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In vitro growth characteristics of simian T-lymphotropic virus type III

(acquired immunodeficiency syndrome/retrovirus/nonhuman primate/T lymphocyte)

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ABSTRACT **The type C retrovirus simian T-lymphotropic virus type III (STLV-III) has been isolated recently from immunodeficient macaque monkeys at the New England Regional Primate Research Center. The present studies were done to define the *in vitro* growth characteristics of this agent. STLV-III replicates efficiently in interleukin 2-dependent T-cell cultures of macaque peripheral blood lymphocytes (PBL), less efficiently in such cultures of human and gibbon PBL, and inefficiently in baboon PBL. No replication, as assessed by measuring reverse transcriptase activity in these culture supernatants, could be detected in similarly maintained cultures of chimpanzee, squirrel monkey, and cotton-top tamarin PBL. Like the human acquired immunodeficiency syndrome (AIDS) virus, human T-cell lymphotropic virus III/lymphadenopathy-associated virus (HTLV-III/LAV), STLV-III replicates in $T4^+$ but not $T8^+$ lymphocytes and its infection of macaque and human lymphocytes can be blocked with monoclonal anti-T4 antibodies. STLV-III differs from the human AIDS virus, however, in its apparent inability to grow in the Epstein–Barr virus-transformed B lymphocytes tested, the differing range of nonhuman primate T-cell populations that support its growth, and its less striking toxicity for T lymphocytes. These studies provide further characterization of an agent that will be extremely important in facilitating the development of vaccines and antiviral therapy for AIDS.**

Individuals affected by the acquired immunodeficiency syndrome (AIDS) show a profound depression of their cell-mediated immune function and develop opportunistic infections and neoplasms (1). The number of reported cases of this usually fatal syndrome continues to rise at an alarming rate (2). With the recent demonstration of human T-cell lymphotropic virus (HTLV)-III/lymphadenopathy-associated virus (LAV) as the etiologic agent in this disease, it has become clear that the control of AIDS will require the development of new antiviral treatment modalities and the development of a safe and efficacious vaccine to protect against this syndrome. To achieve these ends it is of critical importance to define an animal model for AIDS.

A T-cell tropic retrovirus with striking similarities to HTLV-III/LAV has been isolated recently at the New England Regional Primate Research Center (NERPRC) from macaque monkeys (3). This newly isolated agent has been named simian T-lymphotropic virus type III (STLV-III) of macaques. By electron microscopy, it has a cylindrical nucleoid and buds in a fashion typical of type C retroviruses (3). Radioimmunoprecipitation analysis of STLV-III-infected cells, using sera from humans who are seropositive for HTLV-III/LAV, has revealed cross-reactive virus-specific proteins of 160 kDa, 120 kDa, 55 kDa, and 24 kDa, all similar in size to the major *gag* and *env* proteins encoded by HTLV-III/LAV (4).

In the present studies we have defined some of the *in vitro* growth characteristics of STLV-III of macaques. We have shown that, like HTLV-III/LAV, STLV-III of macaques is T-cell tropic *in vitro* and its infection of lymphocytes can be blocked by monoclonal antibodies directed against the T4 structure. STLV-III differs from HTLV-III/LAV in the range of primate species lymphocyte populations that are susceptible to its infection and the degree to which it appears to be cytopathic *in vitro*. These studies provide further characterization of an agent that will be extremely important in facilitating the development of therapeutic interventions in AIDS.

MATERIALS AND METHODS

Animals. Nine healthy adult humans, four chimpanzees (*Pan troglodytes*), two gibbons (*Hylobates lar*), two baboons (*Papio papio*), two rhesus monkeys (*Macaca mulatta*), three Taiwanese macaques (*Macaca cyclopis*), five cynomolgus monkeys (*Macaca fascicularis*), two squirrel monkeys (*Saimiri sciureus*), and two cotton-top tamarins (*Saguinus oedipus*) served as blood donors. Chimpanzee and gibbon blood was kindly provided by the Yerkes Primate Center (Atlanta, GA); the other nonhuman primates that served as blood donors were chosen from the colony at the NERPRC.

Viruses. The STLV-III isolate utilized in this study was obtained from a rhesus monkey (Mm 251-79) with a poorly differentiated lymphocytic lymphoma (5, 6). It was expanded in a culture of human phytohemagglutinin (PHA)-stimulated lymphocytes that was maintained with interleukin 2 (IL-2)-containing medium. These culture supernatants were passed through a 0.45-μm filter prior to use.

Two HTLV-III/LAV-infected cell lines were kindly provided by M. Hirsch (Massachusetts General Hospital, Boston, MA). One was an H9 cell line and the other was a HUT 78 cell line infected with HTLV-III/LAV derived from two different patients with AIDS; they are referred to as HTLV-III-1 and HTLV-III-2 in these studies. Culture supernatants of these cell lines were filtered prior to use.

***In Vitro* Infection with Viruses.** Peripheral blood lymphocytes (PBL) from animals were isolated on a Ficoll-Diatrizoate gradient and stimulated with 0.1% PHA (Difco, Detroit, MI) for 3 days. PHA-stimulated PBL were washed with phosphate-buffered saline (P_i/NaCl) and incubated in a

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Abbreviations: AIDS, acquired immunodeficiency syndrome; HTLV-III/LAV, human T-cell lymphotropic virus III/lymphadenopathy-associated virus; IL-2, interleukin 2; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte(s); PHA, phytohemagglutinin; RT, reverse transcriptase; STLV-III, simian T-lymphotropic virus type III; NERPRC, New England Regional Primate Research Center.

human lymphocyte culture supernatant containing STLV-III [2×10^4 cpm/ml of reverse transcriptase (RT) activity] at a cell concentration of 2×10^6 per ml for 2 hr at 37°C. The cells were then washed three times with P_i/NaCl and adjusted to a concentration of 7.5×10^5 per ml in RPMI-1640 medium (GIBCO) supplemented with 10% fetal calf serum (Sterile Systems, Logan, UT) and 20% crude IL-2 supernatant prepared as described (7). These cells were then placed in culture at 37°C in a humidified atmosphere with 5% CO_2 in air. The culture medium was changed every 3–4 days, and cell concentrations were adjusted to 7.5×10^5 per ml. Culture supernatants were passed through 0.45-μm filters and stored at −70°C. Infections of lymphocytes with HTLV-III/LAV were performed in the same fashion.

RT Assay. RT activities of culture supernatants were measured as described (3). Briefly, 1.4 ml of each supernatant was centrifuged in a 1.5-ml Eppendorf tube at $12,000 \times g$ for 90 min. The supernatant was removed and pelleted virus was incubated on ice for 10 min with 20 μl of dissociation buffer (0.01 M Tris·HCl, pH 7.3/0.2% Triton X-100/0.001 M EDTA/0.05 M dithiothreitol/0.06 M KCl). Fifteen microliters of dissociated virus solution was mixed with 60 μl of assay mixture {0.05 M Tris·HCl, pH 8.3/0.007 M $MgCl_2$/0.06 M KCl/0.08 mg of poly(rC)·oligo(dG) primer per ml/0.007 M dithiothreitol/3.3 μCi of [α-^{32}P]dGTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham)} and incubated at 37°C for 60 min. Sixty microliters of each sample was dropped onto a Whatman 3 disk. Each disk was washed in a beaker with 5% trichloroacetic acid/2% sodium pyrophosphate, rinsed, and dried, and radioactivity of the disk was measured.

Blocking of STLV-III Infection with Monoclonal Antibodies (mAbs). Three mAbs, 19Thy5D7 (anti-T4A), 12T4D11 (anti-T4B), and 7PT3F9 (anti-T8), were used for blocking of STLV-III infection *in vitro*. These antibodies were kindly provided by S. Schlossman and E. Reinherz (Dana–Farber Cancer Institute, Boston, MA). PBL from one human and one cynomolgus monkey were stimulated with PHA for 3 days. The PHA-stimulated PBL were then divided into four aliquots and incubated with anti-T4A (1:250), anti-T4B (1:250), anti-T8 (1:125), or P_i/NaCl at room temperature for 30 min. The cells were then washed with P_i/NaCl and suspended in a supernatant containing STLV-III. After incubation at 37°C for 2 hr, the cells were washed three times with P_i/NaCl and placed in culture in RPMI-1640 medium supplemented with 20% IL-2-containing supernatant, 10% fetal calf serum, and the same mAb (1:400) at a cell concentration of 7.5×10^5 per ml. The culture medium was changed and the cell concentration was adjusted to 7.5×10^5 per ml every 3–4 days.

RESULTS

Susceptibility of Various Primate Species PBL to Infection with STLV-III of Macaques. Initial studies were performed to determine the growth characteristics of STLV-III in primate lymphocytes. In doing these studies we sought to determine first whether cell-free virus can infect lymphocytes *in vitro*, then the range of primate species PBL that support the growth of this agent, and finally the effect of STLV-III on the kinetics of T-cell growth.

In preliminary studies PHA-stimulated human PBL were incubated for 2 hr with a cell-free supernatant containing STLV-III of macaques and then washed three times. When grown in culture in the presence of IL-2, these cells themselves generated RT activity (data not shown), indicating that cell-free STLV-III can infect human T lymphocytes *in vitro*.

PHA-stimulated PBL from eight different nonhuman primate species were exposed to STLV-III *in vitro* and maintained in culture with IL-2, and RT activity in the culture supernatants was measured. The results of these studies are summarized in Table 1. The kinetics of the generation of RT activity was similar in the T-cell cultures derived from PBL of humans, gibbons, and macaques. In all such cultures peak RT activity in supernatants was observed after 7–13 days of culture. The peak RT activity varied among T-cell cultures from individuals of the same species, but, in general, T-cell cultures of the rhesus monkey, the species from which this virus was isolated, generated the highest peak. Taiwanese macaque and cynomolgus monkey T-cell populations generated less RT activity than those of the rhesus monkey. Peak RT activity generated by human and gibbon T-cell cultures was lower than that generated by infected T cells of macaque monkeys. In the culture supernatants of baboon T-cell cultures, significant RT activity was detected, but with a later and lower peak than that observed in T-cell cultures of other species that support the growth of STLV-III. T-cell cultures generated from PBL of chimpanzees, squirrel monkeys, and cotton-top tamarins, after incubation with cell-free STLV-III, did not produce detectable RT activity during 1 month of *in vitro* growth.

The relationship between the kinetics of the RT activity measured in supernatants of T-cell cultures of various representative species and the growth of cells in these cultures is shown in Fig. 1. Although growth of the T lymphocytes of humans, chimpanzees, macaques, and baboons was comparable under these culture conditions, early peaks in RT activity were observed in the supernatants of the human and macaque but not the chimpanzee and baboon cell cultures. Moreover, although lymphocytes of both the gibbon and squirrel monkey grew quite well in culture with human IL-2,

Table 1. *In vitro* growth of STLV-III in PBL of various primate species

Family	Species	No. of PBL samples tested	No. of samples showing growth	Mean peak RT activity in culture supernatants, cpm*		Median no. of days in culture at RT peak
				Infected	Uninfected	
Hominidae	Man	9	9	25,584 ± 13,911	264 ± 95	10.1
Pongidae	Chimpanzee	4	0	331 ± 53	247 ± 26	—
Hylobatidae	Gibbon	2	2	25,452 ± 15,776	154 ± 11	7
Cercopithecidae	Baboon	2	2	2,038 ± 1,275	245 ± 109	24.5
	Rhesus monkey	2	2	110,465 ± 14,714	477 ± 144	13
	Taiwanese macaque	3	2	46,496 ± 21,314	374 ± 99	8.3
	Cynomolgus monkey	5	5	53,579 ± 35,527	322 ± 124	7.8
Cebidae	Squirrel monkey	2	0	846 ± 97	662 ± 159	—
Callitrichidae	Cotton-top tamarin	2	0	763 ± 16	570 ± 30	—

*Data are expressed as mean ± SD. The SD reflects the differences between viral replication in individual cell populations and the fact that assays were performed at differing stages of decay of the isotope. [^{32}P]dGTP was always utilized within 7 days of radioactive labeling. Cell populations from different primate species were frequently cultured in parallel and RT assays were performed simultaneously to verify that species-specific differences in RT levels were reproducible.

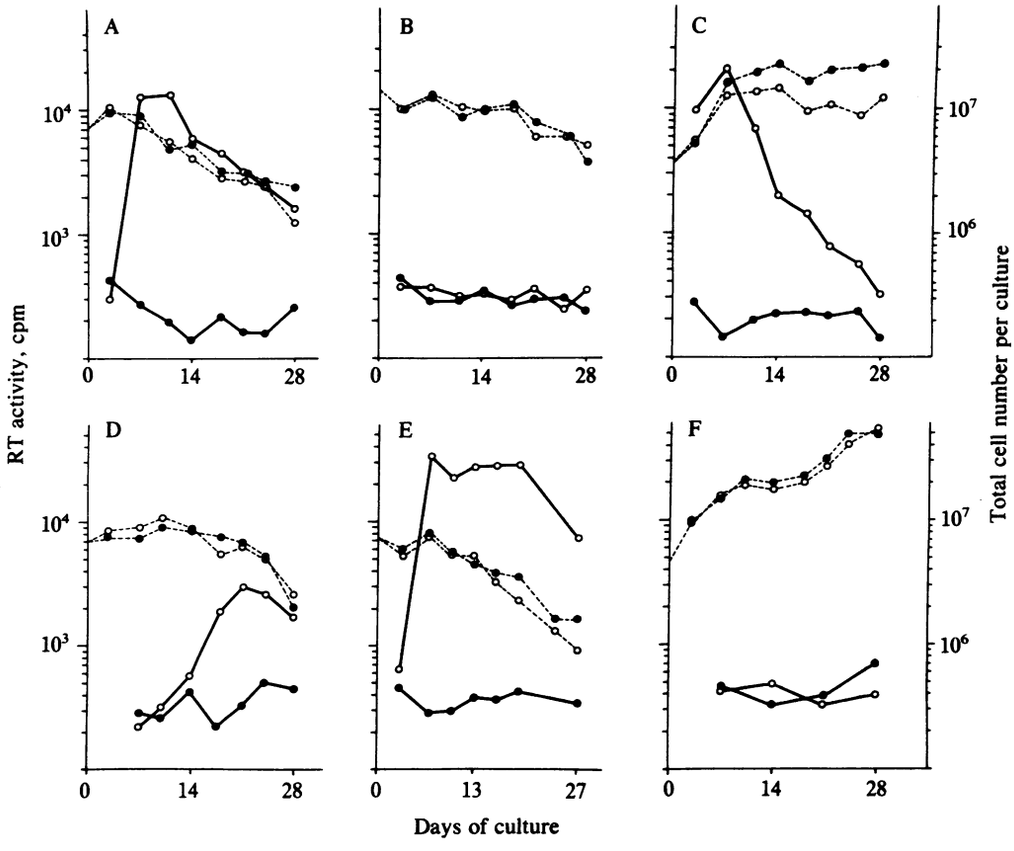

FIG. 1. Kinetics of RT activity in the supernatants (solid line) and cell growth (broken line) of STLV-III-infected PBL (○) and uninfected PBL (●) from selected primate species. (*A*) Man. (*B*) Chimpanzee. (*C*) Gibbon. (*D*) Baboon. (*E*) Cynomolgus monkey. (*F*) Squirrel monkey. Cell concentrations were adjusted to 7.5×10^5 per ml in each culture flask every 3–4 days.

the kinetics of the generation of RT activity in gibbon lymphocyte cultures were similar to those seen in cultures of human lymphocytes, whereas no RT activity was detected in those of the squirrel monkey. Thus, the differences in the kinetics of RT activity generated in lymphocyte cultures of the various primate species are not attributable merely to differences in the *in vitro* growth of these lymphocyte populations. A slight depression in the numbers of infected T cells compared with uninfected T cells was observed in one of nine human, three of nine macaque, one of two gibbon, and one of two baboon cell cultures studied.

RT activity was not detectable in Epstein–Barr virus-transformed B-cell lines (LCL) derived from two humans, a common marmoset and an owl monkey during 1 month of cultivation after exposure to STLV-III (data not shown).

Role of T4 Determinant in STLV-III Infection of T Lymphocytes. We have shown previously that STLV-III efficiently replicates in $T4^+$ but not $T8^+$ human lymphocytes (3). We therefore assessed the role that the T4 determinant plays in this infection of T cells with STLV-III. Monoclonal anti-T4 antibodies were utilized to block STLV-III infection of unfractionated human and macaque T-cell populations. In these studies two monoclonal anti-T4 antibodies were utilized, one of which (anti-T4A) recognizes a conserved epitope on both the human and macaque T4 molecule, whereas the other (anti-T4B) recognizes an epitope of T4 expressed on the human but not the macaque monkey T4 molecule (8). These two anti-T4 (anti-T4A, anti-T4B) and one anti-T8 mAb were tested for their ability to block STLV-III infection of either human or cynomolgus monkey T cells *in vitro* (Fig. 2). Replication of the virus was minimal in the culture of human T lymphocytes treated with anti-T4A or anti-T4B, whereas untreated and anti-T8-treated T cells generated high RT activity in the culture supernatant (Fig. 2*A*). STLV-III infection of cynomolgus monkey lymphocytes was inhibited by anti-T4A, the antibody that binds to the macaque monkey T4 molecule. STLV-III infection of cynomolgus monkey T cells was not blocked by anti-T4B or anti-T8 (Fig. 2*B*). This finding suggests that the T4 molecule or a physically related structure on the T-cell surface membrane serves as a binding site for STLV-III.

Comparison of Infectivity of STLV-III and HTLV-III/LAV

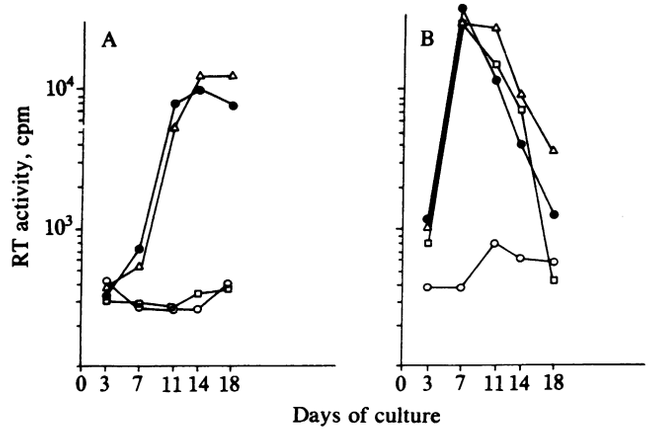

FIG. 2. Blocking of STLV-III growth *in vitro* with anti-T4 and anti-T8 mAbs was tested in PBL from man (*A*) and the cynomolgus monkey (*B*). PBL were treated with anti-T4A (○), anti-T4B (□), and anti-T8 (△), or P_i/NaCl (●) prior to exposure to STLV-III and were cultured with medium containing the respective mAb.

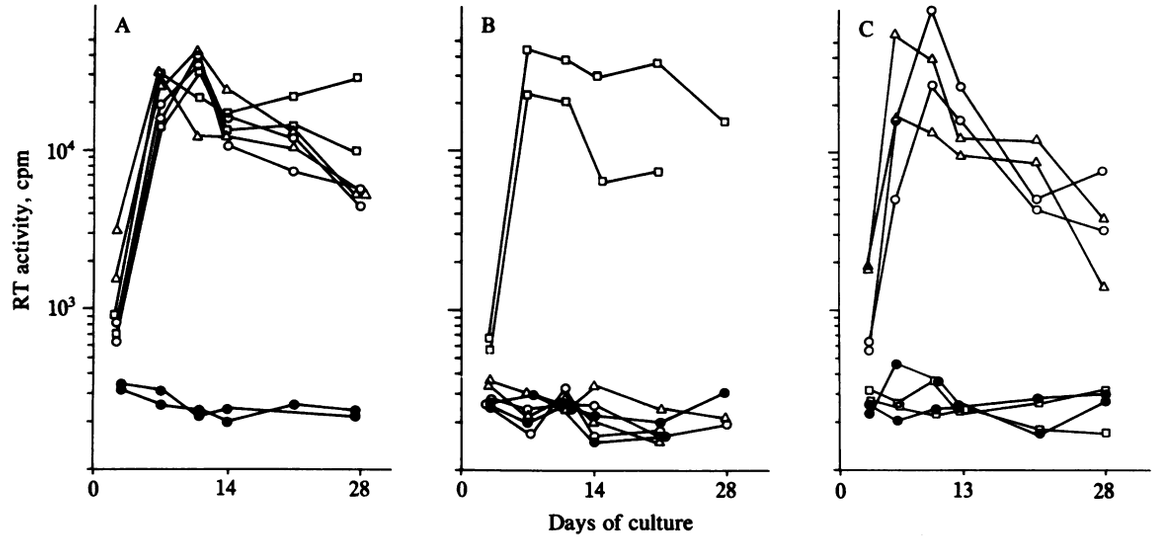

FIG. 3. Replication of HTLV-III and STLV-III in PBL from two men (*A*), two cynomolgus monkeys (*B*), and two chimpanzees (*C*). PBL from each were exposed to supernatant containing HTLV-III-1 (○), HTLV-III-2 (△), STLV-III (□), or control medium (●) for 2 hr on day 0.

in Vitro. We then sought to determine whether STLV-III has properties of infectivity that differentiate it in any way from HTLV-III/LAV. STLV-III and two different isolates of HTLV-III/LAV were incubated with a human Epstein–Barr virus-transformed B-cell line (LCL). Significant RT activity was found in the culture supernatant of the LCL cells exposed to HTLV-III-1 or HTLV-III-2 but was not detectable in the culture of the LCL cells exposed to STLV-III during 1 month in culture (data not shown).

These two different isolates of HTLV-III/LAV and STLV-III were then used to infect T-cell cultures generated from human, cynomolgus monkey, and chimpanzee PBL. Both HTLV-III/LAV and STLV-III replicated well in human T cells (Fig. 3*A*). STLV-III-infected cynomolgus monkey T cells produced large amounts of RT activity in the culture supernatants. However, significant RT activity was not detected in the culture supernatant of cynomolgus monkey T cells exposed to either of the two HTLV-III/LAV-containing supernatants (Fig. 3*B*). Chimpanzee T-lymphocyte cultures supported the *in vitro* replication of HTLV-III/LAV but not STLV-III (Fig. 3*C*).

Finally, the *in vitro* growth of human, chimpanzee, and cynomolgus monkey T cells infected with HTLV-III/LAV and STLV-III was determined in a number of experiments. A representative experiment of this type is shown in Fig. 4. The total cell number in cultures of human T lymphocytes infected with either of the two HTLV-III/LAV-containing supernatants was significantly lower than that of uninfected human T cells. The difference between cell growth of uninfected and infected human T cells with STLV-III was minimal (Fig. 4*A*). Similarly, the cell number in chimpanzee T-cell cultures infected with HTLV-III/LAV decreased significantly over time, whereas that of uninfected and STLV-III-infected chimpanzee T cells did not (Fig. 4*C*). In cultures of cynomolgus monkey T cells, no significant differences in cell growth were observed in T-cell populations exposed to HTLV-III-1, HTLV-III-2, STLV-III, or the control medium (Fig. 4*B*).

DISCUSSION

The present studies confirm that cell-free STLV-III of macaques can infect primate lymphocytes and document the range of species whose T cells support the growth of this agent. They demonstrate that, like HTLV-III/LAV, STLV-III is tropic for $T4^+$ lymphocytes. STLV-III differs from the human AIDS virus, however, in its apparent inability to grow in the LCL tested, the differing range of nonhuman primate

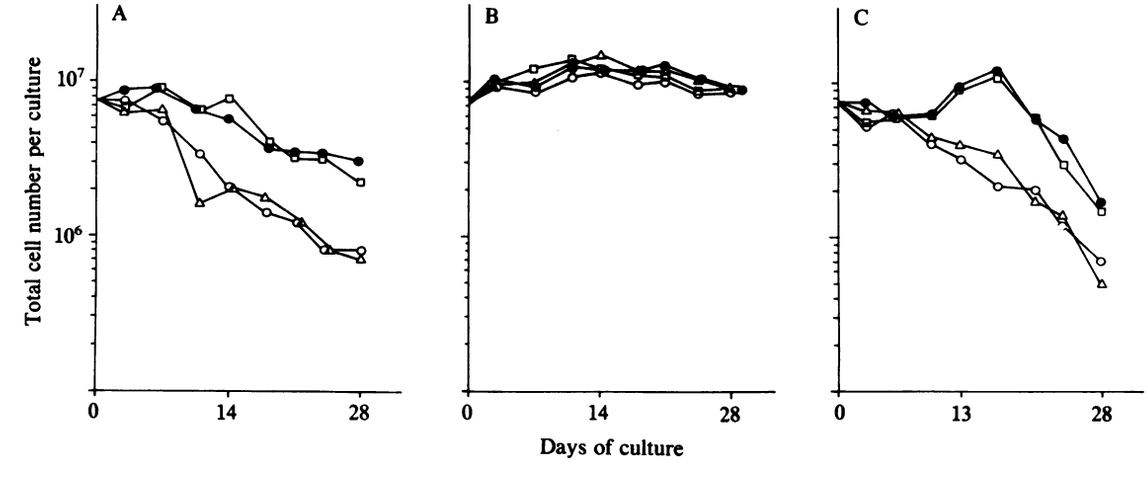

FIG. 4. Kinetics of cell growth of PBL exposed to HTLV-III-1 (○), HTLV-III-2 (△), STLV-III (□), or control medium (●) from a human (*A*), a cynomolgus monkey (*B*), and a chimpanzee (*C*).

T-cell populations that support its growth, and its less striking toxicity for T lymphocytes.

All of the isolates of STLV-III to date have been obtained from animals with evidence of the macaque immunodeficiency syndrome. Macaques with this syndrome develop profound T-lymphocyte dysfunction and eventually die of lymphomas or opportunistic infections by a spectrum of agents similar to those seen in human AIDS (5, 6, 9–12). It is noteworthy that the three species of macaque monkeys housed at the NERPRC exhibit very different manifestations of the macaque immunodeficiency syndrome. *M. cyclopis* have had an extremely high mortality rate from this syndrome due to opportunistic infections but no cases of lymphoma have been seen in this species. Fatal opportunistic infections and lymphomas have occurred in *M. mulatta*. Lymphomas but no opportunistic infections have been observed in *M. fascicularis*. The present studies provide no data to explain these species-specific differences in disease manifestations. STLV-III replication is supported comparably by lymphocytes of these three macaque species and there is no evidence that this virus is more toxic to lymphocytes of one than those of another macaque species.

Our studies of the range of primate species whose lymphocytes support STLV-III growth have yielded surprising results. Since a significant phylogenetic distance exists between the Old World macaques and such New World species as the squirrel monkey and cotton-top tamarin, it is not surprising that STLV-III does not grow in lymphocytes of New World monkeys. However, the finding that a chimpanzee T-cell population does not efficiently support the replication of this agent is not readily explicable since STLV-III grows well in lymphocytes of Old World monkeys, gibbons, and man. These differences in capacity to support productive infections of T lymphocytes do not relate to the expression by these populations of the T4 antigen. Chimpanzee, squirrel monkey, and cotton-top tamarin T cells clearly express the T4 determinant (8). In fact, the chimpanzee and cotton-top tamarin even express the epitope of T4 recognized by anti-T4A, a mAb that blocks STLV-III infection of susceptible T-cell populations. T lymphocytes derived from the chimpanzee, squirrel monkey, and cotton-top tamarin, nevertheless, do not support a productive infection *in vitro* by STLV-III.

For a productive infection, STLV-III must bind, penetrate, and efficiently replicate in a lymphocyte. These events cannot be differentiated with the experimental approach used in the present studies. Further experiments will be needed to determine at which stage the STLV-III infection of chimpanzee and New World primate T cells is aborted. However, the present studies indicate that the expression of T4 alone is not sufficient to allow a productive infection of a primate lymphocyte population with this retrovirus.

Recent studies have indicated that considerable structural heterogeneity exists among HTLV-III/LAV isolates, particularly in the *env* region of the virus genome (13). Therefore, it is important to determine whether STLV-III is merely a minor variant of HTLV-III/LAV or a related virus with some structural and biologic similarities to the human AIDS virus. Though the definitive answer to that question awaits detailed molecular characterization of STLV-III, the demonstration in the current studies of its inability to grow in LCL, its relative lack of *in vitro* toxicity for human T cells, and the differing range of nonhuman primate T-cell populations that support its growth suggest that STLV-III does, in fact, differ from the human AIDS virus.

The current studies provide important further characterization of an agent and a host species that hold promise as a critical model for the testing of antiviral treatments and vaccine development for human AIDS. Our recent demonstration that the inoculation of STLV-III into rhesus monkeys resulted in a wasting syndrome, immunologic abnormalities that include a decrease in $T4^+$ PBL, overwhelming adenovirus infections, and primary retroviral encephalitis further supports the notion that this model will be of critical importance in the development of effective therapies for AIDS (14).

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