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$CD4^+$ blood lymphocytes are rapidly killed *in vitro* by contact with autologous human immunodeficiency virus-infected cells

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ABSTRACT **We have investigated the ability of human immunodeficiency virus (HIV)-infected cells to kill uninfected $CD4^+$ lymphocytes. Infected peripheral blood mononuclear cells were cocultured with autologous ^{51}Cr-labeled uninfected cells. Rapid death of the normal CD4-expressing target population was observed following a brief incubation. Death of blood $CD4^+$ lymphocytes occurred before syncytium formation could be detected or productive viral infection established in the normal target cells. Cytolysis could not be induced by free virus, was dependent on gp120–CD4 binding, and occurred in resting, as well as activated, lymphocytes. $CD8^+$ cells were not involved in this phenomenon, since HIV-infected CEMT4 cells ($CD4^+$, $CD8^-$ cells) mediated the cytolysis of uninfected targets. Reciprocal isotope-labeling experiments demonstrated that infected CEMT4 cells did not die in parallel with their targets. The uninfected target cells manifested DNA fragmentation, followed by the release of the ^{51}Cr label. Thus, in HIV patients, infected lymphocytes may cause the depletion of the much larger population of uninfected $CD4^+$ cells without actually infecting them, by triggering an apoptotic death.**

Much effort has been focused on the issue of how a relatively small number of human immunodeficiency virus type 1 (HIV-1)-infected cells results in the ultimate destruction of all $CD4^+$ lymphocytes (reviewed in ref. 1), yet the mechanisms causing this immunodeficiency remain unclear. Experimental observations *in vitro* describe several phenomena that might independently contribute to the cell death *in vivo*. Cytopathicity can be a direct consequence of viral replication inside infected lymphocytes (2–5). Induction of apoptosis by HIV-1 has been suggested as a cause of the depletion of infected $CD4^+$ cells (6–12). However, the small minority of lymphocytes infected at any given time and the dramatic decline in the number of $CD4^+$ cells during the course of the disease, in spite of the replenishment of these cells (as indicated by the rise in $CD4^+$ lymphocytes occurring after anti-HIV therapy), have suggested that large numbers of uninfected $CD4^+$ cells also may be destroyed. Fusion of HIV-infected lymphocytes to uninfected $CD4^+$ lymphocytes and death of all cells in the resulting syncytia have been observed *in vitro* (13, 14). In addition to direct cytopathic effects, autoimmune responses stimulated by HIV antigenic determinants crossreacting with normal cellular antigens might lead to the elimination of uninfected cells (15–17). Destruction of uninfected $CD4^+$ lymphocytes has been attributed, in part, to immune responses directed against passively acquired HIV proteins or peptides (18–20), or to the crosslinking of CD4 by gp120 concomitant with stimulation of the T-cell receptor (TCR) (21). Moreover, $CD8^+$ cytotoxic T lymphocytes from HIV-infected subjects have been reported to attack normal $CD4^+$ cells (22).

In this study, we demonstrate that rapid destruction of autologous, uninfected $CD4^+$ lymphocytes by HIV-infected cells can occur *in vitro* via a direct mechanism not requiring productive viral infection of the $CD4^+$ target cells or the formation of syncytia and not involving immune mechanisms. To analyze this phenomenon, we have quantitated cell lysis by means of isotope release and the occurrence of apoptosis in the target population.

MATERIALS AND METHODS

Antibodies and Reagents. Anti-human monoclonal antibodies (mAbs) were the following: anti-CD4 (T4, Coulter; OKT4 and OKT4A, Ortho Diagnostics), anti-CD8 (T8, Coulter), anti-CD19 (B4, Coulter), anti-CD16 and anti-CD25 (Becton Dickinson), and anti-CD3 (OKT3, Ortho). Soluble CD4 was obtained through the AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases; phytohemagglutinin from Difco; staphylococcal enterotoxin A from Toxin Technology (Sarasota, FL); 3′-azido-3′-deoxythymidine (AZT) from Sigma; and soluble gp120 from MicroGeneSys (Meriden, CT).

HIV-1 Infection and Cell Culture. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of HIV-1 seronegative donors by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Cells, in RPMI 1640 medium containing 10% (vol/vol) fetal bovine serum (culture medium), were stimulated for 3 days with either 5 μg of phytohemagglutin per ml or 1 μg of staphylococcal enterotoxin A per ml (23) and infected by incubation for 4 h with 100 median tissue culture infective doses ($TCID_{50}$) of HIV-1 clinical isolates v615 or v142. Both isolates form syncytia in the standard MT-2 assay. CEMT4 cells were infected by coculture with HIV-infected CEMT4 cells.

Target Cell Preparation. Autologous $CD4^+$ and $CD8^+$ blasts were prepared from normal PBMC by negative selection with a mixture of mAbs and magnetic beads coated with anti-mouse IgG (Dynal, Great Neck, NY). Target cells were >95% pure as assessed by flow cytometry. The cells were then cultured for 2–3 days with phytohemagglutinin. In some experiments, freshly purified, unstimulated, $CD4^+$ autologous lymphocytes were used as targets.

Infected-Cell-Mediated Killing (ICMK) Assay. A total of 100 μl of target $CD4^+$ cells ($3–4 \times 10^5$ cells per ml) labeled with ^{51}Cr, were added to 100 μl of the effector cells at various effector to target cell ratios in 96-well, round-bottom microtiter plates. Percent lysis was calculated as in a standard ^{51}Cr-release assay (22). After overnight incubation, spontaneous release of the target cells averaged 15–30%.

DNA Fragmentation Assay. Cell DNA fragmentation was determined as described (24–26). Briefly, uninfected CEMT4 cells were labeled overnight with 10 μCi of [^3H]thymidine per

Abbreviations: ICMK, infected-cell-mediated killing; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cells; mAb, monoclonal antibody; AZT, 3′-azido-3′-deoxythymidine; TCR, T-cell receptor.

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ml (1 Ci = 37 GBq), resuspended in unlabeled culture medium for 1 h, washed, and plated with infected CEMT4 cells in a manner identical to the ^{51}Cr assay described above. After the appropriate incubation period, the cell mixture was treated with 0.4% Triton X-100, allowing fragmented but not intact DNA to exit from the cells. Following centrifugation, [^{3}H]thymidine in the supernatant was measured by scintillation counting. The percentage of fragmented DNA was calculated as follows: [(cpm of the supernatant − cpm of background)/(cpm of the total lysate − cpm of background)] × 100. Background DNA fragmentation was determined by culturing target cells in the absence of effector cells and was between 2% and 18%. To determine total [^{3}H]thymidine incorporation, cells were lysed with 1% SDS buffer.

Flow Cytofluorometric Analysis of Apoptotic Cells. Apoptotic nuclei were stained with propidium iodide as described (27) and quantitated by flow cytometry.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. Uninfected CEMT4 cells, labeled with [^{125}I]5-iododeoxyuridine, were incubated for 4 h at a ratio of 1:5 with infected or uninfected CEMT4 cells. The cells were subsequently harvested, and DNA fragmentation was analyzed by gel electrophoresis, as outlined (28).

RESULTS

Killing of Normal CD4$^+$ Cells by HIV-Infected Autologous Lymphocytes. Activated PBMC from seronegative donors were infected with an inoculum of a clinical HIV-1 isolate. Infected and control, uninfected cells were maintained in culture several days, extensively washed, and tested for their cytotoxicity against autologous, uninfected, ^{51}Cr-labeled CD4$^+$ blasts (Fig. 1*A*). After 16 h of coculture, a high level of target cell lysis was observed when infected PBMC were used as effector cells, as measured by the isotope release from the uninfected CD4$^+$ cell population, and was dependent on the ratio of infected to radiolabeled, uninfected cells. In contrast, no cytotoxicity was observed when uninfected PBMC were used as effectors. At the same time point, the whole surfaces of flat-bottom wells containing the cocultures were monitored for syncytia by phase-contrast microscopy. Syncytia were defined as giant cells with a cytoplasm greater than four lymphocyte cell diameters (14). The wells in which killing occurred did not contain syncytia or cells with ballooning cytoplasm. ICMK also occurred following infection of PBMC with 10 additional HIV-1 clinical isolates, including isolates that had been grown only in PBMC for 2 weeks (data not shown). Purified, infected CD4$^+$ lymphocytes demonstrated an enhanced level of cytotoxic activity per number of lymphocytes against autologous target cells when compared with an equal number of unseparated PBMC (data not shown).

Killing of uninfected, ^{51}Cr-labeled CEMT4 cells by CEMT4 cells infected with two different HIV-1 isolates was also observed (Fig. 1*B*), providing additional evidence that in this system the destruction of CD4$^+$ cells is not mediated by CD8$^+$ cells. Cell death was not due to free virus since supernatant culture media from heavily infected cells were not cytotoxic when incubated with target cells.

To determine whether resting CD4$^+$ cells are susceptible to lysis by infected, autologous PBMC, CD4$^+$ lymphocytes were purified by negative selection with magnetic beads and a combination of mAbs, including anti-CD25, a mAb specific for activated cells. Immediately after purification, CD4$^+$ cells were labeled with ^{51}Cr and used as targets. Autologous serum was used in the medium during CD4$^+$ purification, labeling, and the ^{51}Cr-release assay to avoid potential stimulation by fetal bovine serum. The uninfected targets were readily killed without any need for previous activation (Fig. 1*C*).

Experiments on the kinetics of killing demonstrated that cytolysis began within a few hours, before a replicative cycle of

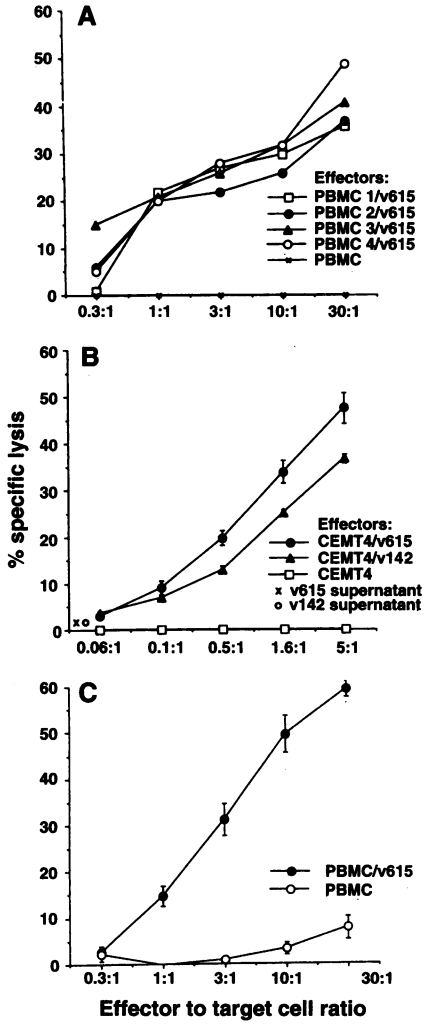

FIG. 1. ICMK of normal CD4$^+$ lymphocytes and CEMT4 cells. (*A*) PBMC from four seronegative donors were infected *in vitro* with an HIV-1 clinical isolate. Their cytolytic activity against autologous CD4$^+$ blasts was analyzed in a 16-h assay. The cytotoxicity of uninfected PBMC (-x-) represents the mean of the four donors. (*B*) Lysis of normal CEMT4 cells by CEMT4 cells infected with two clinical HIV-1 isolates (v615 and v142) and by the supernatants from the infected cells. (*C*) Resting, uninfected CD4$^+$ lymphocytes are killed by HIV-infected autologous PBMC.

HIV could be completed. Fig. 2 depicts the amount of lysis observed upon coculturing uninfected CD4$^+$ cells and infected autologous PBMC for 2, 4, 8, and 20 h. ICMK increased with time, but significant level of lysis occurred within the first 2 h. In absence of infected cells, negligible background levels of cytotoxicity were observed. PBMC were usually used as effectors 5–7 days postinfection, when the release of p24 in the cell cultures (supernatant p24 concentration > 80 ng per 10^6 cells per ml) and the cell-surface expression of HIV antigens reached high levels, concurrent with a low expression of surface CD4 antigen. Cultures of PBMC contained approximately 20–30% of cells positive for HIV envelope protein, as assessed by flow cytometry.

Experiments with human PBMC employed autologous combinations of infected and uninfected lymphocytes to mimic the *in vivo* state and to avoid a mixed lymphocyte reaction, which theoretically could result in cytotoxicity. However, the T cell line CEMT4 (HLA: $A_{1,W19}$, B_{W6}, C−) infected with HIV kills uninfected CD4$^+$ HeLa cells (HLA: A26, A3, Bw35).

Infected Cells Are Not Sacrificed During ICMK. Experiments with reciprocal isotope labeling were performed to

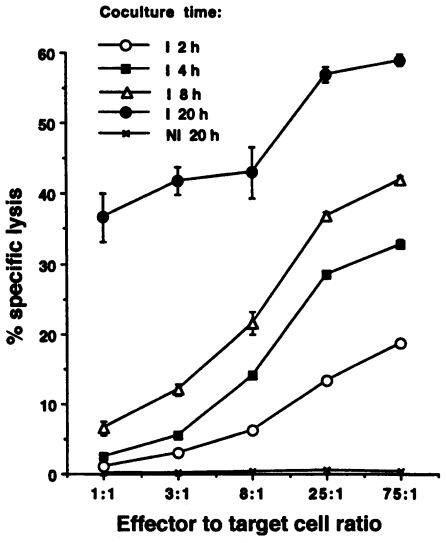

FIG. 2. Kinetics of the lysis of CD4+ blasts incubated for various times with autologous, infected PBMC (I). The percent lysis values obtained by using noninfected PBMC (NI; -x-) are the values obtained at 20 h.

determine if the infected effector cells also die in parallel while killing CD4+ uninfected targets (Table 1). The data indicate that the infected cells are not sacrificed in detectable numbers following contact with their uninfected targets, even though the latter cells are killed. The results shown were obtained after 3 h of coculture, since the spontaneous isotope release of the highly infected CEMT4 cells increased with longer incubations. Clearly, the infected cells were dying; however, the level of ^{51}Cr release from infected cells was not increased by the coculture with uninfected cells at various ratios and at different times of incubation (data not shown).

ICMK Is Dependent on CD4–gp120 Binding. To examine the role of the gp120–CD4 interaction in the ICMK phenomenon, blocking experiments were performed. Treatment of the uninfected CD4+ blasts with 50 μg of recombinant gp120 abolished cytolysis (Fig. 3*A*). The inhibition was concentration dependent, since 5 μg and 0.5 μg caused respectively 89% and 15% reduction of target cell lysis. Cytotoxicity also was impaired by preincubation with OKT4A, a mAb recognizing an

Table 1. Unidirectional killing

Effector to target cell ratio	Effector cells/target cells, % target cell lysis		
	Uninfected/ ^{51}Cr-uninfected	Infected/^{51}Cr-uninfected	^{51}Cr-infected/ uninfected
30:1	0.40 ± 0.4	20.01 ± 1.2	0.57 ± 2.1
10:1	0.50 ± 0.3	16.59 ± 1.7	1.54 ± 1.9
3:1	0.81 ± 0.8	8.62 ± 1.1	−0.10 ± 2.7
1:1	0.51 ± 0.6	3.47 ± 1.1	−2.70 ± 2.0

^{51}Cr release from labeled CEMT4 cells, either uninfected or v615-infected, was measured after 3 h of coculture with unlabeled CEMT4 cells. The results are expressed as mean of three experiments ± SEM. The average spontaneous ^{51}Cr release from uninfected cells was 9% and from infected cells was 28%.

epitope on CD4 involved in the gp120-binding site, while OKT4, a mAb reacting with CD4 outside the gp120-binding site, was significantly less effective. OKT3, a mAb against a TCR-associated protein, did not have inhibitory activity. When the infected PBMC were preincubated with polyclonal anti-HIV-1 immunoglobulins (HIV-Ig) or with serum from some but not all AIDS patients, the lysis of the target cells was greatly diminished (Fig. 3*B*). In contrast, serum from a seronegative person was ineffective. Soluble CD4 caused >60% inhibition of target cell death. When uninfected CD8+ blasts were used as targets, no cytotoxic activity was observed (Fig. 3*C*).

Importantly, the addition of AZT, an inhibitor of reverse transcriptase, did not inhibit specific ^{51}Cr release (Fig. 3*A*). These results, combined with the rapid killing demonstrated in Fig. 2, in which >25% of the uninfected lymphocytes were lysed after 4 h, argue against a requirement for productive infection of the target cells as the cause of their death in this system.

Contact Between Infected and Uninfected Cells Causes DNA Fragmentation in the Uninfected Cells. The death of lymphocytes initiated by several different processes often involves apoptosis. To investigate changes in the DNA of the uninfected target cells during ICMK, CEMT4 cells were stained with propidium iodide, and the percentage of apoptotic cells was measured by flow cytometry (Table 2). The characteristic fluorescent peak generated by hypodiploid DNA, a marker for apoptosis, significantly increased when infected and uninfected cells were stained after being cocultured for 4 h at 37°C, in comparison to the fluorescent peak

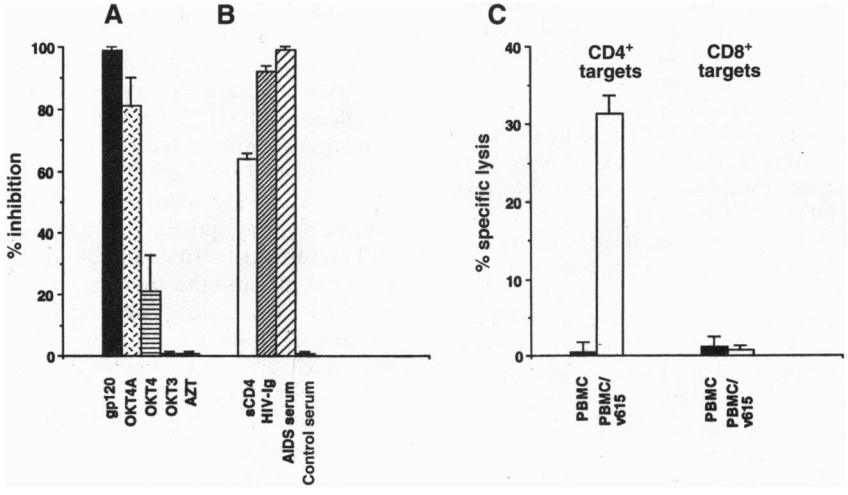

FIG. 3. Inhibition of ICMK. (*A*) Uninfected, ^{51}Cr-labeled CD4+ blasts were preincubated for 45 min at 37°C with recombinant gp120 (50 μg/ml); with the mAbs OKT4, OKT4A, or OKT3 (0.5 μg/ml); or AZT (10 μM) before adding autologous, infected PBMC as effector cells. The cytotoxicity was assessed after 16 h of incubation. The HIV strain used in these experiments is sensitive to <1 μM AZT. (*B*) Infected PBMC were preincubated with recombinant CD4 (10 μg/ml), HIV-Ig (1/40 final dilution), serum from an AIDS patient, or serum from a seronegative person (1/4 final dilution). (*C*) Lysis of CD4+ and CD8+ target lymphocytes.

Table 2. Flow cytometric analysis of apoptotic nuclei

Cells	Treatment: Coculture	Treatment: $ZnSO_4$	% apoptotic cells
CEMT4	−	−	9
CEMT4/v615	−	−	49
CEMT4 + CEMT4/v615	−	−	31
CEMT4 + CEMT4/v615	+	−	71
CEMT4 + CEMT4/v615	+	+	44

A total of 10^6 infected (CEMT4/v615) and 10^6 uninfected (CEMT4) cells were stained with hypotonic propidium iodide fluorochrome solution after 4 h of coculture in presence or absence of 2 mM $ZnSO_4$. Alternatively, infected and uninfected cells were incubated separately and mixed immediately before staining. The percent apoptotic cells in the mixture of infected and uninfected cells without coculture (31%) approximates the calculated value for a 1:1 mixture of CEMT4 (9%) and CEMT4/v615 (49%).

given by the two populations mixed immediately before staining. Addition of $ZnSO_4$, an endonuclease inhibitor, during the 4-h incubation reduced the percentage of cells undergoing apoptosis approximately to the level observed in the culture of infected cells alone.

Since HIV-infected cells also undergo apoptosis (refs. 6–12 and Table 2), to distinguish apoptosis occurring in the uninfected cells from the apoptosis occurring in the infected cells, we analyzed DNA fragmentation of the target cells by [^{3}H]thymidine release (24–26). This assay permits quantitation of the percentage of uninfected cells that become apoptotic after contact with infected cells. Specific DNA fragmentation and ^{51}Cr release from uninfected CD4$^+$ cells were determined after 1 and 4 h of incubation. The results of a representative experiment are shown in Fig. 4*A*. The percent of ^{3}H-labeled DNA present as fragments, indicative of apoptosis, increased with increasing numbers of infected cells added to a constant number of labeled target cells. When uninfected CEMT4 were used as effectors, the amounts of ^{3}H and ^{51}Cr released from the target cells were similar to the corresponding spontaneous releases (data not shown). The kinetics of DNA fragmentation and ^{51}Cr release clearly revealed that DNA fragmentation is an early event, preceding the lysis of the cells as measured by ^{51}Cr release.

The occurrence of apoptosis in the uninfected cells also was visualized by resolving the fragmented DNA on an agarose gel (Fig. 4*B*). DNA extracted from ^{125}I-labeled, uninfected CEMT4 cells incubated with HIV-infected CEMT4 cells shows the characteristic pattern of nucleosome-size fragments (Fig. 4*B*, lane 2), which is not present in the DNA of an equal number of CEMT4 cells incubated with uninfected autologous cells (Fig. 4*B*, lane 1).

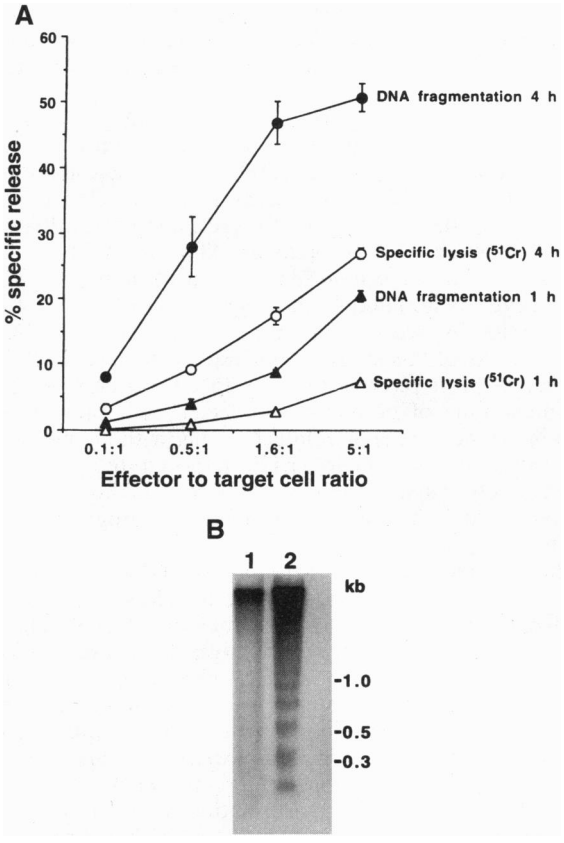

FIG. 4. ICMK is mediated by apoptosis. (*A*) DNA fragmentation (as measured by [^{3}H]thymidine release) and specific lysis (as measured by ^{51}Cr release) of uninfected CEMT4 cells following 1 h or 4 h of incubation with HIV-1-infected CEMT4 cells. (*B*) Agarose gel electrophoresis of target cell DNA. Fragmented DNA from [^{125}I]-5-iododeoxyuridine-labeled, uninfected CEMT4 cells after 4 h of incubation with uninfected (lane 1) or HIV-infected (lane 2) CEMT4 cells was examined by electrophoresis through 1.8% agarose gels, and labeled DNA was visualized by autoradiography.

DISCUSSION

Our results indicate that normal, uninfected CD4$^+$ T lymphocytes may die rapidly by an apoptotic pathway following contact with HIV-infected cells before syncytium formation can be observed and without the establishment of a productive viral infection in the target cells. ICMK occurred when using CD4$^+$ T cell lines and CD4$^+$ lymphocytes from uninfected individuals, indicating that immune responses to HIV are not required.

The absence of detectable cellular fusion after overnight incubation of infected PBMC and autologous uninfected CD4$^+$ lymphocytes parallels previous studies showing that infected, mitogen-stimulated PBMC died in the absence of syncytium formation (29) and that death in HIV-infected CD4$^+$ cultures was caused by apoptosis and was not due to cellular fusion (26). Our hypothesis is that, although syncytia formation and ICMK share a requirement for gp120–CD4 binding, ICMK occurs before syncytia are formed and that ICMK and syncytia formation may be separable phenomena. Indeed, several experimental conditions inhibiting syncytia formation do not decrease the death of uninfected cells, such as incubation in medium containing anti-CD7 antibody or depleted of calcium ions (B.N., unpublished data). Furthermore, syncytia have rarely been seen in lymphoid tissues from patients (30).

ICMK is dependent on the binding of gp120 expressed by the infected cells to the CD4 antigen on the normal cells. Addition of soluble gp120 to the assay inhibits the cytolysis. This result, taken together with the inability of infected culture supernatants to lyse CD4$^+$ cells, indicates that to trigger cell death in this system gp120 must be cell bound and not in soluble form.

Several characteristics serve to distinguish this phenomenon from other causes of apoptosis in HIV-1 disease. Uninfected cell lysis is not blocked by the addition of cycloheximide or cyclosporin A to the cytotoxicity assay (B.N., unpublished data), thus differentiating ICMK from the apoptosis occurring in the lymphocytes of HIV-infected individuals following *in vitro* activation which is inhibited by these compounds (10). Target cells need not be activated, antibody to gp120 is not required, and only CD4$^+$ cells are killed. Others have found that both CD4$^+$ and CD8$^+$ lymphocytes from infected patients undergo apoptosis when cultured *in vitro* (9, 11). Apoptosis of CD8$^+$ cells was observed in several types of lentiviral infections in primates, but apoptosis of CD4$^+$ cells occurred only in infections that resulted in diseases [HIV-1 in humans, simian immunodeficiency virus (SIV) in macaques] (31). Mechanisms

related to cellular activation but not depending on the contact between infected and uninfected cells may be responsible for the apoptosis occurring in $CD8^+$ cells, as suggested by a recent study (32).

Banda *et al.* (21) reported that the binding of the CD4 receptor to recombinant gp120 crosslinked with a polyclonal anti-gp120 antibody primed $CD4^+$ cells for apoptosis; additional activation of the cells with an antibody to the TCR was necessary for death to occur. However, apoptosis initiated by recombinant envelope protein gp120 was not observed by others (26). In our system, destruction of uninfected $CD4^+$ lymphocytes is initiated by the engagement of the CD4 molecule in the absence of ligands for the TCR. The CEMT4 cells used in parts of this study do not express surface TCR (data not shown). It is possible that the gp120 concentrated in the cell membrane of productively infected cells may crosslink CD4 in the membrane of uninfected cells, without needing the concentrations of soluble gp120 required to demonstrate effects in other systems *in vitro*. This crosslinking of the CD4 receptor could then initiate a signal for programmed cell death.

The relevance of these observations on HIV-1 pathogenesis *in vivo* remains to be established, but ICMK can serve as a mechanism by which a small number of infected cells may result in the destruction of a much larger number of uninfected $CD4^+$ cells. Heavily infected cultures were used in our study to induce the death of large numbers of uninfected cells in the limited time of observation; however, HIV-expressing cells equivalent to those used in these experiments are present in patients, especially in lymphoid organs, where the virus is sequestered in the early phase of the disease (33, 34). We hypothesize that in the environment of the lymphoid tissues, infected cells come in contact with normal quiescent $CD4^+$ lymphocytes and trigger their death before the latter become productively infected and before syncytia are formed.

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