr. George Shaw for help with Southern hybridization experiments, and the capable technical assistance of Ms. E. Read-Connole, Mr. S. Kueberuwa, Mr. K. Veren, and Ms. L. Bruch.

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