High Viral Burden and Rapid CD4⁺ Cell Depletion in Human Immunodeficiency Virus Type 1-Infected SCID-hu Mice Suggest Direct Viral Killing of Thymocytes In Vivo

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The mechanism of CD4⁺ cell loss in lymphoid organs is unknown. In this study, human immunodeficiency virus (HIV) infection of human fetal thymus/liver implants in severe combined immunodeficient mice was used to investigate the mechanism of HIV-induced depletion of CD4-bearing cells in vivo. The implants were assessed for depletion of CD4⁺ thymocytes, apoptosis, and viral burden. We detected two phases of CD4 cell depletion, an initial rapid phase and a more gradual later phase. Compared to mock-infected implants, HIV-infected implants did not demonstrate detectable increases in the levels of apoptosis while severe depletion of CD4-bearing cells was ongoing. During peak loss of CD4⁺ cells, high viral burden was observed, suggesting that loss of CD4⁺ cells in this in vivo system is due to direct killing of infected thymocytes. Increased levels of apoptosis were observed during the later phase of thymocyte depletion; however, these apoptotic cells lacked CD4. This finding suggests that a second indirect mechanism may be responsible for the destruction of CD4⁺ CD8⁺ thymocytes in vivo. Taken together, these results suggest that CD4⁺ and CD4⁺ cells may die by different mechanism(s).

Of all of the mechanisms proposed for loss of CD4⁺ cells in human immunodeficiency virus type 1 (HIV-1) infection, apoptosis has been one of the most extensively studied, yet its role in T-cell depletion remains controversial. Evidence supporting a role for apoptosis comes in part from the observation that in vitro activation of T cells from HIV-1-seropositive individuals results in elevated levels of apoptosis (14, 16, 27). In addition, cell lines and T-cell cultures infected with HIV-1 in vitro demonstrate apoptosis (22, 41) that, in some cases, can be blocked by the addition of neutralizing antibody to gp120 (41). While apoptosis is observed in in vitro-stimulated peripheral blood mononuclear cells (PBMC) from HIV-infected donors, it occurs in CD8⁺ as well as CD4⁺ cells (13, 14, 27). These findings raise questions regarding the mechanism and in vivo relevance of the observed in vitro apoptosis. Recent studies have documented apoptosis in the lymph nodes of HIV-1-infected humans and macaques (10, 28). However, the cells undergoing apoptosis were not HIV-1 infected, and the levels of apoptosis did not correlate with disease progression or viral burden, suggesting that the observed apoptosis may have been the result of normal immune responses to HIV-1 by both CD4⁺ and CD8⁺ T cells or of other indirect mechanisms.

The SCID-hu mouse is a small animal model which has been successfully used to examine HIV-1 pathogenicity in vivo (2, 4, 18, 19, 21, 38). The SCID-hu mouse is constructed by implanting human fetal thymus and liver under the kidney capsule of a severe combined immunodeficient (SCID) mouse, resulting in a conjoint organ (Thy/Liv) which functionally resembles a normal human thymus (26, 29). Infection of the Thy/Liv implant with HIV-1 results in the loss of CD4-bearing cells (2, 4, 38) and gross histological abnormalities similar to those observed in humans (15, 31, 33). The probable absence of an

immune response in the Thy/Liv implant makes the SCID-hu mouse a useful model for addressing questions of early HIV-1 infection and differentiating the effects of the virus from the impact of the responding immune system. The SCID-hu mouse model also eliminates variables such as donor effects and opportunistic infections that can confound clinical studies, as all control and experimental Thy/Liv implants can be derived from the same fetal donor, ensuring that they are genetically identical. Opportunistic infections are prevented by housing the SCID mice under pathogen-free conditions. In addition, clonal virus stocks can be used as the virus source to minimize strain variation (19).

In this study, we used SCID-hu mice to investigate the role of apoptosis in HIV-1-induced CD4⁺ cell depletion in the human thymus. We found, contrary to the findings reported by others (4, 39), that elevated levels of apoptosis were not present while CD4⁺ cells were undergoing rapid depletion. Rather, high proviral burden and high numbers of productively infected cells were observed, suggesting that CD4⁺ cell depletion is due to direct killing of infected thymocytes. Elevated levels of apoptosis were observed only when the majority of CD4⁺ cells had already been depleted and were observed predominantly in the CD4⁺ CD8⁺ subset. This finding suggests that apoptosis of CD8⁺ cells at this late stage of CD4⁺ cell depletion is due to indirect mechanisms such as disruption of the cytokine network and/or the thymic microenvironment.

MATERIALS AND METHODS

Construction of SCID-hu mice. C.B.-17 mice homozygous for the SCID genetic defect (5, 6) were bred at the University of California, Los Angeles, housed in a biosafety level 3 animal facility, and maintained free of antibiotics. SCID-hu mice were constructed as previously described (2, 10, 13, 14, 16, 22, 26–29, 41). All implants for apoptosis studies reported here were constructed from the same fetal donor (donor 80) to minimize variation, although similar results, not presented here, have been obtained with implants derived from several different donors.

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Preparation of virus stocks and infection of SCID-hu mice. Stocks of HIV-1_{NL4-3} (1) were obtained by electroporation of infectious proviral DNA (7) into mycoplasma-free CEM cells. Thy/Liv implants were infected with 100 infectious units (as determined by limiting dilution analysis on PBMC). In this study, this corresponded to an inoculum of 5 ng of viral p24. Sixteen Thy/Liv implants were infected by direct injection on a staggered basis (three implants per day). Four additional implants were injected with medium (mock infected). All implants were sequentially biopsied two to three times as a group at weekly intervals (by removing approximately 25% of the tissue), resulting in a series of infected and mock-infected biopsy samples ranging from 19 to 34 days postinfection.

In situ terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL). Biopsy samples were fixed in 10% neutral buffered formalin (Fisher, Pittsburgh, Pa.) and paraffin embedded. Tissue sections were cut to 6 μ m and then deparaffinized by heating to 60°C for 30 min. The sections were hydrated by two washes in xylene (Fisher), two washes in 100% ethanol, and then one wash each in 95% ethanol, 85% ethanol, 70% ethanol, and finally double-distilled water. Nick end labeling of DNA strand breaks in the tissues was performed according to the protocol of Gavrieli et al. (11), with the minor exception that the aminoethyl carbazole reaction was carried out at room temperature for 15 to 30 min. Tissue sections were counterstained with Probe hematoxylin (Biomedica Corp., Foster City, Calif.). As a positive control, tissue sections from a normal Thy/Liv implant, irradiated with 650 rads 24 h previously, were stained in parallel with the mock- and HIV-1-infected tissues.

Flow cytometric TUNEL assay (TUNEL flow). Single-cell suspensions of SCID-hu thymocytes were assayed for DNA strand breaks by a protocol adapted from that of Gorczyca et al. (12). Biotinylated dUTP (Boehringer Mannheim, Indianapolis, Ind.) was incorporated into strand breaks by using a TdT kit (Boehringer Mannheim) according to the manufacturer's directions. Fluorescein isothiocyanate (FITC)-conjugated avidin (Caltag, Burlingame, Calif.) was used to detect the incorporated biotinylated dUTP. For cell cycle analysis, the cells were resuspended in 20 μ g of 7-amino-actinomycin D (7-AAD) dye (Calbiochem, San Diego, Calif.) per ml in phosphate-buffered saline. Single-cell suspensions were divided equally between two tubes and assayed in parallel. Both tubes received the same reagents, with the exception of the TdT enzyme, which was added to only one of the tubes in the pair (TdT⁺ samples). Samples were subjected to flow cytometry using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif. [BDIS]) within 2 h of staining. Events (5×10^3 to 10×10^3) were acquired and analyzed by using the Lysis II program (BDIS). Initially, a gate was set on the forward and side scatter profiles to exclude debris. Placement of the statistical region for analysis of the TdT⁺ samples was determined by the TdT⁻ control. As a positive control for apoptosis, thymocytes were cultured overnight in dexamethasone (5×10^{-5} M in RPMI 1640 with 10% fetal calf serum; Sigma, St. Louis, Mo.) and acquired and analyzed in parallel.

Cell surface staining in combination with 7-AAD staining for apoptosis. Single-cell suspensions obtained from biopsy samples were stained with CD4 and CD8 monoclonal antibodies (BDIS) conjugated to phycoerythrin and FITC, respectively. At all time points, fluorescence-labeled mouse immunoglobulin G1 monoclonal antibodies (BDIS) were used as isotype controls. After staining for cell surface expression of CD4 and CD8, the cells were costained with 20 μ g of 7-AAD dye (Calbiochem) as described by Schmid et al. (34) to identify early and late apoptotic cells by dye uptake. Cells were then fixed in 1% paraformaldehyde containing actinomycin D (20 μ g/ml in phosphate-buffered saline; Boehringer Mannheim). Data were acquired on a FACScan flow cytometer (BDIS) within 24 h of staining. As a positive control for apoptosis, thymocytes were cultured overnight in dexamethasone as described above. Initially a gate was set on the forward and side scatter profiles to exclude debris. Placement of the statistical regions was determined by the dexamethasone-treated control samples.

Annexin V staining. Thymocytes were stained with Annexin V-FITC for external expression of phospholipid phosphatidylserine, using an APOTEST kit (Brand Applications BV, Maastricht, The Netherlands) according to the manufacturer's instructions. Cells were costained with propidium iodide (Calbiochem, Cambridge, Mass.) to identify dead cells. A total of 5×10^3 to 10×10^3 events were acquired. Dexamethasone-cultured thymocytes (as described above) served as a positive control. Statistical regions were set as described for 7-AAD samples.

Quantitative PCR. Thymocytes were lysed in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl) and subjected to phenol-chloroform extraction and ethanol precipitation. Total nucleic acids obtained from this procedure were then subjected to quantitative PCR using radio-end-labeled primers specific for the viral R and U5 regions of the long terminal region (M667 and AA55) and human β -globin sequences as previously described (19, 45, 46). Quantitation was achieved by comparison to standard curves consisting of known amounts of cloned proviral DNA or uninfected normal human lymphocyte DNA following analysis with an Ambis radioanalytic imaging system as previously described (19).

Limiting dilution coculture of SCID-hu thymocytes. Single-cell suspensions were prepared from Thy/Liv implants infected 21 days previously. These Thy/Liv implants were derived from a fetal donor different from that for the implants used for the apoptosis analysis. However, they were infected with the same amount of virus. The cells were washed, resuspended in medium, and counted, and the indicated number of thymocytes was cocultured in quadruplicate in 96-well tissue culture plates with 10^5 phytohemagglutinin-stimulated human

FIG. 1. HIV-1-induced depletion of CD4-bearing cells. Mock-infected (open circles) and HIV-infected (solid symbols) implants were biopsied at the indicated times postinfection and assayed by flow cytometry for percent CD4-bearing cells. The solid triangle, square, and diamond symbols represent implants biopsied twice during the rapid phase of CD4⁺ cell depletion (triangles, mouse 80-3; squares, mouse 80-1; diamonds, mouse 80-2). Percent CD4 cells includes both immature CD4⁺ CD8⁺ and mature CD4⁺ CD8⁻ cells. Each symbol represents an individual biopsy sample.

PBMC. Supernatants were collected after 10 days of coculture and analyzed for p24^{gag} content by enzyme-linked immunosorbent assay (Coulter, Hialeah, Fla.).

RESULTS

Kinetics of HIV-induced CD4⁺ cell depletion. Infection of Thy/Liv implants with 100 infectious units of HIV-1_{NL4-3} results in hypocellularity, loss of cortical-medullary junctions, and depletion of CD4-bearing cells (2, 4, 18). These histological changes are observed in approximately one-third of implants at 3 weeks postinjection and in 100% at 6 weeks postinjection (3, 19). To investigate the mechanism(s) of CD4⁺ cell depletion, it was important to examine implants at different stages of CD4⁺ cell loss, as apoptotic events may occur exclusively either early or late during CD4⁺ cell depletion. To this end, we began by better defining the kinetics of CD4⁺ thymocyte depletion (Fig. 1). Thy/Liv implants were either mock or HIV-1 infected and biopsied as described in Materials and Methods, such that a series of postinfection biopsy samples ranging from 19 to 34 days was generated. As in a normal human thymus, 85 to 96% of all thymocytes in mock-infected implants expressed the CD4 surface molecule, which included both the CD4⁺ CD8⁺ (immature) and CD4⁺ CD8⁻ (mature) subsets. No change in histological appearance or CD4⁺ cell number has been observed as a result of multiple biopsies. However, by 19 days postinfection, one of three HIV-1-infected implants demonstrated CD4⁺ cell depletion, with a subset distribution of only 56% CD4⁺ cells, as determined by flow cytometry (Fig. 1 and Table 1). The depletion of CD4⁺ cells proceeded rapidly from this time point, such that by 23 days postinfection, depletion was observed in 100% of implants. By days 24 and 25, implants demonstrated as few as 18% CD4⁺ cells. Although the depletion curve appears to plateau near this value, several implants demonstrated an almost total loss of CD4⁺ cells by 27 days postinfection (Fig. 1 and Table 1). Cross-sectional analysis of the data revealed that an average of 10% CD4⁺ cells are lost per day between days 19 and 25 postinfection. This high rate of loss is supported by longitudinal analysis of three implants that were sequentially biopsied twice between days 19 and 25. Implants 80-1, 80-2, and 80-3 lost 17, 28, and 9%, respectively, CD4⁺ cells over 2 days, averaging a loss of 9% CD4⁺ cells per day. The observed

TABLE 1. Levels of apoptosis in mock- and HIV-1-infected implants^a

Implant	Infection	Days p.i.	% CD4 cells	TUNEL flow		7-AAD		Annexin V	
				No. live	No. apoptotic	No. live	No. apoptotic	No. live	No. apoptotic
80-16	Mock	28	96	99	1	98	1	87	2
80-19	Mock	NA	96	98	1	94	4	91	3
80-16	Mock	20	95	98	2	96	3	94	2
80-19	Mock	NA	94	ND	ND	ND	ND	93	3
80-16	Mock	34	94	ND	ND	ND	ND	90	2
80-13	Mock	21	94	ND	ND	ND	ND	93	2
80-6	Mock	31	94	99	1	99	1	94	1
80-13	Mock	36	93	98	<1	96	2	92	1
80-6	Mock	23	93	99	1	97	1	87	3
80-13	Mock	29	92	99	1	95	3	91	2
80-6	Mock	38	85	ND	ND	ND	ND	90	4
80-17	NL	19	90	99	1	97	1	93	1
80-9	NL	22	89	99	<1	99	1	90	1
80-21	NL	19	88	95	3	97	1	89	1
80-12	NL	20	81	98	1	98	1	82	3
80-14	NL	20	69	98	1	97	2	83	3
80-15	NL	20	68	98	2	96	1	90	1
80-11	NL	21	60	97	1	96	2	83	3
80-20	NL	19	56	98	<1	97	1	85	2
80-8	NL	21	54	96	3	97	1	89	2
80-2	NL	22	46	99	<1	97	2	86	3
80-10	NL	21	40	ND	ND	95	3	84	2
80-1	NL	23	35	98	1	96	3	87	2
80-5	NL	23	33	97	1	98	1	88	3
80-3	NL	23	31	96	1	97	1	81	3
80-14	NL	28	30	92	4	83	2	86	7
80-17	NL	27	27	95	<1	96	2	85	6
80-12	NL	28	23	93	6	88	1	90	3
80-3	NL	25	22	96	<1	98	1	ND	ND
80-12	NL	34	19	85	3	ND	ND	83	7
80-4	NL	22	19	ND	ND	97	1	89	2
80-1	NL	25	18	96	<1	98	1	ND	ND
80-2	NL	24	18	82	10	94	3	ND	ND
80-5	NL	31	16	89	6	96	2	75	8
80-9	NL	30	16	96	3	83	1	85	4
80-21	NL	27	11	96	2	93	4	70	7
80-15	NL	28	9	95	7	90	9	76	8
80-8	NL	29	8	91	3	99	1	87	4
80-11	NL	29	4	ND	ND	76	8	76	11
80-20	NL	27	4	86	11	93	3	66	16
80-4	NL	30	2	85	11	92	1	76	9

^a Implants were assessed by flow cytometry for percent CD4⁺ cells and by the assays indicated for the levels of apoptosis. Percent CD4⁺ cells includes both immature CD4⁺ CD8⁺ and mature CD4⁺ CD8⁺ cells. Implants are listed in order of decreasing percentages of CD4⁺ cells, and all biopsy samples from HIV-infected implants are shown. p.i., postinfection; ND, not done; NL, HIV-1_{NL4-3}. Most mock-infected animals received medium on the days indicated in lieu of virus. However, animal 80-19 did not; therefore, days postinfection is not applicable (NA).

loss of CD4⁺ cells, as measured by flow cytometry, is not due solely to down-regulation of surface CD4 by HIV, as severe hypocellularity was observed histologically, and increased levels of cell debris were observed by flow cytometry following infection (data not shown). Thus, depletion of CD4⁺ cells begins approximately 3 weeks postinfection and proceeds rapidly, with the majority of CD4⁺ cell depletion occurring within a 6-day period, providing an optimal window in which to examine the mechanism(s) of CD4⁺ cell depletion.

Levels of apoptosis during CD4⁺ cell depletion. To examine the role of apoptosis in the HIV-1-induced depletion of CD4-bearing cells, all biopsy samples from mock- and HIV-1-infected implants were assayed for the presence of apoptotic cells by using the *in situ* TUNEL (11), TUNEL flow (12), 7-AAD (34), and Annexin V-FITC (43) assays. As a positive control for all assays, apoptosis was induced in an uninfected Thy/Liv implant by irradiation with 650 rads 24 h prior to

biopsy. *In situ* TUNEL staining of the irradiated implant revealed high levels of apoptotic cells at the cortical-medullary junction and the subcapsular region (Fig. 2A). Sections stained in the absence of TdT were negative for apoptotic cells (data not shown), demonstrating the specificity of TUNEL staining. Staining of single-cell suspensions from the irradiated implant by TUNEL flow, 7-AAD, and Annexin V-FITC techniques revealed high levels of apoptosis (Fig. 3), demonstrating that all four techniques can detect *in vivo*-induced apoptosis of human thymocytes and that similar results are obtained with each assay. Induction of necrosis by heat treatment of implants *ex vivo* resulted in only very low levels of apoptosis, as determined by all four assays (Fig. 2B and 3), demonstrating that these assays are specific for apoptosis and not necrotic cell death. Heat treatment successfully induced thymocyte death, as evidenced by the high percentage of dead cells detected by 7-AAD and propidium iodide staining.

FIG. 2. Determination of apoptosis by in situ TUNEL. TUNEL was performed on formalin-fixed tissue sections obtained at biopsy from implants subjected to in vivo irradiation with 650 rads 24 h previously (A), heat treated at 60°C ex vivo for 5 min (B), mock infected 31 days previously (implant 80-6) (C), and infected with HIV-1_{NL4-3} 22 days previously (implant 80-2) (D). Implant 80-2 had 46% CD4-bearing cells at this time and ~33,000 copies of HIV-1 genome per 10⁵ human cells. Two days later, this implant had only 18% CD4⁺ cells and ~2,100 copies of HIV-1 genome per 10⁵ human cells. Arrows indicate apoptotic cells.

Staining of thymocytes from mock-infected implants revealed that 1 to 4% of cells were apoptotic, consistent with levels found in normal murine thymus (40). TUNEL staining revealed that these cells were located primarily in the cortex, with fewer cells scattered in the medulla (Fig. 2C). The amount and pattern of apoptotic cells in mock-infected implants did not vary substantially between biopsy samples, even as a result of multiple biopsies (Fig. 3, Table 1, and data not shown).

The levels of apoptosis present in HIV-1-infected implants were dependent on the extent of CD4⁺ cell depletion. Implants with greater than 30% CD4⁺ cells did not demonstrate significantly higher levels of apoptosis than mock-infected implants (Kruskal-Wallis test, $P = 0.8169$ performed on the Annexin V data) (Fig. 3 and Table 1). Only implants with 30% CD4⁺ cells or less had significantly elevated levels of apoptosis (Kruskal-Wallis test, $P = 0.0003$) (Table 1). However, the majority of apoptotic cells at these late stages of CD4⁺ cell depletion was CD4⁻ CD8⁺, with only a small percentage of apoptotic cells expressing surface CD4 (Fig. 4). The high percentage of apoptotic CD8⁺ cells suggests that at the late stages of CD4⁺ cell depletion, apoptosis is most likely due to the loss of thymic integrity and/or disruption of the cytokine network. Taken together, these results demonstrate that during rapid CD4⁺ cell decline, the levels and distribution of apoptosis are

not altered, and they suggest that other mechanisms of CD4⁺ cell death may be operative in infected implants.

Viral burden in infected implants. Our previous studies indicated that high viral loads are required for CD4⁺ cell depletion (2, 3, 18, 19). To investigate the relationship between viral burden and CD4⁺ cell loss, the HIV-1-infected implants shown in Fig. 1 were analyzed for HIV-1 DNA by quantitative PCR (Fig. 5A). Proviral loads peaked at 23 days postinfection, when rapid CD4⁺ cell depletion was occurring. Implants biopsied at this time point contained an average of 71,000 copies of HIV-1 genomes per 10⁵ cells and had lost about two-thirds of all CD4-bearing cells. Proviral load then declined, and by 28 days postinfection, implants contained an average of only 10,500 copies of HIV-1 DNA per 10⁵ cells. These results demonstrate that immediately prior to and during CD4⁺ cell depletion, proviral load is high, with up to 70% of human cells containing HIV-1 DNA, assuming a single provirus per cell. The eventual drop in virus load following CD4 cell depletion is consistent with previous studies by us and others (2, 4, 18, 19) and most likely reflects the loss of infected target cells.

Because CD4⁺ cells are the primary target of HIV-1 and we did not sort CD4⁺ and CD8⁺ cells prior to analysis, it is difficult to assess the relationship between viral burden and CD4⁺ cell depletion once cell loss has become significant.

FIG. 3. Determination of apoptosis by flow cytometry. Single-cell suspensions from implants treated as indicated were aliquoted and assayed for apoptosis by TUNEL flow, 7-AAD, and Annexin V-FITC. The number shown in each flow panel represents the percentage of apoptotic cells in that implant, as determined by the indicated assay. The location of the number indicates the region which contains the apoptotic cells. The implants are the same as those shown in Fig. 2, with the exception of the heat-treated implant. For the heat-treated cells, single-cell suspensions were made from a normal implant and then treated at 60°C for 5 min. P.I., propidium iodide; FSC, forward angle light scatter.

Therefore, proviral load per 10⁵ CD4⁺ cells was also determined. When adjusted for the percentage of CD4⁺ cells present, proviral load still peaked at 23 days postinfection, with an average of ~200,000 copies of HIV-1 DNA per 10⁵ CD4⁺ cells (Fig. 5B). This seemingly high proviral burden, as related to CD4⁺ cell number, may reflect localization of provirus in phenotypically CD4⁺ cells and/or multiple copies of the HIV-1 genome per CD4⁺ cell. However, these data strongly suggest

that proviral load in CD4⁺ cells is very high at a time when CD4⁺ cells are being rapidly depleted. By 28 days postinfection, when CD4⁺ cell depletion had slowed, proviral load decreased but still averaged ~56,000 copies of HIV-1 DNA per 10⁵ CD4⁺ cells. These results demonstrate that during rapid CD4⁺ cell depletion, enough virus is present to infect virtually every CD4⁺ cell.

The quantitative PCR technique used in the assay described

FIG. 4. Distribution of apoptotic cells during the slow phase of $CD4^+$ cell depletion. Apoptosis was assessed in implant 80-15 infected 28 days previously, at which time $CD4^+$ cells constituted 9% of total thymocytes. Cells were costained for surface markers CD4 and CD8, and apoptosis was determined by 7-AAD staining. The phenotype of apoptotic cells was determined by gating on the 7% apoptotic cells and analyzing this population for CD4 and CD8 surface staining. Similar results were obtained following analysis of several other implants. PE, phycoerythrin; P.I., propidium iodide; FSC, forward angle light scatter.

above cannot differentiate defective provirus from functional viral genomes, nor can it distinguish whether multiple proviral copies are present in only a limited number of cells. Thus, even though proviral load was very high, levels of productively infected cells remained unclear. To determine how much of the proviral load in HIV-infected implants represents productively infected cells, limiting dilution coculture was performed on cells obtained from five Thy/Liv implants infected 21 days previously with the same virus inoculum given the implants shown above. In an assay analogous to a cell-associated 50% tissue culture infective dose assay, limiting dilution was performed in quadruplicate for all five implants. Thus, we generated a total of 20 coculture wells for each number of infected cells tested. Wells scoring positive for production of viral p24 were noted, and the total number of wells receiving productively infected cells was plotted against the number of input thymocytes per well (Fig. 6A). As can be seen, 50% of the total wells were positive for virus production when an average of four thymocytes was added to the wells. The data were also plotted to reflect the Poissonian distribution of productively infected cells (Fig. 6B). By this analysis, one in nine thymocytes was able to transmit virus to cocultured cells. This is a minimum estimate, as thymocytes are, in general, difficult to culture in vitro, and transfer of infection would likely be poor. However, taken together, our data demonstrate that at least 10% of thymocytes were productively infected. When limiting dilution data from individual implants were assessed, some variation between implants was seen (not shown). Only one implant had fewer than 10% productively infected cells (1 to 2%), while the remaining four had a minimum of 10% or more virus-produc-

ing cells. The highest level of infection was observed in one implant in which 75% of the thymocytes were productively infected. We have also performed limited in situ hybridization studies for virus expression and see areas of the thymus in which essentially every remaining thymocyte is expressing viral RNA (not shown). Taken together, these results demonstrate that viral burden and active virus replication in HIV-1-infected implants could be high enough to effect the observed depletion of $CD4^+$ cells by direct nonapoptotic killing mechanisms.

DISCUSSION

We have investigated the mechanism(s) of HIV-1-induced $CD4^+$ cell depletion in the SCID-hu mouse, and our results are consistent with direct killing of HIV-1-infected thymocytes. Throughout the period of rapid $CD4^+$ cell depletion, proviral burden was consistent with infection of the majority of $CD4^+$ cells. In addition, large numbers of cells were actively producing virus. It is thus not necessary to evoke killing of bystander cells to elicit the observed rate of thymocyte loss. It is now believed that viral burden in lymphoid tissues of HIV-infected humans is high enough to account for a majority of the observed $CD4^+$ T-cell loss by direct killing of infected cells (9, 30). This view is further supported by the observation that productively infected PBMC have a shorter life span than latently infected PBMC and undergo rapid turnover (17, 44), suggesting that expression of viral proteins leads to death of the infected cell. Several mechanisms of HIV-1-induced direct killing, including destruction of the plasma membrane during viral budding (23, 25), syncytium formation (24, 36), and par-

FIG. 5. (A) Proviral burden during CD4⁺ cell depletion. The number of HIV-1 genomes in 10^5 human cells was determined by quantitative PCR at the indicated times postinfection. Each diamond represents an individual implant. (B) Proviral burden in CD4⁺ cells. Proviral burden from samples shown in panel A was normalized to CD4⁺ cell number, as determined by flow cytometry. Each symbol represents proviral burden in an individual implant.

asitism of cellular transcriptional and translational machinery (35, 37), have been postulated. Cao et al. recently reported that the expression of envelope proteins alone is sufficient to cause killing of the expressing cell by a nonapoptotic mechanism (8). In our studies, at a time when $\sim 10\%$ of CD4⁺ cells are lost per day, no elevation in the levels of apoptosis was observed. Given that a cell can complete the apoptotic cycle within 3 to 6 h, an elevation of only ~ 2 to 4% apoptotic cells over background could account for the numbers of cells lost per day. While these levels may be difficult to detect on an individual basis, none of the 14 biopsy samples taken during the rapid phase of CD4⁺ cell depletion demonstrated any increase over background. Our results suggest that apoptosis either is not involved or occurs at such low levels that it is unlikely to be the major mechanism of CD4⁺ cell loss. While we cannot formally rule out that rapid apoptosis and nearly immediate cell death are occurring, no elevation of apoptotic levels is observed in HIV-1-infected thymocyte suspension cultures depleted of macrophages, even though cell death is occurring (42a). These data suggest that the lack of observable apoptosis is not due solely to rapid clearance of apoptotic cells. Taken together, our results suggest that depletion of CD4⁺ cells is predominantly due to direct infection rather than indirect mechanisms, as reported by others (39).

Our finding that HIV-1-induced CD4⁺ deletion occurs without extensive apoptosis is supported by two other recent studies that examine the lymph nodes of simian immunodeficiency virus-infected macaques (10) and HIV-1-infected individuals

FIG. 6. Detection of cell-associated virus 3 weeks postinfection. The number of thymocytes producing infectious virus in Thy/Liv implants 21 days postinfection was determined by limiting dilution coculture. Cells from each of five infected Thy/Liv implants were cocultured in quadruplicate with stimulated PBMC for 10 days, and virus production was assessed by enzyme-linked immunosorbent assay for p24^{gag}. (A) The total number of p24^{gag}-positive wells resulting from individual cocultures of all five Thy/Liv implants is plotted against the indicated input number of thymocytes per well. The bisecting line indicates the point at which 50% of the wells scored positive for p24^{gag}. (B) To reflect the Poissonian distribution of productively infected cells, the fraction of wells positive for p24^{gag} is plotted on a log scale against the indicated number of thymocytes per well, also shown on a log scale. The bisecting line indicates when the fraction of negative wells reaches 0.37.

(10, 28). Although a small increase in apoptosis was observed in these systems, it was restricted primarily to uninfected cells (10) and occurred in both CD4⁺ and CD8⁺ T cells (10, 28), as well as in B cells (28). The level of apoptosis in the lymph nodes of HIV-1-infected individuals correlates with the degree of lymphoid tissue activation (28), suggesting that the observed apoptosis may be a result of activation-induced cell death (AICD) rather than virus-induced cell death. AICD results in apoptosis of mature T cells exposed to repeated antigenic stimulation. One function of AICD is to maintain lymphocyte homeostasis following antigen-specific lymphocyte activation and proliferation. AICD has been documented in several viral infections (32, 42). The absence of elevated apoptosis in the SCID-hu mouse may therefore reflect the probable absence of HIV-induced stimulation of the thymocytes. This lack of a specific immune response in the SCID-hu mouse would also exclude apoptotic events elicited by cytotoxic T cells or antibodies.

Our findings are in contrast to an earlier report by Su et al. (39), in which high levels of apoptosis were observed in HIV-1_{NL4-3}-infected implants in SCID-hu mice. The reason for the discrepancy between the two reports is not clear. However, donor-derived effects are not likely, as we have previously used

implants from multiple donors in several experiments, and the results are consistent with those presented here (data not shown). Our data also demonstrate a higher viral burden than the 10% infected cells reported by Su et al. (39). Whether this higher viral load is responsible for the discrepancy in apoptotic levels is unclear. Our results agree with those of Su et al. (39) in that there is an increase in apoptosis in the late stages of CD4⁺ cell depletion. However, apoptosis at this late stage is primarily in CD4⁺ CD8⁺ cells and therefore most likely reflects perturbation of the thymic microenvironment and/or disruption of the cytokine network and not a direct effect of HIV-1 on the infected cell. Alternatively, we have recently noted that late in infection, HIV can infect immature CD4⁺ CD8⁺ thymocytes, which subsequently differentiate into productively infected CD4⁺ CD8⁺ cells (20). It is conceivable that these cells are eventually killed by HIV via an apoptotic mechanism.

Understanding the mechanism(s) of HIV-1-induced cell death has important implications for designing therapeutic strategies, especially those that rely on antiviral approaches such as drug or gene therapy. If a significant number of uninfected bystander cells are killed by HIV-1, successful gene therapy strategies must target 100% of all CD4-bearing cells. This frequency cannot be achieved with current technologies. Our results, however, strongly suggest that direct virus killing accounts for the majority of observed CD4⁺ cell death. This further suggests that cells expressing antiviral genes might have a selective advantage *in vivo*.

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