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Shared Antigenic Epitopes on the V3 Loop of HIV-1 gp120 and Proteins on Activated Human T Cells

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Proliferation of HIV-1 in the infected host is characterized by a progressive loss of $CD4^+$ T lymphocytes and consequent dysregulation of the immune system. Both direct and indirect mechanisms have been proposed. We show here that proteins with molecular weights 35, 48, and 110 kDa on stimulated primary human T cells are recognized by neutralizing antibodies against the V3 loop of HIV-1 gp120. Recognition is specific since it can be blocked by a recombinant HIV-1 gp120. Furthermore, these V3 monoclonal antibodies, as well as sera from AIDS patients that recognized these V3-like proteins, induced killing of HIV-1-infected as well as uninfected T cells. This killing was also inhibited by HIV-1 gp120 V3 peptides. These results indicate that the V3 loop shares epitopes with proteins on stimulated T cells. This may be an additional autoimmune mechanism contributing to $CD4^+$ T cell disappearance in AIDS. V3 antibodies have been proposed as potential prophylactic agents. However, if such vaccines were based on certain epitopes, they might induce cross-reacting immune responses with cellular proteins. Vaccine candidates should be evaluated for such potential effects. © 1998 Academic Press

The hallmark of HIV-1 progression is the loss of $CD4^+$ T cells (Fauci, 1993). However, the exact mechanisms by which HIV-1 kills $CD4^+$ T cell remain unresolved. Direct $CD4^+$ T cell killing by HIV-1 is one possibility (Yu *et al.,* 1994). A high turnover of HIV-1 was associated with depletion of T cells (Ho *et al.,* 1995; Wei *et al.,* 1995) and plasma HIV-1 RNA levels were shown to be a predictor for AIDS progression (Mellors *et al.,* 1996; O'Brien *et al.,* 1996). Indirect mechanisms such as apoptosis may induce killing of T cells (Gougeon and Montagnier, 1993). HIV-1 proteins, such as tat and gp120, induced apoptosis (Li *et al.,* 1995; Westendorp *et al.,* 1995). Other mechanisms such as cross-linking of CD4 proteins (Banda *et al.,* 1992), $CD8^+$ cytotoxic T lymphocyte attack of $CD4^+$ T cells binding gp120 (Koenig *et al.,* 1988), the presence of a superantigen encoded by the virus (Berberian *et al.,* 1993), or participation of the immune system via autoimmunity including molecular mimicry have been suggested as playing a role in killing $CD4^+$ T cells (Levy, 1994). Molecular mimicry occurs when pathogens share antigenic sites with normal host cells (Fujinami and Oldstone, 1989). For example, monoclonal antibodies (mAb) to viral proteins can bind host components (Srinivasappa *et al.,* 1986; a hepatitis B virus polymerase peptide that shares six consecutive amino acids with rabbit myelin basic protein led to autoimmune disease (Fujinami and Oldstone, 1985).

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Retroviral infections, including HIV-1, may induce autoimmune responses by molecular mimicry. Supportive evidence for this possibility includes the homology between HIV-1 proteins such as envelope with IL-1, IL-2 receptor, MHC class I and II, Fas, and proteins of normal astrocytes (reviewed by Silvestris *et al.,* 1995). The homology of regions of gag, tat, and nef with human normal proteins has been described (reviewed by Silvestris *et al.,* 1995). Previously, we identified proteins in the human brain containing epitopes that resembled the V3 loop of HIV-1 gp120 (Trujillo *et al.,* 1993). Three prominent human brain proteins of 35, 55, and 110 kDa reacted with monoclonal antibodies against the V3 loop. The V3 loop of gp120 is important in AIDS pathogenesis and in potential therapy (Emini *et al.,* 1992). In this current work, we had two goals. First, we sought to determine the expression, localization, and regulation of V3-like proteins in normal human $CD4^+$ T lymphocytes. Second, we wanted to know whether anti-V3 antibodies could kill uninfected T cells, an important question since depletion of $CD4^+$ T lymphocytes cells is a primary feature of AIDS.

RESULTS

Detection of HIV-1 gp120 V3-like protein in T cells

To determine whether T cell protein would react with a V3 mAb, we used a Western blot (WB) analysis of the lysate of an established T cell line, SupT1. The lysate was tested against a panel of monoclonal and polyclonal anti-HIV-1 antibodies. Only the V3 mAbs (NEA

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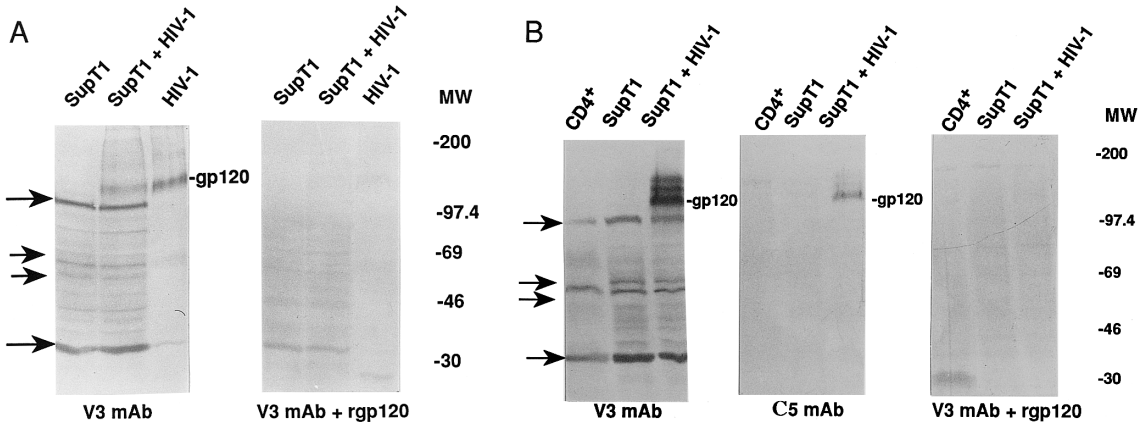

FIG. 1. Western blot analysis of $CD4^+$ T lymphocytes with anti-HIV-1 gp120 V3 loop antibody. (A) WB analysis of a SupT1 $CD4^+$ T lymphocyte cell line, SupT1 infected with HIV-1_{IIIb}, and HIV-1_{IIIb} viral lysates that were probed with V3 mAb. The arrows indicate proteins of 35, 45, 50, and 110 kDa. Binding between V3 mAb and T cell proteins was completely blocked with recombinant HIV-1 gp120. (B) WB analysis of normal $CD4^+$ T lymphocytes probed with V3 mAb. Cell lysates of normal $CD4^+$ lymphocytes, SupT1 cells, and SupT1 infected with HIV-1_{IIIb} were probed with V3 mAb. Arrows indicate proteins of 35, 50, and 110 kDa. A control HIV-1 gp120 475–486 mAb did not recognize any T cell proteins, but did recognize the HIV-1 gp120 protein. Blocking experiments using rgp120 completely inhibited the binding between V3 mAb and T cell proteins.

9205, raised against amino acids 308 to 322, and NEA 9284, raised against amino acids 307 to 330) reacted with two prominent proteins of approximately 35 and 110 kDa (Trujillo *et al.,* 1996a). In addition, other less prominent proteins reacted with the V3 mAb, including one of 45 and one of 50 kDa. These experiments were repeated five times and yielded identical results (data not shown).

To confirm that T cells share a common epitope with HIV-1 envelope protein, the uninfected SupT1 cell line, the SupT1 cell line infected (Trujillo *et al.,* 1996b) with the HIV-1_{IIIb} strain (AIDS Research and References Reagent Program, National Institutes of Health, Bethesda, MD), and the viral lysate of HIV-1_{IIIb} were probed with V3 mAb in a WB analysis. As shown in Fig. 1A, the V3 mAb cross-reacted with normal T cell proteins as well as the HIV-1 gp120. To determine whether the human T cell proteins with molecular weights 35, 45, 50, and 110 kDa were specific to the V3 loop, a blocking experiment using recombinant gp120 from a baculovirus expression system (ABT, Cambridge MA) was performed. As shown in Fig. 1B, binding of the V3 mAb with the human T cell proteins was blocked with 10 μg/ml of rgp120, consistent with previous studies of human brain proteins (Trujillo *et al.,* 1993). Similarly, 10 μg/ml of rgp120 inhibited binding of V3 mAb to gp120 in the viral lysate (data not shown). As an internal control, 10 μg of bovine albumin/ml was tested for nonspecific inhibition. V3 mAb was not blocked by this protein. These experiments were repeated with lysates of other $CD4^+$ T lymphocyte cell lines including H9; Jurkat; Hut78; Molt3; and Molt4 (American Type Culture Collection, Rockville, MD). All yielded identical results (data not shown).

To determine whether V3 mAb would react with normal human $CD4^+$ T lymphocytes, as well as T-cell lines, PBL from a healthy HIV-1-negative blood donor were purified with Lymphocyte Separation Medium (LSM) (Organon Teknika, Durham, NC). The cells were enriched and activated with PHA (Sigma, St. Louis MO) and a subset of $CD4^+$ cells were collected by affinity chromatography column (Biolex Lab Inc., Canada). As shown in Fig. 1B (left), the V3 mAb reacted with the same 35- and 110-kDa proteins in both the $CD4^+$ and the SupT1 lymphocytes. Initially, the $CD4^+$ lymphocytes appeared to display only one protein of 48 kDa recognized by the V3 mAb. However, additional experiments with $CD4^+$ lymphocytes when the gel was run for a longer time revealed two proteins of 45 and 50 kDa, as was seen on SupT1 lymphocytes. As an Ig isotype-negative control, a mouse mAb that was upstream from the V3 loop (amino acids 476 to 486) corresponding to the C5 region of gp120 was used. It did not recognize any T cell proteins, but did recognize the HIV-1 gp120, confirming V3 loop specificity (Fig. 1B, center). In data not shown, 10 μg/ml of rgp120 inhibited binding of C5 mAb to gp120 of the cell lysate. Also confirming the specificity of the V3 loop was the observation that binding between the human T cell proteins and V3 mAb was completely blocked with 10 μg of rgp120 (Fig. 1B, right).

Increased expression of V3-like proteins in activated T cells

Interestingly, we found that V3 mAb reacted more than 10-fold more (measured by densitometry) with proteins isolated from a tumor T cell line (SupT1) than with proteins from normal human $CD4^+$ T lymphocytes, despite standardization of the protein extracts

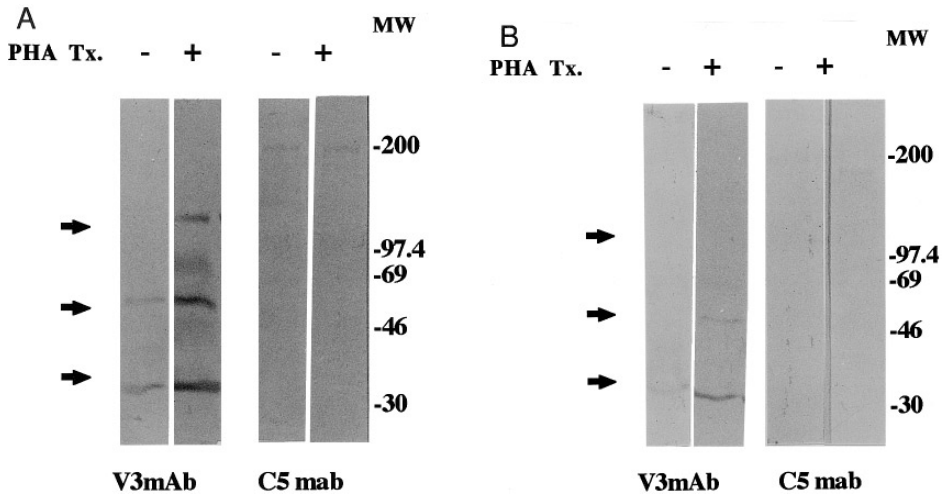

FIG. 2. Western blot and flow cytometry analysis of normal $CD4^+$ T lymphocytes before and after PHA activation. (A) Cell lysates of activated and nonactivated $CD4^+$ T lymphocytes were probed with V3 mAb. Arrows indicate proteins of 35, 50, and 110 kDa. An HIV-1 gp120 475–486 (C5) mAb was used as a negative control. (B) $CD8^+$ T cells were purified, activated, fractionated, and analyzed by WB. The binding of V3 mAb to $CD8^+$ T cell proteins remained minimal despite PHA activation.

(Bio-Rad Protein Assay, Melville, NY). This may be because tumor cell lines have high mitotic activity, while lymphocytes *in vivo* are quiescent (Zack, 1995). We then determined if activation of human T cells altered the expression of the V3-like proteins. PBL from a normal blood donor were obtained and divided into two aliquots. One served as a control and the other was activated with PHA for 24 h. $CD4^+$ T cells were obtained by an affinity chromatography column and cell lysates from control and activated cells were fractionated by SDS–PAGE and analyzed by WB. As shown in Fig. 2A, the binding of V3 mAb to human T cell proteins was increased 10-fold as measured by densitometry, 24 h postactivation. As a negative isotype antibody control, C5 mAb was also tested. Both control and PHA-activated $CD4^+$ T cells yielded negative results. In addition, a subset of $CD8^+$ T lymphocytes were similarly analyzed. The $CD8^+$ T cells were purified and activated, and the proteins fractionated and analyzed by WB. The binding of V3 mAb to $CD8^+$ T cell proteins remained minimal despite PHA activation (Fig. 2B).

To confirm the upregulation of the V3-like proteins by activation, $CD4^+$ T cells were combined with fluorescent V3 mAb and analyzed with flow cytometry. As shown in the top panel of Fig. 3, low V3 mAb binding was seen in nonactivated cells. Importantly, after 24 h activation with PHA, T cells expressed a dramatic increased in V3-like proteins. When the C5 mAb antibody was used as an isotype IgG-negative control, fluorescence was low and a negligible increase was noted after activation (Fig. 3, bottom panel). These data confirm that activation is specific and causes only expression of more V3-like proteins on $CD4^+$ T cells.

Immunostaining of uninfected T cells by HIV-1 gp120 V3 antibodies

To determine the anatomic distribution of the T cell V3 epitope, SupT1 lymphocyte cells were immunostained with fluorescent V3 mAb and analyzed by laser confocal microscopy. Imaging of the immunolabeled cells revealed V3-like epitopes in the cytoplasmic region and on cell surfaces (Fig. 4A). When unfixed $CD4^+$ SupT1 cells were labeled with V3 mAb without prior acetone treatment, the immunolabeled V3 mAb was primarily seen on the cell surface (Fig. 4B). Ig isotype (C5 mAb), used as a control, produced no significant binding (Fig. 4C). SupT1 cells incubated with anti-mouse FITC secondary antibody were also not labeled (data not shown). We then analyzed one sample from seven AIDS patients which had reacted with V3-like proteins (Trujillo *et al.,* 1996a). Uninfected T cells incubated with serum from a HIV-1-positive patient revealed cell surface and cytoplasmic distribution of antigen (Fig. 4C). As a negative control, serum from an HIV-1-negative individual was used; there was no significant binding (data not shown). V3 mAb and serum antibodies from the HIV-1-positive patient stained similar regions of the T cells (Figs. 4A and 4D).

Killing of T cells by V3 antibody binding and complement

These results indicate that the V3 loop of HIV-1 gp120 shares epitopes with T cells. We suggest that an immune response to the V3 loop in HIV-1-positive patients could generate cross-reactive antibodies that bind to $CD4^+$ T cells. This interaction plus activation of complement could kill infected or uninfected $CD4^+$ T cells. To determine if binding of the V3 mAb to T cell proteins could

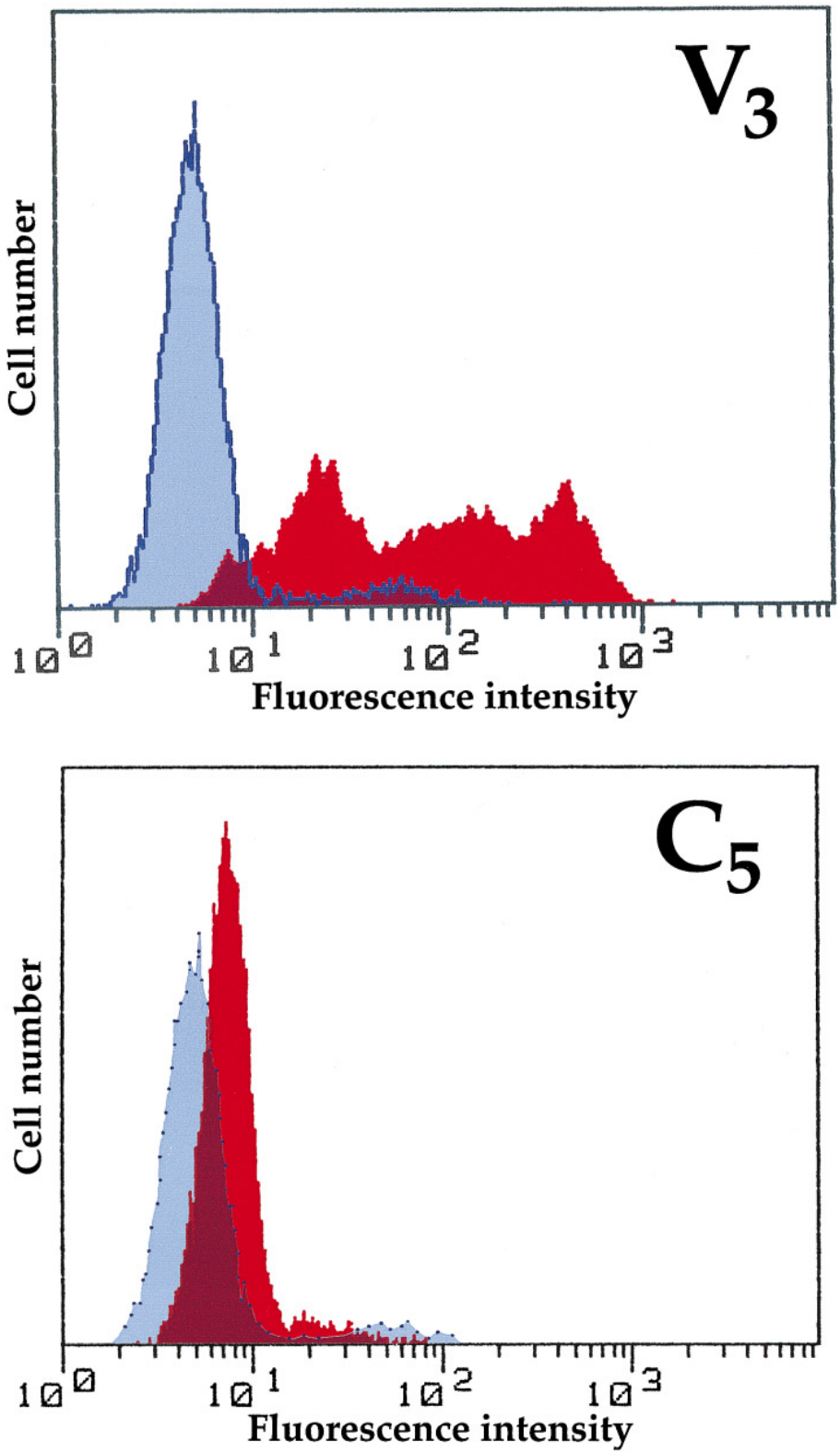

FIG. 3. Flow cytometric analysis of V3 mAb binding to the surface of normal $CD4^+$ T lymphocytes before and after PHA activation (top). The blue color indicates the nonactivated $CD4^+$ T lymphocytes, which shows minimal binding of the V3 mAb to the cell surface. As shown by the red color, 24 h after PHA treatment a significant shift to the right was noted indicating much greater V3 mAb binding. When the C5 mAb antibody was used as an isotype IgG-negative control (bottom), fluorescence was low and minimal increase was noted after PHA activation.

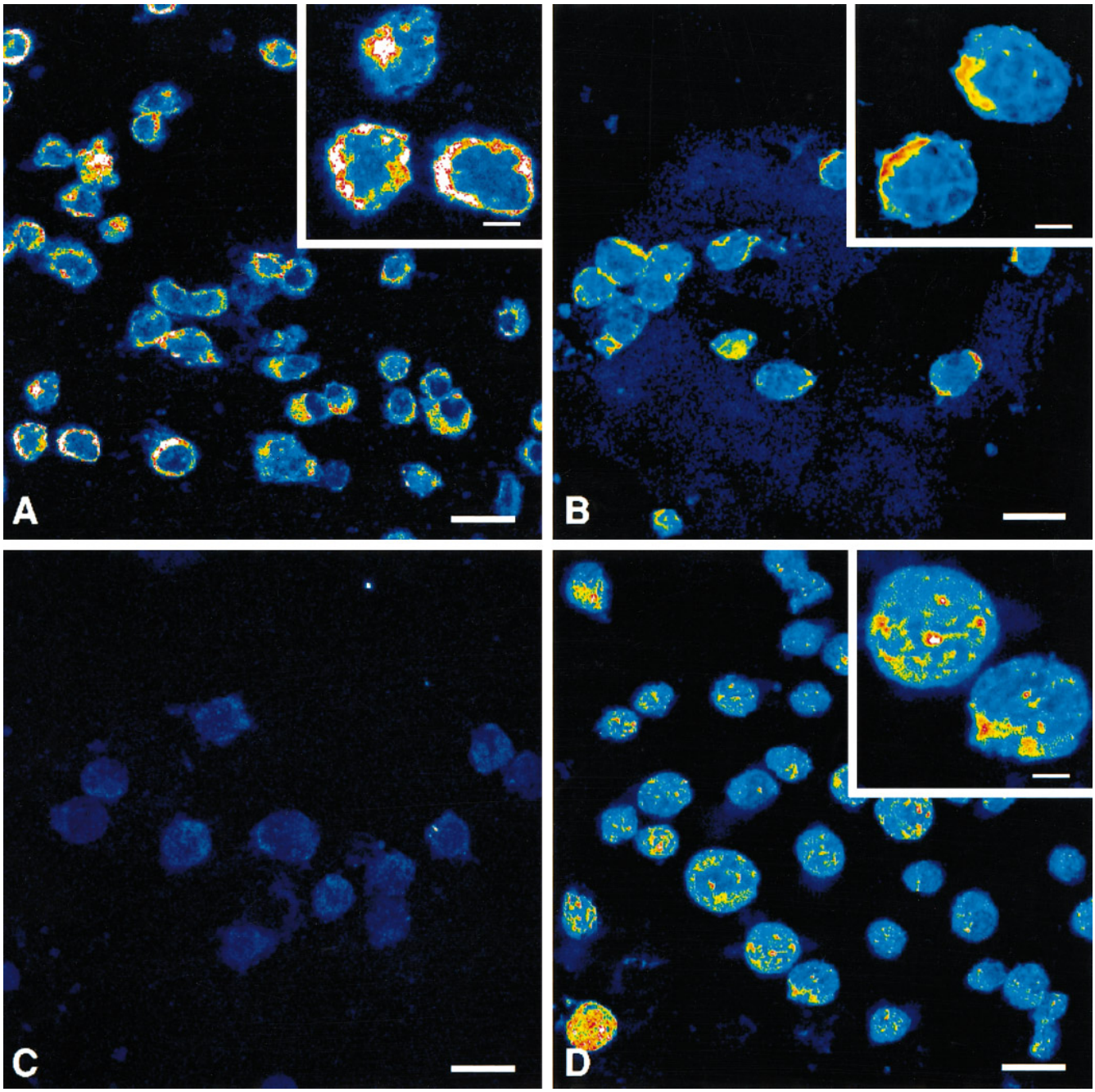

FIG. 4. CD4$^+$ SupT1 T lymphocytes immunostained using mouse monoclonal antibodies against V3, isotype Ig control, C5, and sera from HIV-1-positive and negative individuals. (A) Confocal micrographs of immunolabeled cells with V3 mAb reveal V3 fluorescence in the cytoplasmic region and on cell surfaces. (B) Confocal micrographs of immunolabeled living cells without acetone fixation reveal that V3 is located primarily on cell surfaces. (C) When used as an IgG isotype control, C5 mAb produced no labeling. (D) Cells incubated with HIV-1+ serum reveal a surface and cytoplasmic distribution of the V3 antibody. Bar, 20 μm. Inset bars, 5 μm.

elicit T cell killing, we studied CD4$^+$ SupT1 cells in a complement-dependent cytotoxic assay. As shown in Fig. 5A, V3 mAb plus complement killed 70% of the CD4$^+$ SupT1 cells. When serum from an HIV-1-infected patient containing antibodies which recognized the V3-like proteins in a WB analysis was used, 70% killing of uninfected T cells was also observed. As a positive antibody control, anti-CD4 monoclonal antibodies, OKT4A (Ortho Diagnostic Inc., Raritan, NJ), were used with complement and 60% of CD4$^+$ cells were killed. As an isotype antibody-negative control, we used a C5 mAb plus complement. Only 10% killing was observed; when V3 mAb, C5 mAb, or complement alone was used we saw a background of 10% killing of CD4$^+$ T cells. Similarly, sera of an

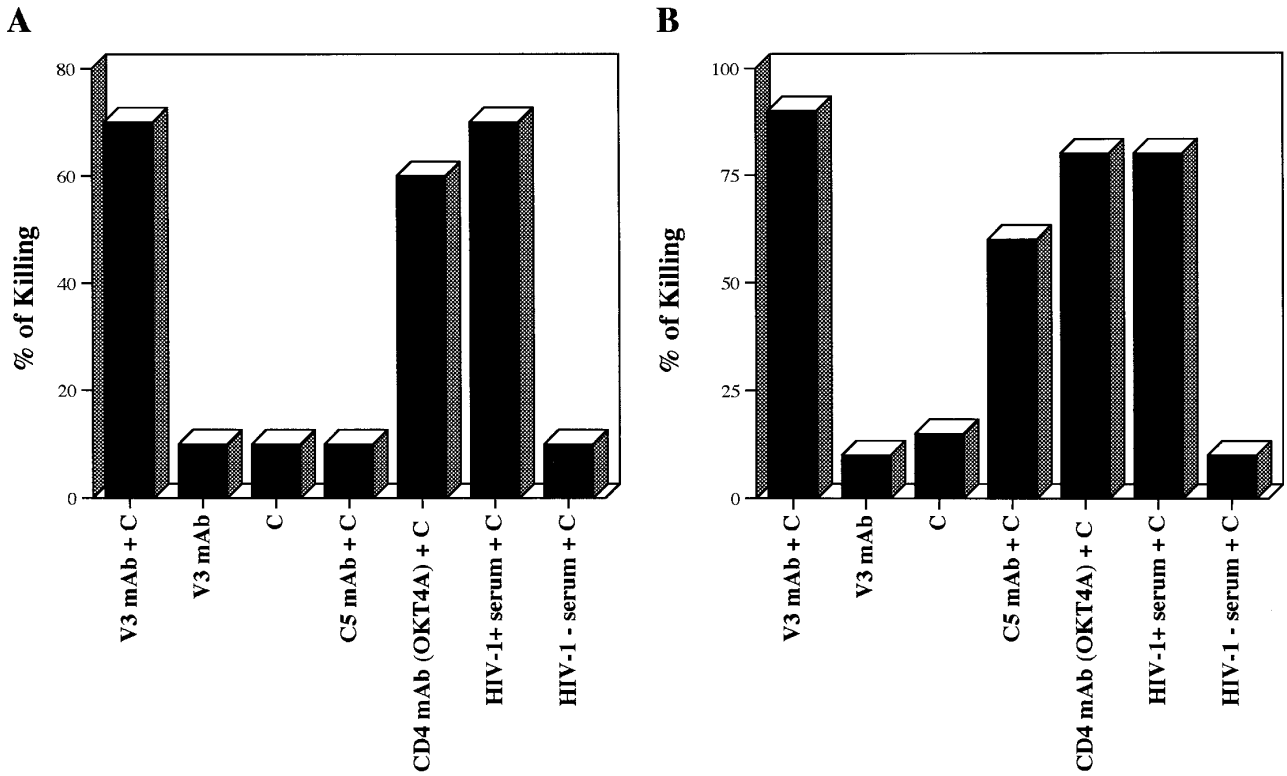

FIG. 5. Killing of $CD4^+$ SupT1 T lymphocytes by V3 mAb binding and guinea pig complement. (A) $CD4^+$ SupT1 T lymphocytes were incubated with V3 mAb and resuspended in guinea pig serum complement. These experiments were repeated with V3 mAb alone, complement alone, and C5 mAb plus complement. As a positive control, a CD4 monoclonal antibody, $OKT4_A$, was used. Serum from an HIV-1-positive patient that recognized similar proteins in WB analysis was analyzed as well as serum from a seronegative person. (B) The $CD4^+$ SupT1 T lymphocytes were infected by $HIV\text{-}1_{IIIb}$, and a complement lysis experiment was performed as indicated above.

HIV-1-seronegative individual showed negative results when repeated twice.

Following HIV-1 infection, viral envelope proteins can become incorporated into T cell membranes (Robey *et al.,* 1985). Since this could enhance T cell killing by V3 mAb, we repeated the same experiment after infecting the $CD4^+$ SupT1 cells (Trujillo *et al.,* 1996b) with $HIV\text{-}1_{IIIb}$ strain (Fig. 5B). Now V3 mAb plus complement induced 90% killing of $CD4^+$ T cells. This increase of $CD4^+$ killing by V3 mAb may reflect HIV-1 V3 epitopes on T cell membranes following incorporation of HIV-1 envelope. This explanation is supported by the fact that the C5 mAb, which recognizes the amino acids 476 to 486 of gp120, now induced 60% $CD4^+$ T cell killing (Fig. 5B). When V3 mAb without complement or when complement only was used, 10 to 15% killing was observed. Serum from an HIV-1-infected patient and CD4 mAb induced approximately 80% killing of $CD4^+$ T cells. Again, sera of an HIV-1-seronegative individual showed only 10% killing. This experiment was repeated twice with similar results. In addition, when fresh activated $CD4^+$ T cells were used with V3 mAb plus complement, approximately 60% killing was observed (data not shown).

To exclude the possibility of human T cell killing that might reflect the use of guinea pig complement, the same complement-dependent cytotoxic assay was performed in the presence of human complement. As shown in Figs. 6A and 6B, V3 mAb plus human complement killed approximately 65 and 70% of uninfected and HIV-1-infected T cells, respectively. To further determine whether this reactivity was specific to complement, inactivation of human complement for 1 h at 55°C was performed. The V3 mAb in the presence of inactivated human complement did not induced killing (Fig. 6A). To determine whether the killing of T cells by V3 mAb plus human complement was specific to the V3 loop, a blocking experiment using HIV-1 V3 peptide was performed. As shown in Figs. 6A and 6B, the cytotoxic effect of the V3 mAb on the T-cells was blocked by 10 μg/ml of V3 peptide.

More significantly, we wanted to exclude the possibility that human HIV-1 antibodies may be directed against other proteins besides the V3-like peptides. Thus we again conducted a blocking experiment using V3 peptide. As shown in Fig. 6A, there was an over 70% reduction of killing of uninfected T cells. Similarly, in Fig. 5B, 50% reduction of killing of HIV-1-infected T cells was observed. When V3 mAb, C5 mAb, or complement alone

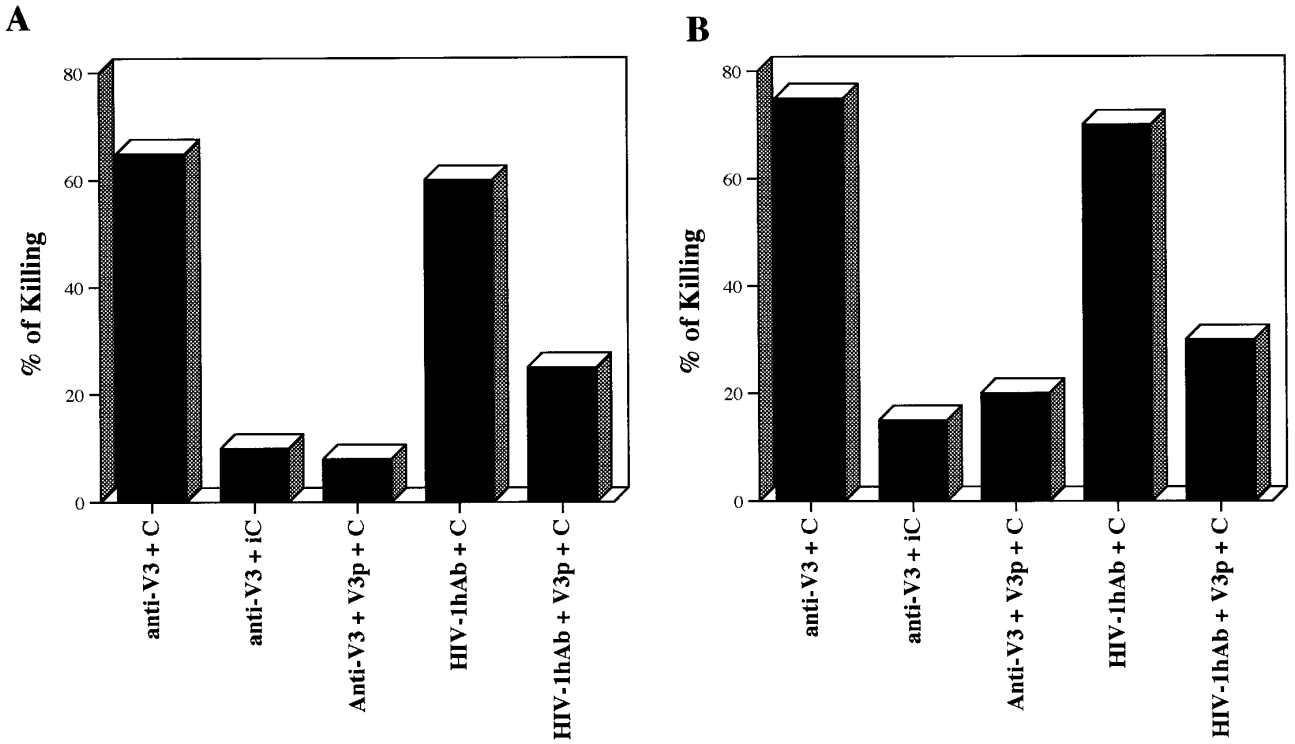

FIG. 6. Killing of $CD4^+$ SupT1 T lymphocytes by V3 mAb binding and human complement. (A) $CD4^+$ SupT1 T lymphocytes were incubated with V3 mAb and resuspended in human complement. As a negative control, the human complement was inactivated for 1 h at 55°C. Binding between V3 mAb as well as human HIV-1 antibodies with $CD4^+$ SupT1 T lymphocytes was blocked with a V3 peptide. These experiments were repeated with V3 mAb alone, complement alone, or with serum from a seronegative person. (B) The $CD4^+$ SupT1 T lymphocytes were infected by $HIV\text{-}1_{IIIb}$, and a complement lysis experiment was performed as indicated above.

was used we observed a background of approximately 10% killing of both uninfected and HIV-1-infected T cells (data not shown). Similarly, sera of an HIV-1-seronegative individual showed only 10% killing (data not shown).

DISCUSSION

These experiments demonstrate that the V3 loop of HIV-1 gp120 shares epitopes with two prominent T cell proteins of approximately 35 and 110 kDa. Other less prominent T cell proteins that reacted included one of 45 and one of 50 kDa. Importantly, T cell protein homology appears to be specific to HIV-1 gp120 since these interactions can be blocked by rgp120 and V3 peptides. Use of antibodies against the V3 loop and C5 region further suggest that T cell binding specificity is confined to the V3 loop. Activation of normal $CD4^+$ T lymphocytes by PHA upregulates these V3-like epitopes. We also showed that V3 mAb plus complement induced killing of $CD4^+$ T lymphocytes in an *in vitro* assay. Collectively, these results suggest that HIV-1 V3 loop epitopes shared with T cell proteins may contribute to the depletion of T cells in AIDS.

The V3 loop has been a focus for AIDS research (Foley and Korber, 1995). It is formed by a disulfide bridge between Cys residues 301 and 336. Although most of the amino acids of the V3 loop are variable among different strains of HIV-1, a Gly-Pro-Gly-Arg sequence at the tip of the loop is highly conserved among isolates derived from Europe and North America (Foley and Korber, 1995). Several studies have also demonstrated that the principal neutralizing domain (PND) of HIV-1 lies within the V3 loop (LaRosa *et al.,* 1990; Javaherian *et al.,* 1990). This highly conserved tip also forms a binding site for antibodies that can inhibit fusion of HIV-1 with cells and thus block HIV-1 infection. The V3 loop has been identified as a fusion domain (Freed *et al.,* 1992) and an anti-V3 antibody can by itself prevent *in vitro* infection (Skinner *et al.,* 1988). Furthermore, an anti-V3 antibody was able to convey protection in two chimpanzees given HIV-1 (Emini *et al.,* 1992).

Our findings suggest that autoimmunity may contribute to T cell depletion in AIDS patients. But if this mechanism exists, why don't HIV-1 asymptomatic individuals with anti-V3 antibodies inevitably have $CD4^+$ T cell depletion? Perhaps, the extent of T cell killing depends on how activated the T cells are and the nature of the antibody. It has been shown that sera of AIDS patients contained autoantibodies that reacted with stimulated

$CD4^+$ T cells (Stricker *et al.,* 1987). We observed upregulation of V3-like epitopes in activated $CD4^+$ T lymphocytes. Nonactivated $CD4^+$ T lymphocytes only expressed small amounts of the V3-like proteins. Then, the binding of cross-reactive antibodies to $CD4^+$ T cells may be minimal and have no pathologic consequences. Similarly, we observed minimal binding between the V3 mAb and $CD8^+$ T lymphocytes, also consistent with AIDS pathogenesis. However, any mitogenic stimulus, such as concomitant viral or bacterial infections, may activate $CD4^+$ T cells. This activation, characteristic of late stages of HIV-1 infection, may lead to upregulation of V3-like epitopes. Then, it becomes more likely that cross-reactive V3 antibodies target $CD4^+$ T cells and induce killing. These findings may also be relevant to cell-mediated immunity. The V3 loop is a main epitope for cytotoxic T lymphocyte (CTL) responses (Takahashi *et al.,* 1992). Therefore, activated T cells may also become targets for killing by CTL. This V3 loop cross-reactivity with human cells is not unique to $CD4^+$ T cells. Indeed our first observation of molecular mimicry was made in human central nervous system cells, especially neurons (Trujillo *et al.,* 1993, 1996a).

Our data demonstrating molecular mimicry between the V3 loop and $CD4^+$ T cells suggest that the presence of neutralizing V3 antibodies may enhance the progression to AIDS by contributing to elimination of $CD4^+$ T cells. Consistent with this hypothesis is the observation that neutralizing antibodies are present in symptomatic stages of AIDS-related complex and AIDS (Weiss *et al.,* 1985; Boucher *et al.,* 1989).

These findings have two implications. First, cells which have V3-like epitopes such as neurons and T cells may be susceptible to cell damage and/or killing when anti-V3 antibodies are elicited. This phenomenon is probably proportional to the extent of their own activation. Second, these observations may have consequences for selecting vaccination strategies. The V3 loop is the principal neutralizing domain of HIV-1 and anti-V3 antibody may prevent HIV-1 infection (Emini *et al.,* 1992; Javaherian *et al.,* 1990; Skinner *et al.,* 1988). However, our finding of cross-reactivity between V3 mAb and human T cell proteins suggests that some V3 antibodies might also induce autoimmune disease. Envelope vaccine of equine infectious anemia virus caused autoimmune side effects (Wei *et al.,* 1995) and immunotherapy with V3 mAb in humans has generated autoimmune side effects (G. Bratt *et al.,* 1991, abstract 2147, presented at VIIth Int. Conf. AIDS, Florence Italy; J. Hinkula *et al.,* 1993, abstract PO-A28-0674, presented at IXth Int. Conf. AIDS, Berlin Germany). Furthermore, Hinkula *et al.,* (1994) have shown decreased $CD4^+$ T cell counts in some patients receiving passive immunotherapy with V3 mAb. But genetic modifications of HIV-1 vaccines that minimize mimicry epitopes, while preserving others which are useful, should lead to more appropriate HIV-1 vaccines.

In summary, our results indicate that the V3 loop of HIV-1 gp120 shares common epitopes with proteins on human T cell surfaces. Both V3 mAb and sera of AIDS patients induced killing of uninfected $CD4^+$ T cells. An immune response against an immunodominant viral epitope (such as the V3 loop) may cross-react with surface components of normal T cells. This may be a mechanism which contributes to $CD4^+$ T depletion in AIDS. further identification of V3-related T cell proteins will give better insight into the pathogenesis of AIDS and lead to optimal treatments and vaccines.

MATERIALS AND METHODS

Antibodies

Several monoclonal and polyclonal anti-HIV-1 antibodies were used, including polyclonal rabbit anti-HIV-1 gp120 (American Bio-Technologies, Inc., Cambridge, MA); monoclonal anti-gp120 V3 loop made from amino acids 308 to 322 (mAb) (NEA-9205, NEN, Boston, MA); monoclonal anti-gp120 V3 loop made from amino acids 307 to 330 (NEA-9284, NEN); anti-gp41 (NEA-9303), anti-p24 (NEA-9306); and anti-gp120 C5 loop made from amino acids 475 to 486 (NEA-9201). We also used sera from 7 of 26 HIV-1-positive subjects which recognized the V3-like proteins (Trujillo *et al.,* 1996a). According to the Centers for Disease Control Classification of HIV infection the 7 HIV-1-positive subjects were classified as CDC IV (CDC, 1993).

Virus and cells

SupT1 cells, a $CD4^+$ T cell line, were obtained from the AIDS Research and References Reagent Program. The H9, Jurkat, Hut78, Molt3, and Molt4 $CD4^+$ T cell lines were obtained from the American Type Culture Collection. These $CD4^+$ T cell lines were maintained at 37°C in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1% antibiotic. HTLV-IIIb isolate (HIV-1_{IIIb}) was obtained from the AIDS Research and References Reagent Program. Cell-free supernatant from SupT1 cells persistently infected with HIV-1_{IIIb} was filtered through a 0.45-μm membrane and spun in an ultracentrifuge at 20,000 *g* for 2 h with 20% sucrose in phosphate-buffered saline. The virus pellet was lysed and mixed with reduced sample bufffer. The mixture was boiled for 2 min and fractionated electrophoretically on a 12.5% sodium dodecyl sulfate–polycrylamide gel (SDS–PAGE). PBLs were obtained from a normal blood donor and were separated using LSM (Organon Teknika, Durham, NC). $CD4^+$ T lymphocytes were separated by an affinity chromatography column (Biotex Laboratories Inc., Alberta, Canada).

Western blot (WB) analysis

Viral and T cell proteins were fractionated by SDS–PAGE (Laemmli, 1970). Proteins were transferred passively to a nitrocellulose membrane (0.22-mm pore size, Bio-Rad). Immunoblot analysis was performed as described previously (Barin *et al.,* 1985). In a WB analysis, lysates of a SupT1 $CD4^+$ T lymphocyte cell line, SupT1 infected with $HIV\text{-}1_{IIIb}$ (Trujillo *et al.,* 1996b), and $HIV\text{-}1_{IIIb}$ were probed with V3 mAb at a dilution of 1:500 (10 μg/ml). Cell lysates of normal $CD4^+$ T lymphocytes, SupT1 cells, and SupT1 infected with HIV-1IIIb were probed with V3 mAb at a dilution of 1:500 (10 μg/ml). A second mouse monoclonal antibody that was upstream from the V3 loop corresponding to the C5 region (amino acids 475 to 486) served as an additional control (10 μg). The immnoreactivity of V3 mAb and T cells was measured by densitometry of the nitrocellulose membrane (15-100 Digital Imaging System; Sun Bio Science, Branford, CT).

Blocking experiments

The blocking experiment used recombinant HIV-1 gp120 from a baculovirus expression system (ABT Inc., Cambridge MA); 10 μg of gp120/ml was incubated with V3 mAb at 1:500 dilution (10 μg/ml) for 1 h at 37°C. As an internal control, bovine albumin, 10 μg/ml, was tested for nonspecific inhibition. It was >95% pure as estimated by analysis of Coomassie blue-stained SDS–PAGE under reduced and nonreduced conditions. It was recognized by monoclonal and polyclonal anti-gp120 antibodies and bound CD4 in ELISA or FACS analyses.

Ten micrograms per milliliter of an HIV-1 gp120 V3 loop peptide (C-N-T-R-K-R-I-R-I-Q-R-G-P-G-R-A-F-V-T-I-G-K) was used for blocking experiments in the complement cytotoxic assay (Intracel Corp., Cambridge, MA).

T cell activation and flow cytometry analysis

Lymphocytes were incubated for 24 h at 37°C in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 1% antibiotic, plus 10 μg/ml of PHA to activate them. $CD4^+$T lymphocytes were separated by an affinity chromatography column (Biotex Laboratories Inc.). Cell lysates of activated and control $CD4^+$ T lymphocytes were probed with 10 μg/ml of V3 mAb; 10 μg/ml of C5 mAb was used as a negative control. The immunoreactivity of V3 mAb and T cells was measured by densitometry of the nitrocellulose membrane (15–100 Digital Imaging System; Sun Bio Science). For the flow cytometric analysis, PBL were obtained as mentioned previously, and cells were sorted by FACS analysis (Becton–Dickinson, San Jose, CA), which yielded 95 to 98% $CD4^+$ T lymphocytes. The cells were divided into two aliquots; one was maintained as a control and the other was activated with PHA for 24 h. $CD4^+$ T lymphocytes were incubated with 10 μg/ml V3 mAb and cells were exposed to anti-mouse IgG-FITC. The C5 mAb and FITC alone served as negative controls. Measurements were analyzed using a Becton–Dickinson software program. This program plots logarithmic fluorescent values against cell number.

Indirect immunofluorescence, microscopy, and image analysis

SupT1 cells were put on glass slides and fixed with acetone for 10 min. The slide was blocked with 5% sheep serum and 3% bovine serum albumin in PBS for 30 min at 37°C. First, antibodies V3 mAb of the isotype Ig control or the C5 mAb were applied for 30 min at 37°C. Albumin in PBS was used as a negative control. These antibodies were followed by FITC "Isomer" conjugate goat IgG fraction to mouse Ig F(ab')2(Organon Teknika Corp., Durham, NC) for 30 min at 37°C. Immunostained cells were briefly washed in PBS, then sealed in microwell chambers, and examined using a Sarastro 2000 confocal laser scanning microscope (CLSM) (Molecular Dynamics, Sunnyvale CA) fitted with a 25 mW argon-ion laser. The microscope was configured for single channel fluorescent imaging with 514 nm excitation, 535 nm primary beam splitter, 10% laser transmission, and 18 mW laser power. Images were recorded in 1024 image size format. A 60X 1.4 numeric aperture objective was used.

Complement cytotoxic assay

This assay was performed as previously described (Duerst *et al.,* 1991). The SupT1 cell pellet (6×10^6) was incubated with anti-V3 or anti-C5 loop HIV-1 monoclonal antibodies. We also used serum from an HIV-1-positive or HIV-1-negative individual (dilution 1:20) for 45 min at 4°C. Afterward the cell–antibody complex was washed twice with 1× Dulbeccos' phosphate-buffered saline (PBS) (Life Technologies Inc. Grand Island, NY) and resuspended in guinea pig serum complement (dilution 1:1) (Sigma Chemical Co., St. Louis, MO) or human complement (from a healthy HIV-1-negative blood donor) (for 20 min at 4°C. Then, the cell–antibody–complement complex was incubated for 30 min at 37°C. Finally, the complex was washed twice in 1× PBS and the cytotoxicity index was determined by trypan blue exclusion (Life Technologies Inc.).

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