Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4297–4301, June 1986
Cell Biology

Differential response to the cytopathic effects of human T-cell lymphotropic virus type III (HTLV-III) superinfection in T4$^+$ (helper) and T8$^+$ (suppressor) T-cell clones transformed by HTLV-I

ANITA DE ROSSI*, GENOVEFFA FRANCHINI†, ANNA ALDOVINI†, ANNAROSA DEL MISTRO*, LUIGI CHIECO-BIANCHI*, ROBERT C. GALLO†, AND FLOSSIE WONG-STAAL†‡

*Laboratory of Oncology, School of Medicine, University of Padova, Via Gattamelata 64, 35100 Padova, Italy; and †Laboratory of Tumor Cell Biology, Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD 20892

Communicated by Albert B. Sabin, January 31, 1986

ABSTRACT **We isolated six human T-cell lymphotropic virus type I (HTLV-I)-transformed T-cell clones carrying the phenotypic markers of helper and suppressor T cells. Five of the transformed T-cell clones produced infectious HTLV-I, but one (clone 55) contained a defective provirus and was therefore not competent for viral replication. To test whether there is interference between HTLV-I and the cytopathic virus HTLV-III in infection and/or their biological effects, we superinfected these T-cell clones with HTLV-III. The recipient cells that we used displayed either the OKT4 or the OKT8 membrane antigens (helper or suppressor phenotype, respectively). The superinfection was successful in all cases, regardless of phenotype of the recipient cells and status of viral production. Both HTLV-III and HTLV-I were expressed by the infected cell lines containing complete HTLV-I proviruses, as demonstrated by electron microscopy and immunofluorescence. However, only HTLV-III in the virus mixture obtained from the culture supernatants was transmitted to the human neoplastic T-cell line H9. The nonproducer clone 55 did not express HTLV-I upon superinfection with HTLV-III. HTLV-III exerted its cytopathic effect on all but one of the superinfected T-cell clones 15–20 days after infection. The exception, clone 67, is also the only cell clone that expresses the phenotypic marker of suppressor T lymphocytes (OKT8); the other clones carry the OKT4 antigen, correlated with helper functions. The virus released from the superinfected clone 67 is cytopathic for fresh peripheral and umbilical-cord blood lymphocytes, suggesting that cellular factors, rather than a genetic change in the virus, may be responsible for the lack of cytopathic effect of HTLV-III on the suppressor T-cell clone 67.**

The identification of the human T-cell lymphotropic viruses (HTLV) type I, II, and III (1–5) in the last decade has provided the etiological link of human retroviruses and certain human diseases. HTLV-I is recognized as the etiological agent of adult T-cell leukemia and lymphoma (6–8), which is endemic in some regions of the world (9). A less certain disease association exists for HTLV-II, originally isolated from a patient with hairy-cell leukemia (3). A third human retrovirus, HTLV-III [also referred to as lymphadenopathy-associated virus (LAV)] (4, 5) has recently been identified as the causative agent of a new epidemic affecting the immune system, acquired immunodeficiency syndrome (AIDS) (10).

A common feature of these retroviruses is their tropism for T cells *in vivo*. In fact, they all have been isolated initially from a subset of T cells, carrying the OKT4 antigen (11, 12), from the peripheral blood of the infected patients. HTLV-I has also been shown to infect occasionally the OKT8$^+$ T-cell subset (13) as well as B cells (14–16) and human neoplastic cell lines (17). The host range of HTLV-III appears to be more restricted to cells expressing the OKT4 antigen, but preliminary data suggest that certain non-lymphocyte cells in the brain may be infected by the virus *in vivo* (18). The biological effect of HTLV-III differs from that of HTLV-I and HTLV-II. While HTLV-I and HTLV-II immortalize T cells efficiently *in vitro* (19–21), HTLV-III infection of human T cells correlates with depletion of OKT4$^+$ cells both *in vivo* and *in vitro* (22).

HTLV-I, HTLV-II, and HTLV-III also share another notable feature, the presence of *trans*-activator (*tat*) genes. The genes *tat*-I and *tat*-II, thought to be responsible for the immortalization of T cells by HTLV-I and -II (23, 24), have been shown to encode protein products capable of activating the transcription initiated within their respective long terminal repeats (LTRs) (23, 24). A gene, *tat*-III, that activates expression of genes linked to the LTR has also been identified in HTLV-III-infected cells (25, 26). The *tat* genes of HTLV-I, -II, and -III are expressed *in vivo*, as shown by the detection of natural antibodies in infected people (ref. 27 and unpublished data). In addition to *tat*-III, HTLV-III contains two genes with no counterparts in other retroviruses. Although the functions of these genes, termed *sor* and 3′-*orf*, are not known, the protein products have been identified and also shown to be immunogenic *in vivo* (29–31). Additional studies are needed to determine which, if any, viral gene(s) may be involved in the cytopathic effect of HTLV-III.

Some individuals have been shown to have an immune response against viral proteins of both HTLV-I and HTLV-III (32–35), indicating that they might have been infected by both viruses. We infected human T cells with both HTLV-I and HTLV-III to study viral interference, host range, biological effect, and expression of both viruses. We show here that clones of HTLV-I-transformed cells expressing either suppressor (OKT8$^+$) or helper (OKT4$^+$) phenotypes can be infected by HTLV-III. However, the suppressor T-cell clone is resistant to the cytopathic effect of the virus.

MATERIALS AND METHODS

Cell Lines and Viruses. HTLV-I-infected clones were obtained from normal peripheral blood leukocytes infected by cell-free virions produced by the C91/PL cell line (13). Fourteen days after infection, the recipient cells were layered on hardened agarose medium in Petri dishes (36). Colonies originated from single cells were removed from the soft agar

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Abbreviations: HTLV, human T-cell lymphotropic virus; LTR, long terminal repeat; kb, kilobase(s).

‡To whom reprint requests should be addressed.

with a Pasteur pipette and transferred to fresh medium in 96-well plates. Initially, the HTLV-I cellular clones were maintained in culture with RPMI 1640 medium (GIBCO) supplemented with 20% fetal bovine serum (Biofluids, Rockville, MD), 10% interleukin 2 [IL-2; 640 half-maximal units/ml (Advanced Biotechnologies, Silver Spring, MD)], 2 mM L-glutamine, and 50 μg of gentamicin per ml. The concentration of exogenous IL-2 was progressively decreased, and 5% IL-2 was used in these experiments. H9 and H9/HTLV-III cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and gentamicin. The supernatant from H9/HTLV-III cells centrifuged at 2000 × *g* for 20 min was collected and filtered through a 0.22-μm Millipore filter and used as source of virus.

Viral Infection. Five million cells were pelleted by centrifugation, incubated for 1 hr at 37°C with Polybrene (10 μg in 100 μl), and then exposed to 1 ml of the filtered supernatant containing HTLV-III. The mixtures were incubated for 1 hr at 37°C, after which 4 ml of fresh complete medium was added.

DNA Isolation and Filter Blot Hybridization. High molecular weight DNA was purified by proteinase K digestion followed by extraction with phenol/chloroform (1:1, vol/vol). Cellular DNA (5–10 μg) was digested with restriction enzymes, under the conditions suggested by the supplier, and fractionated by electrophoresis in 0.8% agarose gels in Tris/borate/EDTA buffer. The DNA fragments were transferred to nitrocellulose filters by the procedure described by Southern (37). The filters were hybridized with 2×10^8 cpm of nick-translated DNA comprising the complete genome of HTLV-I (38) or HTLV-III (39) as described (40).

Assay for Cell Surface Makers and Virus Expression. Viable cells were examined for the expression of various surface markers (T3, T4, T8, Tac) as described (41), by use of fluorescence-activated cell sorter (FACS II, Becton Dickinson). Cells fixed in methanol/acetone (1:1) were analyzed by indirect immunofluorescence for the presence of HTLV-I and HTLV-III core proteins, using monoclonal antibodies specific for the HTLV-I *gag*-encoded protein, p19 (42), and the HTLV-III *gag*-encoded proteins, p15 and p24 (43, 44). Extracellular particulate reverse transcriptase activity was assayed using oligo(dG·rC)template-primer (44). Electron microscopy of thin sections of cells producing HTLV-I and HTLV-III was carried out as reported (45).

RESULTS

Characterization of HTLV-I-Transformed Cell Clones. We obtained six T-cell clones transformed with HTLV-I by cloning, in soft agar, peripheral blood lymphocytes infected with cell-free HTLV-I (13). Five of them produced viral particles, as demonstrated by electron microscopy, reverse transcriptase assay, and the detection of the HTLV-I core antigen p19 (Table 1); the sixth clone was infected and expressed p19 but did not produce virus. This clone (clone 55) has been shown to contain a single-copy provirus from which the entire envelope (*env*) gene has been deleted (46), whereas all the other cell lines contained at least one complete HTLV-I provirus, as summarized in Table 1.

Superinfection of HTLV-I-Transformed Helper and Suppressor T-Cell Clones by HTLV-III. The supernatant of a T-cell line (H9/IIIB) productively infected by HTLV-III (12) was used to infect the HTLV-I-infected T-cell lines as described in *Materials and Methods*. Superinfection with HTLV-III was successful in all cases, including cells expressing the $OKT8^+$ phenotype. We further characterized the cloned cell lines 62, 67, and 55 for the following reason. Both lines 62 and 67 are productively infected with HTLV-I. However, line 62 expresses the OKT4 antigen, while line 67 expresses the OKT8 antigen (Table 2). The 55 cell line expresses OKT4 antigen but does not produce viral particles. All the cell lines express Tac antigen (Table 2), a feature common among HTLV-I-infected T cells (41). We studied the kinetics of HTLV-III infection by measuring extracellular reverse transcriptase activity and the expression of the HTLV-III core proteins p15 and p24 (43). In each of the three cell lines, approximately 80% of the cells reacted with antiserum against HTLV-III p15 and with antiserum against HTLV-III p24 in an immunofluorescence assay 30 days postinfection. All clones also remained positive (>90%) for the HTLV-I core protein p19. The levels of reverse transcriptase activity were consistent with the values often detected for HTLV-III-infected cells. Electron microscopy revealed that cell lines 62/III and 67/III produced both HTLV-I and HTLV-III particles. Fig. 1 shows a picture of one cell section of line 67/III in which one can discriminate clearly between HTLV-I and HTLV-III virions with their distinct characteristic morphologies. Infection of the $OKT8^+$ 67 cells by HTLV-III was reproducible.

Table 1. Viral expression of the T-cell clones transformed by HTLV-I

	Parameters of HTLV-I infection			
Clone	p19	RT*	Viral particles	HTLV-I genome†
62	+	+	+	Complete (8.5 kb)
90/G9	+	+	ND	Complete (8.5 kb)
67	+	+	+	Complete (8.5 kb)
62a	+	+	+	Complete and defective (8.5 kb and 6.2 kb)
52/B10	+	+	ND	Complete and defective (8.5 kb and 2.9 kb)
55	+	−	−	Defective (6.4 kb)

ND, not done.
*RT, reverse transcriptase activity in culture medium.
†Determined by Southern blot hybridization; kb, kilobases.

Resistance of the Suppressor T-Cell Clone to the Cytopathic Effect of HTLV-III. The cytopathic effect of HTLV-III on the HTLV-I-transformed cells was measured by growth kinetics in culture. A positive cytopathic effect was indicated by a steeper decline in cell number as compared to growth of cells not superinfected with HTLV-III (Table 3). The cells of the cloned lines 62 and 55 were clearly susceptible to the cytopathic effect of the virus (Fig. 2), following growth curves similar to those of normal T cells either infected with HTLV-III virions or transfected with a biologically active HTLV-III DNA clone (22). In contrast, clone 67 was completely resistant to the cytopathic effect (Fig. 2). The phenotypic markers of clone 67 were consistent with those of suppressor T cells prior to infection with HTLV-III, and analysis of the same cells 2 months after superinfection showed no relevant changes in the surface markers (84.6% $T3^+$, 0.9% $T4^+$, 74.8% $T8^+$, 84.1% Tac^+).

To ascertain whether the lack of cytopathic effect was due to changes in the property of the virus rather than a feature of $OKT8^+$ cells (which are infrequently infected by HTLV-III), we transmitted the virus from the supernatant of clone

Table 2. Phenotypic markers of HTLV-I-infected clones

	% positive cells*			
Clone	T3	T4	T8	Tac
62	71.0	86.6	1.8	67.6
67	77.8	0.6	82.8	79.9
55	60.5	87.0	1.6	47.5

Determined by fluorescence-activated cell-sorting analysis with monoclonal antibodies OKT3, OKT4, OKT8, and anti-Tac.

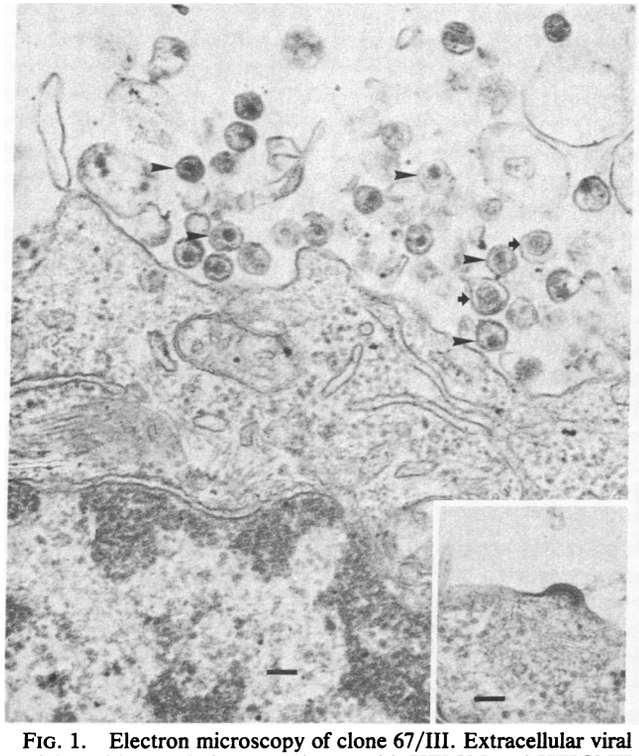

FIG. 1. Electron microscopy of clone 67/III. Extracellular viral particles with morphology consistent with HTLV-I and HTLV-III are shown. Arrowheads mark HTLV-III virions. Arrows point to HTLV-I virions. (*Inset*) An HTLV-III virion budding at the cell margin. (Bars = 100 μm.)

67/III to normal peripheral blood and umbilical-cord blood lymphocytes. A dramatic decline in surviving cells occurred 15–20 days after infection of the cultured lymphocytes obtained from both sources (data not shown). Thus, the virus released from clone 67/III retained its cytopathic effect on appropriate target cells, suggesting that cellular factors play a role in determining the cytopathic effect of HTLV-III.

Table 3. Kinetics of HTLV-III superinfection of HTLV-I-infected clones

Clone	Days after infection	Parameters of HTLV-III infection			
		p15*	p24*	RT†	CPE‡
62	3	0.1	0.5	0.015	−
	6	8.0	15	0.091	±
	10	20	30	0.018	±
	15	30	50	ND	+
	23	50	70	0.40	++
	30	70	80		+++
67	3	0.5	1	0.025	−
	6	10	20	0.130	−
	10	40	40	0.290	−
	15	40	50	1.500	−
	23	80	80	ND	−
	30	85	90	1.400	−
	71	90	90	6.700	−
55	3	2	5	0.015	−
	6	15	30	0.210	±
	10	30	40	ND	+
	15	50	60	8.900	++
	23	50	60	ND	++
	30	70	80	4.100	+++

More than 90% of the cells of all three clones expressed HTLV-I antigen p19 at all time points.
*Percent positive cells.
†Reverse transcriptase activity (pmol of [^{32}P]dCMP incorporated per ml of culture supernatant). ND, not done.
‡Cytopathic effect: +++, <10% viable cells; ++, ≈30% viable cells; +, ≈60% viable cells; ±, >80% viable cells.

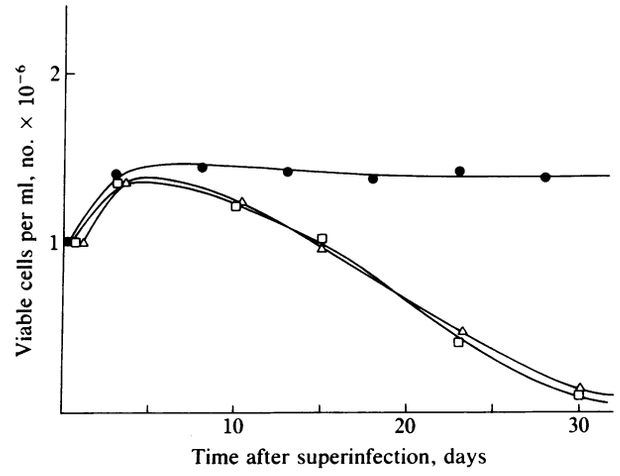

FIG. 2. Growth rate of the cell lines 67 (●), 62 (□), and 55 (△) after HTLV-III infection. Cells (10^6 per ml) were infected with HTLV-III at time 0. Cells of clone 67 were passaged when they reached a density of 1.4×10^6 per ml (every 5 days). Cells of clones 62 and 55 were passaged every 5 days during the first 10 days; after that they were not passaged because of the decreasing number of viable cells.

Analysis of the State of Integration of the HTLV-I and HTLV-III Genomes in the Doubly Infected Cells. It has been observed that the persistence of unintegrated viral DNA is a hallmark for cytopathic retroviruses (28). We analyzed the DNAs of the infected cells by Southern blot hybridization using molecular clones of HTLV-I and HTLV-III DNA. When DNAs from lines 62/III, 67/III, and 55/III were digested with *Sst* I and hybridized to an HTLV-I probe, an 8.5-kb band, representing a nearly complete virus genome, was observed for lines 62/III and 67/III, whereas cell line 55/III showed only a 6.4-kb band, corresponding to a defective provirus (Fig. 3 *Left*). Hybridization of the same *Sst* I-digested DNAs with an HTLV-III probe (Fig. 3 *Right*)

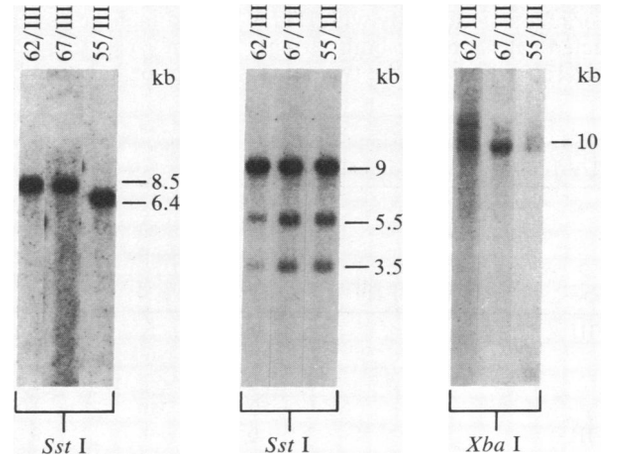

FIG. 3. Southern blot analysis HTLV-III-superinfected clones 62/III, 67/III, and 55/III. High molecular weight DNA (5–10 μg per lane) was cleaved with *Sst* I or *Xba* I, electrophoresed in 0.8% agarose gel, and transferred to nitrocellulose filters (37). The filters were hybridized with a ^{32}P-labeled HTLV-I DNA (*Left*) and with a ^{32}P-labeled HTLV-III DNA (*Right*). Each DNA probe comprises the complete genome of the respective virus. Phage λ DNA digested with *Hin*dIII was used as size markers.

yielded the expected 9-, 5.5-, and 3.5-kb bands (39). We cleaved the same DNAs with *Xba* I, an enzyme that does not cut the HTLV-III LTRs, to ascertain whether we could detect unintegrated HTLV-III genomes. In *Xba* I-digested DNAs, a smear of hybridization and a faint band at 10 kb were detected (Fig. 3 *Right*), indicating the presence of polyclonally integrated proviral DNA as well as unintegrated linear viral DNA in the recipient cell lines 62/III and 67/III, similar to the infected control cells H9/IIIB (39). Thus, no difference in the state of viral DNA was observed in cells that are susceptible (clone 62) or resistant (clone 67) to the cytopathic effect of HTLV-III.

Selective Transmission of HTLV-III to H9 Cells. Filtered supernatant media from the cell lines 62/III, 67/III, and 55/III were used to infect the human neoplastic cell line H9, which is highly susceptible to HTLV-III (12). The H9 recipient cells were tested for reverse transcriptase activity and for the expression of HTLV-I p19 antigen and HTLV-III p15 and p24 antigens in a time-course study over 45 days. In all three cases, infection by HTLV-III was evidenced both by extracellular reverse transcriptase activity and by immunofluorescence with the anti-p15 and anti-p24 antibodies on the recipient H9 cells (Table 4). In contrast, immunofluorescence was not observed with the antibodies directed against HTLV-I p19. Blot hybridization of *Sst* I-cloned DNAs of the H9/62-III, H9/67-III, and H9/55-III cells, using HTLV-I and HTLV-III probes, indicated that the HTLV-I provirus was absent in the H9 recipients (Fig. 4), although it was readily detectable in the DNA of line 67/III used as a positive control. The internal *Sst* I HTLV-III viral bands were detected, confirming transmission of HTLV-III to the H9 cells. *Xba* I digestion of the same DNAs again yielded a 10-kb band as well as a smear upon hybridization to HTLV-III probe, indicating the presence of unintegrated as well as polyclonally integrated viral DNA (Fig. 4). Thus, we conclude that HTLV-III but not HTLV-I from the three doubly infected cell lines was transmitted to H9 cells. This result is consistent with earlier observations that cell free transmission of HTLV-III is more efficient than that of HTLV-I.

DISCUSSION

We have shown that HTLV-I-transformed T cells can be superinfected efficiently with HTLV-III and that the doubly infected cells produce both virions. Not only was there no interference between the two viruses, but prior infection by

Table 4. Viral transmission to H9 cells from supernatants of HTLV-I- and HTLV-III-infected clones

Clone	Days after infection of H9	Parameters of HTLV-III production in H9 indicator cells		
		p15*	p24*	RT†
62/III	3	0	5	0.05
	7	15	30	ND
	12	30	50	3.10
	45	80	90	ND
67/III	3	0	0	0.029
	7	15	30	4.300
	12	60	70	3.100
	45	85	90	10.100
55/III	3	0	0.5	0.027
	7	20	40	0.040
	12	30	50	ND
	45	80	90	8.500

The H9 cells did not express HTLV-I antigen p19 at any time.
*Percent positive cells.
†Reverse transcriptase activity (see Table 3 for units). ND, not done.

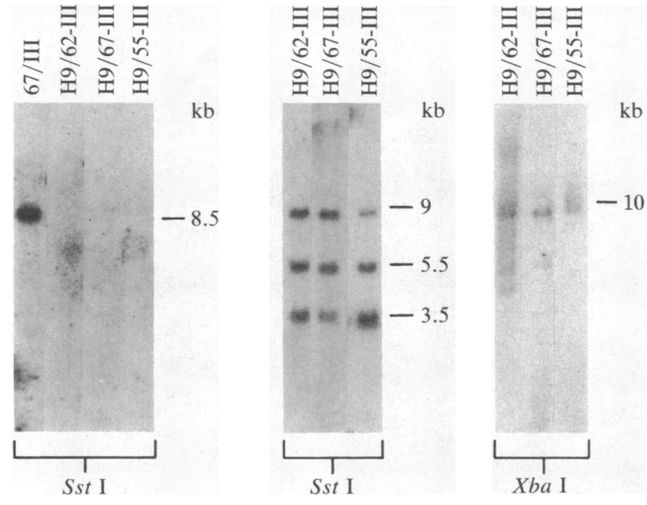

FIG. 4. Southern blot analysis of the DNAs of the H9 cells infected with virus from the supernatants of 62/III, 67/III, or 55/III cells. High molecular weight DNA (5–10 μg) was cleaved with *Sst* I or *Xba* I, electrophoresed in 0.8% agarose gel, and transferred to nitrocellulose filters. The filters were hybridized with a ^{32}P-labeled HTLV-I DNA (*Left*) and with a ^{32}P-labeled HTLV-III DNA (*Right*). *Sst* I-digested 67/III DNA served as positive control for HTLV-I hybridization.

HTLV-I seemed to facilitate HTLV-III expression as demonstrated by the detection of viral antigens earlier after infection and in greater levels as compared to HTLV-III infection of normal peripheral blood lymphocytes (5). It is possible that the preexisting HTLV-I infection induces cellular factors that favor HTLV-III infection; an alternative explanation is that HTLV-I infection preselects a cloned population of susceptible target cells for HTLV-III. This result raises the possibility that patients with a latent HTLV-I infection may be predisposed to subsequent infection with HTLV-III.

It has been suggested that an essential, though not necessarily sufficient, component of the receptor for HTLV-III is a molecule bearing an epitope of the OKT4 antigen (47, 48). Accumulating data also indicate that HTLV-III may infect non-T cells (21, 47, 48) that aberrantly express T4. The successful infection of a T-cell clone expressing only the OKT8$^+$ suppressor phenotype, reported here, indicates that alternative receptors may be used by HTLV-III. It is not known whether such receptors are specifically induced by HTLV-I infection. Alternatively, undetectably low levels of T4 antigen may have been coexpressed in this T8$^+$ cell line.

The finding that the OKT8$^+$ clone is resistant to the cytopathic effect of HTLV-III, although the virus recovered from these cells is still cytopathic on fresh T cells, suggests that an interaction of viral and cellular factors may be involved in the manifestation of the cytopathic effect induced by HTLV-III in some infected cells. The detection of high levels of unintegrated viral DNA in both T4$^+$ and T8$^+$ cell clones indicates that accumulation of unintegrated viral DNA does not directly induce cell killing. The mechanism by which HTLV-III exerts its cytopathic effect is still unclear. At least two hypotheses can be proposed. One is that the *tat* III gene may activate genes that lead to terminal differentiation. If so, such genes would have to be specific for the T4 (helper) lineage. Alternatively, a viral gene product may directly function in the cell killing. This then would also be highly specific for the T4 target cell. Our data also suggest that the OKT8$^+$ cells could act as a reservoir for viral replication *in vivo*, although there is no direct evidence to support this.

We are very grateful to Dr. A. Fisher for her suggestions and to Dr. B. Kramarsky for performing the electron microscopic analysis of the infected cells. This research was supported by the National Cancer Institute and by Consiglio Nazionale delle Ricerche, Progetto Finalizzato Oncologia, and Associazione Italiana per la Ricerca sul Cancro.

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