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The Effect of Deletion of the V3 Loop of gp120 on Cytotoxic T Cell Responses and HIV gp120-Mediated Pathogenesis

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New strategies for improving the efficacy of HIV vaccines are of significant importance. In this study, we analyzed the effect of deletion of the hypervariable V3 loop of gp120 on envelope (env)-specific CTL responses in PBMC of HIV-infected individuals. We showed increased CTL activities against conserved epitopes of the env glycoprotein in cultures induced with the ∆V3 mutant compared with those stimulated with the full-length env gene products. In contrast to the wild-type env, the ∆V3 mutant-expressing cells were resistant to Ab-dependent cell-mediated cytotoxicity, formed no syncytia, and neither underwent nor induced apoptosis in CD4+ cells. Thus, the ∆V3 mutant may redirect immune responses toward conserved epitopes of gp160, has longer expression time due to increased resistance to Ab-dependent cell-mediated cytotoxicity, and does not trigger cytotoxic effects associated with apoptosis and syncytium formation. This approach may apply to other Ags of HIV, where deletions of highly variable or immunosuppressive epitopes may improve the efficacy of HIV vaccines. The Journal of Immunology, 1998, 160: 5676–5683.

Dear editor,

I am interested in the ongoing debate regarding the role of CTL responses in the control of HIV infection. While some studies have suggested that CTL responses play a central role in the control of HIV replication and in determining disease progression (1, 2), others have shown that strong gp160-specific CTL responses have a rapid reduction of acute viremia and antigenemia, and high levels of CTL activity have been associated with long-term survival (3, 4). Furthermore, definite CTL responses have been observed in Gambian sex workers who remain seronegative (5) and in an HIV-exposed, but uninfected, infant (6). However, natural immunity or that induced by immunization with attenuated pathogens or whole proteins with multiple immunogenic sites may not always result in the desired effect. For example, the unequivocal demonstration of CTL escape mutations arising specifically within regions of the virus encoding immunodominant CTL epitopes has been suggested to contribute to viral spread and the inability of anti-HIV immunity to prevent the onset of AIDS (7–9). Also, the secondary responses induced by the mutated epitopes in vivo are likely to drive chronic immune activation, leading to accelerated exhaustion of the immune system’s regenerative capacity (10). The presence of cell-free and cell-associated env glycoprotein may contribute to deletion of bystander CD4+ cells by apoptosis or syncytium formation (11–14). These effects could potentially be avoided if it were possible to direct immunity to selected regions of HIV proteins that are highly conserved and delete regions that may actually promote the spread of infection in vivo.

Among the variable regions of gp120, the V3 loop induces both humoral and cellular responses against HIV, but also undergoes frequent mutations and plays a central role in the env-CD4 interactions that are detrimental to the host (11, 15, 16). In this study, we investigated the effect of the V3 loop deletion on env-specific CTL responses, ADCC-mediated lysis of env-expressing cells, syncytium formation, and apoptosis. We showed that stimulation of PBMC derived from HIV-infected individuals with the V3 loop-deleted env gene products improves env-specific CTL responses without triggering effects that are cytotoxic to CD4+ T cells.

Materials and Methods

Patients

PBMC were obtained from heparinized blood of HIV-infected adults followed in the Clinical Office of the Division of Infectious Diseases at Thomas Jefferson University Hospital (Philadelphia, PA). PBMC of HIV-seronegative donors were obtained from buffy coats (NABI, Miami, FL). The HIV-infected individuals were at early stages of disease, with CD4+ T cell counts ranging from 300 to 600 cells/mm³. All patients were undergoing antiretroviral therapy with a protease inhibitor (Crixivan, Merck Research Laboratories West Point, PA) in combination with nucleoside reverse transcriptase inhibitors.

All HIV-infected patients expressed HLA-A2 Ag determined by the standard microlymphocytotoxicity assay (One Lambda, Canoga Park, CA; Biotest Diagnostics, Denville, NJ) according to the manufacturer’s directions.

Recombinant vaccinia viruses (vv)

The HIV-1IIIB isolate was the source of the full-length env gene and the ∆V3 loop mutant cloned in the pSCL1-based vector under the control of a

6 Abbreviations used in this paper: env, human immunodeficiency virus envelope glycoprotein; ADCC, antibody-dependent cell-mediated cytotoxicity; vv, vaccinia virus; LCL, lymphoblastoid cell line; vac, vaccinia; CTLp, cytotoxic T lymphocyte precursors; HmAb, human monoclonal antibody; MmAb, mouse monoclonal antibody; sCD4, soluble CD4; PI, propidium iodide.

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Synthetic early/late vv promoter (17). The vv-DV3 env mutant with the Δp24-to deletion (16) was constructed by ligation of fragments obtained by PCR amplification from the pSVIII-env plasmid (a gift from Dr. J. Sodroski, Dana-Farber Cancer Institute, Boston, MA). One fragment was generated by PCR with the synthetic oligonucleotide containing the Sall site and the CCACC Kozak's sequence in front of the ATG codon (5'-AGGAGAATGAGGAGGATGAGGTA3'; antisense) and with the oligonucleotide (5'-ACAGTGACCCCTAAATAGACTGGTC-3'; sense) containing the KpnI side. The second fragment was derived from KpnI and BamHI digests of the pSVIII-env plasmid, and the third fragment was generated by PCR with the synthetic oligonucleotide containing the BamHI site at its 5' end (5'-AAGGATCTGACATATGCTG-3'; sense) and the antisense primer (5'-TTCGGCGGCGCTTATAGCA AAATCTCTTCC-3'; antisense) containing the Taa stop codon followed by the NotI site. The three fragments were ligated into the Sall and NotI sites of the pSCh1-based vector (a gift from Dr. L. Eisenlohr, Thomas Jefferson University) to generate plasmid pSCh-DV3. A similar approach was used to generate plasmid with the wild-type env gene (pSCh-WTP) using recombinant plasmid pFII (15) provided by Dr. B. Cullen (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC). Plasmids pSCh-DV3 and pSCh-WTP were used to generate recombinant vv-DV3 and vv-WTP clones by homologous recombination as previously described (17).

Synthetic peptides

The env peptides D1 (KLTPLCVTL, amino acids 120–128), D2 (LLNAAVTL, amino acids 131–138), and D3 (G3-523, amino acids 567–574) have been previously described (18, 19). They were synthesized using standard F-moc methodology (20), purified by reverse phase HPLC, and characterized by amino acid analysis and laser desorption mass spectroscopy at the Tiswort Institute (Philadelphia, PA).

Target cell lines

Autologous B lymphoblastoid cell lines (LCLs) were established for use as target cells by incubation of PBMC with supernatant from the EBV-producing murine cell line B95.8 (American Type Culture Collection, Rockville, MD) as previously described and Jurkat cell line transfected with the gene for HLA-A2, here designated JAU2 (21), was obtained from Dr. Linda Sherman (The Scripps Research Institute, La Jolla, CA).

Analysis of env-specific CTL responses in vitro

PBMC isolated by centrifugation over Ficoll-Hypaque (density, 1.077 g/cm³) were cryopreserved at different time points during the study. Cryopreserved PBMC were thawed and expanded in vitro by Ag-specific or anti-CD3 mAb stimulation as previously described (2, 18, 23). Briefly, cells were adjusted to a concentration of 3 × 10⁶ cells/ml and cultured in flat-bottom wells of 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ) in RPMI 1640 medium supplemented with 10% FCS along with recombinant human interleukin-2 (50 U/ml). Approximately 2 wk later, cultures were tested for cytotoxicity against JAU2 cells infected with various peptides expressing the env gene products or coated with the env-specific D1 peptide.

Cytotoxicity assays

Standard ¹¹¹I Cr release assays were performed as previously described (24). Briefly, JAU2 cells were infected overnight at a multiplicity of infection of 100 for the syncytium assay (vac) alone or with vv expressing either the wild-type or the ΔV3 loop env gene products and labeled with Na₂¹¹¹I CCl₄O₂ (DuPont-New England Nuclear, Boston, MA), or were coated with the 51 peptide (1 μM) for 1 h during ¹¹¹I Cr labeling. After washings, 10⁵ target cells were combined with in vitro stimulated T cell lines established from each patient. After 4 h, supernatants were harvested, and radioactivity was measured in a 1470 Wizard gamma counter (Wallac, Gaithersburg, MD). Spontaneous Cr release was always <15% of the maximum release. Specific lysis was calculated as: 100 × (experimental release – spontaneous release)/maximum release – spontaneous release). The percent HIV-specific lysis was calculated by subtracting the percent specific lysis against vac-infected target cells from the percent specific lysis against vv-env-infected target cells.

Limiting dilution assay of CTL precursors

Precursor frequencies of D1 peptide-specific CTL were determined using limiting dilution analysis (2, 24). Briefly, cryopreserved PBMC were thawed and diluted from 18,000 to 667 cells/well in 24 replicate wells of 96-well microtiter plates. Stimulator cells were autologous LCLs infected with vv-DV3 or vv-WTP and subsequently inactivated with 0.5% paraformaldehyde on ice. Fixed stimulator cells (10⁶) and autologous irradiated PBMC (10⁷; 6000 rad) were added to each well, and the plates were incubated at 37°C for 10 days in medium with rIL-2 (50 U/ml). Wells were then split and assayed for cytotoxicity on ¹¹¹I Cr-labeled JAU2 cells (5 × 10⁴) coated with the D1 peptide at a concentration of 1 μM as well as control target cells without peptide. The fraction of nonresponding wells was defined as the number of wells in which ¹¹¹I Cr release did not exceed 10% specific lysis. The CTLp frequency and 95% confidence limits were calculated using the maximum likelihood method (24, 25). CTLp frequencies were expressed as the number of CTLp per 10⁶ PBMC. The D1 peptide-specific CTLp frequencies were computed as differences between CTLp frequencies determined on D1 peptide-coated target vs control targets.

Antibodies

MAbs against the HIV env glycoprotein were comprised of F105, a human mAb (HmAb) directed against an epitope composed of four discontinuous regions of gp120 that partially overlap the binding site for the CD4 receptor (26); G3-523, a mouse mAb (MmAb) directed against an epitope within the V3 region (27); 697-D, a MmAb directed against an epitope located within the C-terminal domain of gp120 (28); 50-69II, a HmAb directed against a linear epitope (GRAF) located within the V3 loop (28); 697-D, a HmAb that recognizes an epitope within the V2 region of gp120 (28); and 50-69II, HmAb against epitope within the gp41 region of the env glycoprotein (28). HmAb F105 was provided by Dr. M. Posner (New England Deaconess Hospital, Boston, MA). MmAb G3-523 was provided by Dr. M. Fung (Tanox Biosystems, Houston, TX), and HmAb 697-D, 694/98-D, 670-D, and 50-69II were gifts from Drs. S. Zolla-Pazner and M. Gorny (Veterans Affairs Medical Center, New York, NY). Polyclonal Ig from sera of HIV-infected donors was obtained by purification over protein G column (Pharmacia Biotech, Piscataway, NJ).

Immunoprecipitation

Immunoprecipitation of env glycoproteins from vv-DV3- and vv-WTP-infected cells was conducted using a Nonidet P-40 buffer (0.5% Nonidet P-40, 0.5 M NaCl, and 10 mM Tris-HCl, pH 7.5) to lyse cells after overnight labeling with 60 μCi/ml of [¹³S] cysteine and [³⁵S] methionine (DuPont-New England Nuclear) in cysteine- and methionine-free DMEM. Radiolabeled cell lysates were precipitated with a mixture of sera from HIV-infected individuals followed by polyclonal rabbit anti-human Ig (Organon Teknika, West Chester, PA) and protein A-Sepharose CL-4B (Pharmacia Biotech) followed by Protein A-Sepharose CL-4B (Pharmacia Biotech). The precipitates were washed with Nonidet P-40 buffer and analyzed on 8% SDS-polyacrylamide gels.

Indirect flow cytometry

JAU2 cells infected for 12 h with vv-DV3 or vv-WTP were incubated for 30 min with a mixture of sera from HIV-infected patients or G3-523 mAb (1 μg/ml) in PBS buffer containing 2% FCS and 0.1% sodium azide (PBS/FS). After washing in PBS/FS, the cells were stained with a 1/40 dilution of FITC-conjugated F(ab')₂ goat anti-human or anti-mouse Ig (Organon Teknika Co., West Chester, PA) fixed with 1% paraformaldehyde, and analyzed on a Coulter Cyanfluorograf System, PROFILE II. To measured the binding of soluble CD4 (scCD4) to the cellular gp120, scCD4 (1 μg/ml; DuPont, Wilmington, DE) was incubated with vv-DV3, vv-WTP, or vacc-infected LCL at 4°C; washed; and stained with OKT4 mAb (provided by Dr. Giorgio Trinchieri, The Wistar Institute, Philadelphia, PA) followed by FITC-conjugated second antibody (29).

ADCC

Target cells consisted of JAU2 cells infected with vac, vv-DV3, or vv-WTP and ¹¹¹I Cr labeled. PBMC from HIV-seronegative donors were used as effector cells (ET cell ratio of 50:1) in the presence of env-specific Abs. The percent specific lysis was calculated according to the formula [(cpm experimental – cpm spontaneous release)/(cpm maximum – cpm spontaneous release)] × 100. ADCC specific lysis was calculated by subtracting the background reactivity with the vac-infected target only from the percent cytotoxicity of the vv-DV3- or vv-WTP-infected target cells. All experimental conditions were run in triplicate, with SD ≤ 5%.

Syncytium-forming assay and induction of apoptosis

For the syncytium-forming assay, SupT-1 cells were infected with vac, vv-WTP, and vv-DV3. Control cultures included uninfected cells and cells chronically infected with the HIV-IIIIB isolate plated alone or mixed at a 1:1 ratio. The number of giant cells in each well was determined under an inverted microscope at different time points after infection.
Induction of apoptosis was analyzed during infection of SupT-1 cells with vac, vv-ΔV3, and vv-WTP env glycoproteins as well as in uninfected CD4+ T lymphocytes stimulated with anti-CD3 mAb in the presence of THP-1 cells expressing the env glycoproteins (30). IL-2-dependent CD4+ T cell lines were established by repeated stimulation of CD4+-enriched PBL derived from HIV-seronegative donors with anti-CD3 mAb (100 ng/ml) in the presence of irradiated APC. THP-1, a monocytic cell line (American Cell Type Culture Collection, Rockville, MD), was infected with vac, vv-ΔV3, or vv-WTP for 12 h and fixed with 0.5% paraformaldehyde. For activation-induced apoptosis, IL-2-dependent CD4+ T cell blasts (5 × 10^6 cells/ml) were preincubated with vv-env-expressing THP-1 cells (10^6 cells/ml) for 2 h before stimulation with 100 ng/ml anti-CD3 mAb for 72 h (30).

Detection of apoptosis-associated chromatin degradation and DNA content analysis

The percentage of cells undergoing apoptosis was quantitated by a flow cytometric method for determining fragmented nuclei with propidium iodide (PI) staining and DNA gel electrophoresis. Briefly, cells were fixed with 70% ethanol for 3 h at 4°C and centrifuged at 800 g for 10 min, the precipitates were dried and resuspended in 10 mM Tris, 1 mM EDTA (pH 7.4), and 0.5% SDS and treated with 8 l of isopropanol and 100 l of 5 M sodium chloride at -20°C. After centrifugation at 27,000 g for 15 min, the precipitates were dissolved and resuspended in 10 nM Tris, 1 mM EDTA (pH 7.4), and 0.5% SDS and treated with 8 l of RNase A (5 mg/ml) at 37°C for 30 min. Electrophoresis was conducted in a 0.75% agarose gel, and fragmented DNA was visualized under UV light after staining with ethidium bromide.

Results

Expression of ΔV3 and wild-type env glycoproteins in vv-infected cells

We constructed a set of recombinant vv with complete and V3 loop-deleted env genes to examine the effect of V3 loop deletion on cellular responses in PBMC of HIV-infected individuals. The expression of the mutated and wild-type env glycoproteins was first evaluated by immunoprecipitation of cell lysates derived from vv-infected JA2 cells using a mixture of sera from HIV-infected individuals. Figure 1 shows that both precursor and processed forms of the wild-type and the ΔV3 mutant were precipitated from the infected cells with similar efficiencies. To estimate the level of cell surface expression of the full-length and the mutant glycoprotein, we selected two clones, designated vv-WTP-2 and vv-ΔV3, for infection of JA2 cells and immunofluorescence studies.

Staining of vv-ΔV3- or vv-WTP-2-infected JA2 cells with a mixture of sera from HIV-infected individuals followed by flow cytometric analysis revealed that both forms of env glycoproteins were expressed on the cell surface and retained the ability to be recognized by the env-specific Abs present in the sera (Fig. 2A). As expected, cells expressing the ΔV3 mutant did not bind mAb G3-523 directed against a linear epitope located within the V3 loop of gp120. However, they exhibited ~60% less efficient binding to sCD4 compared with those expressing the wild-type env glycoprotein (Fig. 2B). These results are consistent with previous findings that deletion of the V3 loop induces conformational changes in the CD4 binding region of gp120 due to a structural relationship between the V3 loop and the fourth conserved (C4) region of the gp120 glycoprotein (32).

CTL activity of PBMC stimulated with the 7ΔV3 mutant or env-encoded peptides

The env-specific CTL responses in PBMC of HIV-infected individuals were first examined in bulk cultures stimulated by a CD3-specific mAb and screened against ^51^Cr-labeled JA2 cells infected with vv-WTP-2. Responses that were >10% specific lysis in repeated experiments were considered positive. Six of 10 patients who exhibited env-specific CTL activity after polyclonal stimulation were further analyzed for responses to the full-length and mutated env gene products (Fig. 3). PBMC were cultured for approximately 2 wk with autologous LCLs expressing either the
WTP-2 or 7ΔV3 env glycoprotein and analyzed for CTL activity against vv-WTP-2-infected JA2 cells. In donors 417, 410, and 428, the respective env-specific CTL activities in cultures stimulated with the 7ΔV3 mutant were as high as 40, 61, and 55% at an E: T cell ratio of 40:1. In these cultures, the CTL activities were ~two-fold higher than those detected in cultures stimulated with the complete env gene products over a relatively broad range of E: T ratios. Responses to the 7ΔV3 mutant were also higher in patient 521, whereas in patients 450 and 540, there was <10% difference in the env-specific CTL activity between individual cultures.

The heterogeneity of the env-specific CTL responses could be related to differences in the potency of APC to induce CTL responses to variable vs conserved regions of env glycoproteins. In subsequent studies, we compared CTL activities against vv-WTP-2- and vv-7ΔV3-infected JA2 cells stimulated with env-encoded peptides corresponding to the conserved N- and C-terminal regions of the env glycoprotein (peptides D1 and D2, respectively) and the V3 loop (peptide I10). Peptides D1 and D2 matched the optimally active synthetic peptides recognized by epitope-specific CTL, and both conformed to the A2 consensus motif (18), whereas the I10 peptide lacks the A2 anchor residues but possesses structural features that confer promiscuous A2 binding (33). The results of three separate experiments revealed consistently higher lysis of the 7ΔV3- than the WTP-2-expressing target in D1-stimulated cultures derived from patients 521 and 410, and low to background response levels in PBMC stimulated with the D2 peptide (Fig. 4). In patient 417, small increases in lysis of vv-7ΔV3-infected JA2 cells were detected in D1-stimulated cultures compared with lysis directed against targets expressing the WTP-2 env. The responses were similar in PBMC stimulated with the D2 peptide. The CTL responses against vv-WTP-2-infected JA2 cells in I10-stimulated PBMC were diminished in all donors and were either undetectable or at background levels against JA2 cells expressing the 7ΔV3 mutant.

Responses to the conserved epitope of gp120 in PBMC stimulated with the wild-type or the mutated env glycoprotein were further characterized by quantitating the D1-specific CTLp frequency using limiting dilution analysis (2, 24). Varying concentrations of PBMC derived from patients 410, 428, 450, and 544 were stimulated in vitro with autologous LCLs expressing either the WTP-2 or the 7ΔV3 env glycoprotein and tested for lysis of JA2 cells coated with the D1 peptide. As shown in Table I, frequencies of D1-specific CTLp in PBMC stimulated with the 7ΔV3 mutant were higher than those in WTP-2-stimulated cultures (p = 0.04). In patient 410, stimulation of PBMC with the 7ΔV3 mutant resulted in an increased frequency of the D1-specific CTLp compared with that detected in cultures stimulated with the WTP-2 env gene products (180 vs 102 CTL/10^6 PBMC). The D1-specific CTL responses were at background levels in WTP-2-stimulated PBMC derived from patients 450 and 544, but they were detectable after stimulation with the mutated env gene products. In patient 428, the D1-specific CTLp frequency in PBMC stimulated with the 7ΔV3 mutant was also higher than that in WTP-2-stimulated cultures, but the 95% confidence limits suggested no difference between these responses.

**ADCC against vv-7ΔV3 mutant-infected targets**

The susceptibility of cells expressing the 7ΔV3 mutant and WTP-2 env glycoproteins to ADCC-mediated lysis was analyzed using PBMC of HIV-seronegative donors and polyclonal Ig purified from sera of HIV-infected individuals or env-specific mAbs. The results of experiments with PBMC from three individuals and polyclonal anti-gp160 Abs revealed ~20% specific lysis against vv-WTP-2-infected target cells at an E: T cell ratio of 50:1 (Fig. 5). Among five mAbs specific for epitopes within the V2 and V3 loops (697-D and 694/98-D, respectively), the CD4 binding site (F105), the C-terminus (670-D), and the gp41 region of gp160 (50-69II), the highest ADCC activities against vv-WTP-2-infected target were mediated by 694/98-D and F105 mAbs. Abs directed to other regions of gp120 had less effect on the ADCC-mediated lysis. In contrast, the specific lysis against vv-7ΔV3-infected target cells in the presence of polyclonal anti-gp160 Abs or the F105 mAb was <4% (Fig. 5), and no ADCC activity was detected with other anti-gp160 mAbs.

**Syncytium formation and induction of apoptosis by the 7ΔV3 mutant**

Examination of SupT-1 cells infected with vv-WTP-2 under a light microscope revealed that 80 to 90% cells formed giant cells 24 h after infection. No syncytia were observed in cultures infected with either the CR19 vv alone or the vv-7ΔV3 mutant, consistent with
the previous findings that V3 is required for the syncytium-forming ability of the virus (32). The apoptotic process associated with syncytium formation was analyzed in individual cultures 16 h after infection. Analysis of DNA content by staining of nuclei with PI indicated that infection of cells with the full-length env glycoprotein resulted in the appearance of 20% of cells with a subG1 DNA content representing apoptotic cells (Fig. 6A), concomitant with decreases in the proportion of cells in the G1 phase of the cell cycle (from 39–15%). In contrast, the numbers of apoptotic cells in uninfected cultures and cultures infected with vac or the 7ΔV3 mutant were <5%. Apoptosis of Sup-T1 cells expressing the full-length env glycoproteins was also confirmed by visualization of nucleosome-sized DNA multimers of 180 to 200 bp to form the characteristic stepladder appearance after size separation on agarose gel (Fig. 6B). Sup-T1 cells infected with vac or expressing the 7ΔV3 mutant failed to induce DNA fragmentation over background levels in isolated low m.w. DNA.

The induction of apoptosis during infection of SupT-1 cells with vv-WTP-2 is consistent with results of previous studies that demonstrated that full-length env glycoprotein is responsible for syncytium formation and apoptosis (12, 13). Because HIV may only rarely induce apoptosis in infected cells in vivo (14), we analyzed the effect of a deletion of the V3 region on activation-induced apoptosis in uninfected CD4+ cells. For these experiments, CD4+ T cell blasts of HIV-seronegative donors were incubated with APC

Table I. D1-specific CTLp frequencies in WTP-2- and 7ΔV3-induced PBMC

<table>
<thead>
<tr>
<th>Patient</th>
<th>CTLp (95% Confidence Intervals)</th>
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<tbody>
<tr>
<td></td>
<td>WTP-2</td>
</tr>
<tr>
<td>410</td>
<td>102 (85–119)</td>
</tr>
<tr>
<td>428</td>
<td>93 (28–157)</td>
</tr>
<tr>
<td>450</td>
<td>20 (11–28)</td>
</tr>
<tr>
<td>544</td>
<td>12 (10–14)</td>
</tr>
</tbody>
</table>

*Precursor frequencies of D1-specific effector cells in WTP-2- and 7ΔV3-stimulated cultures are expressed as number of CTLp per 106 PBMC. Values in parentheses represent 95% confidence limits of the calculated frequencies. The D1-specific CTLp frequencies were computed as differences between CTLp frequencies determined on D1-coated target vs control target cells without peptide. The CTLp frequencies on control targets ranged from 0.1 to 25 CTL/106 PBMC. There was significant difference (p = 0.04) in frequencies of D1-specific CTLp in PBMC stimulated with the full-length vs the mutated env gene products, as determined by a paired t test analysis (50).

FIGURE 4. CTL activity against vv-WTP-2- and vv-7ΔV3-infected JA2 cells in PBMC of HIV-infected patients stimulated with the env-encoded peptides. PBMC of HIV-infected individuals were stimulated with env-specific peptides (0.5 μM) and tested on day 14 in a 51Cr release assay against target cells infected with vv-WTP-2 (○) or vv-7ΔV3 (■). Cells infected with vac were used as a negative control, and CTL activity was computed as differences between CTL responses to env vs the control. Results are representative of three separate experiments.

FIGURE 5. ADCC-mediated lysis of JA2 cells infected with vv-WTP-2 (●) or vv-7ΔV3 (○) in the presence of env-specific Abs and PBMC of an HIV-seronegative individual. ADCC-specific lysis was calculated by subtracting the background reactivity with the vac-infected target only from the percent cytotoxicity of the vv-7ΔV3- or vv-WTP-2-infected target cells. The E:T cell ratio was 50:1. Results are representative of three separate experiments and are the means of triplicate values with SD <5%.
expressing either the ΔV3 or the complete env glycoprotein before stimulation with anti-CD3 mAb. Induction of apoptosis, monitored after 72 h by staining of nuclei with PI and flow cytometric analysis, revealed <7% of fragmented DNA in cultures infected with vac or the vv-7ΔV3 mutant. On the other hand, T cells preincubated with APC expressing the full-length env glycoprotein before activation revealed DNA fragmentation approximately 3 times the control level (Fig. 6C).

**Discussion**

In this study we investigated the effect of deletion involving the V3 loop of the env glycoprotein on cellular responses in PBMC derived from HIV-infected patients. Consistent with previous findings (32), we showed that deletion of the V3 loop did not abrogate the processing and transport of the env glycoprotein to the cell surface. However, it was associated with decreases in sCD4 binding to the cellular gp120 and lack of induction of both syncytia and apoptosis, supporting the observations that deletion of V3 induces conformational changes in the HIV env glycoprotein relevant to the membrane fusion process (12, 32). These findings together with the observation that the majority of neutralizing Abs (30–80%) in HIV-infected patients recognize linear V3 loop sequences (34) may also explain the increased resistance of the ΔV3 env mutant to ADCC-mediated lysis.

The enhanced CTL responses in cultures stimulated with the ΔV3 env gene products compared with those induced with the full-length env glycoprotein suggest an increased antigenicity of the 7ΔV3 mutant. Previous studies have shown that processing and transport of the V3-deleted mutant env glycoprotein are accelerated compared with the wild-type counterpart (32). This may have increased the pool of immunogenic peptides presented by the target cells and improved their recognition by CTLs. It is also possible that the binding of the full-length gp120 to the CD4 receptor on uninfected T lymphocytes may interfere with function of CD4+ T cells, thus reducing help needed for optimal induction of CTL activity (35, 36). In addition, it has been demonstrated that free I10 peptide can inactivate murine CD8+ CTLs by a self-veto mechanism that involves simultaneous occupancy of the MHC class I
molecule and the TCR on the same CTL (19). The possible implication of this phenomenon for HIV pathogenesis in humans is that when virus-infected cells are lysed and the digested intracellular proteins released into the T cell environment, the V3 loop-related self-epitope effect may inhibit the induction of CTL responses.

The immunogenicity of the V3 loop-deleted gp160 remains to be determined in mice and nonhuman primates before the possible application of such mutated env gene products for effective vaccination and therapeutic strategies. Such altered HIV constructs could be injected as plasmid DNA (37) or expressed in autologous cells by intracellular immunization (38) before injection into the host. In either case, cells expressing the mutated env gene products are likely to escape from the ADCC-mediated lysis in vivo and could be injected as plasmid DNA (37) or expressed in autologous vaccination and therapeutic strategies. Such altered HIV constructs

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