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E-316-84

PATENT OFFICE
APR 23 1984

APPLICATION FOR UNITED STATES PATENT

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Title: Method of Continuous Production of Retroviruses (HTLV-III) from Patients with AIDS and pre-AIDS

Abstract of the Disclosure

A cell system is disclosed for the reproducible detection and isolation of human T-lymphotropic retroviruses (HTLV-family) with cytopathic effects (HTLV-III) from patients with the acquired immune deficiency syndrome (AIDS), pre-AIDS and in healthy carriers. One neoplastic aneuploid T-cell line derived from an adult with lymphoid leukemia, and termed HT, was susceptible to infection with the new variants of HTLV, which are transformed and providing T-cell populations which are highly susceptible and permissive for HTLV-III, and convenience for large scale production, isolation, and biological detection of the virus.

The present invention describes a cell system for the reproducible detection and isolation of human T-lymphotropic retroviruses (HTLV-family) with cytopathic or cell killing effects (HTLV-III) from patients with the acquired immune deficiency syndrome (AIDS), pre-AIDS and in healthy carriers. One neoplastic aneuploid T-cell line derived from an adult with lymphoid leukemia, and termed HT, was susceptible to infection with the new variants of HTLV, providing T-cell populations which are highly susceptible and permissive for HTLV-III, and convenience for large scale production, isolation, and biological detection of the virus.

Background of the Invention

The disclosure of this invention is contained in the following journal articles: Gallo et al., "Detection, Isolation, and Continuous Production of Cytopathic Human T-Lymphotropic Retroviruses (HTLV-III) from Patients with AIDS and pre-AIDS," Science, in press; and Gallo et al., "Human T-Lymphotropic Retrovirus, HTLV-III, Isolated from AIDS Patients and Donors at Risk for AIDS," in press.

Epidemiologic data strongly suggest that acquired immune deficiency syndrome (AIDS) is caused by an infectious agent which is apparently horizontally transmitted by intimate contact or blood products. Though the disease is manifested by opportunistic infections, predominantly Pneumocystis carcinii pneumonia and Kaposi's sarcoma, the underlying disorder affects the patient's cell-mediated immunity with absolute lymphopenia and reduced helper T-lymphocyte ($OKT4^+$) subpopulation(s). Moreover, before a complete clinical manifestation of the disease occurs, its prodrome, pre-AIDS, is frequently characterized by unexplained chronical lymphadenopathy and/or leukopenia involving a helper T cell subset. This leads to the severe immune

deficiency of the patient, suggesting that a specific subset of T-cells is the primary target for an infectious agent. Although patients with AIDS or pre-AIDS are often chronically infected with cytomegalovirus or hepatitis B virus, for various reasons these appear to be opportunistic or coincidental infections apparently not linked to the immunological response deficiency. It is believed that the cause of AIDS may be a virus from the family of human T-cell lymphotropic retroviruses (HTLV) which, prior to the present invention, comprised two major well characterized subgroups of human retroviruses, called human T-cell leukemia/lymphoma viruses, HTLV-I and HTLV-II. The most common isolate, HTLV-I, is mainly obtained from patients with mature T-cell malignancies. Seroepidemiological studies, in vitro biological effects, and nucleic acid hybridization data indicate that HTLV-I is etiologically associated with these malignancies, affecting adults primarily in the south of Japan, the Caribbean and Africa. HTLV of subgroup II (HTLV-II) was first isolated from a patient with a T-cell variant of hairy cell leukemia. 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 HTLV of both subgroups can sometimes be found in patients with AIDS.

Evidence suggests that the retrovirus(es) of the HTLV family is an etiological agent of AIDS based on the following: (1) there is precedence for an animal retrovirus cause of immune deficiency (feline leukemia virus in cats); (2) retroviruses of the HTLV family are T-cell tropic; (3) they preferentially infect "helper" T-cells ($OKT4^+$); (4) they have cytopathic effects on various human and mammalian cells as demonstrated by their induction of cell syncytia formation; (5) they can alter some T-cell functions; (6) in some cases infection may result in selective T-cell killing; and (7) they are

transmitted by intimate contact or through blood products. The presence of antibodies directed to cell membrane antigens of HTLV infected cells has been shown in sera of more than 40% of patients with AIDS [Essex et al., Science, 220:859 (1983)]. This antigen has since been defined as part of the envelope of HTLV [Schüpbach, et al., Science, in press; and Lee, et al., Proc. Nat. Acad. Sci. USA, in press].

The original detection and isolation of the various HTLV isolates were made possible by two earlier developments: the discovery of T-cell growth factor (TCGF), also called Interleukin 2 (Il-2), which enabled the routine selective growth of different subsets of normal and neoplastic mature T-cells [Ruscetti, et al., J. Immunol., 119:131 (1977); and Poiesz, et al., Proc. Nat. Acad. Sci. USA, 77:6134 (1980)] and the development of sensitive assays for detection of retroviruses based on reverse transcriptase assays. The methods of HTLV isolation and transmission involved a cocultivation procedure using permissive T-cells for the virus. The use of normal human T-cells in cocultivation experiments preferentially yielded HTLV of both subgroups with immortalizing (transforming) capability for some of the target T-cells.

However, HTLV variants (now termed HTLV-III), lack immortalizing properties for normal T-cells and mainly exhibit cytopathic effects on the T-cells and is now believed to be the cause of AIDS. In fact, such variants were frequently but only transiently detected using these normal T-cells as targets in cocultivation or cell-free transmission experiments. The cytopathic effect was overcome by finding a highly susceptible, permissive cell for cytopathic variants of HTLV, thus preserving the capacity for permanent growth after infection with the virus. The present invention

discloses the identification and characterization of this new immortalized T-cell population and its use in the isolation and continuous high- level production of such viruses from patients with AIDS and pre-AIDS.

Early experiments identified one neoplastic aneuploid T-cell line, termed HT, derived from an adult with lymphoid leukemia, that was susceptible to infection with the new cytopathic virus isolates.

This cell line is a sensitive target for transmission of these virus isolates (HTLV-III) and it allows continuous large-scale virus production and development of specific immunologic reagents and nucleic acid probes useful for comparison of these new isolates among themselves and with HTLV-I and HTLV-II. In addition to their differences in biological effects that distinguish them from HTLV-I and HTLV-II, HTLV-III also differs from these known HTLV subgroups in several immunological assays and in morphology. However, these new retroviruses are T4 lymphotropic and exhibit many properties similar to HTLV-I and II, including similar properties of the reverse transcriptase, cross reactivity of structural proteins as determined by heterologous competition radioimmune assays with patients' sera and with animal hyperimmune sera, and induction of syncytia. These new retrovirus isolates are collectively designated HTLV-III, together with detectable differences in some of their proteins and genetic information, HTLV-III's ability to kill T-cells clearly separates these variants from other members of the HTLV family.

Statement of Deposit

A cell line corresponding to the present invention, and denoted H9/HTLV-III$_B$, has been deposited in the ATCC (under ATCC No. CRL 8543) on April

19, 1984, prior to the filing of this patent application. This deposit assures permanence of the deposit and ready accessibility thereto by the public. H9 is a representative and preferred cell line in accordance with the invention.

Utility Statement

The cell line which is a product of the present invention (H9/HTLV-III$_B$) is presently useful for the production of vaccines for the relief of AIDS and for the detection of antibodies to the virus in blood samples.

General Description

A susceptible cell line HT was tested for HTLV before in vitro infection and it was negative by all criteria, including lack of proviral sequences. Continuous production of HTLV-III is obtained after repeated exposure of parental HT cells (3×10^6 cells pretreated with polybrene) to concentrated culture fluids containing HTLV-III harvested from short term cultured T-cells (grown with TCGF) which originated from patients with pre-AIDS or AIDS. The concentrated fluids were first shown to contain particle associated reverse transcriptase (RT). When cell proliferation declined, usually 10 to 20 days after exposure to the culture fluids, the fresh (uninfected) HT parental cells are added to cultures. Culture fluids from the infected parental cell line was positive for particulate RT activity and about 20% of the infected cell population was positive in an indirect immune fluorescent assay (IFA) using serum from a hemophilia patient with pre-AIDS (patient E.T.). Serum from E.T. also contained antibodies to proteins of disrupted HTLV-III but did not

A susceptible cell line HT was tested for HTLV

react with proteins of HTLV-I or HTLV-II infected cells.

Specific Disclosure

As has been mentioned above, an aneuploid HT-cell line exhibited the desired prerequisites for the continuous propagation of HTLV-III. This cell line is a neoplastic aneuploid T-cell line derived from an adult patient with lymphoid leukemia, selected for its mature T-cell phenotype [$OKT3^+$ (62%), $OKT4^+$ (39%) and $OKT8^-$], as determined by cytofluorometry using a fluorescence-activated cell sorter. Cultures of these cells are routinely maintained in RPMI/1640 with 20% fetal calf serum and antibiotics. These cultures are shown in Example 1, Table 1. Clone H9 is preferred, with Clone H4 being secondarily preferred.

HTLV-III culture fluids are isolated from cultured cells of patients with acquired immune deficiency syndrome (AIDS). Peripheral blood leukocytes from these patients are banded in Ficoll-Hypaque, incubated in growth media (RPMI 1640, 20% fetal bovine serum 0.29 mg/ml glutamine) containing 5 ug/ml phytohemagglutinin (PHA-P) for 48 hours, at 37°C in a 5% CO_2 atmosphere. The leukocytes are then refed with growth medium containing 10% purified T cell growth factor (TCGF); optionally, some of the cells also received rabbit antibody to alpha interferon. Cells and growth media from these lymphocytes are then assayed for the presence of HTLV subgroups I-III. Samples exhibiting more than one of the following were considered positive: repeated detection of a Mg^{++} dependent reverse transcriptase activity in supernatant fluids; virus observed by electron microscopy; intracellular expression of virus-related antigens detected with antibodies from sero-positive donors or with hyperimmune serum; or trans-

mission of particles, detected by reverse transcriptase assays or by electron microscopic observation, to fresh human cord blood, bone marrow, or peripheral blood T-lymphocytes. All isolates not classified as either HTLV-I or HTLV-II by immunological or nucleic acid analysis were classified as HTLV-III. The cells in the HTLV-III producing cell cultures, characterized using established immunological procedures, are predominantly T-lymphocytes (E rosette receptor, OKT/3 and Leu/1 positive, with a T4 phenotype (OKT4, leu 3a positive). This process is also described by Gallo, et al., in Science, 220:865-867 (1983).

The infection of parental HT cells as well as other cloned cell populations occurs by exposure of these cells to concentrated or nonconcentrated culture fluids (cell-free infection) from T-cell cultures from AIDS or pre-AIDS patients, or by cocultivation; that is, HT cells are infected by exposure to HTLV-III containing cultures. The usual cell-free infection procedure is as follows: 2 to 5 x 10^6 cells are treated with polybrene (2 µg/ml) or DEAE dextran for 30 minutes in CO_2 incubator at 37°C, and then exposed to the virus inoculum (0.1 to 1 ml) for one hour in the incubator (CO_2/37°C). The cells are kept in suspension by shaking at regular intervals. After one hour of incubation a regular growth medium is added. The positivity of infected cultures for HTLV-III is assessed after one, two, and three weeks of cultivation.

The infection of HT cells (clones) is also obtained by cocultivation procedure--HT cells are mixed in various proportions (usually 1:5) with short- term cultured T-cells (about 5 to 20 days) from AIDS or pre-AIDS patients. The positivity for HTLV-III was scored by the detection of viral antigens or viral nucleic acid sequences in the infected recipient cells

at various intervals (7, 14, 21 days, etc.) after cocultivation. The mixed cultures are maintained in growth medium for several months.

Example 1

As shown in Table 1 below, single cell HT clones were isolated as described by Popovic, et al., in Neoplasma, 18:257 (1971), and Bach, et al., Immunol. Rev., 54:5 (1981) from a long-term cultured aneuploid HT-cell line exhibiting mature T-cell phenotype ($OKT3^+$ [62%], $OKT4^+$ [39%] and $OKT8^-$) as determined by cytofluorometry using a fluorescence-activated cell sorter. The cultures were routinely maintained in RPMI/1640 with 20% fetal calf serum and antibiotics. The terminal cell density of the parental cell culture, seeded at a concentration of 2×10^5 cells/milliliter of culture media, was in the range 10^6-1.5×10^6 cells/ml after 5 days of culture.

For detection of multinucleated cells, cell smears were prepared from cultures 6 and 14 days after infection and stained with Wright-Giemsa. Cells having more than 5 nuclei were considered as multi-nucleated cells. Cloned cells from uninfected cultures also contained some multi-nucleated giant cells as well; however, the arrangement of multiple nuclei in a characteristic ring formation was lacking and the number of these cells was much less (0.7% to 10%).

Immunofluorescence positive cells were washed with phosphate-buffered saline (PBS) and resuspended in the same buffer at concentration 10^6 cells per milliliter. Approximately 50 λ of cell suspension were spotted on slides, air dried, and fixed in acetone for 10 min. at room temperature. Slides were stored at

Table 1

Response of Cloned T-Cell Populations to HTLV-III Infection

Characteristics	Clones							
	H3	H4	H6	H9	H17	H31	H35	H38
Total cell number (x 10^6)								
6 days after infection	1	1.5	1.5	0.3	0.4	0.3	0.5	1.8
14 days after infection	2.2	7.3	7.5	10.0	4.7	5.0	4.5	3.2
Multinucleated cells (%)								
6 days after infection	24	42	32	7	13	14	30	45
14 days after infection	45	48	45	30	22	45	60	60
Immunofluorescence positive cells (%)								
6 days after infection								
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
14 days after infection								
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 (x 10^4 cpm/ml)								
6 days after infection	2.4	1.8	2.1	4.1	2.6	1.4	1.7	2.5
14 days after infection	16.2	18.1	16.1	20.2	17.1	13.4	15.1	18.2

ND = not done

-20°C until use. Twenty microliters of either hyperimmune 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 min. at 37°C. The fluorescein-conjugated antiserum to rabbit or human immunoglobulin G was diluted and applied to the fixed cells for 30 min. at room temperature. Slides then were washed extensively before microscopic examinations. The uninfected parental cell line as well as the clones were consistently negative in these assays.

To determine reverse transcriptase activity, virus particles were precipitated from cell-free supernatant as follows: 0.4 ml of 4M NaCl and 3.6 ml of 30% (wt/vol.) 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 rpm at 4°C for 30 min. The precipitate was resuspended in 300 ul at 50% (vol/vol) glycerol (25 mM Tris-HCl, pH 7.5/5mM dithiothreitol/150 mM KCl/0.025% Triton X-100. Particles were disrupted by addition of 100 ul of 0.9% Triton X-100/1.5M KCl. Reverse transcriptase (RT) assays were performed as described by Poiesz, et al., Proc. Nat. Acad. Sci. USA, 77:7415 (1980) and expressed in cpm per milliliter culture medium.

Example 2

As shown in Table 2 below, cocultivation with H4 recipient T-cell clone was performed with fresh mononuclear cells from peripheral blood of patients RF and SN, respectively. In the case of patients BK and LS cocultivation was performed with T-cells grown in the presence of exogenous TCGF (10% V/V) for 10 days. The ratio recipient/donor (patients') cells was 1:5. The mixed cultures were maintained in RPMI/1640 (20% FCS and

Table 2

Isolation of HTLV-III from Patients with Pre-AIDS and AIDS

Patient	Diagnosis	Origin	Virus Expression: RT Activity (x 10 cpm)	Virus Expression: IFA with Rabbit Serum (% Positive)	Virus Expression: IFA with Human Serum (ET) (% Positive)	EM
RF	AIDS (heterosexual)	Haiti	0.25	80	33	ND
SN	Hemophiliac (lymphadenopathy)	U.S.	6.3	10	ND	+
3K	AIDS (homosexual)	U.S.	0.24	44	5	+
.S	AIDS (homosexual)	U.S.	0.13	64	19	+
'T	Hemophiliac (lymphadenopathy)	U.S.	3.2	69	ND	ND

RT = reverse transcriptase
IFA = immunofluorescence assays
EM = electron microscopy
ND = not done

antibiotics) in the absence of exogenous TCGF. Cell-free infection of H9 T-cell clone was performed using concentrated culture fluids harvested from T-cell cultures of the patient WT. The T-cell cultures were grown in the presence of exogenous TCGF for two weeks before the culture fluids were harvested and concentrated. Cells of H9 clones were pretreated with polybrene (2 ug/ml) for 20 min. and 2 x 10^6 cells were exposed for one hr. to 0.5 ml of 100-fold concentrated culture fluids positive for particulate RT activity.

HTLV-III virus expression in both cocultured and cell-free infected cell cultures were assayed approximately one month after in vitro cultivation. There was considerable fluctuation in HTLV-III expression (see Table 2). For details of both reverse transcriptase (RT) assays and indirect immunofluorescence assays (IFA) see Example 1.

Example 3

To select for high permissiveness for HTLV-III and to preserve permanent growth and continuous production of virus, extensive cloning of the HT parental T-cell population was performed. A total of 51 single-cell clones was obtained by both capillary and limited dilution techniques using irradiated mononuclear cells from peripheral blood of a healthy donor as a feeder. The growth of these cell clones was compared after HTLV-III infection. A representative example of response to virus infection of 8 T-cell clones which are susceptible to and permissive for HTLV-III is shown in Table 1. In parallel experiments, 2 x 10^6 cells of each T-cell clone were exposed to 0.1 ml of concentrated virus. Then cell growth and morphology, expression of cellular viral antigen(s), and RT activity in culture fluids were assessed 6 and 14 days after infection.

Although all 8 clones were susceptible to and permissive for the virus, there were considerable differences in their ability to proliferate after infection. The cell number decreased by 10% to 90% from the initial cell count within 6 days after infection, and a high proportion of multinucleated (giant) cells were consistently found in all 8 infected clones. The percentage of T-cells positive for viral antigen(s) determined by immunofluorescent assays with serum from AIDS patient (E.T.) and with hyperimmune rabbit serum raised against the whole disrupted HTLV-III ranged from 10% to over 80%. Fourteen days after infection, the total cell number and the proportion of HTLV-III positive cells increased in all 8 clones. The virus positive cultures consistently showed round giant cells which contained numerous nuclei. These multinucleataed giant cells are similar to those induced by HTLV-I and HTLV-II except that the nuclei exhibit a characteristic ring formation. Electron microscopic examinations showed that the cells released considerable amounts of virus.

Example 4

To determine whether HTLV-III is continuously produced by the infected T-cells in long-term cultures, both virus production and cell viability of the infected clone, H4, were followed for several months. Although the virus production fluctuated, culture fluids harvested from the H4/HTLV-III cell cultures at approximately 14-day intervals consistently exhibited particulate RT activity which has been followed for over 5 months. The viability of the cells ranged from 65% to 85% and doubling time of the cell population, which is called H4/HTLV-III, was approximately 30-40 hours. Thus, this permanently growing T-cell population can continuously produce HTLV-III.

The yield of virus produced by 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. The highest RT activity was found at density 1.16 g/ml, similar to other retroviruses.

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APPLICATION FOR UNITED STATES PATENT

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Title: Serological Detection of Antibodies to HTLV-III in Sera of Patients with AIDS and pre-AIDS Conditions

Abstract of the Disclosure

This invention relates to the detection of antibodies in sera of AIDS and pre-AIDS patients and describes the biochemical and immunological analysis of antigens associated with the virus HTLV-III. It is shown that antigens associated with the infection of human cells by this virus are specifically recognized by antibodies from AIDS patients. Specifically, HTLV-III isolated from AIDS patients and transformed by cocultivation with an HT cell line is specifically detected by antibodies from human sera taken from AIDS patients. The method of detection of antibodies preferred is a strip radioimmunoassay (RIA) based on the Western Blot technique or ELISA (an enzyme-linked immunosorbent assay) or indirect immunofluorescence assay.

This invention relates to the detection of antibodies in sera of AIDS and pre-AIDS patients and describes the biochemical and immunological analysis of antigens associated with the virus HTLV-III. It is shown that antigens associated with the infection of human cells by this virus are specifically recognized by antibodies from AIDS patients. Specifically, HTLV-III isolated from AIDS patients and transmitted by cocultivation with an HT cell line is specifically detected by antibodies from human sera taken from AIDS patients. The detection method preferred is a strip radioimmunoassay (RIA) based on the Western Blot technique and ELISA (an enzyme-linked immunosorbent assay).

Statement of Deposit

A cell line corresponding to the present invention, and denoted H9/HTLV-III$_B$, has been deposited in the ATCC (under ATCC No. CRL 8543) on April 19, 1984, prior to the filing of this patent application. This deposit assures permanence of the deposit and ready accessibility thereto by the public. H9 is a representative and preferred cloned cell line in accordance with the invention.

Description of the Figure

The figure illustrates identification of HTLV-III antigens recognized by sera of AIDS patients.

Background of the Invention

A family of T-lymphotropic retroviruses causes T-cell proliferation leukemia, T-cell depletion , and immunosuppression in humans infected by the viruses. These retroviruses are known as the HTLV family of T4 tropic retroviruses. Subgroup HTLV-I causes T-cell

proliferation in leukemia; subgroup HTLV-II induces T-cell proliferation in vitro but its role in disease is unclear. A third group of related virus, collectively designated HTLV-III, has now been isolated from cultured cells of patients with acquired immune deficiency syndrome (AIDS). The biological properties of HTLV-III and immunological analysis of its proteins show that this virus is a member of the HTLV family and closely related to HTLV-II. Sera of 88% of patients with AIDS and of 79% of homosexual men with pre-AIDS, but less than 1% of heterosexual donors have antibodies reactive against antigens of HTLV-III. The major immune reactivity appears to be directed against p41, a 41,000 m.w. protein, believed to be an envelope antigen of the virus.

Acquired immune deficiency syndrome (AIDS) is a relatively recently recognized disease evident in several parts of the world. Its overwhelming prevalence among homosexual men with multiple sexual partners, illegal intravenous drug abusers, hemophiliacs, blood transfusion recipients, and close heterosexual contacts of members of the above high-risk groups strongly suggests that the disease spreads by the transmission of an infectious agent. The primary targets of affliction in the human body are specific subpopulations of T-cells. The severe immune deficiency of these patients results from an unusually low proportion of helper T-cells (T4) in their lymphocyte population, thus reducing the availability of many T4 helper functions, among which is the production of antibodies by B-cells.

Retrovirus infection is known to lead to depressed immune functions in animal systems. Analogizing the human response to these non-animal systems, a human retrovirus with a tropism for T-cells was considered a candidate in the etiology of human AIDS. As mentioned above, several members of a family of human

T-lymphotropic retroviruses (HTLV) have been isolated. One of these isolates was obtained from a black American with an aggressive form of T-cell lymphoma. This virus, designated HTLV-I, has been etiologically linked to the pathogenesis adult T-cell leukemia/lymphoma (ATLL). In vitro infection with HTLV-I can alter T-cell function and, in some cases, leads to T-cell death. Another member of the HTLV family was isolated from a patient with a T-cell variant of hairy cell leukemia and was designated HTLV-II. Isolation of HTLV-I and HTLV-II have been reported from cultured T-cells of patients with AIDS. Isolation of another retrovirus was reported from a homosexual patient with chronic generalized lymphadenopathy, a syndrome that often precedes AIDS and therefore referred to as "pre-AIDS." Proviral DNA of HTLV-I was detected in the cellular DNA of two AIDS patients, and sera of some patients were shown to react with antigens of HTLV-I. The correlation between AIDS and serum antibodies to HTLV-I protein is weak. The present invention shows that the primary cause of the syndrome is a human T-lymphotropic retrovirus variant with limited cross reactivities with the known HTLV-subgroups. These new variants are designated HTLV-III and are the subject of the present invention. Disclosed is the use of this virus in an immunological screening of sera of patients with AIDS, pre-AIDS, and individuals at increased risk for AIDS.

HTLV-III was purified from supernatants of cell cultures supporting the continuous production of these cytopathic viruses. These HTLV variants (HTLV-III) lack immortalizing (transforming) properties for normal T-cells and mainly exhibit cytopathic effects on the T-cell helper. The cytopathic effect was overcome by finding a highly susceptible, permissive cell for cyto-
pathic variants of HTLV, thus preserving the capacity

for permanent growth after infection with the virus. These cell cultures allow for the continuous production of the HTLV-III virus. Cell line HT is infected with HTLV-III virus in order to obtain a reproducible source of the virus. Antigens associated with the virus obtained from these cultures are reacted with human sera from suspected AIDS patients. Assays by the Western Blot technique and ELISA technique determine whether the patient examines positive for AIDS.

General Description

Incorporated by reference is a copending application of the same inventorship entitled, "Method of Continuous Production of Retroviruses (HTLV-III) from Patients with AIDS and pre-AIDS."

Lysates of immortalized human T-cell clones, H9, to which HTLV-III has been transmitted by cocultivation with lymphocytes from AIDS (designated H9/HTLV-III_B) were tested with human sera in a strip radioimmunoassay (RIA) based on the Western Blot technique. The sera used for the analysis were also tested by ELISA with purified HTLV-III. Sera from patients with AIDS and from some homosexuals and heroin-addicts recognized a number of specific antigens not detected by any other means. The most prominent reactions are with antigens of about Mr 41,000. In short, the antigens associated with HTLV-III virus produced by HT cells permits the detection of antibodies in AIDS and pre-AIDS patients. This virus-infected cell line also makes possible the detection of AIDS and pre-AIDS in other samples of human sera, such as donated blood.

As is indicated above, the major immune reactivity or specificity is directed against p41, a

the HTLV-III virus. It is believed that additional purification and refinement of p41 might lead to an even more sensitive ELISA assay. The figure illustrates the noted specificity for p41. Although p41 appears to be the prominent antigen, sera from patients with AIDS, some homosexuals, and heroin addicts recognize a number of specific antigens not detected in normal sera. These antigens are about Mr 65,000 (p65), 60,000 (p60), 55,000 (p55), and 24,000 (p24). Although other antigens were detected, these were the most significant. Example 4 illustrates the specificity of these rections.

Specific Disclosure

HTLV-III was concentrated by ultracentrifugation from virus producer culture supernatants (H9/HTLV-III$_B$) and after careful removal of lipids and cell debris by centrifugation through a cushion of 20% (W/W) sucrose in TNE buffer (10 mM Tris-NaCl, pH 7.4, 0.1 M NaCl and 0.001 mM EDTA) was purified by equilibrium density banding through a linear gradient of 20-60% sucrose (W/W) in TNE. The gradient is divided into several small fractions and the virus band located by assaying aliquots of each fraction for HTLV-III-specific reverse transcriptase activity. This produces the antigen component (HTLV-III) suitable for use in ELISA or Western Blot assay procedures.

Radioimmunoassay techniques for detecting antibodies include radiolabeled assays of the so-called blot technique, such as the Western Blot technique exemplified by Example 2, post.

Also operable and most preferred for the detection of antibodies are the enzyme-linked immunosorbent assay (ELISA) shown in Example 1.

Thirdly, antibodies to HTLV-III may be detected by an indirect immunofluorescence assay. See Example 3 for this technique. This assay is significant because it uses the infected T-cell as a starting material. ELISA and Western Blot techniques start with the HTLV-III virus.

Antibodies to HTLV-III are detected in sera of patients with AIDS and pre-AIDS lymphadenopathy syndrome by the process of the present invention. Example 1 is a detailed description of the present invention using the ELISA technique. Example 2 is a detailed description of the present invention using the Western Blot technique.

In general, the ELISA technique involves reacting a lysate of density-banded HTLV-III to the test sera, blood taken from a human patient. The mixture is then incubated with a peroxidase labeled antibody. Any wells positive for the presence of AIDS antibodies forms a detectable and measurable color product.

As indicated above, antibodies to HTLV-III may also be detected in sera of patients with AIDS or pre-AIDS by means of the Western Blot technique. HTLV-III is lysed and electrophoretically fractionated on a polyacrylamide slab gel. Protein bands on the gel are then electrophoretically transferred to a nitrocellulose sheet. Strip solid phase radioimmunoassays have been performed. Test sera obtained from human patients suspected of contraction with AIDS is then added to tubes containing the above described strips. Another antibody of ^{125}I labeled goat anti-human immunoglobulin is added to the reaction strips which are then exposed to X-ray film. Strips positive for the presence of AIDS antibodies exhibit wide bands at the 41,000 Mr location.

ing a lysate of density-banded HTLV-III to the test

Example 1

Antibodies to HTLV-III in Sera of Patients with AIDS and pre-AIDS Lymphadenopathy Syndrome

Wells of 96-well plates were coated overnight with a lysate of density-banded HTLV-III at 0.5 µg protein per well in 100 µl 50 mM sodium bicarbonate buffer, pH 9.6. The wells were washed with water and incubated for 20 min. with 100 µl of 5% bovine serum albumin in phosphate buffered saline (PBS). After washing, 100 µl of 20% normal goat serum in PBS were added to each well, followed by 5 or 10 µl of the test sera (blood taken from a human patient), and allowed to react for 2 hr. at room temperature. The wells were washed three times with 0.5% Tween-20 in PBS in order to remove unbound antibodies and incubated for 1 hr. at room temperature with peroxidase labeled goat anti-human IgG at a dilution of 1:2000 in 1% normal goat serum in PBS. Goat anti-human IgG is a second antibody that binds with the antibody antigen complex formed in positive wells. The wells were successively washed 4 times with 0.05% Tween 20 in PBS and 4 times with PBS to remove unbound goat antibody and reacted with 100 µl of the substrate mixture containing 0.05% orthophenylene diamine and 0.005% hydrogen peroxide in phosphate-citrate buffer, pH 5.0. This substrate mixture detects the peroxidase label and forms a color product. The reactions were stopped by the addition of 50 µl of 4N H_2SO_4 and the color yield measured using an ELISA reader which quantifies the color reading. Assays were performed in duplicate; absorbance readings greater than three times the average of 4 normal negative control readings were taken as positive. The results are shown in Table 1.

Table 1

Subjects	No. Positive/ No. Tested	Percent Positive
Patients with AIDS	43/49	87.8
Pre-AIDS	11/14	78.6
Intravenous Drug abusers	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	0
Patients with Rheumatoid arthritis	0/1	0
Patients with Systemic lupus erythematosus	0/6	0
Patients with acute mononucleosis	0/4	0
Patients with lymphatic leukemias	0/8	0

Example 2

Western Blot analysis of the test sera was conducted as follows. HTLV-III was lysed and fractionated by electrophoresis on a 12% polyacrylamide slab gel in the presence of sodium dodecylsulfate (SDS). The protein bands on the gel were electrophoretically transferred to a nitrocellulose sheet, according to the procedure of Towbin et al., Proc. Natl. Acad. Sci. USA, 76:4350 (1979). Strip solid phase radioimmunoassays were then performed. The sheet was incubated at 37° for 2 hr. with 5% bovine serum albumin in 10 mM Tris-HCl, pH 7.5 containing 0.9% NaCl and cut into 0.5 cm strips. Each strip was incubated for 2 hr. at 37° and 2 hr. at room temperature in a screw cap tube containing 2.5 ml of buffer 1 (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2 M NaCl, 0.3% Triton X-100 and 2 mg/ml bovine serum albumin and 0.2 mg/ml of human antibody fractions, Fab). Test sera (25 µl), taken from human patients with AIDS or exhibiting pre-AIDS symptoms, were then added to individual tubes containing the strips and incubation continued for 1 hr. at room temperature and overnight in the cold. The strips were washed three times with solution containing 0.5% sodium deoxycholate, 0.1 M NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride and 10 mM sodium phosphate, pH 7.5. The strips were incubated for 1 hr. 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 anti-human immunoglobulin (µ chain and Fc fragment) (1.25×10^6 cpm) were added to the reaction mixture and the incubation continued for 30 min. at room temperature. The strips were wshed as described, dried, mounted and exposed to X-ray film. The figure indicates graphically the results of these experiments. Strip 1 is test sera from an adult with T-cell leukemia; Strip 2 is a normal donor; Strip 3 is a mother of a child with AIDS; Strips

4 and 6-10 are AIDS patients; and Strip 5 is a patient with pre-AIDS.

Example 3

Fixed Cell and Live Cell Indirect Immunofluorescence Assay for Antibodies to HTLV-III

Indicator cell - HTLV-III infected negative cells; negative control - uninfected T-cells.

Infected cells were washed with phosphate buffered saline (PBS) and resuspended in PBS at 10^6 cells/ml. Approximately 50 ml of cell suspension were spotted on a slide, air dried, and fixed in acetone for 10 min. at room temperature. Slides were stored at -20°C until ready for use. 20 ml of the test human serum diluted 1:10 in PBS were added to the fixed cells and incubated for 1 hr. at 37°. Slides were washed and reacted for 30 min. at room temperature with a dilute solution of fluorescein-conjugated goat anti-human IgG. Slides were again washed and examined under a microscope for fluorescence. A negative control used uninfected parental cells. The above describes a fixed cell system in which the antibody antigen reaction is sought for both inside and outside the cell.

For live cell immunofluorescence assay all the above reactions were done in a tube instead of on the slide, but without chemical fixation of the cells. After reaction with the fluorescein conjugated anti-human antibody, the cells were added to the slide and examined under a microscope for antibody-antigen reaction on the surface of the cell.

10 min. at room temperature. Slides were stored at

The results of each of these assays show a strong fluorescence reaction specifically with sera of AIDS and pre-AIDS patients.

Example 4

Sera of patients with clinically documented AIDS, Kaposi's sarcoma, sexual contacts of AIDS patients, intravenous drug abusers, and homosexual men and heterosexual donors were tested for their reactivity to HTLV-III. The system employed was ELISA. Lysates of sucrose density banded HTLV-III were coated on 96-well microtiter plate wells. Test sera were diluted with a dilute solution of normal goat serum, added to the wells, and allowed to react for 2 hr. or overnight at room temperature. The antibodies in the human sera were detected by the reaction of the primary immune complex with peroxidase labeled goat anti-human immunoglobulins followed by the development of a colored peroxidase reaction product. The results obtained are presented in Table 1. Of 49 clinically diagnosed AIDS patients, 43 (88%) showed serum reactivity in this assay. Fourteen homosexual men with pre-AIDS were also tested for antibodies to HTLV-III. Of these, 11 (79%) were positive. Among 17 homosexual men with no clinical symptoms of AIDS, 7 scored positive. Of these, at least one was known to be a long-time sexual partner of a diagnosed case of AIDS. One had persistent fatigue and was exhibiting early signs of AIDS. One of the three intravenous drug abusers that were positive for serum antibodies to HTLV-III was also a homosexual.

In contrast, only 1 of 186 controls tested reacted positive in this test. They included 3 with hepatitis B virus infection, 1 with rheumatoid arthritis, 6 with systemic lupus erythematosus, 4 with acute mononucleosis and 8 with various forms of

lymphatic leukemias and lymphomas, some of whom were positive for HTLV-I. The remaining test subjects were normal donors of unknown sexual preference, including laboratory workers ranging in age from 22 to 50.

Example 5

To investigate the specificity of the reactions, lysates of the HTLV-III-infected cell clones were analyzed in comparison with lysates of the same cell clones before viral infection. No antigen was found reactive in the uninfected clones, with the exception of a Mr 80,000 band in H17 which bound antibodies from all human sera tested, but not from rabbit or goat serum. Antigens newly expressed after viral infection and recognized by the human serum used for this analysis include p65, p55, p41, p39, p32, and p24. In addition, a large protein of approximately Mr 130,000 and one of 48,000 were detected. With normal human serum, none of the antigens was detected. These results show that the antigens detected are either virus-coded proteins or cellular antigens specifically induced by viral infection.

S.N. 6-602,945

WE CLAIM

1. A method for the detection of antibodies of HTLV-III in patients with AIDS or risk of AIDS (pre-AIDS) which comprises utilizing a radioimmunoassay system of detection for antibodies.

2. The method according to Claim 1 wherein the HTLV-III is used in the presence of HT neoplastic aneuploid T-cell.

3. The method according to Claim 1 wherein the method of detection is selected from ELISA, Western Blot and indirect immunofluorescence assay methods using HTLV-III.

4. The method according to Claim 1 wherein a 41,000 m.w. fraction of HTLV-III is utilized.

5. A method of diagnosing antibodies in sera and synthetic blood products of AIDS and pre-AIDS patients by use of an enzyme linked immunosorbent assay (ELISA) using extract of HTLV-III.

6. The method of Claim 5 wherein HTLV-III was concentrated by ultracentrifugation from the virus culture supernatants; lipids were removed by centrifugation through 20% (W/W) sucrose in TNE buffer and the gradient was divided into fractions and virus bands located by assaying aliquots of each fraction for HTLV-III-specific reverse transcriptase activity.

7. A therapeutic AIDS specific test kit for antibodies detection comprising a compartmented enclosure containing a plurality of wells, plates which are coated prior to use with HTLV-III and ELISA materials for enzyme detection consisting of normal goat serum and

4. The method absorbing IgG and nuclear

change indicator consist-14-of orthophenylene diamine and hydrogen peroxide in phosphate citrate buffer.

8. The kit according to Claim 7 wherein the HTLV-III is present in the form of a lysate.

9. A therapeutic AIDS specific test kit for detecting antibodies using the Western Blot technique comprising a container, a cover, and therein containing a nitrocellulose sheet and a polyacrylamide slab gel in the presence of sodium dodecylsulfate, and additionally surfactants as well as pH modifiers and bovine serum albumin and human Fab, and additionally this Western Blot analysis container also contains a supply of dilute normal goat serum and I^{125} labeled goat antihuman immunoglobulin and a source of HTLV.

10. An AIDS specific test kit for detecting antibodies using the indirect immunofluorescence assay comprising a compartmental container, human test serum containing HTLV-III, phosphate buffered saline, and fluorescein-conjugated goat antiserum IgG.

11. A method of testing for HTLV-III in AIDS and pre-AIDS in sera of human patients according to Claim 1 wherein said patients are specially selected being in the pre-AIDS stage or initial stage of the illness.

S.N. 6-602,946

WE CLAIM

1. A cell line containing HTLV-III designated H9/HTLV-III$_B$, ATCC Acsession CRL 8543.

2. A method for continuous production of HTLV-III virus which comprises infecting highly susceptible, permissive cells consisting of a neoplastic aneuploid T-cell line with said virus, said cells preserve the capacity for permanent growth after the infection with said virus.

3. The method of Claim 2 wherein said virus consists of cytopathic variants of HTLV.

4. The method of Claim 2 wherein said infecting comprises cocultivating said virus with said cells.

5. The method of Claim 2 wherein said infecting comprises cell-free infection of said cells with said virus.

6. The method of Claim 2 wherein said cells are neoplastic aneuploid T-cells derived from an adult with lymphoid leukemia.

7. A method of producing a cell line containing an antigen of HTLV-III which comprises infecting a T-cell line derived from lymphoid leukemia and susceptible to infection with HTLV-III, said cell line capable of continuous large-scale production of HTLV-III.

8. The method of Claim 7 wherein said cell line is a neoplastic aneuploid T-cell line.

9. The method of Claim 8 wherein said infecting

9. The method of Claim 7 wherein said HTLV-III are variants of human T-lymphotropic retrovirus, exhibit cytopathic effects and are non-transforming.

10. A process for producing a cell line H9/HTLV-III$_B$ which comprises infecting a target T-cell with HTLV-III virus, said infecting process overcomes the normal cytopathic effect of HTLV-III and preserves the immortal growth capacity of the target cell.

11. A process for immortalizing HTLV-III virus which comprises cocultivating said virus with a target T-cell.

12. The process of Claim 11 wherein said target T-cell is a neoplastic aneuploid T-cell susceptible to infection with HTLV-III.

13. A process for the continual production of HTLV-III by infecting T-cells in high producing long-term cultures which comprises cocultivating HTLV-III virus with an HT-clone, said clone being an aneuploid T-cell line derived from lymphoid leukemia.

14. The process in Claim 13 wherein said clone is a mature T-cell phenotype of OKT3$^+$ (62%), OKT4$^+$ (39%) and OKT8$^-$.