Crosslinking CD4 by Human Immunodeficiency Virus gp120 Primes T Cells for Activation-induced Apoptosis

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Summary

During human immunodeficiency virus (HIV) infection there is a profound and selective decrease in the $CD4^+$ population of T lymphocytes. The mechanism of this depletion is not understood, as only a small fraction of all $CD4^+$ cells appear to be productively infected with HIV-1 in seropositive individuals. In the present study, crosslinking of bound gp120 on human $CD4^+$ T cells followed by signaling through the T cell receptor for antigen was found to result in activation-dependent cell death by a form of cell suicide termed apoptosis, or programmed cell death. The data indicate that even picomolar concentrations of gp120 prime T cells for activation-induced cell death, suggesting a mechanism for $CD4^+$ T cell depletion in acquired immune deficiency syndrome (AIDS), particularly in the face of concurrent infection and antigenic challenge with other organisms. These results also provide an explanation for the enhancement of infection by certain antibodies against HIV, and for the paradox that HIV appears to cause AIDS after the onset of antiviral immunity.

The immunodeficiency that defines AIDS is due primarily to a progressive decline in the number and function of $CD4^+$ T cells. The mechanism of this decline is debated, though lytic infection of cells targeted by interaction of CD4 with the envelope glycoprotein of the HIV virion, gp120, is an obvious model (1–4), and recent data suggest an apoptotic mechanism of cell death after HIV infection (5). However, previous studies have found that only 1 in 1–10 $\times$ 10^4 PBMC actively express HIV-1 in patients with AIDS (6–10), and immune dysfunction is seen early in infection, before a significant proportion of $CD4^+$ cells has been eliminated (11–15). Thus, it is likely that mechanisms other than direct viral destruction contribute to $CD4^+$ T cell loss and to the anergy associated with $CD4^+$ T cell–dependent immune responses.

Mouse splenic T cells pretreated with anti-CD4 antibodies die by apoptosis when stimulated through the α/β TCR (16). Apoptosis is an active form of physiologic cell death, requiring RNA and protein synthesis, which is characterized by the activation of endogenous endonucleases that cleave chromatin DNA between nucleosomes (17, 18). Here we report that crosslinking of gp120 on human $CD4^+$ T cells followed by signaling through the TCR results in activation-induced cell death. This cell death has the characteristic features of apoptosis, including the histologic changes of nuclear and cytoplasmic condensation and DNA fragmentation into nucleosome-sized multimers of 200 bp. Our data provide a mechanism for the recent observation that $CD4^+$ T cells from HIV-infected individuals are primed in vivo for suicide by apoptosis, upon TCR activation by both superantigen and MHC class II–restricted antigens (19).

Materials and Methods

Isolation of $CD4^+$ T Cells. Human T cells were separated from Ficoll-Hypaque–isolated PBMC by rosetting with 2-aminoethyl-isothio-uronium bromide hydrobromide (AET)-treated SRBC, as described (20). $CD4^+$ cells were isolated by incubation of the rosetted cells with affinity-purified anti-CD8 antibody (OKT8, 20 μg/ml, CRL 8014; American Type Culture Collection, Rockville, MD), followed by negative selection on goat anti-mouse (GAM)[1] Ig-coated panning plates (100 μg/ml; Jackson Immunoresearch, Westgrove, PA) (21). Isolated cells were ~90% $CD4^+$ by flow

[1] *Abbreviation used in this paper:* GAM, goat anti-mouse.

cytometric analysis (89.52 ± 1.65%, n = 5). The 10% non-$CD4^+$ cells were shown by FACS® analysis (Becton Dickinson & Co., Mountain View, CA) to be ~6% $CD8^+$ T cells, 2% NK cells, and 2% B cells. Results were similar using $CD4^+$ T cells isolated entirely by negative selection using monosized superparamagnetic beads with covalently bound sheep anti-mouse Ig (Dynal Inc., Great Neck, NY), to bind cells incubated with a cocktail of mAbs directed against CD8 (OKT8, 20 μg/ml), Ia (HB 55, 20 μg/ml), and CD16 (3G8, 20 μg/ml; the generous gift of Dr. J. C. Unkeless, Mount Sinai School of Medicine, New York) (data not shown). Similar results were also obtained using antigen-specific $CD4^+$ T cell clones (data not shown), suggesting that the contaminating non-$CD4^+$ cells are not required for the induction of apoptosis.

Priming and Induction of Apoptosis. $CD4^+$ T lymphocytes (4×10^6 cells/sample) were isolated, as described above, and irradiated with 500 rad γ-irradiation or incubated on ice with or without the primary antibodies anti-CD4 (Leu-3a, 20 μg/ml; the generous gift of the Sloan-Kettering Institute, New York) or the HIV surface glycoprotein, gp120, in a balanced salts solution. Two gp120 preparations were tested in separate experiments. Recombinant gp120 ($rgp120_{SF2}$, 40 μg/ml; references 22–24) crosslinked with polyclonal anti-gp120 (anti-env $2\text{-}3_{SF2}$, 40 μg/ml; reference 25) and recombinant gp120-HSV chimeric protein (gp120-HSV; 10 μg/ml; reference 26) crosslinked with anti-gp120-HSV mAb (5B6, 10 μg/ml; reference 26) were bound at uniform, high-density levels, comparable to the binding of the ligand, CD4, by Leu-3a, an antibody that binds the same epitope as gp120 (27). Crosslinking of the mAbs Leu-3a and 5B6 was achieved using GAMIg (100 μg/ml) incubated at 37°C for 30 min. Though the priming signal through CD4 does not decay for at least 8 h (data not shown), in all experiments shown, the anti-TCR antibody (BMA-031, 100 μg/ml; reference 28) was added immediately after CD4 crosslinking and was incubated with cells on ice for 45 min. To allow crosslinking and, hence, activation through the TCR, cells were incubated in IMDM with 5% inactivated FCS in 24-well plates (10^6 cells in 1.5 ml per well; Costar, Cambridge, MA) precoated with GAMIg. Unless otherwise noted, cells were harvested and analyzed after 72 h of incubation. Long-term culture of human T cells was required to see optimal induction of double-stranded DNA breaks to produce the ladder characteristic of nucleosome-sized DNA fragments (data not shown; and Fig. 3). Cell aliquots were stained with 0.2% trypan blue and counted. 500 cells were counted per sample per time point.

DNA Fragmentation. Three methods were adopted for analysis of the DNA laddering characteristic of apoptotic cell death: one analyzing low molecular weight DNA (Fig. 1), a second analyzing total DNA by ethidium bromide staining of DNA separated by agarose gel electrophoresis (Figs. 2 and 4, *top*), and a third analyzing total DNA by Southern blotting after size separation (Figs. 2 and 4, *bottom*). Isolated low molecular weight DNA fragmentation was determined as previously described (18) with minor modifications. Briefly, the cell pellet was lysed in 0.5 ml hypotonic buffer, pH 8.0 (5 mM Tris-HCL, 20 mM EDTA, 0.5% Triton X-100), and centrifuged at 27,000 *g* for 10 min. Fragmented DNA in the supernatant was precipitated overnight at −20°C in 650 μl isopropanol and 100 μl 5 M sodium chloride. After centrifugation at 27,000 *g* for 15 min, the precipitates were collected, air dried, and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.4, 0.5% SDS, and kept at 37°C for 24 h. 8 μl of 5 mg/ml RNase A DNase-free was added to each sample and incubated at 37°C for 30 min. Electrophoresis was carried out in a 0.75% agarose gel for 3 h at 90 V. DNA fragmentation in total DNA was determined as previously described (29). Briefly, cells were lysed in 20 μl of 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5 mg/ml proteinase K, and incubated at 50°C for 1 h. 10 μl 0.5 mg/ml RNase A was then added to each sample and incubated at 50°C for 1 h. Samples were heated to 65°C for 10 min before loading onto dry wells of a 2% agarose gel. Total DNA was analyzed by Southern blotting after size separation on agarose gels (30, 31). The gel was transferred to nitrocellulose in 0.4 M NaOH in order to hydrolyze any contaminating RNA. The membrane-bound DNA was probed with ^{32}P-labeled human placental DNA.

Quantitation of DNA Fragmentation. DNA fragmentation was quantitated by a modification (18) of the method originally described by Wyllie (17). This method has been shown to be highly specific for DNA (e.g., with 1,000-fold lower sensitivity for RNA) and sensitive to microgram quantities of DNA (32). Briefly, cell pellets were lysed with 0.5 ml hypotonic buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Lysates were kept on ice for 15 min, then centrifuged at 27,000 *g* to separate intact chromatin (pellet) from low molecular weight DNA fragments (supernatant). The pellet was resuspended in 0.5 ml hypotonic buffer. Pellet and supernatant were separately precipitated overnight at 4°C in 12.5% TCA. The precipitates were collected after centrifugation at 27,000 *g* for 15 min, and resuspended in 480 μl TCA, followed by hydrolysis at 90°C for 10 min. DNA in both pellet and supernatant was assayed colorimetrically (570 nm) using the diphenylamine (DPA) reagent, as previously described (32).

Results and Discussion

We first asked if human peripheral T cells undergo apoptosis in response to crosslinking of CD4 followed by activation through the TCR. Induction of apoptosis may be demonstrated by visualization of nucleosome-sized DNA multimers of 180–200 bp to form the characteristic "step ladder" appearance after size separation on agarose gels, as seen in γ-irradiated cells (Fig. 1, lane *6*) (16–18). As previously reported for murine immature thymocytes (33) and mature splenic T cells (16), the culture of human T cells bearing the CD4 antigen with anti-CD4 antibodies under crosslinking conditions, in the absence of subsequent stimulation, failed to induce DNA fragmentation over background levels in isolated low molecular weight DNA (Fig. 1, lanes *1* and *2*). Crosslinking of anti-TCR antibody, or simultaneous exposure to anti-TCR and anti-CD4 antibodies, also failed to induce fragmentation over background (Fig. 1, lanes *3* and *4*, and Fig. 2, lane *1*), arguing against proliferation-induced lymphokine depletion as the mechanism of cell death (34). The bands observed at 1.0 and 0.2 kb in Fig. 1 are RNA species that are resistant to RNase under these extraction conditions (see legend to Fig. 2). In contrast, when cells were pretreated with anti-CD4 antibody under crosslinking conditions and then activated by incubation with anti-TCR antibody, DNA fragmentation increased over background, and a nucleosomal pattern of degradation was seen in isolated low molecular weight DNA (Fig. 1, lane *5*) (18), and confirmed in analyses of total DNA (Fig. 2, lane *2*) (29), and by quantitation of DNA fragmentation (Fig. 3 *A*) (32). This priming for activation-induced apoptosis by anti-CD4 antibody may in part explain why these antibodies produce antigen-specific and transplantation tolerance in vivo (35).

We then asked if crosslinking of CD4 by gp120 would

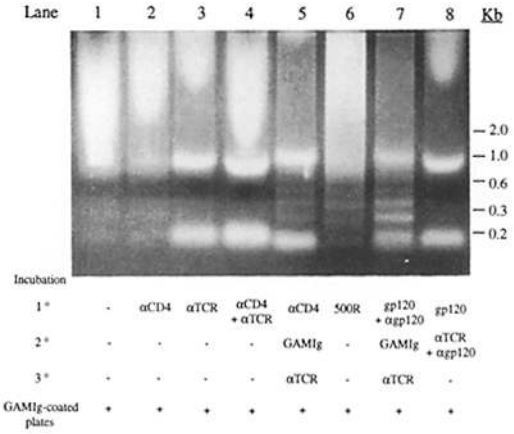

Figure 1. Activation-induced apoptosis in human CD4+ peripheral T cells after CD4 ligation by anti-CD4 antibody or by gp120. Agarose gel electrophoresis of DNA fragments isolated from human CD4+ T cells after treatment of the cells with: lane *1*, untreated; lane *2*, anti-CD4 antibody; lane *3*, anti-TCR antibody; lane *4*, simultaneous anti-CD4 and anti-TCR; lane *5*, anti-CD4 crosslinked with GAMIg, then incubated with anti-TCR; lane *6*, 500 rad γ-irradiation; lane 7, gp120-HSV plus anti-gp120-HSV mAb, crosslinked with GAMIg, then incubated with anti-TCR; lane *8*, gp120-HSV, then simultaneous incubation with anti-TCR and crosslinking anti-gp120-HSV. Cells were then cultured for 72 h on plates coated with GAMIg. DNA fragments (oligonucleosomal fragments) appear as a DNA ladder, with the molecular sizes approximate multiples of 200 bp. The bands observed at 1.0 and 0.2 kb are RNA species that are resistant to RNase under these extraction conditions (see legend to Fig. 2). Data are representative of four experiments performed using peripheral T cells from four different uninfected donors.

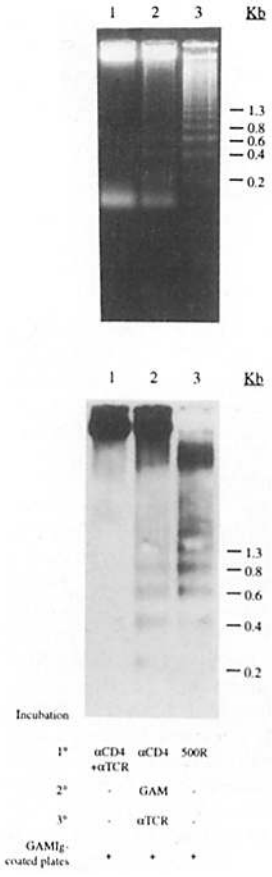

Figure 2. Crosslinking CD4 by anti-CD4 antibody primes T cells for activation-induced apoptosis. Agarose gel electrophoresis of total DNA (*top*) and Southern blotting of the gel, probed with ^{32}P-labeled human placental DNA (*bottom*) after treatment of cells with: lane *1*, simultaneous anti-CD4 and anti-TCR; lane *2*, anti-CD4 crosslinked with GAMIg, then incubated with anti-TCR; lane *3*, 500 rad γ-irradiation. Cells were then cultured for 72 h on plates coated with GAMIg. The bands observed <0.2 kb (*top*, lanes *1* and *2*) are RNA species that are resistant to RNase, as suggested by the absence of these bands (*bottom*) after Southern blotting under alkaline conditions.

prime T cells for activation-induced apoptosis. Crosslinking of recombinant gp120 (22–24) by polyclonal anti-gp120 antibody (25) did not result in increased DNA fragmentation over background levels in analyses of total DNA (Fig. 4, lane *5*). This was confirmed using a second gp120/anti-gp120 preparation, gp120-HSV crosslinked with anti-gp120-HSV mAb (26) (data not shown). Ligation of TCR after binding of gp120-HSV to CD4, but before crosslinking with anti-gp120-HSV antibody, also failed to induce fragmentation over background (Fig. 1, lane *8*). In contrast, when cells were pretreated with gp120-HSV plus anti-gp120-HSV antibody, under crosslinking conditions, and then activated by incubation with anti-TCR antibody, DNA fragmentation was increased over background, with a nucleosomal pattern of DNA degradation seen in isolated low molecular weight DNA (Fig. 1, lane 7). The induction of DNA fragmentation was also seen with cellular activation through TCR, after pretreatment with recombinant gp120 and polyclonal anti-gp120 antibody, in analyses of total DNA (Fig. 4, lane *3*).

Apoptosis was confirmed histologically by the characteristic and definitive changes of nuclear and cytoplasmic condensation (data not shown) (17). Apoptosis was measured quantitatively by assessing the proportion of fragmented DNA (Fig. 3) (32). Less than 5% of total DNA was fragmented under control conditions, despite prolonged in vitro culture (data not shown). Crosslinking of bound gp120 or TCR alone did not significantly increase this percentage (Fig. 3 *A*). In contrast, crosslinking of CD4 with gp120, followed by activation through the TCR, resulted in DNA fragmentation four times that of background levels, a level equivalent to that seen after pretreatment with anti-CD4 antibody. Significant levels (23.19 ± 4.02%) of DNA fragmentation were seen as early as 12 h after activation in cells primed by CD4 ligation, increasing over at least the next 60 h to ~30% of total DNA (30.80 ± 1.18%) (Fig. 3, *B* and *C*). Studies have shown that in a single cell the process of DNA fragmentation is all-or-nothing; cells that are half-apoptotic are not seen (36). That these apoptotic cells actually go on to die was confirmed by trypan blue uptake in gp120-primed and irradiated CD4+ peripheral T cells (Fig. 3 *C*) and in gp120-primed antigen-specific CD4+ T cell clones (data not shown). Thus, some human peripheral CD4+ T cells pretreated with gp120 and anti-gp120 antibody (20–30% under these assay conditions) die by apoptosis when activated through the TCR.

Does this in vitro priming for apoptosis by gp120 have any in vivo relevance? Recent work of Groux et al. (19) describes activation-induced apoptosis of CD4+ T cells from HIV-infected asymptomatic individuals. How might priming occur in vivo? Though not addressed in this study, recirculating CD4+ T cells could be directly primed and crosslinked by gp120 expressed on viral particles or infected cells (37). Alternatively, it is possible that circulating gp120 and anti-gp120 prime in vivo as they do in vitro. The presence of anti-gp120 antibodies in HIV-infected patients has been well documented (38–48), as is the formation of a population of antibodies that has a promoting rather than an inhibiting or neutralizing effect on virus pathogenicity, in vitro

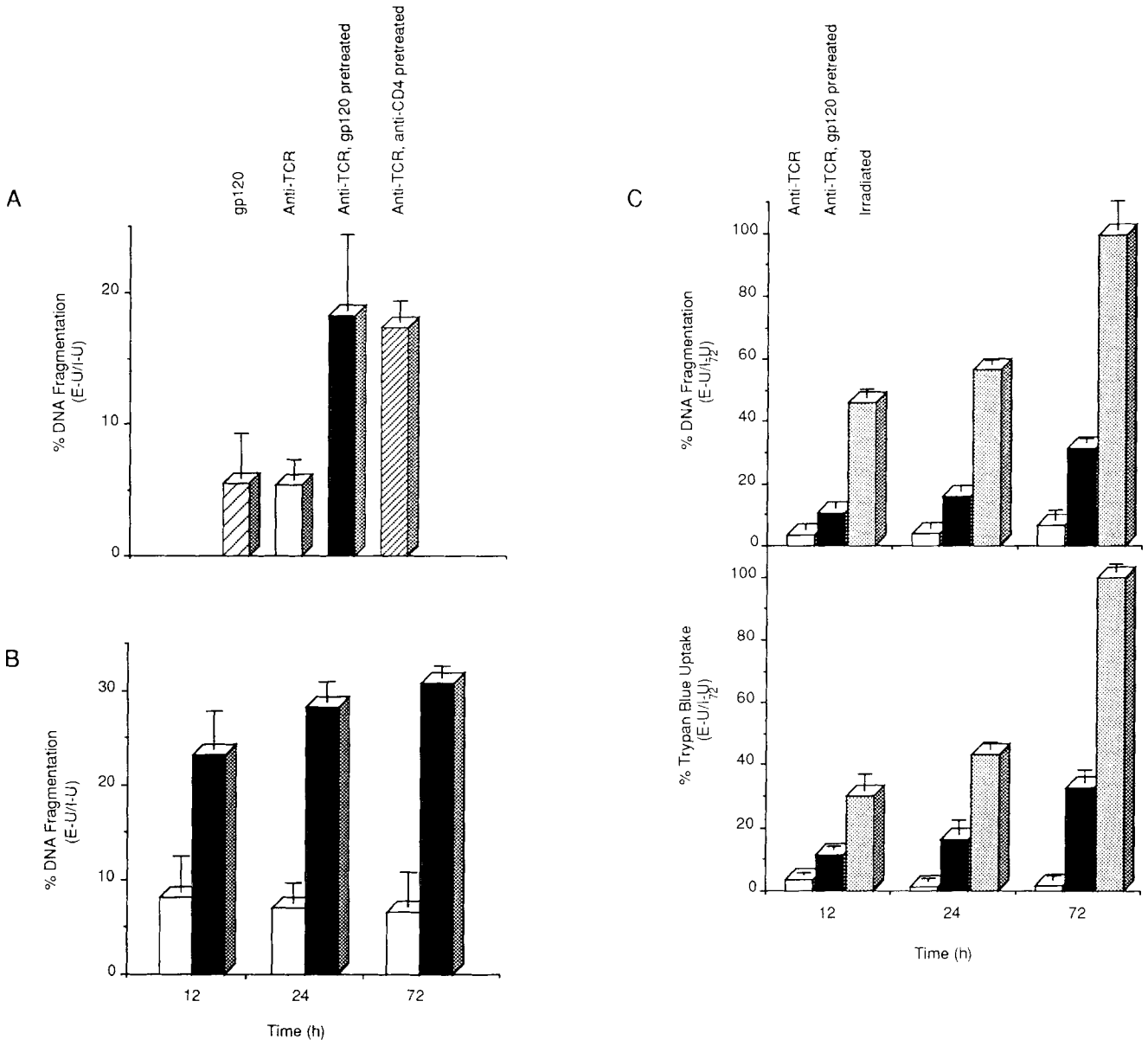

Figure 3. Quantitation of DNA fragmentation in response to activation through the TCR after ligation of CD4 by gp120. (*A*) Quantitation of fragmented DNA. CD4+ T lymphocytes were isolated from peripheral blood of healthy human donors, as described in Materials and Methods, and incubated with: (▨) gp120 plus anti-gp120 antibody; (□) anti-TCR antibody (BMA-031); (■) gp120 crosslinked with anti-gp120 antibody, then incubated with anti-TCR; (▨) anti-CD4 antibody (Leu-3a) crosslinked with GAMIg, then incubated with anti-TCR. Cells were then cultured for 72 h on plates coated with GAMIg. DNA fragmentation was quantitated as described in Materials and Methods. Results are shown as percent DNA fragmentation: $DNA_{supernatant}/DNA_{supernatant} + DNA_{pellet}$, relative to the irradiated control. Data are expressed as E − U/I − U, where E = experimental, U = untreated, and I = irradiated control (66). Actual values are as follows: untreated, 5.22 ± 0.67%; gp120 + anti-gp120, 7.45 ± 1.24%; anti-TCR, 7.38 ± 0.40%; gp120 + anti-gp120 + anti-TCR, 12.25 ± 1.21%; anti-CD4 + anti-TCR, 11.43 ± 0.75%; irradiated, 46.33 ± 5.87%. Percent DNA fragmentation of the anti-TCR, gp120-pretreated sample (■) was significantly different from the gp120 (▨) and anti-TCR (□)-treated samples (p = 0.02 and 0.01, respectively, by a univariate repeated measures analysis of variance; reference 67). In addition, percent DNA fragmentation of the anti-TCR, gp120-pretreated sample (■) was significantly different from the sum of the gp120 (▨) and anti-TCR (□)-treated samples (p = 0.04 by a paired t test), suggesting that the effect of priming is not merely additive with that of activation. Percent DNA fragmentation of the gp120 (▨) and anti-TCR (□)-treated samples was not significantly different from the untreated sample. (*B*) Kinetics of DNA fragmentation in response to T cell activation after priming by CD4 ligation. CD4+ T lymphocytes were isolated and incubated with anti-TCR antibody (BMA-031) (□) or with gp120 crosslinked with anti-gp120 antibody, then incubated with anti-TCR (■). Data are expressed as in *A*. Actual values are as follows: 12-h incubation: untreated, 2.37 ± 0.47%; anti-TCR, 3.45 ± 0.51%; gp120 + anti-gp120 + anti-TCR, 5.48 ± 0.54%; irradiated, 15.82 ± 0.84%. 24-h incubation: untreated, 2.51 ± 0.28%; anti-TCR, 3.17 ± 1.0%; gp120 + anti-gp120 + anti-TCR, 7.18 ± 0.51%; irradiated, 19.05 ± 0.36%. 72-h incubation: untreated, 6.67 ± 0.82%; anti-TCR, 8.32 ± 1.28%; gp120 + anti-gp120 + anti-TCR, 14.43 ± 0.28%; irradiated, 32.9 ± 2.29%. (*C*) Comparison of the kinetics of DNA fragmentation and cell death, as measured by trypan blue uptake, in response to irradiation or to T cell activation after priming by CD4 ligation. CD4+ T lymphocytes were isolated and irradiated with 500 rad γ-irradiation (▨), or incubated with anti-TCR antibody (BMA-031) (□) or with gp120 crosslinked with anti-gp120 antibody, then incubated with anti-TCR (■). Data are expressed relative to the

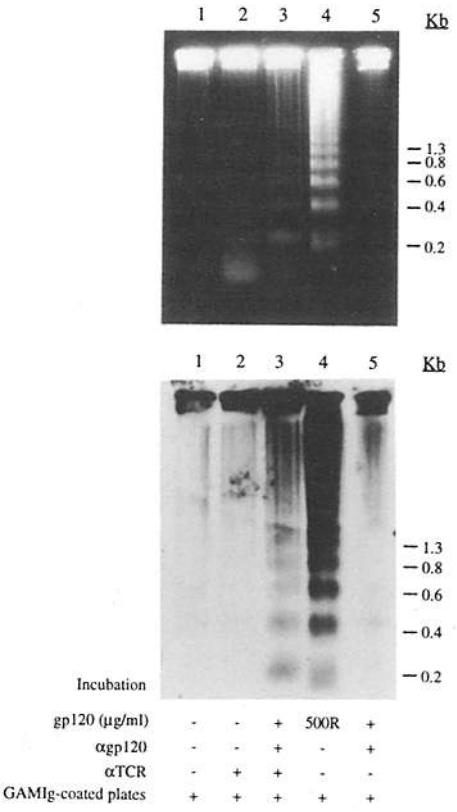

Figure 4. Crosslinking CD4 by HIV gp120 primes T cells for activation-induced apoptosis. Agarose gel electrophoresis of total DNA (*top*) and Southern blotting of the gel probed as Fig. 2 (*bottom*) after treatment of cells with: lane *1*, untreated; lane *2*, anti-TCR; lane *3*, gp120 crosslinked with polyclonal anti-gp120 antibody, then incubated with anti-TCR; lane *4*, 500 rad γ-irradiation; lane *5*, gp120 crosslinked with polyclonal anti-gp120. Cells were then cultured for 72 h on plates coated with GAMIg. Though not quantitative, the loading of DNA in all lanes appeared to be equivalent, as confirmed by densitometric scanning (data not shown), suggesting equivalent cell numbers and recovery of DNA from all samples. Data are representative of three experiments performed using peripheral T cells from three different uninfected donors. These three donors were different from the four used in the experiments detailed in Fig. 1.

(49–51). Such enhancing antibodies may act in part by crosslinking bound gp120 (52, 53), thus priming cells for apoptosis in the presence of an activating antigen or infectious agent. There is evidence that gp120 is shed from HIV-infected cells in vitro (54, 55) and in vivo (56), and, in recent work, gp120 has been measured in the blood of patients with AIDS at levels of 12–92 ng/ml (57). In the in vitro system reported here, as little as 10 ng/ml (10^{-9} M) gp120 primed $CD4^+$ T cells for activation-induced apoptosis (data not shown). This level of gp120 is equal to the dissociation constant of the gp120-CD4 interaction (10^{-9} M) (14) and represents binding of ~10% of CD4 on the cell surface. Thus, even picomolar concentrations of gp120 in vivo could prime T cells for activation-induced apoptosis.

One of the major contributing factors to the pathogenicity of HIV infection is the ability of the virus to suppress immunologic function (11, 15). This immune suppression renders the host susceptible to a myriad of secondary infections, which are frequently directly responsible for morbidity and mortality. The primary mechanism by which HIV compromises immunity is by subversion of the immune system so that it eliminates $CD4^+$ T cells. Interestingly, this may not be predominantly due to direct viral infection, since only ~ 1 in 1–10 × 10^4 PBMC are productively infected in HIV-seropositive individuals (6–10). Alternatively, in vitro systems have demonstrated antibody-dependent cell-mediated cytotoxicity via recognition of CD4-bound gp120 (58). Similarly, cytotoxic T cells, specific for viral peptides, mediate in vitro elimination of cells that have internalized gp120 or virus via CD4 (59). While these mechanisms may delete $CD4^+$ T cells, it has been suggested that they in fact serve to protect against viral spread. Here, we have identified a mechanism by which "innocent bystander" T cells, bound with circulating or cell surface gp120, may self-destruct upon initiation of an appropriate response to the pathogens that coinfect AIDS patients, or to the superantigens that may be expressed either by these pathogens (60), or by HIV itself (61–63). Our observation that this priming for apoptosis requires crosslinking of bound gp120 by anti-gp120 antibody may in part explain the paradox that HIV appears to cause AIDS after the onset of antiviral immunity (64, 65). Though this study does not address the question of apoptotic cell death in HIV-infected individuals, our observation that even picomolar concentrations of gp120 prime for activation-induced apoptosis suggests that this mechanism of cell death may be active in vivo. In addition, our data provide a mechanism for the recent observation that $CD4^+$ T cells from HIV-infected asymptomatic individuals and AIDS patients are primed, in vivo, for suicide by apoptosis, upon TCR activation by both superantigen and MHC class II–restricted antigen (19). This work suggests that deletion of $CD4^+$ T cells upon activation may contribute to the progressive depletion of $CD4^+$ T cells in AIDS. Importantly, the active nature of the apoptotic process suggests that this mechanism of cell death in AIDS may potentially be suppressed.

irradiated control values after 72 h of incubation ($E - U/l_{72} - U$) in order to show the relative increases in DNA fragmentation and trypan blue uptake with time. Data for percent DNA fragmentation were as noted in *B*. Data for trypan blue uptake: 12-h incubation: untreated, 5.81 ± 0.06%; anti-TCR, 7.19 ± 0.72%; gp120 + anti-gp120 + anti-TCR, 12.84 ± 1.17%; irradiated, 18.70 ± 2.17%. 24-h incubation: untreated, 9.07 ± 0.73%; anti-TCR, 9.59 ± 1.12%; gp120 + anti-gp120 + anti-TCR, 15.15 ± 1.66%; irradiated, 25.51 ± 1.05%. 72-h incubation: untreated, 13.73 ± 1.78%; anti-TCR, 14.3 ± 1.36%; gp120 + anti-gp120 + anti-TCR, 24.39 ± 2.67%; irradiated, 46.55 ± 2.51%. Levels of DNA fragmentation and trypan blue uptake at 0 h (i.e., immediately after $CD4^+$ T cell isolation) were 1.19 ± 0.14% and 4.84 ± 0.07%, respectively. Data shown in *A* are from replicates of four independent experiments using four different uninfected donors. Data shown in *B* and *C* are representative of three independent experiments.

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