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Persistent Noncytopathic Infection of Normal Human T Lymphocytes with AIDS-Associated Retrovirus

Abstract. *Infection of normal peripheral blood T cells by the acquired immune deficiency syndrome (AIDS)–associated retrovirus (ARV) was evaluated in long-term cultures of helper-inducer T cells (T4 cells). Cells that were inoculated with ARV and maintained in medium supplemented with interleukin-2 remained productively infected with this virus for more than 4 months in culture, although they showed no cytopathic effects characteristic of acute ARV infection. The presence of replicating virus was demonstrated by reverse transcriptase activity of culture fluids and by viral antigens and budding particles detected on cells by immunofluorescence and electron microscopy. Virus produced in these cultures remained infectious and could induce cytopathic effects and viral antigens in uninfected lymphoid cells. The finding that normal lymphocytes may be productively infected by an AIDS retrovirus in the absence of cell death suggests that a range of biologic effects may occur after infection in vivo.*

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A group of newly recognized human retroviruses termed lymphadenopathy-associated virus (LAV) (*1*), human T-cell lymphotropic virus type III (HTLV-III) (*2*), or AIDS-associated retrovirus (ARV) (*3*) has been strongly implicated in the etiology of the acquired immune deficiency syndrome (AIDS). This disease is clinically characterized by the progressive loss of T lymphocytes of the helper phenotype (T4 lymphocytes), as defined by the monoclonal antibodies OKT4 or Leu-3a (*4*). These AIDS-associated retroviruses have shown a tropism for T4 lymphocytes in vitro, possibly through a specific interaction with the 62,000 molecular weight glycoprotein antigen CD4 (*5–8*). Infection of T4 cells with these viruses results in cell death. This observation supports the role for these viruses in the pathogenesis of AIDS (*3, 5, 9*). However, in view of the prolonged period between viral exposure and disease (*10*), as well as the functional abnormalities in isolated T4 cells from patients with AIDS (*11*), the possibility exists that noncytotoxic infection of T4 cells may also occur. Such an observation has been made for established T-cell lines (*3, 9*). To evaluate more fully the range of biologic effects that follow viral infection, we evaluated infection of normal human T4 lymphocytes by ARV and report the stable production of this virus in long-term cultures of these cells.

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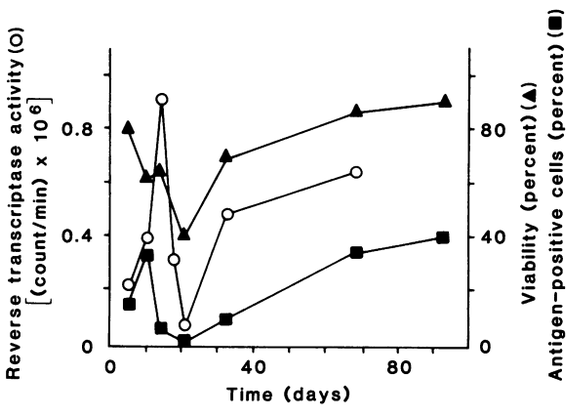
The experimental conditions and results are summarized in Fig. 1. T lymphocytes were enriched for T4 cells by affinity chromatography and cultured in phytohemagglutinin (PHA) for 3 days. Cells were then incubated for 4 hours with filtered fluid containing ARV-2 (*3, 12*), washed, and suspended in medium supplemented with lectin-free interleukin-2 (IL-2; 10 percent by volume). Cytopathic effects, including the presence of multinucleated cells and degenerative cellular forms characteristic of acute ARV (or HTLV-III/LAV) infection (*3, 5, 9*), were observed between 5 and 12 days after the addition of virus. The presence of retrovirus was documented during this period both by reverse transcriptase (RT) activity in culture supernatants and by ARV antigens detected in lymphocytes by immunofluorescence (IFA) (*3, 12*); for the latter assay, serum from a patient with AIDS-related complex was used as a source of antibody. Up to 35 percent of cells showed viral antigens by IFA at this time. Simultaneously, more than 90 percent of these lymphocytes ceased to react with the OKT4 monoclonal antibody, as has been described for other T4-positive lymphocytes and cell lines infected with the AIDS retrovirus (*5, 6, 12*). Control (uninfected) T4 cells cultured under identical conditions continued to show more than 85 percent



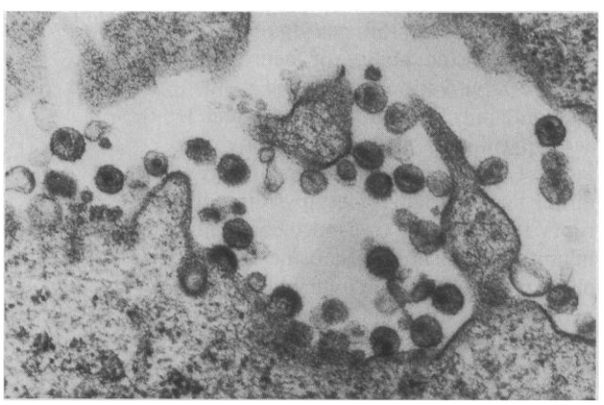


Fig. 1 (left). Persistance of ARV infection in cultures of T4 lymphocytes (*15*). Results of a representative experiment are shown. Fig. 2 (right). Electron micrograph of ARV-infected T4 cells from long-term cultures. T cells 93 days after infection with ARV-2 are shown (× 90,000); the numerous budding and mature particles are characteristic of ARV.

OKT4 reactivity and displayed no evidence of viral antigens or RT in their culture fluids.

Late effects of ARV infection were evaluated in cultures maintained at a concentration of 0.5 to 1.0 × 10^6 cells per milliliter in RPMI 1640 supplemented with IL-2. The medium was 50 percent exchanged twice weekly, and cultures were pulsed every 3 weeks with PHA (10 μg/ml). In four separate experiments, cells were maintained for 50 to 130 days. Throughout this period, cells continued to require exogenous IL-2 for growth. Two representative experiments are described. In experiment 1 (Fig. 1), RT activity could be detected for more than 2 months in the culture fluid and reached 675 × 10^3 count/min 68 days after addition of virus. Moreover, on days 68 and 93, 35 to 40 percent of cells showed ARV as detected by IFA. Cell viability at this time was greater than 85 percent as determined by trypan-blue exclusion. Electron microscopy of this long-term culture on day 93 revealed numerous mature and budding viral particles with the characteristic morphology of ARV (Fig. 2).

In experiment 2, RT activity—which was markedly elevated (4586.4 × 10^3 count/min) on day 10 after the addition of virus—was 71.4 × 10^3 count/min on day 98. At that time, cell viability was 90 percent, and analysis of these cultured cells for viral antigens showed that 5 percent were brightly fluorescent (Fig. 3). [^{3}H]Thymidine incorporation on day 105 was 9148 count/min per 5 × 10^4 cells compared to 974 count/min per 5 × 10^4 cells in fresh, nonstimulated peripheral blood lymphocytes. This observation documents ongoing DNA synthesis in late cultures of ARV-infected cells.

In contrast to cells during the initial phase of infection, no multinucleated or degenerative forms were present after 1 month in these long-term cultures. No RT activity or viral antigen–positive cells were detected in control cultures of T4 cells maintained under identical conditions for up to 100 days.

To determine whether the lymphocytes from these long-term cultures produced infectious virus biologically similar to the initial ARV used, we filtered supernatants from cultures from experiment 1 on day 78 and from experiment 2 on day 93 and added the filtrate to HUT-78 cells as described (*3*). Within 10 days, multinucleated giant cells and degenerative cellular forms were observed. Moreover, the cells became strongly positive for viral antigens as determined by IFA. This observation demonstrates that lymphocytes that were able to proliferate for more than 3 months without morphologic cytopathic changes produced ARV that was still cytopathic for uninfected cells.

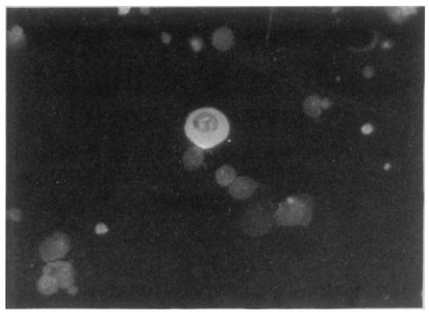


Fig. 3. Immunofluorescence microscopy of ARV-infected T4 cells from a long-term culture. Cytospins of T4 lymphocytes 105 days after addition of ARV were prepared and fixed in methanol:acetone (1:1). Cells were stained with a 1:10 dilution of serum from a patient with AIDS-related complex and then with a 1:20 dilution of F(ab)'$_2$ goat antibody to human immunoglobulin G conjugated with fluorescein isothiocyanate (Tago). Note the bright fluorescence present on an ARV-infected cell.

Surface marker studies of cultured lymphocytes were performed with monoclonal antibodies and immunofluorescence flow cytometry. Before infection, cell populations in both experiments were 95 percent T cells as determined by their reactivity with OKT11, and more than 89 percent were positive for OKT4 (a representative experiment is described in Table 1). Infection with ARV resulted in a loss of reactivity with OKT4 but not OKT3 or OKT11. Of cells from both experiments analyzed 78 and 85 days after infection, more than 98 percent were positive for OKT11 and only 10 percent were positive for OKT8. They remained unreactive with OKT4, although at these times only 35 percent (experiment 1) and 5 percent (experiment 2) were positive for viral antigens, as determined by IFA. In contrast, 98 percent of control lymphocytes remained positive for OKT4, OKT3, and OKT11. The observation that the expression of the T3 antigen did not change after ARV infection contrasts with a previous report that described a loss of T3 from T4 cells infected with LAV (*5*).

These studies indicate that T4 lymphocytes from normal donors can be productively infected with ARV and live for more than 4 months in vitro without showing cytopathic effects. Virus produced in these cultures remained infectious and could induce cytopathic effects and viral antigens in uninfected lymphoid cells. Infected T cells from these long-term cultures showed no expression of the T4 antigen, even though in two experiments only 35 percent and 5 percent of cells were positive for viral antigens by IFA. Moreover, control cultures maintained for similar periods continued to exhibit the T4 antigen. This observation on the infected T cells suggests either expansion of a minor population of uninfected cells with this phenotype or modulation of the T4 antigen on these cells in the absence of virus production. The expression of this antigen could be effected at the cell surface by the direct interaction with virus produced by other cells or by a perturbation in regulation of the T4 molecule in infected cells independent of virus production. Folks et al. have described a T-cell line that became negative for T4 antigen expression after infection by HTLV-III, although these cells produced virus only after the addition of iododeoxyuridine (*13*). A similar latent infection may also have occurred in the T4-negative T cells in these cultures of infected normal peripheral blood lymphocytes.

Previous reports have described the cytotoxic effects of the AIDS retrovirus on T4 lymphocytes in vitro (*2, 3, 5*). They have supported the concept that these viruses play a central role in the pathogenesis of AIDS, which is characterized clinically by a progressive loss of

Table 1. Reactivity of monoclonal antibodies with cultured peripheral blood T cells after infection with ARV-2 (results of a representative experiment). Affinity-enriched T4 cells were inoculated with ARV, and monoclonal antibody analysis of cell-surface antigens was performed at the indicated times by indirect immunofluorescence staining and flow cytometry with the use of a Spectrum III fluorescence cell analyzer (Ortho) as described (*14*). Antibodies included OKT3 (mature T cells), OKT4 (helper-inducer T cells), OKT8 (suppressor-cytotoxic T cells), and OKT11 (sheep erythrocyte receptor). All cells were fixed in 4 percent paraformaldehyde before analysis. Values in parentheses indicate percentages of antibody-positive cells in cultures of T cells not infected with virus that were maintained under identical conditions.

Monoclonal antibody	Antibody-positive cells (%)			
	Day 0	Day 10	Day 44	Day 85
OKT3	95	86 (96)	94 (99)	99 (95)
OKT4	89	27 (88)	4 (99)	2 (98)
OKT8	3	19 (3)	16 (1)	10 (10)
OKT11	95	97 (99)	97 (99)	98 (99)

T4 lymphocytes. The present finding that T4 lymphocytes may be productively infected in the absence of cell death suggests that a range of biologic effects may occur after infection. This fact may have relevance both to the prolonged latent period between virus exposure and disease and to possible abnormalities in T4 cell function (*11*) that may contribute to immune deficiency. Moreover, persistance of viable infected cells would permit continual spread of virus in the host. The mechanisms that regulate viral production and produce either cell death or alterations in cellular function after ARV infection remain essential to our understanding of the pathogenesis of AIDS.

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15. Peripheral blood lymphocytes were depleted of monocytes by adherence to plastic, and the T cells were isolated by rosetting with neuraminidase-treated sheep erythrocytes and subsequent separation over lymphocyte-separation medium (Litton Bionetics). Cells (65×10^6) were concentrated in 0.5 ml of phosphate-buffered saline with 10 percent fetal bovine serum (FBS) and incubated for 30 minutes at 4°C with 50 μl of OKT8 (Ortho Diagnostic Systems) and Leu-M3 (Becton-Dickinson) monoclonal antibodies. Cells were then washed and passed over Sepharose 6-MB (Pharmacia, 10-ml column) conjugated with $F(ab)'_2$ goat antibody to mouse immunoglobulin G (Cappel). The resulting OKT8/Leu-M3–depleted cells, more than 90 percent of which were T4 positive, were incubated for 3 days with PHA (Gibco, 10 μg/ml) in RPMI 1640 with penicillin (100 U/ml) and streptomycin (100 μg/ml) with 20 percent FBS. Cells were then washed and placed in 1 ml of filtered (0.45 μm) supernatant from an HUT-78 cell line producing ARV-2 (10^4 infectious particles per milliliter) (*3*). After incubation for 4 hours at 37°C, cells were washed and maintained in medium supplemented with lectin-free IL-2 (Electronucleonics). At designated times, RT activity was determined on 3-ml samples of filtered culture supernatants (*3*). Viral antigens on methanol:acetone–fixed cells from a patient with AIDS-related complex were detected by IFA as described in the legend of Fig. 2. The serum used had anti-ARV activity as determined by Western blot analysis of viral antigens and no detectable reactivity with normal T lymphocytes. No reactivity of this serum was seen in control cultures of T4 cells not infected with ARV. Cell viability was determined by trypan-blue exclusion.

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Characterization of gp41 As the Transmembrane Protein Coded by the HTLV-III/LAV Envelope Gene

Abstract. *Radiolabeled amino acid sequencing was used to characterize gp41, an antigen of HTLV-III/LAV, the virus believed to be the etiological agent of the acquired immune deficiency syndrome. This antigen is the one most commonly detected in immunoblot assays by sera of patients with AIDS or AIDS-related complex (ARC) and other individuals infected with HTLV-III/LAV. A mouse monoclonal antibody that was reactive with gp41 precipitated a 160-kilodalton protein (gp160) in addition to gp41, but did not precipitate a 120-kilodalton protein (gp120) from extracts of metabolically labeled cells producing HTLV-III. Extracts of infected cells that had been labeled with tritiated leucine or isoleucine were immunoprecipitated with the monoclonal antibody. The immunoprecipitates were fractionated by polyacrylamide gel electrophoresis and the p41 was eluted from the gel bands and subjected to amino-terminal radiolabeled amino acid sequencing by the semiautomated Edman degradation. Leucine residues occurred in cycles 7, 9, 12, 26, 33, and 34 among 40 cycles and isoleucine occurred in cycle 4 among 24 cycles analyzed. Comparison of the data with the deduced amino acid sequence of the* env *gene product of HTLV-III precisely placed gp41 in the COOH-terminal region of the* env *gene product. Gp160 is thus the primary* env *gene product and it is processed into gp120 and gp41.*

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Human T-cell lymphotropic (leukemia) virus type-III (HTLV-III) has been isolated from several patients with acquired immune deficiency syndrome (AIDS), AIDS-related complex (ARC), and from asymptomatic carriers of infection (*1*). The lymphadenopathy associated virus (LAV) (*2*) and other isolates (*3*) from similar sources appear to be closely similar to HTLV-III (*4–6*). Many data indicate that HTLV-III/LAV is the etiological agent of AIDS (*1, 7–11*).

By use of the Western blot technique (*12*) investigators have identified several proteins in HTLV-III preparations that appear to be major targets of antibody reaction with sera from patients with AIDS and from individuals infected with HTLV-III (*7, 13*). Most of the serum samples that have high titers of antibodies react against HTLV-III proteins of molecular weights 120,000 (120K), 66K, 51K, 41K, 31K, 24K, and 17K (*7, 13*). However, the antigen most consistently correlated with seropositivity to HTLV-III is the 41K protein (p41) (Fig. 1A) (*7*). Less frequently, p24, the major HTLV-III core protein, is the only antigen recognized. This difference may have a bearing on the level of intracellular virus replication or the degree of exposure of virus antigens as a result of the lytic activity of the virus on the target cell. P41 is not only the most consistently detected antigen, but reactivity to it is also the most persistent, being present even at late stages of the disease. Whether p41 is a product of an HTLV-III gene or a cellular protein induced by viral infection is an important question.

We developed mouse hybridomas secreting monoclonal antibodies to p41 and used these antibodies to isolate and characterize p41. BALB/c mice were immunized with successive intraperitoneal inoculations of HTLV-III (100 μg) that had been purified on density gradients and lysed by detergent treatment. The lysates were emulsified in complete Freund's adjuvant for the first inoculation and in incomplete adjuvant for the following four booster doses given 1 week apart. Three days after a final intraperitoneal inoculation with disrupted virus in phosphate-buffered saline, splenic lymphocytes were fused with the NS-1 mouse myeloma line. The cell fusion, cell culturing, and cloning of hybridoma lines were essentially as described (*14*). Cellular fluids from growing hybrids were screened for antibody to HTLV-III proteins by an enzyme-linked immunosorbent assay (ELISA) (*7*). Lysates of HTLV-III were coated on 96-well microtiter plates and allowed to react with the antibody overnight. The resulting antigen-antibody complexes were then revealed by reaction with peroxidase-conjugated goat antibody to