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Infection of human endothelial cells by human T-lymphotropic virus type I

(syncytium/Kaposi sarcoma/acquired immunodeficiency syndrome)

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ABSTRACT **We studied the effects of human T-lymphotropic virus type I (HTLV-I) on human endothelial cells *in vitro*. During cocultivation with an HTLV-I producer cell line (C91/PL), endothelial cells formed characteristic multinucleated syncytial giant cells. Inoculation with concentrated cell-free supernatant fluid from C91/PL cultures produced similar cytopathic effects, which were neutralized by pretreatment with HTLV-I specific human serum. HTLV-I antigens were detected in the cytoplasm of the multinucleated cells by indirect immunofluorescence. When endothelial cells showed maximal cytopathic changes, reverse transcriptase activity was demonstrated in the supernatant fluid and HTLV-I was isolated by cocultivation with peripheral blood mononuclear cells. This study demonstrates that HTLV-I tropism is not limited to lymphoid cells but extends to human endothelial cells as well.**

Human T-lymphotropic virus type I (HTLV-I), also called human T-cell leukemia virus, was initially isolated and characterized in 1980 (1). It is the first retrovirus associated with a human tumor, the adult T-cell leukemia/lymphoma (2). This neoplasm occurs in clusters in southwestern Japan (3), the Caribbean (4), central Africa (5), and southeastern United States (6). *In vitro*, HTLV-I is tropic for human lymphocytes, especially those with the T_4 (helper-inducer) surface phenotype (7), although infection of human osteogenic sarcoma cells (8), lung cells (FL), and cervical carcinoma cells (HeLa) (9) has also been reported.

Endothelial cells are the putative target cells for Kaposi sarcoma (10, 11), a tumor commonly seen in the acquired immunodeficiency syndrome (AIDS) (12). We have been studying the pathogenesis of Kaposi sarcoma by evaluating *in vitro* susceptibility of endothelial cells to viruses implicated in the cause of AIDS. Recently, we have demonstrated that endothelial cells can be productively infected by cytomegalovirus (13). Retroviruses have also been implicated in the pathogenesis of AIDS. Although the lymphadenopathy-associated virus (LAV) and the closely related or identical HTLV-III have received the greatest attention (14–18), HTLV-I has also been isolated from patients with AIDS (19). We now report our findings of infection of human umbilical vein endothelial cells by HTLV-I.

MATERIALS AND METHODS

Cells and Virus. Early passage endothelial cells were obtained from R. Weinstein (Beth Israel Hospital, Boston). These cells were harvested from a human umbilical vein (20) and grown on a human fibronectin matrix while maintained on medium 199 supplemented with fetal calf serum (10%), endothelial cell growth factor (100 μg/ml), Hepes buffer (10 mM), L-glutamine (2 mM), penicillin (250 units/ml), and streptomycin (250 μg/ml) (21). All cells expressed factor VIII-related antigen as demonstrated by direct immunofluorescence using fluoresceinated anti-human factor VIII antibody (Atlantic Antibody, Scarborough, ME). C91/PL, an HTLV-I producer T-cell line, was provided by R. Gallo (National Cancer Institute, Bethesda, MD) and maintained on RPMI medium 1640 containing fetal calf serum (20%), Hepes buffer, L-glutamine, and antibiotics.

Inoculation of Endothelial Cells with Cell-Associated HTLV-I. Endothelial cells grown to a density of 1×10^6 cells in 25-cm^2 flasks (37°C, 5% CO_2 in air) were cocultivated with 1×10^6, 5×10^5, and 2×10^5 C91/PL cells that had been pretreated with mitomycin C (100 μg/ml, 30 min). These cell concentrations corresponded to multiplicities of infection (mois) of 1.0, 0.5, and 0.2 C91/PL cell/endothelial cell, respectively. Similarly, a control culture of 1×10^6 endothelial cells was inoculated with 1×10^6 cells of CEM, also known as CCRF-CEM, a virus-negative T-lymphoblastoid cell line (22). After two media changes (4–5 days), the nonadherent C91/PL or CEM cells were lost from the cultures. The endothelial cells were not pretreated with enhancing agents such as Polybrene or DEAE-dextran.

Inoculation of Endothelial Cells with Cell-Free HTLV-I. Cell-free HTLV-I pellets were obtained by ultracentrifugation (100,000 × *g*, 60 min, 4°C) of 100 ml of preclarified (10,000 × *g*, 15 min) supernatant fluid from C91/PL cultures. The virus pellets were resuspended in a total of 2.5 ml of medium 199 and filtered through a 0.45-μm Nalgene filter. Endothelial cells (1×10^6 per 25 cm^2) were incubated with 1 ml of this concentrated suspension for 2 hr at 37°C before complete medium was added. A parallel culture was similarly inoculated, but the inoculum was pretreated for 60 min at 37°C with 1 ml of 1:10 dilution of heat-inactivated (56°C, 60 min) HTLV-I specific serum (3740), provided by M. Essex (Harvard School of Public Health, Boston). This serum, obtained from a Japanese person from an HTLV-I endemic area, had a titer exceeding 1:1000 by indirect immunofluorescence for HTLV-I membrane antigens (23).

Indirect Immunofluorescence. Endothelial cells were trypsinized and resuspended in phosphate-buffered saline before attachment to glass slides. After acetone fixation, cells were covered for 30 min with a 1:100 dilution of serum 3740 or a 1:250 dilution of murine monoclonal antibody against HTLV-I protein p19, obtained from B. Haynes (Duke University School of Medicine, Durham, NC). After three washes, the appropriate undiluted goat anti-human IgG-fluorescein isothiocyanate conjugate (Electronucleonics, Bethesda, MD) or a 1:40 dilution of rabbit anti-mouse $F(ab')_2$-fluorescein isothiocyanate conjugate (Cappel Laboratories, Cochranville, PA) was added for 30 min. After three additional washes, the slides were mounted and examined under a Zeiss ultraviolet microscope.

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Abbreviations: AIDS, acquired immunodeficiency syndrome; CPE, cytopathic effects; HTLV-I and HTLV-III, human T-lymphotropic virus types I and III, respectively; LAV, lymphadenopathy-associated virus; moi, multiplicity of infection; PBMC, peripheral blood mononuclear cells.

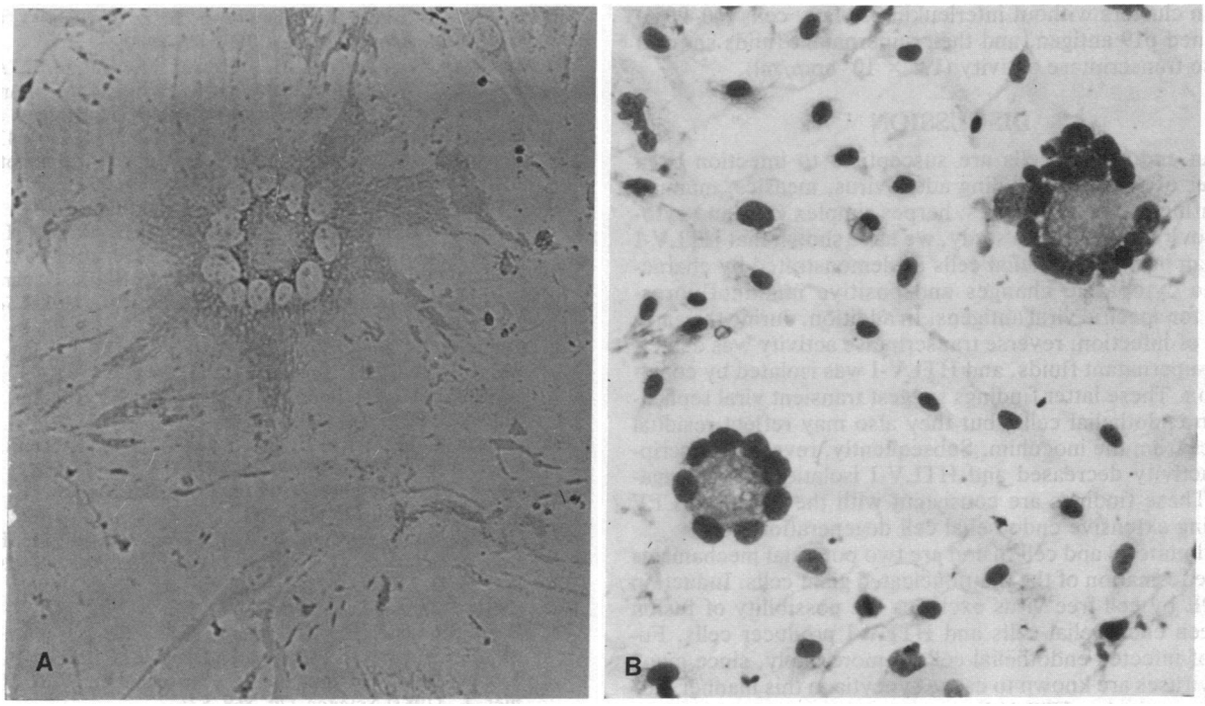

FIG. 1. (*A*) Multinucleated giant cells seen in endothelial cell culture 3 days after addition of HTLV-I. (×190.) (*B*) Multinucleated giant cells stained with the May–Grunwald–Giemsa stain. (×170.).

Reverse Transcriptase Assay. Reverse transcriptase assays were performed according to the method described by Poiesz *et al.* (1), using poly(A)-oligo(dT)$_{12-18}$ as template primer and 10 mM Mg^{2+} instead of Mn^{2+} as the cation. The activities were expressed in counts per minute per milliliter of culture medium.

Isolation of HTLV-I from Endothelial Cells. Attempts were made to isolate HTLV-I from endothelial cells exhibiting cytopathic changes following inoculation with exogenous virus. These were done by passage of affected cells (2.5×10^5) onto additional endothelial cells (1×10^6) and by cocultivation with peripheral blood mononuclear cells (PBMC). PBMC were obtained by Ficoll-Hypaque separation of peripheral venous blood donated by healthy volunteers. Following treatment with phytohemagglutinin (25 μg/ml), 2×10^6 PBMC were cocultivated with 1×10^6 endothelial cells for 4 days. Subsequently, the PBMC were removed and maintained on RPMI medium 1640 supplemented with 20% fetal calf serum and 10% lectin-free interleukin 2 (Electronucleonics), plus Hepes buffer, L-glutamine, and antibiotics. After 4–6 weeks, the cultures were examined for altered cellular morphology, interleukin 2-independent growth, reverse transcriptase activity, and HTLV-I p19 antigen. If all these features were present, the culture was considered positive for HTLV-I.

RESULTS

Cytopathic Effects (CPE). Multinucleated giant cells and syncytia were seen in the endothelial cell cultures beginning 3 days after inoculation of C91/PL cells at a moi of 1.0 (Fig. 1*A*). Cell nuclei, numbering 2–16, were often arranged in a characteristic ring formation (Fig. 1*B*). Mitotic figures were not observed. Over the ensuing 5–7 days, the CPE increased to involve ≈50% of the cells in culture. After day 14, the multinucleated giant cells showed signs of degeneration with extensive vacuolization before detaching from the monolayer. Similar CPE were observed for cultures inoculated with C91/PL cells at lower mois (0.5 and 0.2) or with concentrated cell-free supernatant fluid of C91/PL. No CPE were seen in endothelial cells inoculated with HTLV-I that had been incubated with neutralizing antiserum. Inoculation of endothelial cells with control CEM cells did not result in any morphologic changes.

Detection of Viral Antigen. Between 5 and 17 days after inoculation, HTLV-I antigens were detected in the cytoplasm of the multinucleated cells by indirect immunofluorescence using human HTLV-I specific serum or mouse HTLV-I p19 monoclonal antibody. Nuclear fluorescence was not seen. In cultures without CPE, the immunofluorescence tests were consistently negative for HTLV-I antigens.

Detection of HTLV-I. Results of our attempts to detect HTLV-I in the endothelial cells inoculated with C91/PL at moi of 1.0 are summarized in Table 1. On the 8th day following inoculation, ≈50% of the cells were multinucleated, and reverse transcriptase activity was detected in the supernatant fluid. Passage of the infected cells onto additional endothelial cells resulted in similar CPE within 5–7 days. However, passage of endothelial cells 14 and 28 days after infection did not induce syncytia formation. Similarly, HTLV-I was isolated from endothelial cells by cocultivation with PBMC on day 8, but not on days 14 and 28. The HTLV-I positive PBMC showed characteristic morphologic changes (24) and

Table 1. Methods for detection of HTLV-I in endothelial cells after inoculation with C91/PL cells

Method	Time after inoculation of C91/PL cells (moi = 1.0)		
	Day 8	Day 14	Day 28
Passage of infectious virus			
To endothelial cells*	+	−	−
To PBMC†	+	−	−
Reverse transcriptase activity, ×10^3 cpm/ml	2.1	0.3	0.3

*2.5×10^5 endothelial cells exhibiting CPE were scraped off and passed onto 1×10^6 additional endothelial cells.
†1×10^6 endothelial cells exhibiting CPE were cocultivated with 2×10^6 PBMC.

grew in clusters without interleukin 2. Many cells (60–90%) contained p19 antigen, and their supernatant fluids showed reverse transcriptase activity (1.2×10^4 cpm/ml).

DISCUSSION

Human endothelial cells are susceptible to infection by a number of viruses, including adenovirus, measles, mumps, parainfluenza, enteroviruses, herpes simplex (25), and cytomegalovirus (13). In this study, we have shown that HTLV-I also can infect endothelial cells as demonstrated by characteristic cytopathic changes and positive immunofluorescence for specific viral antigens. In addition, during the early phase of infection, reverse transcriptase activity was detected in supernatant fluids, and HTLV-I was isolated by cocultivation. These latter findings suggest transient viral replication in endothelial cells, but they also may reflect residual viruses from the inoculum. Subsequently, reverse transcriptase activity decreased and HTLV-I isolations were negative. These findings are consistent with the observed CPE showing extensive endothelial cell degeneration.

Endomitosis and cell fusion are two potential mechanisms for the formation of the multinucleated giant cells. Induction of CPE by cell-free virus excludes the possibility of fusion between endothelial cells and HTLV-I producer cells. Fusion of infected endothelial cells is more likely, since many retroviruses are known to cause syncytia in this manner (26–29). In particular, HTLV-I can induce some human neoplastic cells to form multinucleated cells by fusion (9, 30). The absence of mitotic figures in the endothelial cells suggests that endomitosis is not a likely mechanism.

HTLV-I is tropic for human lymphocytes, particularly those with the T_4 surface phenotype (7). Recently, Clapham *et al.* showed that human osteogenic sarcoma cells can also be productively infected, indicating that the spectrum of this virus is not strictly limited to lymphoid cells (8). Our current study demonstrates that normal human endothelial cells are also susceptible to HTLV-I infection. Endothelial cells may be important in immunoregulation by interacting with circulating lymphocytes *in vivo* (31–33). It is, thus, possible that endothelial cells may be infected by virus-bearing T cells during natural infections. The infected endothelial cells, in turn, may serve as a reservoir to further propagate the virus.

Endothelial cells are the putative target cells for Kaposi sarcoma (10, 11), a neoplasm commonly seen in AIDS. HTLV-I was previously implicated in the pathogenesis of AIDS (19, 23, 34), but recent studies suggest that a related human retrovirus, HTLV-III or LAV, is the probable etiologic agent (14–18). Nevertheless, HTLV-I has been isolated from patients with AIDS and may play a role in some of the manifestations observed. In view of our current findings, it will be important to study the effects of HTLV-III and/or LAV on human endothelial cells as well.

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