The third hypervariable region, or V3 loop, represents the principal neutralizing domain of the gp120 envelope glycoprotein of human immunodeficiency virus type 1 (HIV-1). Sequential viral isolates from a laboratory worker (LW) accidentally infected with HIV-1 in 1985 were analyzed using type-specific neutralizing monoclonal antibodies directed to the V3 loop. A single amino acid substitution, Ala → Thr at position 21 in the V3 loop of HIV-1LW isolated in 1987, was shown to determine the loss of the neutralizing epitope recognized by one of the monoclonal antibodies (M77). However, this antibody efficiently recognized linear V3 loop peptides containing either the Ala or Thr residue at position 21, indicating that a local change in conformation was responsible for the epitope loss in the native gp120. Molecular modeling studies, experimentally supported by different amino acid replacements at position 21, indicated that the Ala → Thr substitution leads to a drastic change in the domain of the V3 loop, which contains the complementary surface for antibody binding. These results provide evidence for the first time that a conformation-dependent epitope within the V3 loop of HIV-1 is involved in the generation of neutralization escape mutants in vivo.

The primary translational product of the envelope gene of the human immunodeficiency virus type 1 (HIV-1) is a gp160 which is processed to gp120 and gp41 as the external and transmembrane glycoproteins, respectively (Veronese et al., 1985; Allan et al., 1985). Because of its location on the surface of the virion and of the infected cell, gp120 contains epitopes naturally accessible to the immune system and thus represents a prime candidate for the development of vaccine strategies. Indeed, a domain of gp120, situated in the third hypervariable region, has been identified as dominant for development of high titred type-specific neutralizing antibodies against HIV-1 (Jahavarian et al., 1986; Rusche et al., 1988; Palker et al., 1988). This principal neutralizing domain lies within a loop formed by a disulfide bridge between 2 conserved cysteines. A distinctive characteristic of this loop is the extensive degree of genetic variability found among individual isolates of HIV-1 (Robert-Guroff, 1990). This hypervariability, a likely consequence of strong immune selective pressures in vivo, suggests that mutations in the primary amino acid sequence of the neutralizing loop become prevalent as viruses which escape neutralization are selected.

Different approaches have been followed in order to identify amino acid changes which are critical for neutralization. One approach involved the in vitro generation of neutralization escape mutant viruses under selective pressure of human sera from HIV-infected individuals (Robert-Guroff et al., 1986) or monoclonal antibodies to the V3 loop (McKeating, 1989). In vitro studies with human neutralizing antibodies were the first to demonstrate that HIV mutants are selected by immune pressures (Robert-Guroff et al., 1986). In addition, these studies identified a point mutation in gp41 (Ala → Thr replacement at position 582) which resulted in neutralization resistance (Reitz et al., 1988) but was apparently not part of the antibody binding site (Wilson et al., 1990), indicating the involvement of discontinuous epitopes in HIV neutralization. Molecular analysis of mutant viruses obtained by in vitro selection with monoclonal antibodies demonstrated amino acid changes within the V3 loop itself (McKeating et al., 1989), but more interestingly also in regions distantly located from the loop, suggesting an interaction of this domain with other regions of gp120 (McKeating et al., 1989). Another approach involved the analysis of sequential viral isolates obtained from chimpanzees experimentally infected with a laboratory strain of HIV-1 (HIV-1LH). The in vivo passage of this isolate resulted in the generation of variant viruses which were resistant to neutralization by V3 loop-specific monoclonal antibodies (Nara et al., 1990). Sequence analysis of the V3 loops from these mutants did not reveal any amino acid difference between the neutralization-resistant and -sensitive viruses, suggesting that the crucial changes did not directly involve the antibody binding site (Nara et al., 1990).

In this report, we studied the immunological reactivity and neutralizing capability of two monoclonal antibodies to the V3 domain of HIV-1 in vivo, with biologically active molecular clones from sequential viral isolates obtained from a laboratory worker who became accidentally infected with HIV-1 (Weiss et al., 1988). This unique in vivo human model allowed us to identify a single change in the V3 loop primary amino acid sequence which is crucial for neutralization resistance by V3 loop-specific monoclonal antibodies. Moreover, we have performed molecular modeling studies, experimentally supported by amino acid replacements, to show that this single amino acid substitution may cause a drastic change in the domain of the V3 loop which provides the complementary surface for antibody binding.

EXPERIMENTAL PROCEDURES

Neutralization Assay of Cell-free Virus—Ascitic fluids from the two monoclonal antibodies were filtered through a 0.2-μm filter and di
luted in complete RPMI 1640 medium. Aliquots of the respective antibody were incubated with HIV-1IM virus stock containing 25,000 cpm reverse transcriptase activity in 100 μl final volume for 60 min at 4 °C and then for 15 min at room temperature. CEM cells (1 x 10⁶) were then added to the virus/antibody mixture and incubated for 60 min at 37 °C. The cells, still in the presence of virus and antibody, were supplemented with 2 ml of complete RPMI medium and transferred to six-well plates. Two ml of RPMI medium were added to each well after 24 h, and the reverse transcriptase activity of the supernatant was determined on the first day when syncytia were clearly visible by microscopic examination.

Syncytium Inhibition Assay—The syncytia assay was performed in 96-well plates by coculturing 1 x 10⁶ uninfected cells with 5 x 10⁵ virus-infected cells. Monoclonal antibodies were added at different dilutions to the mixture of cells, and the total volume of medium was adjusted to 0.2 ml. The plates were incubated for 40 h at 37 °C, and the number of multinucleated giant cells was determined by microscopic examination.

Radiolabeling of Cells and Immunoprecipitation of HIV-1 Proteins—Transfected HeLa-tat cells or infected SupT1 were suspended for 1 h at 37 °C in cysteine-free media. [³⁵S]Cysteine was then added to a final concentration of 200 μCi/ml, and the cells were incubated for 18 h at 37 °C.

After labeling the cells were washed in phosphate-buffered saline and disrupted in phosphate-buffered saline containing 0.5% NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS). The lysates were absorbed for 3 h at 4 °C with protein A-Sepharose and protein A-Sepharose bound to rabbit anti-mouse light chain antisera, and then clarified by ultracentrifugation. Radioimmuno precipitation analyses were performed by the addition to 0.5 ml of labeled extract of either 10 µl of serum from an HIV-infected individual and 0.2 ml of a 10% suspension of protein A-Sepharose or 1 µl of ascites containing the appropriate antibody and 0.2 ml of a 10% suspension of protein A-Sepharose bound to rabbit anti-mouse light chain antisera. The samples were incubated for 18 h at 4 °C. Immunoprecipitates were collected by centrifugation, washed repeatedly, re suspended in Laemmli sample buffer (Laemmli, 1970), heated for 2 min at 90 °C, and analyzed by SDS-polyacrylamide gel electrophoresis.

Indirect Immunofluorescence and Fluorocytometric Analysis—Indirect immunofluorescence analysis was performed on live cells with primary antibodies at 5 μg/ml and with fluorescein isothiocyanate-conjugated goat antiserum to murine IgG (Sigma) as a second layer antibody. Controls were incubated with a mouse IgG, irrelevant antibody, then stained with the same fluorescein isothiocyanate-conjugated antiserum. After staining, the cells were fixed with 2% paraformaldehyde and analyzed with a Facscan analyzer (Becton Dickinson Immuno- cytometry). The results are expressed as arbitrarily normalized histograms (i.e. relative number of cells versus fluorescence intensity). At least 10,000 events were accumulated in all the experiments.

Construction of V3 Loop Cassette and env Chimera—A 2.7-kbp SalI-BamHI restriction enzyme fragment from pHXB2gpt, a molecular clone of HIV-1IM, containing the V3 loop region, was sub cloned into the cognate sites of gEM4 (Promega, Madison, WI). A 0.6-kbp V3-containing BglII fragment from this subclone was further sub cloned into pSP72 (Promega). Two PCR amplifications were performed on this fragment, such that one amplified fragment consisted of the region from the 5' BglIII site to the 5' terminus of the V3 loop and contained MluI and PstI sites introduced at the downstream end of the fragment by a tail on the antisense PCR primer. The other PCR fragment consisted of the region from the 3' end of the V3 loop, where PstI and HpaI sites were introduced at the upstream end of the fragment by a tail on the sense PCR primer. The PCR fragments were purified, digested with BglII plus PstI, and coligated into pSP72. The 0.5-kbp BglII fragment from the resultant clone was ligated with the 5.1-kbp BglII fragment of the Slall-BamHI env subclone of pXHB2gpt. The resulting plasmid has unique MluI and HpaI restriction sites, whose introduction did not alter the amino acid sequence, forming a cassette into which oligonucleotides representing V3 coding regions and containing an MluI 4-base overhang at one end and a blunt end at the other end can be ligated. This construction is summarized in Fig. 4. The V3 loop coding region of LW12.3 (Lori et al., 1992), an infectious molecular clone from the virus isolated in 1987 from a laboratory worker accidentally infected with HIV-1IM and hereinafter designated LW87, was synthesized as a set of four partially overlapping nucleotides, phosphorylated with T4 kinase, annealed, and ligated into the MluI and HpaI sites of the cassette plasmid. The resultant 2.7-kbp SalI-BamHI fragment, now containing

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**FIG. 1. Scheme depicting the sequence for the V3 loop of the pHXB2 molecular clone of HIV-1IM and the location of the Thr (LW virus from 1987) for A (HXB2) substitution.** Also designated are the binding sites for M77 and 0.5β monoclonal antibodies.

**Fig. 2. A, inhibition of syncytia formation between HIV-1IM (panels 1 and 2) or LW87 (panels 3 and 4) infected and uninfected SupT1 cells. The assays were performed without the addition (panels 1 and 3) and in the presence of M77 (panels 2 and 4) as described under "Experimental Procedures." B, neutralization assays with cell-free virions from HIV-1IM (△), HXB2 (□), and LW87 (■) were performed with the indicated dilutions of M77 ascites as described under "Experimental Procedures." The neutralizing activity of the antibody is expressed as the percent reduction of reverse transcriptase levels in the supernatants of antibody-treated wells compared with those of the control.
FIG. 3. Reactivity of M77 and 0.5β monoclonal antibodies with gp120. SupTl cells infected with HIV-1IIIB (A) or LW87 (B) and HeLa-tat cells transfected with pHXB2 (env LW85-2) (C) were labeled with [35S]cysteine. The clarified lysates from cells and virus containing supernatants were then immunoprecipitated with an HN-1 positive human serum (lane 1), M77 (lane 2), and 0.5β (lane 3).

FIG. 4. Derivation of chimeric viruses. The construction of the cassette for insertion of foreign V3 loops into the infectious clone pHXB2gpt, described under “Experimental Procedures,” is shown above. The SalI-BamHI 2.7-kbp fragment, containing most of the env gene, was subcloned. A BglII fragment containing the V3 region was subcloned, altered by PCR to replace the V3 loop coding region with cloning sites, and put back into the SalI-BamHI fragment. The resultant SalI-BamHI construct was used as the cassette and inserted back into pHXB2gpt after introduction of the LW87 V3 loop.
Loop Neutralization Escape Mutant in Vivo

We compared the ability of these antibodies to neutralize infection by HIV-1IIIB, HXB2, and LW12.3 (Lori et al., 1992), an infectious molecular clone derived from a biological clone (no. 12) of the virus isolated in 1987 from a laboratory worker accidently infected with HIV-1IIIB in 1985 (Weiss et al., 1988) and hereinafter designated LW87. The only amino acid difference within the M77 and 0.5β binding sites between LW87 and the HXB2 resides at position 21 in the sequence of the V3 loop, where an alanine has been substituted for a threonine (Fig. 1). As expected, M77 completely blocked the formation of syncytia and neutralized the infectivity of both the IIIB and HXB2 viruses (Fig. 2). However, M77 did not inhibit the formation of syncytia between SupT1 cells infected with LW87 and uninfected SupT1 (Fig. 2A). Moreover, M77 failed to neutralize cell-free infection of CEM cells by the same virus (Fig. 2B). In contrast, 0.5β inhibited infection by LW87 in both experiments (data not shown).

Failure of M77 to Recognize Native gp120 from LW87—Since lack of neutralization is not necessarily associated with lack of

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Results

We compared the ability of these antibodies to neutralize infection by HIV-1IIIB, HXB2, and LW12.3 (Lori et al., 1992), an infectious molecular clone derived from a biological clone (no. 12) of the virus isolated in 1987 from a laboratory worker accidently infected with HIV-1IIIB in 1985 (Weiss et al., 1988) and hereinafter designated LW87. The only amino acid difference within the M77 and 0.5β binding sites between LW87 and the HXB2 resides at position 21 in the sequence of the V3 loop, where an alanine has been substituted for a threonine (Fig. 1). As expected, M77 completely blocked the formation of syncytia and neutralized the infectivity of both the IIIB and HXB2 viruses (Fig. 2). However, M77 did not inhibit the formation of syncytia between SupT1 cells infected with LW87 and uninfected SupT1 (Fig. 2A). Moreover, M77 failed to neutralize cell-free infection of CEM cells by the same virus (Fig. 2B). In contrast, 0.5β inhibited infection by LW87 in both experiments (data not shown).

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immunological recognition, we performed radioimmunoprecipitation assays to determine if M77 was able to react with LW87 gp120 in its native form. SupT1 cells infected with HIV-1LW and LW87 were metabolically labeled with [35S]methionine overnight. Labeled proteins from cellular and viral lysates were then immunoprecipitated with M77, 0.5β, and an HIV-1 antibody-positive human serum as a positive control.

M77 efficiently precipitated gp120 from both cellular and viral extracts of HIV-1LW-infected cells, but failed to do so with both cellular and viral extracts of LW87 infected cells (Fig. 3, A and B). In contrast, 0.5β immunoprecipitated gp120 from extracts of both types of infected cells (Fig. 3, A and B). Similar results were also obtained by live cell immunofluorescence labeling followed by fluorocytometric analysis (data not shown). Thus, lack of neutralization of LW87 by M77 is explained by the inability of the antibody to react with native gp120 from this virus. These results also suggest that antibodies 0.5β and M77, despite partially overlapping epitopes in the V3 loop, differ in their binding specificity since 0.5β reacted with native gp120 from the LW virus isolated in 1987. However, a decrease in the efficiency of immunoprecipitation of gp120 from LW87 virus was noticeable for 0.5β, suggesting a lowered binding affinity for its epitope.

To substantiate and extend the observation obtained with the molecular clone LW87, we repeated the previously described experiments with a biological clone, designated LW87 no. 17, obtained from the same 1987 LW isolate grown in H9 cells. Again in contrast to 0.5β, M77 failed to neutralize infection or to recognize gp120 from LW87 no. 17-infected cells in both radioimmunoprecipitation and live cell immunofluorescence labeling followed by fluorocytometric analysis (data not shown), demonstrating that an independent biological clone had lost the same neutralizing epitope.

To determine whether M77 recognized gp120 from an earlier LW virus isolated shortly after infection in 1985 (LW85), which has the same V3 loop as the molecular clone HXB2, we tested a chimeric clone with the complete env of a clone of LW85 inserted in HXB2 (HXB2/env LW85-2), a kind gift by Drs. Beatrice Hahn and George Shaw, University of Alabama, Birmingham, AL. HeLa-tat cells were transfected with HXB2(env LW85-2) and labeled with [35S]methionine. Both M77 and 0.5β efficiently immunoprecipitated HXB2/env LW85-2 gp120 from labeled cellular and viral extracts (Fig 3C). Moreover, both monoclonal antibodies were able to completely neutralize the infectivity of this chimeric virus (data not shown).

The Ala → Thr Change Is Crucial for Neutralization Resistance and Recognition of Native gp120 by M77—In addition to the Ala → Thr replacement in the hypervariable loop, the LW virus isolated in 1987 is divergent from HXB2 in more than 10 positions throughout the gp120 sequence. To determine whether the Ala → Thr substitution was critical for the immunological reactivity and neutralizing capability of M77, we inserted the loop sequence of LW87 into HXB2, as described in detail under “Experimental Procedures” and shown in Fig. 4. The resulting infectious chimera was designated HXB2(V3LW87). HeLa-tat cells were transfected with HXB2(V3LW87) or with HXB2 as wild type control and co-cultivated with uninfected CEM cells 36 hours after transfection. Approximately 3 days later, when formation of syncytia was readily visible, CEM cells were collected and subjected to live cell immunofluorescence labeling followed by fluorocytometric analysis. The 0.5β antibody stained the surface of both HXB2(V3LW87) and HXB2-infected cells as did the positive control M90, another monoclonal antibody which reacts with an exposed conserved epitope in gp120 unrelated to the V3 loop (Fig. 5). In contrast, the M77 antibody positively reacted with wild type HXB2-infected cells, but failed to stain the surface of HXB2(V3LW87) infected cells. These results clearly indicated that the Thr for Ala change was sufficient to determine the loss of the M77 epitope, ruling out that other amino acid changes between HXB2 and LW87 outside the V3 loop were necessary for loss of recognition of the epitope by M77.

Recognition of Linear V3 Loop Peptides by M77—As mentioned above, the only amino acid difference between the LW87 and HXB2 V3 loops consists of the substitution of a Thr for an Ala at position 21. When we assayed the ability of M77 and 0.5β to bind in enzyme-linked immunosorbent assay to V3 linear peptides containing either Ala or Thr at that position, both antibodies bound to the two peptides (Fig. 6). These results indicated that M77 was still able to bind to the linear form of the epitope and suggested that local change in conformation of the epitope in the functionally folded protein resulted in the loss of neutralization.

Molecular Modeling and Experimental Validation of Theoretical Predictions—Molecular modeling studies using a simulated annealing approach were performed in order to understand the effect of the Ala → Thr substitution on M77 antibody binding. The folded forms of the V3 loop for the Ala and Thr analogs, as well as the extended forms, were computed using the methodology outlined under “Experimental Procedures.” Molecular modeling studies indicated that the Ala → Thr substitution can disrupt the folded epitope at the tip of the V3 loop (Figs. 7 and 8). Fig. 7, A and B, shows ribbon and skeleton models of the V3 loop for the Ala analog. In this folded motif residues 16–28 of the V3 loop, which contain the crucial sequence for M77 binding, form a compact protruding surface in which the A at position 21 resides in the interior of the contact surface and makes close contact with atoms of the neighboring residues (Fig. 7B). Thus, the Ala → Thr substitution should result in an enlargement of the interior to accommodate the bulkier side chain. This, in turn, should enlarge the contact surface of the 16–28 motif with a resultant loss of M77 binding.

The relative stability of the folded form of the V3 loop was
FIG. 7. The ribbon (A) and skeleton (B) models of the V3 loop of the Ala analog in the folded form. The secondary structural states of various amino acids in the sequence are as follows.
Amino acid sequence: CTRPNNTRKrIQRPGRAFYTGKIGMRQAHc
Secondary structure: cttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
The ribbon (A) and skeleton (B) models of the V3 loop of the Thr analog in the extended conformation. This conformation can also be adopted by the Ala analog. This conformation is taken as the reference (unfolded state). The relative stabilities of the folded states of the Ala and Thr analogs are measured from this reference state. In the extended conformation, residues 16–28 occupy a larger surface area than in the folded one.

Fig. 8. Fluorocytometric analysis of CEM cells infected with HXB2 mutants. These are as follows: HXB2 bearing Ala (Ala21), Ser (Ala21 → Ser), Thr (Ala21 → Thr), or Ile (Ala21 → Ile) at position 21 in the V3 loop. The empty profiles represent the reactivity of the cells with an irrelevant isotype-matched monoclonal antibody. The M77 reactivity perfectly correlated with the modeling predictions.

DISCUSSION

The availability of sequential viral isolates from a laboratory worker accidentally infected with the prototype HIV-1 strain IIIb gave us the unique opportunity of studying the influence of naturally occurring mutations on V3 loop-dependent neutralization. Different molecular clones of LW obtained shortly after infection were genetically almost indistinguishable among themselves and from HIV-1IIIb-derived molecular clones. Indeed, within the V3 region, they completely retained the sequence of the HXB2 clone from HIV-1IIIb. An Ala to Thr substitution within the GPGRAF motif at the tip of the loop was detected 1 year after infection and persisted in all subsequent isolations.

We have observed that the HIV-1IIIb-neutralizing monoclonal antibody M77 was unable to neutralize infection by a biologically active molecular clone of LW isolated in 1987 and bearing the Thr for Ala substitution at position 21 within the V3 loop. Lack of neutralization does not always correlate with lack of immunological recognition, since it has been shown that
monoclonal antibodies can bind the V3 loop without neutralization (Nara et al., 1992). In this case, however, lack of neutralization by M77 was due to a direct consequence of the inability of the antibody to bind native gp120, as indicated by immunoprecipitation and fluorocytometric analysis.

The antibody M77 did not react with the chimeric virus obtained after insertion of LW87 V3 loop in HXB2, clearly indicating that the Ala → Thr substitution is sufficient for the abrogation of antibody binding to native gp120. Thus far, only one neutralization resistant escape mutant selected in vitro with monoclonal antibodies to the V3 loop showed an amino acid change within the binding site of the selecting antibody (McKeating et al., 1989). This change involved the substitution of a G for an R at position 13 in the sequence for the V3 loop and clearly affected the binding of the antibody to the linear epitope. In contrast, the majority of the neutralization-resistant mutants which were selected in vitro with monoclonal antibodies to the V3 loop did not show any amino acid sequence variation within the loop itself, suggesting that critical changes affecting virus neutralization reside outside the V3 domain (McKeating et al., 1989). This, in fact, has been proven in the in vitro immune escape system with human antisera (Robert-Guorro et al., 1986), in which a point mutation in the transmembrane portion of the envelope gene resulted in neutralization resistance (Reitz et al., 1988). Subsequent studies showed that this mutation affected neutralization by conformational changes induced by an Ala → Thr change at position 582 in gp120 (Wilson et al., 1990).

These in vitro findings were also supported by results generated in vivo in chimpanzees (Nara et al., 1990). Experimental infection of chimpanzees with HIV-1HIV resulted in the rapid emergence of mutant viruses resistant to neutralization by V3-specific neutralizing antibodies. Sequencing of the envelope gene from these viruses revealed the complete identity of the V3 amino acid sequence between the neutralization resistant mutant viruses and the neutralization-sensitive molecular clones contained in the original inoculum. However, several amino acid substitutions occurred throughout gp120, indicating again that the changes affecting virus neutralization resided outside the V3 loop. In contrast, our data show that the mutation of a single residue within the V3 loop occurring in vivo in a human results in the loss of the binding site for a virus neutralizing antibody.

The antibody M77 was able to bind to the linear form of its epitope, implying that a local change in conformation was actually responsible for the loss of the epitope itself in the functionally folded protein. It is conceivable that M77 reacts with both isolated V3 peptides because these are flexible, do not have structural constraints, and thus have more freedom to adapt to the antibody complementarity region. However, the M77 antibody can only bind the (S,S)-bridged V3 loop Ala analog but not the V3 loop Thr analog. This means that neither Ala nor Thr at the same position of the V3 loop make direct contact with the antibody. If this were the case, the M77 antibody would have discriminated between the Ala and Thr analogs in the linear peptide epitopes. Therefore, it appears that the (S,S)-bridged V3 loop imposes certain stereochemical constraints such that only the Ala analog, but not the Thr analog, can present the antibody-binding domain of the V3 loop in an effective manner to the M77 antibody.

Our molecular modeling studies, experimentally supported by different amino acid replacements, suggested that the Thr for Ala substitution can disrupt the fold anti-epitope at the tip of the V3 loop (amino acids 16–28). In the folded form the amino acids forming the epitope adopt a protruding surface, in which the Ala residue resides inside the interior of the contact surface, while the Ala → Thr substitution requires enlargement of the contact surface resulting in the loss of antibody binding.

This report demonstrates that the V3 loop can adopt a specific structure in the presence of the antibody. Both from secondary structure prediction algorithms and two-dimensional NMR studies on free V3 loops, it has been suggested that the GPGQ sequence at the center of the immunogenic tip can adopt a type II β-turn (LaRoa et al., 1990; Chandrasekhar et al., 1991). However, no evidence has been presented to date that the GPGQ sequence together with the flanking amino acids can form a well defined combining site for antibodies. Amino acids on the surface of this combining site provide direct contact with the antibody binding pocket, whereas the amino acids in the cavity determine the size and shape of the cavity itself while maintaining a specific geometry of the antibody contact domain of the V3 loop.

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