Brief Definitive Report

Human T Lymphocyte Virus 1 from a Leukemogenic Cell Line Mediates In Vivo and In Vitro Lymphocyte Apoptosis

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Summary

HTLV-1 is implicated in the development of diverse diseases. However, most HTLV-1–infected individuals remain asymptomatic. How HTLV-1 infection leads to disparate consequences remains a mystery, despite extensive investigation of HTLV-1 isolates from infected individuals. As in human infection, experimental HTLV-1 infection in rabbits is generally benign, although HTLV-1–infected rabbit T cell lines that mediate lethal leukemia-like disease have been reported. We report here that thymuses from mature outbred rabbits inoculated with a lethal HTLV-1 T cell line (RH/K34) showed morphological and biochemical evidence of apoptosis, whereas thymuses from rabbits inoculated with nonlethal HTLV-1 T cell lines showed no signs of apoptosis. Exposure of rabbit or human lymphocytes to purified virus from RH/K34 caused rapid induction of apoptosis, providing an in vitro correlate to the pathogenic effects. By contrast, virus isolated from a nonlethal cell line mediated dose-dependent lymphocyte proliferation. These data implicate lymphocyte apoptosis as a potential mechanism by which the lethal HTLV-1 cell line causes fulminant disease and provide a means to identify factors contributing to HTLV-1 disease. Results from this HTLV-1 infection model can provide insight into variations in HTLV-1 pathogenicity in human infection.

HTLV-1 infection has been associated with adult T cell leukemia/lymphoma (ATLL) (1, 2) and HTLV-1–associated myelopathy (3). Extensive studies of HTLV-1 genomic variability reveal a high degree of sequence conservation among isolates from distinct endemic areas (4, 5); and no specific mutation can be linked to HTLV-1–associated pathology (6, 7). Rabbits can be infected with HTLV-1 (8, 9), and, in some reported cases, lethal ATLL-like disease occurs (10, 11). As in human infection, primary structural comparisons of HTLV-1–causing asymptomatic infection with virus-mediating lethal disease in rabbits provide no clues to mechanisms of HTLV-1 pathogenicity (12).

Apoptosis, a form of programmed cell death, can be induced by a number of different factors (13–15) and has been implicated in pathogenicity related to infection with certain viruses, including human immunodeficiency virus (16–19), chicken anemia virus (20), and feline leukemia virus (21). To date, apoptosis has not been implicated in HTLV-1 infection, although Debatin et al. (22) report that cells from patients with HTLV-1 leukemia express the surface antigen APO-1 and undergo in vitro apoptosis when exposed to anti–APO-1. Exposure of normal lymphocytes to cell-free HTLV-1 causes cell activation (23–26).

The present report documents that an HTLV-1–infected rabbit T cell line (RH/K34) that caused lethal leukemic-like disease (12) induced apoptosis in lymphoid organs of inoculated rabbits. Furthermore, virus isolated from RH/K34 induced in vitro apoptosis of rabbit and human lymphocytes. In contrast, virus from a nonlethal HTLV-1–infected cell (RH/K30) induced lymphoproliferation in a dose-responsive manner. The lethal property of RH/K34 derives from factors other than the primary structure of the infecting virus; other HTLV-1–infected cell lines harboring the RH/K34 provirus do not cause leukemia (12), and virus isolated from these cells does not induce apoptosis.

Materials and Methods

Animals. New Zealand White rabbits were given intravenous injections of HTLV-1–transformed cells (2–3 × 10^8 cells in 1–2 ml saline) or uninfected rabbit PBMC. Rabbits were monitored daily for temperature and weight. HTLV-1–infected and control animals were necropsied at various times after inoculation and tissues were taken for study.

Cell Lines and Culture. HTLV-1–transformed rabbit T cell lines (RH/K30, RH/K34, RHT-16) were derived and maintained in complete medium, as described previously (27). RPMI K30 and RPMI K34, two human HTLV-1–infected B cell lines, were derived by coculture of RPMI 8226, a human myeloma cell line, with irradiated RH/K30 or RH/K34, respectively.

PBMC isolated from rabbit or human blood, as described (27), were incubated at a density of 2 × 10^6 cells/ml in complete

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medium in the presence, or absence, of purified virus. Cell viability was assessed by trypan blue exclusion.

Cell activation was measured by [^{3}H]thymidine incorporation after incubation with purified virus. Cells were seeded in triplicate in round-bottomed microculture plates at a density of 1.0×10^5 cells/well in 100 μl of medium. Human rIL-2 (100 U/ml; Biosource International, Inc., Camarillo, CA) was added to a second set of cultures. Purified HTLV-1 (50 μl) was added to each well. [^{3}H]thymidine (1 μCi/well; sp act, 6.7 Ci/mmol; Dupont-NEN, Boston, MA) was added on day 3; after 12 h, cells were harvested and thymidine incorporation was measured by liquid scintillation counting. Data reported are the mean of triplicate cultures.

Experiments to determine the effects of brief exposure to virus used techniques described for HIV-1 (17). Rabbit PBMC (5×10^6) were incubated with virus for 1 h at 37°C, washed in PBS containing 2 mM EDTA, and treated with 2.5 mg/ml trypsin for 10 min at 25°C. Digestion was stopped by the addition of complete medium. Cytoplasmic extracts were prepared from an aliquot of the cells, and p24 gag protein concentration was determined by antigen capture ELISA (Coulter Corp., Hialeah, FL). Aliquots of the trypsinized cells were monitored for viability over a 4-d period.

Virus Purification. Virus was purified by a modification of the method of Kimata et al. (25). HTLV-1 cell cultures were centrifuged at low speed, and supernatants were taken and centrifuged at 25,000 rpm for 1 h at 4°C. Pellets were resuspended in PBS, loaded onto 20–60% continuous sucrose gradients in PBS, and centrifuged at 25,000 rpm for 18 h at 4°C. Fractions were tested for sucrose concentration by refractometry and for protein concentration by the BCA protein assay (Pierce Chemical Co., Rockford, IL). Pooled fractions were diluted 10-fold in PBS and centrifuged at 25,000 rpm for 1 h; pellets were resuspended in PBS and virus concentration estimated by determination of p24 gag protein.

Electron and Light Microscopy. Rabbit or human PBMC exposed to HTLV-1 virus particles were washed in medium and pelleted. Thymuses from HTLV-1–infected or control rabbits were fixed in buffered formalin (pH 7.4) and processed. Paraffin sections were stained with hematoxylin and eosin. Cell pellets and thymuses were fixed for 2 h at 4°C in 2.5% glutaraldehyde in Millonig's buffer (285 mosM, pH 7.4), postfixed in 1% osmium tetroxide, dehydrated, embedded into Spurr's resin, sectioned (70–90 nm thickness), stained with uranyl acetate and lead citrate (JFE Enterprise, Brookville, MD), and examined in a transmission electron microscope (EM 10C; Carl Zeiss, Inc., Thornwood, NY).

Flow Cytometry Analysis. Cells were washed in ice-cold PBS containing 0.1% BSA and 0.01% NaN_3, and reacted with FITC-conjugated mAbs (Spring Valley Laboratories, Woodbine, MD) directed against CD4 (Ken-4), CD8 (12.C7), or surface Ig (NRBL). Subsequently, cells were exposed to 1 μg/ml ethidium bromide (Sigma Chemical Co., St. Louis, MO) for 30 min, as described (15). Cells were analyzed by flow cytometry using Lysys II software (Becton Dickinson & Co., Mountain View, CA).

DNA Fragmentation Assays. DNA was extracted from PBMC or from rabbit thymuses as previously described (28), and fractionated on a 1.8% agarose/ethidium bromide gel.

Results and Discussion

Apoptosis in HTLV-1–infected Rabbit Thymus. 12 mature female rabbits were inoculated with a dose (2×10^8) of RH/K34 cells, seven control rabbits were given an equal number of RH/K30 cells and one was inoculated with uninfected rabbit PBMC. Animals inoculated with RH/K34 developed an ATLL-like disease and died 6–10 d after inoculation. Pathological findings included atypical lymphocytes in the circulation and leukemic infiltrates in lymph node, lung, liver, and spleen (Simpson, R. M., manuscript in preparation). Serial necropsy showed that by 4 d after inoculation, there was histopathological evidence of thymocyte depletion in rabbits inoculated with RH/K34 (Fig. 1 *A*). In contrast, rabbits given the same or higher doses of RH/K30 developed low-grade leukemia; no thymic lesions were observed in these animals (Fig. 1 *B*) or in the rabbit inoculated with normal rabbit PBMC. Transmission electron microscopy of thymuses from RH/K34-inoculated rabbits, but not RH/K30-inoculated rabbits, revealed numerous apoptotic thymocytes characterized by condensed cytoplasm containing intact organelles, peripheral chromatin condensation, and nuclear segmentation (Fig. 1 *C*). In addition, DNA from depleted rabbit thymuses show an oligonucleosomal fragmentation pattern characteristic of apoptosis (not shown). The effects of RH/K34 on rabbit thymus are similar to those documented for HIV-1 (16) and for chicken anemia virus (20) infections where thymus depletion results from apoptosis.

HTLV-1 Particles from RH/K34 Trigger Death of Lymphocytes In Vitro. Virus was isolated from HTLV-1 cell lines, RH/K34 and RH/K30, and tested for its effects on lymphocytes. Rabbit PBMC, exposed to purified cell-free RH/K34 virus particles, showed a rapid and marked decrease in cell viability. In contrast, viability of rabbit lymphocytes exposed to RH/K30 virus particles, or to medium alone, remained relatively constant (Fig. 2 *A*).

Like the thymuses of rabbits inoculated with RH/K34 cells, PBMC exposed to virus particles from RH/K34 showed morphological features characteristic of apoptosis. In contrast, cells exposed to RH/K30 virus particles or cultured in medium alone retained a normal appearance (not shown). Further evidence for breakdown of cellular DNA into oligonucleosome-size fragments in cells exposed to RH/K34 virus (Fig. 2 *B*, lane *4*), but not RH/K30 virus or medium alone (lanes *2* and *3*), confirm that virus from RH/K34 triggers death by apoptosis in target cells.

Human PBMC were exposed to RH/K34 or RH/K30 virus particles to determine whether virus passaged in rabbit cells would affect human cells. As with the rabbit PBMC, virus particles from RH/K34, but not RH/K30, induced rapid death of human PBMC in vitro (Fig. 2 *C*).

To determine whether constant exposure to virus is required for the induction of apoptosis, rabbit PBMC were exposed to several different concentrations of virus for 1 h and washed, and extracellular virus was digested by trypsin treatment (17). Virus antigen levels in lysates of cells initially exposed to 50 μg/ml virus dropped to 40 pg of HTLV-1 p24 protein after this treatment, less than 10^{-6} of the viral antigen in the original input. Dose response data (unpublished results and Fig. 3) indicate that constant exposure to such low virus concentrations cause neither stimulation, nor apoptosis of PBMC, within the time frame evaluated. Viability of the HTLV-1–exposed and trypsin-treated cells indicated that 1-h exposure to RH/K34 virus did not induce cell death (Fig. 2 *D*) in

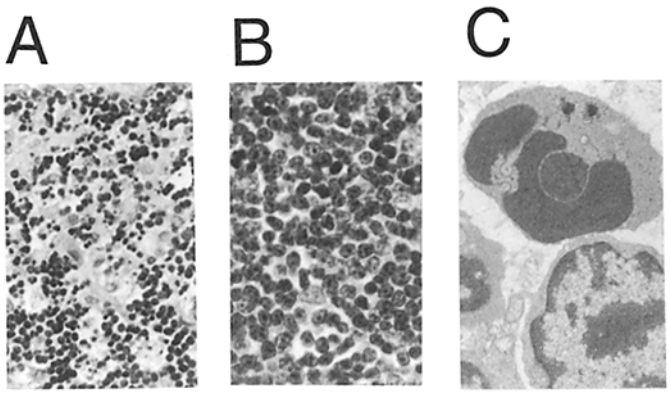

Figure 1. Histopathology of thymuses from rabbits inoculated with the HTLV-1–derived cell lines, RH/K34 and RH/K30. ×200. (*A*) Thymus from rabbit 4 d after inoculation (dai) with RH/K34. Numerous apoptotic thymocytes with dark condensed nuclei surrounded by clear spaces resulted in cortical thymus depletion. (*B*) Thymus from rabbit at 10 dai with RH/K30 showing normal thymic architecture. (*C*) Transmission electron microscopy revealing cytoplasmic and chromatin condensation with nuclear segmentation, characteristic of apoptosis in thymocyte from a rabbit examined 4 dai with RH/K34; for comparison the cell profile at the lower right is unaffected. ×4650.

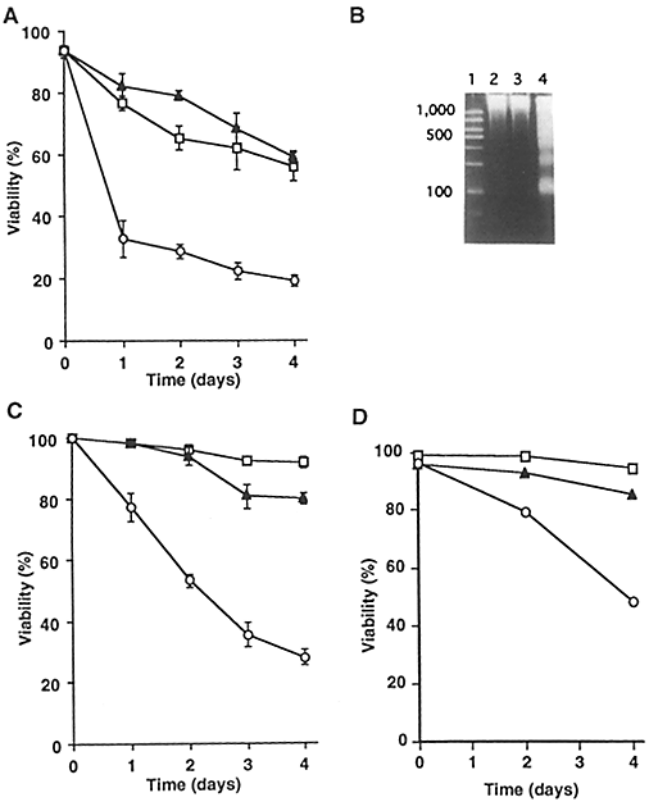

Figure 2. In vitro effects of purified HTLV-1 from RH/K34 and RH/K30 on rabbit and human PBMC. (*A*) Cell viability monitored on cultures of rabbit PBMC incubated in medium alone (*open squares*), in the presence of 50 μg/ml of virus from RH/K30 (*closed triangles*) or RH/K34 (*open circles*). (*B*) DNA fragmentation in rabbit PBMC exposed to RH/K34 virus. DNA was extracted from PBMC cultured for 24 h in medium alone (lane *2*), or in the presence of virus isolated from RH/K30 (lane *3*) or RH/K34 (lane *4*). Lane *1* shows Gel Marker 50–1,000 bp standard (Research Genetics, Huntsville, AL). (*C*) Cell viability measured for human PBMC cultured as in (*A*). (*D*) Viability of rabbit PBMC after brief exposure to 50 μg/ml RH/K30 (*closed triangles*) or RH/K34 (*open circles*) virus or medium (*open squares*). After 1 h exposure to virus or medium at 37°C, cultures were treated with trypsin, washed, and incubated at 37°C.

the same rapid time frame as when rabbit PBMC were constantly exposed (Fig. 2 *A*). At 4 d, cells pulsed with RH/K34 virus particles showed significantly decreased viability, compared to those similarly exposed to RH/K30 virus or to medium alone (Fig. 2 *D*).

Consistent with previous reports that virus isolated from HTLV-1–infected T cells induced proliferation of human lymphocytes (23) and thymocytes (24), RH/K30 virus stimulated thymidine incorporation by rabbit PBMC in a dose-dependent manner (Fig. 3). In marked contrast, RH/K34 virus (in amounts >5 μg p24/ml) inhibited cell activation. Identical results were obtained with PBMC from an HTLV-1–infected rabbit (not shown). The effects of RH/K34 and RH/K30 virus on PBMC were also observed in the presence of exogenous rIL-2; RH/K34 virus particles inhibited IL-2–driven proliferation, while RH/K30 virus acted synergistically to give greater levels of proliferation (Fig. 3). Although IL-2 was reported to inhibit spontaneous apoptosis of T lymphocytes from ATLL patients in culture (29), it did not prevent apoptosis induced by RH/K34 virus in PBMC.

Cell Populations Undergoing RH/K34-mediated Apoptosis. Cells targeted by RH/K34 virus particles were identified by flow cytometric analysis of cell populations stained with ethidium bromide (EtBr) and mAbs to cell surface antigens CD4, CD8, and Ig. In agreement with reports that EtBr stains nucleic acids in cells undergoing apoptosis (15), frequencies of EtBr-positive cells corresponded to the frequency of nonviable cells, as determined by trypan blue exclusion. Analyses indicated that 40% of cells exposed to RH/K34 virus particles were EtBr positive, compared with 5% of cells exposed to RH/K30 virus particles or in medium alone (Fig. 4). A relatively high percentage of apoptotic cells expressed CD4, whereas most $CD8^+$ and Ig^+ cells remained EtBr negative. Interpretations of these experiments must take into account the distribution of surface markers on rabbit lymphocytes. About 10% of rabbit blood lymphocytes bear the CD8 marker, about 28% bear CD4, and as many as 25% are TCR/γδ cells lacking CD4 and CD8 (27). Although it is not possible to define precise targets for the virus from these data, the population undergoing apoptosis contains more T cells than B cells.

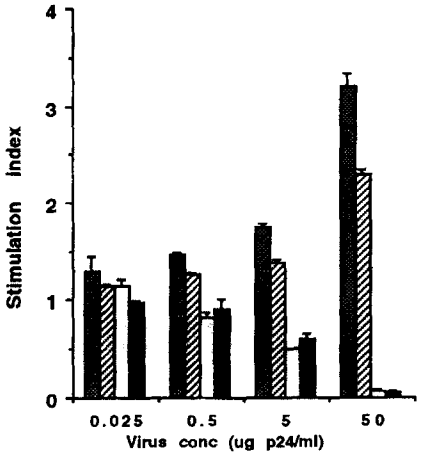

Figure 3. Effects of purified RH/K30 and KH/K34 virus on [^{3}H]thymidine incorporation in cultured rabbit PBMC. Thymidine uptake in presence of different concentrations of RH/K30 (*shaded and striped bars*) or RH/K34 (*open and black bars*). Cultures designated by shaded or solid black bars contained IL-2. Stimulation indices are calculated by reference to PBMC maintained in identical cultures, to which no virus was added.

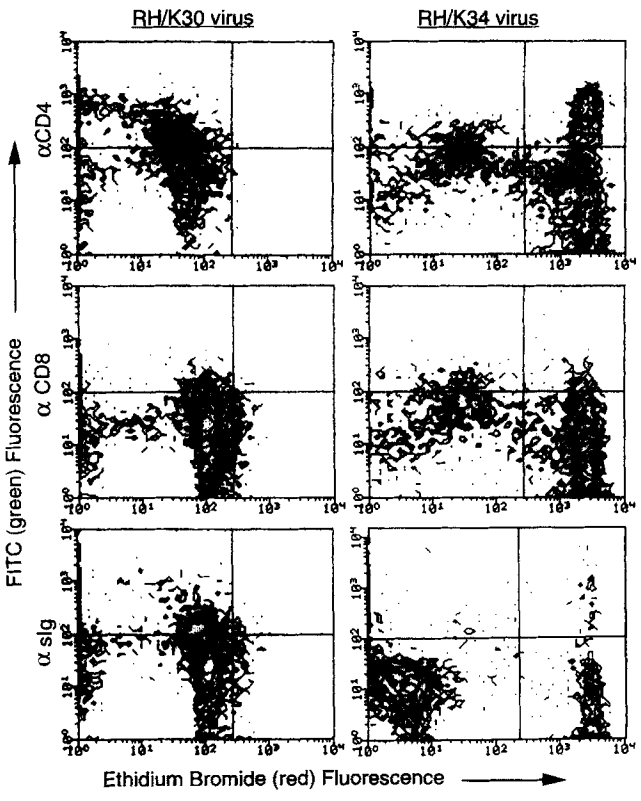

Figure 4. Flow cytometry of rabbit PBMC exposed to virus from RH/K34 or RH/K30. Rabbit PBMC were cultured at 37°C in medium alone or in presence of purified virus particles. Cells were reacted with mAb to rabbit CD4, CD8, or surface Ig and with ethidium bromide and analyzed by flow cytometry. Cells cultured in medium alone at 4°C were used as negative control for EtBr staining.

Host Cell Factors Influence Virus-induced Apoptosis. The relevant differences between the HTLV-1 isolate from RH/K34 that mediates apoptosis and the virus from RH/K30 that causes lymphoproliferation are not known. Explanations based upon structural differences of the virus proteins are difficult to support, since the two viral genomes differ by only 0.2% and have identical envelope sequences (12). In addition, a CD4$^-$, CD8$^+$, TCR/$\alpha\beta$ rabbit T cell line, RHT16, which has an HTLV-1 provirus sequence identical to that of RH/K34, did not cause leukemia in rabbits (12); nor did purified RHT-16 virus induce lymphocyte apoptosis (not shown).

Virus-associated host cell proteins may play a role in virus–cell interactions. Recent reports indicate that cell surface proteins, including class I and II MHC molecules, are incorporated into retrovirus membranes during the budding process (30). Allogeneic differences between MHC proteins on the virus membrane (from the host cell) and those on target cells are not likely to cause lymphoproliferative responses because PBMC preparations from different rabbits, as well as from human subjects, all reacted in a similar fashion. Analysis of the cell lines showed that both RH/K34 and RH/K30 are CD4$^-$, CD8$^-$, TCR/$\gamma\delta$ T cells, and both equally express most markers tested, including TNF-α, LFA3, IL-1-β, CD5, CD25, CD43, and CD44 (27 and unpublished data). However, one potentially significant difference is the expression of the leukocyte common antigen CD45: RH/K34 expresses significantly higher levels of CD45 than RH/K30 (not shown). Since tyrosine phosphorylation has been associated with the cytopathic effects of HIV-1 (31), the expression of this tyrosine phosphatase by RH/K34 may play a role in its pathogenesis. Similar to the present data, phosphatase activity associated with murine leukemia virus appears to correlate with its ability to cause leukemia in mice (32). However, other CD45$^+$ HTLV-1–infected cell lines, including RHT-16, were not lethal in vivo and did not induce apoptosis in vitro. This result suggests that CD45 is necessary, but not sufficient, for HTLV-1 apoptotic potential.

To determine whether the RH/K34 virus produced by human, rather than rabbit, cells would induce apoptosis, irradiated RH/K34 and RH/K30 cells were used as a source for the infection of RPMI 8226, a human myeloma cell line. Two HTLV-1–infected cell lines RPMI K30 and RPMI K34, derived from the human line, expressed high levels of p24 and produced mature HTLV-1 particles, as assessed by electron microscopic analysis (not shown). Rabbits inoculated with 3×10^8 live RPMI K34, or RPMI K30 cells, showed no abnormality in temperature, weight, or other clinical signs of disease. However, both rabbits produced anti–HTLV-1 antibodies from 2 wk after inoculation, and HTLV-1 sequences were detected in PBMC by PCR amplification beginning 8 wk after inoculation (not shown). Furthermore, sucrose gradient–purified virus particles from RPMI K34 supernatants were unable to induce death of rabbit or human PBMC in vitro. Therefore, data from both rabbit and human cell–derived virus emphasize the importance of the infected cell phenotype, as opposed to primary virus structure, in the pathogenic potential of the HTLV-1.

Numerous reports show apoptosis as a routine consequence of virus–host interaction for certain viruses (16–21). By contrast, apoptosis is not commonly associated with exposure to HTLV-1. Induction of apoptosis in both human and rabbit lymphocytes is peculiar to the lethal leukemogenic cell line RH/K34 or its isolated virus. The relevance of these findings to the variable pathogenic effects of human HTLV-1 infection is supported by early observations of thymus depletion in ATLL patients (33). The present data implicate virus-mediated apoptosis as a mechanism for such depletion and allow an in vitro means to investigate pathogenic isolates of HTLV-1.

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