6. R. Redfield *et al.*, *N. Engl. J. Med.*, in preparation. ARC has also been termed pre-AIDS, AIDS syndrome, and lymphadenopathy syndrome.
7. J. Schüpbach *et al.*, *Science* **224**, 503 (1984); M. G. Sarngadharan *et al.*, *ibid.*, p. 506.
8. F. Barré-Sinoussi *et al.*, *Science* **220**, 868 (1983).
9. J. C. Montagnier *et al.*, in *Human T-Cell Leukemia Viruses*, R. C. Gallo, M. Essex, L. Gross, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984), p 363–379.
10. V. S. Kalyanaraman *et al.*, *Science* **225**, 321 (1984).
11. B. Safai *et al.*, *Lancet* **1984-I**, 1438 (1984).
12. D. P. Francis, M. Essex, W. D. Hardy, *Nature (London)* **269**, 252 (1977); D. P. Francis *et al.*, *J. Clin. Microbiol.* **9**, 154 (1979).
13. P. D. Markham *et al.*, *Int. J. Cancer* **33**, 13 (1984).
14. H. Towbin, T. Staehlin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979); M. G. Sarngadharan *et al.*, in preparation.
15. J.E.G. is supported in part by a grant (JFRA 44) from the American Cancer Society. M.G. is supported by PHS contract NOI-CO-23910.
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HTLV-III in Cells Cultured from Semen of Two Patients with AIDS

Abstract. *Epidemiological results suggest that the etiological agent of the acquired immune deficiency syndrome (AIDS) is transmitted primarily through blood products, semen, and saliva. There is evidence that the human T-cell leukemia (lymphotropic) virus type III (HTLV-III) is this agent. HTLV-III has been isolated repeatedly from T cells obtained from peripheral blood or lymph node tissue of AIDS and pre-AIDS patients and of healthy people believed to have been exposed to the virus. In the present study, HTLV-III was detected in and isolated from T cells present in the seminal fluid of AIDS patients. Mononuclear cells from the semen of AIDS patients and normal individuals were cultured in the presence of T-cell growth factor (interleukin-2). After 6 to 8 days, HTLV-III antigens were transiently expressed by the cells from the AIDS patients but not by those from the normal individuals. When the mononuclear cells from the semen of AIDS patients were cocultured with a permissive human T-cell line, cell cultures were produced that expressed high levels of reverse transcriptase activity, showed retroviral particles by electron microscopy, and were positive for HTLV-III–specific antigens when tested by fixed-cell indirect immunofluorescence with the use of monoclonal antibodies to the p24 and p15 antigens of HTLV-III.*

Several findings indicate that the primary cause of the acquired immune deficiency syndrome (AIDS) is an infection with the human T-cell leukemia (lymphotropic) virus type III (HTLV-III) (*1–5*). Almost all patients with AIDS and related conditions have detectable specific serum antibodies to a member or members of this retrovirus family (*4–6*), and 48 isolates of HTLV-III were reported in one study (*3*). More than 95 isolates of HTLV-III have since been obtained (*7*). Epidemiological data indicate that transmission of the AIDS agent is through blood or blood products (*8*) or through intimate contact between homosexual males (*9*) or between heterosexual females and their male partners that have been exposed to the agent (*10*). Such data, obtained mostly from surveys of populations at risk within the United States, together with the observed lymphotropism of the causative virus, led us to suspect that lymphocytes infected with HTLV-III might be found in the semen of AIDS patients. In the study reported here we attempted to answer the following question: Can the lymphocytes from the seminal fluid of AIDS patients be grown in vitro and, if so, can HTLV-III be detected in and isolated from these cells?

Semen was obtained from two patients with AIDS and stored frozen in 10 percent dimethyl sulfoxide in liquid nitrogen. Both patients had disseminated Kaposi's sarcoma, low numbers of circulating T4 lymphocytes, and reversed T4/T8 ratios (0.5 and 0.2, respectively). Semen was also obtained from three healthy heterosexual males. The semen was collected under sterile conditions, thawed at 37°C, and subjected to low-speed centrifugation. Cell pellets were resuspended in RPMI 1640 medium containing 10 percent fetal calf serum. After thawing, a mononuclear cell–enriched fraction was isolated over Ficoll-Hypaque (Seromed, München, FRG). The number of cells obtained in this manner varied from 0.8×10^5 to 3×10^5 per milliliter in the semen of normal donors. The mononuclear cell–enriched fractions from AIDS patients 1 and 2 yielded 0.06×10^5 and 0.9×10^5 cells per milliliter, respectively. These fractions contained 70 percent of adherent macrophage-monocytes and 20 to 30 percent lymphocytes and residual spermatozoa (Fig. 1). The lymphocytes from the normal individuals consisted of 17 percent $T4^+$ cells (OKT4), 30

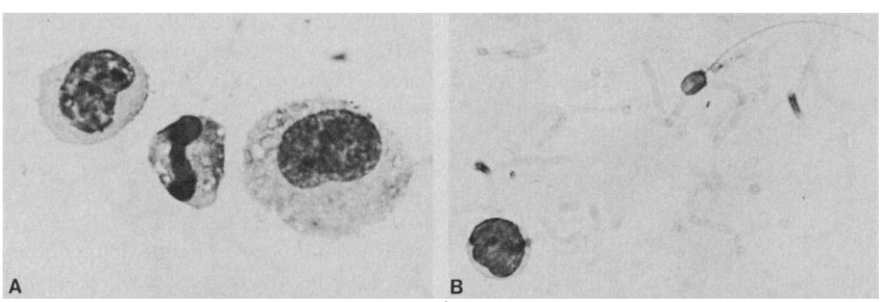


Fig. 1 (above). Mononuclear cells grown from the semen of AIDS patients. A mononuclear cell–enriched fraction was obtained after centrifugation over a Ficoll-Hypaque gradient. Portions were then smeared on a slide and stained (Giemsa-Wright). Note the lymphocytes in (A) and residual spermatozoa in (B). Fig. 2 (right). Reverse transcriptase activity in H9/AIDS semen cell cocultures. The RT was assayed in cell-free culture supernatants. Culture fluid (1 ml) was centrifuged for 10 minutes at 300*g*. Virus particles were precipitated from cell-free supernatants and the RT assay was performed as described (*3*). Samples were collected at different periods (days 2 to 14) after initiation of coculture. Culture fluids from H9 and H9/HTLV-III cells were collected according to the same procedure. Results are expressed in counts per minute per milliliter of culture medium. H9 is the cloned human T-cell leukemic line and H9/HTLV-III is the same cell infected with and producing HTLV-III. H9-AS1 and H9-AS2 are the cocultures of H9 cells with mononuclear cells derived from the semen of AIDS patients 1 and 2.

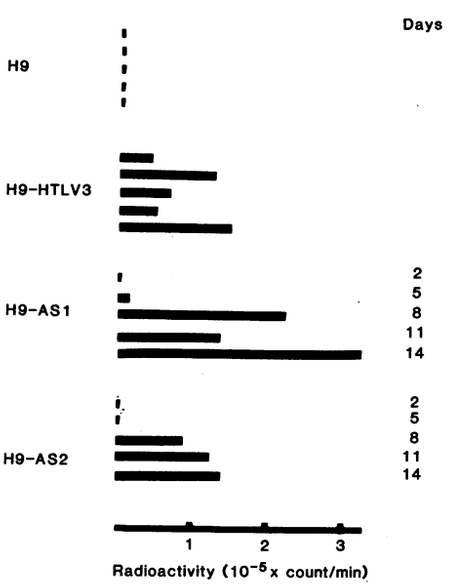
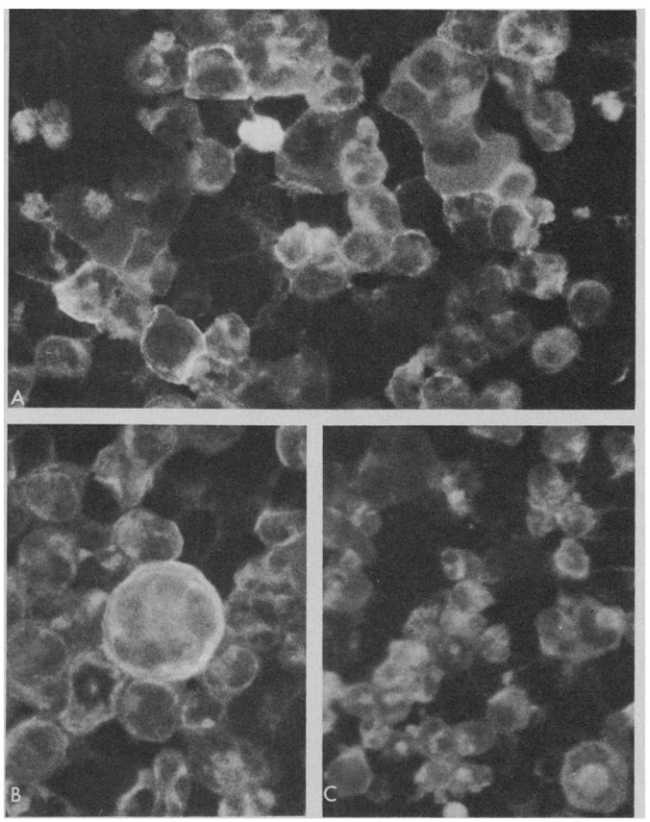


Table 1. HTLV-III Antigens in H9/AIDS semen cocultures. The presence of HTLV-III antigens was assayed by fixed-cell indirect immunofluorescence as described (*3*). In brief, cells of the H9 clone were cocultured with mononuclear cells derived from semen of AIDS patients (H9/AIDS) previously cultured for 15 days and from the reference (control) H9 and H9/HTLV-III lines. They were then spotted on a slide, dried, and fixed in acetone for 10 minutes at room temperature. Twenty microliters of either rabbit polyclonal antiserum to HTLV-III (diluted 1:2000 in phosphate-buffered saline) or murine monoclonal antibody to p15 (1:100) or to p24 (1:100) was applied and the cells were incubated for 50 minutes at 37°C (*16*). After three washes the fluorescein-conjugated antiserum (1:100) was applied and the cells were incubated for 30 minutes at room temperature. Technical controls consisted of (i) spots treated with rabbit serum or with a murine monoclonal antibody to immunoglobulin in place of the specific antibodies and (ii) spots treated only with the fluorescein conjugate. No fluorescence was detected in these control samples.

Antibody reagent	Cells positive (%)*			
	H9/ AIDS semen 1	H9/ AIDS semen 2	H9	H9/ HTLV-III
Rabbit polyclonal antiserum to disrupted HTLV-III	20	22	0	42
Monoclonal antibody to HTLV-III p15 (*14*)	20	26	0	75
Monoclonal antibody to HTLV-III p24 (*14*)	30	40	0	90

*Percentage of positive cells. This number represents the mean percentage found on three samples.



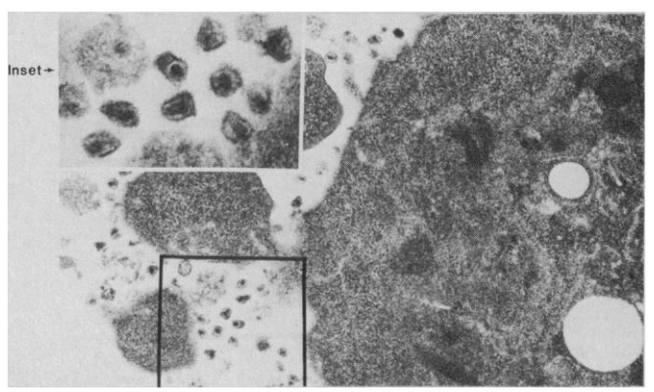


Fig. 3 (top). Detection of HTLV-III antigens by fixed-cell indirect immunofluorescence. Cells were treated as described in Table 1. (A and B) Cells from H9/AIDS semen coculture. (C) Cells from H9. Note the fluorescence localized on the surface of cells in (A) and (B). In (C) the cells are all negative. Fig. 4 (bottom). Retrovirus particles observed by electron microscopy at the surface of a cell from a 15-day-old H9/AIDS semen cell coculture. The cell preparation was fixed in glutaraldehyde-osmium and embedded in Epon. Ultrathin sections were prepared, mounted in grids, and stained with uranyl acetate lead citrate. Magnification: ×10,000; inset in upper left corner, ×76,000.

percent $T8^+$ cells (OKT8) and 14 percent mature B cells (OKB7). The lymphocytes in the AIDS patients consisted of 20 to 50 percent $T4^+$ cells and 2 to 18 percent $T8^+$ cells. The nonproliferating, uncultured semen mononuclear cells from the healthy donors and the two AIDS patients did not express HTLV-III antigens by fixed-cell indirect immunofluorescence with the use of either rabbit polyclonal antiserum to HTLV-III (*2*) or murine monoclonal antibody to the p15 and p24 proteins of HTLV-III (Table 1).

Portions (10^3) of mononuclear cells from the semen of the AIDS patients and the normal donors were seeded in 200 μl round-bottom wells (Nunk) containing culture medium. Cells were activated by phytohemagglutinin (0.1 percent; Gibco) for 24 hours and cultured in the presence of semipurified lectin-free T-cell growth factor interleukin-2 (IL-2) and a feeder layer containing 1.5×10^5 irradiated (4000 rad) lymphoid cells as described (*11*).

Proliferating cultures, monitored by inverted microscopy, were transferred and seeded at 3×10^5 to 4×10^5 cells per milliliter in round-bottom tubes (Falcon) containing 1.5 ml of culture medium and 10 percent IL-2. These cells proliferated only in the presence of IL-2; they were subcultured periodically and growth was maintained for at least 6 weeks under these conditions.

These cultured cells were tested for HTLV-III antigens by fixed-cell indirect immunofluorescence with the use of rabbit antiserum to HTLV-III. By day 6 a few HTLV-III–positive cells were detectable in cultures from the two AIDS patients. However, the positive reaction was transient; it was not observed on cells cultured 12 days or longer. No positive cells were observed at any time in cultures derived from the semen of the three normal individuals.

Cells cultured from the semen of the AIDS patients were cocultivated with clone H9, an HTLV-III permissive T-cell line derived from a parental human leukemic T-cell line (*2*). Cocultivation was initiated after the primary semen mononuclear cells had been cultured for 6 days, at which time HTLV-III–positive cells were present. The medium containing IL-2 was then replaced by normal culture medium containing 0.5 percent sheep antiserum to human α-interferon (α-IFN) (neutralizing titer, 6 IU at 10^{-5} dilution), and 1.5×10^4 H9 cells were added per 200-μl well. The cocultures, H9/AIDS, were transferred after 48 hours to 2-ml round-bottom tubes and seeded at a concentration of 3×10^5 cells per milliliter in culture medium containing

antiserum to α-IFN. The cultures were subcultured when the population reached a concentration of 10^6 cells per milliliter.

Ten days after the initiation of cocultivation the cell population was examined by light microscopy and the cells were tested for various immunological markers. The results were compared with those obtained with the reference H9 and H9/HTLV-III cell lines (*2, 3*). We observed a number of large cells (12 to 20 μm in diameter) with basophilic cytoplasm surrounding a large Golgi region and containing an indented nucleus; giant multinucleated syncytial cells; and numerous cells in mitosis. This morphological pattern is very similar to that seen with the reference H9/HTLV-III cell line, and all three types of cultured cells (the coculture, the H9/HTLV-III reference line, and the uninfected H9 reference line) showed a similar lymphocytic phenotype. No cells expressed T4 or T8 antigens in the cocultures. Thirty-five to 45 percent of the cells expressed HLA-DR antigen (OKI_2). The uninfected H9 cell cultures differed by showing less cellular degeneration and fewer multinucleated cells.

Cocultures were monitored for reverse transcriptase (RT) activity. Such activity was detectable as of day 8 of the coculture (Fig. 2). In contrast, RT activity was not detectable in long-term cultures (12 to 40 days) of semen T cells (not shown). Furthermore, 20 to 40 percent of the cells from 15-day-old cocultures expressed HTLV-III antigens (Table 1 and Fig. 3). Cell samples from the cocultures were also examined by electron microscopy. Retrovirus particles were observed at the surface of some cells (Fig. 4).

This study shows that semen from normal individuals and from patients with AIDS contains a number of mononuclear cells that proliferate at low cell densities in vitro in the presence of IL-2 and a feeder cell layer. We found that some cells from semen derived from the two AIDS patients contained HTLV-III that replicated in the culture system. However, expression was transient. Semen cell cultures older than 12 days contained neither HTLV-III antigens nor RT activity. In previous studies, long-term cultures derived from blood, bone marrow, or lymph node cells from AIDS patients were also frequently negative for HTLV-III (*3*). When the semen mononuclear cells from the primary short-term cultures were cocultured with H9, definitive results were obtained because of transmission and amplification of the virus. That the virus isolated belongs to the HTLV-III subgroup is indicated by its morphology (Fig. 4) and, more important, by the positive results with HTLV-III specific antisera (*12*). Thus, these results are consistent with the epidemiological data implicating semen as a source of the AIDS etiological agent (*9, 10*) and with results (*2–5, 8–10, 12–15*) indicating that HTLV-III is the agent.

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References and Notes

1. F. Barré-Sinoussi *et al.*, *Science* **220**, 868 (1983).
2. M. Popovic, M. G. Sarngadharan, E. Read, R. C. Gallo, *ibid.* **224**, 497 (1984).
3. R. C. Gallo *et al.*, *ibid.*, p. 500.
4. M. G. Sarngadharan *et al.*, *ibid.*, p. 506.
5. B. Safai *et al.*, *Lancet* **1984-I**, 1438 (1984).
6. V. S. Kalyanaraman *et al.*, *Science* **225**, 321 (1984).
7. S. Z. Salahuddin, P. Markham, M. Popovic, R. Gallo, in preparation.
8. J. W. Curran *et al.*, *N. Engl. J. Med.* **310**, 69 (1984); J. Groopman *et al.*, in preparation.
9. J. Goedert *et al.*, *Lancet*, in press; H. W. Jaffe *et al.*, *Ann. Intern. Med.* **99** 145 (1983).
10. J. Groopman *et al.*, in preparation.
11. D. Zagury *et al.*, *J. Immunol. Methods* **43**, 67 (1981).
12. J. Schüpbach *et al.*, *Science* **224**, 503 (1984).
13. S. Broder and R. C. Gallo, *N. Engl. J. Med.*, in press.
14. D. Mathez *et al.*, *Lancet*, in press.
15. M. G. Sarngadharan, L. Bruch, M. Popovic, R. C. Gallo, in preparation.
16. The rabbit polyclonal antiserum has been described (*15*). The monoclonal antibodies to p15 (BT2) and p24 (BT3) were provided by M. G. Sarngadharan [see (*17*)].
17. F. Veronese *et al.*, in preparation.
18. We acknowledge the contributions of Professor Chany, Dr. Lebon, and Mme. Robert (Hôpital Saint Vincent de Paul, Paris) for their help in performing RT assays and providing goat antiserum to human α-IFN. We also thank Professor Caulet and Dr. Tessier (Riems) for providing normal semen from volunteers. This work was supported in part by grants from ARC-Villejuif; Ligue Nationale Contre le Cancer; DRET and Institut Jean Godinot.

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HTLV-III in the Semen and Blood of a Healthy Homosexual Man

Abstract. *Human T-lymphotropic virus type III (HTLV-III) is the probable etiologic agent for the acquired immune deficiency syndrome (AIDS). HTLV-III was isolated from semen and blood of a healthy homosexual man whose serum contains antibodies to HTLV-III. The finding of virus in semen supports epidemiologic data that suggest that AIDS can be transmitted sexually. In addition, the demonstration of HTLV-III in the blood and semen of a healthy individual establishes an asymptomatic, virus-positive carrier state which may be important in the dissemination of HTLV-III and, consequently, AIDS.*

The acquired immune deficiency syndrome (AIDS) was first recognized in 1981 as a generally fatal disorder of cell-mediated immunity manifested clinically by opportunistic infections or Kaposi's sarcoma (*1–3*). More than 6000 cases of AIDS have been reported to the Centers for Disease Control to date. Epidemiologic data in male homosexuals (*4, 5*) and female sexual partners of men with the syndrome (*6*) suggest that the disease can be transmitted sexually, possibly through contact with semen. Recently, a novel retrovirus, the human T-lymphotropic virus type III (HTLV-III), has been shown to be the likely etiologic agent for AIDS (*7, 8*). The lymphadenopathy-associated virus (LAV), initially isolated in France (*9*), appears to be closely related or identical to HTLV-III (*10*). Both viruses are now frequently isolated from blood of persons with AIDS or the AIDS-related complex (ARC) (*11*). Here we report the isolation of HTLV-III from semen of a healthy homosexual man who is seropositive for HTLV-III.

The subject, a 30-year-old homosexual male, has been evaluated at the Massachusetts General Hospital every 3 months since June 1983 as a participant in a prospective study of homosexual men with or without AIDS. His past medical history includes gonorrhea, hepatitis, and sexual contacts in 1982 with a man who subsequently developed Kaposi's sarcoma. However, he has shown no constitutional or localized signs or symptoms of AIDS. His peripheral blood leukocyte counts, lymphocyte proliferative responses to concanavalin A, and allogeneic cytotoxicity responses have been consistently normal. The subject's T helper/T suppressor (T4/T8) ratios have ranged from 1.0 to 2.4 (normal 1.7 ± 0.5) on five serial determinations. Four se-