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Another discontinuous epitope on glycoprotein gp120 that is important in human immunodeficiency virus type 1 neutralization is identified by a monoclonal antibody

(AIDS/envelope glycoprotein/conformation/CD4)

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ABSTRACT **To define the domains in the envelope glycoprotein important for antibody neutralization of the human immunodeficiency virus type 1 (HIV-1), monoclonal antibodies (mAbs) were generated by immunizing mice with purified glycoprotein gp120 of the III_B isolate. One mAb, G3-4, reacted with the gp120 of homologous (III_B) and heterologous (RF) isolates. In addition, mAb G3-4 efficiently neutralized both III_B and RF viruses *in vitro*, as well as four of nine primary HIV-1 isolates. In competition immunoassays, mAb G3-4 and soluble CD4 were found to inhibit one another in binding to gp120. However, no competition was seen between mAb G3-4 and mAbs directed to the third variable region or the fourth conserved region of gp120. In particular, mAb G3-4 did not compete with our human mAb I5e, which identifies a discontinuous epitope on gp120 involved in group-specific neutralization of HIV-1 and in gp120–CD4 binding. Epitope-mapping studies on mAb G3-4 with synthetic or unglycosylated recombinant peptides were negative, suggesting that its epitope may be discontinuous. Indeed, this hypothesis was confirmed by showing the loss of mAb G3-4 serologic reactivity when gp120 was first denatured. We conclude that the site recognized by mAb G3-4 represents another discontinous epitope on gp120 important for neutralization of HIV-1.**

Identification of the envelope domains involved in antibody neutralization of the human immunodeficiency virus type 1 (HIV-1) is a critical step in developing a vaccine for AIDS. Several continuous epitopes on glycoprotein gp120 recognized by neutralizing antibodies have been determined (1–10), including the loop structure in the third variable region (V3) that is believed to be the principal neutralization determinant (1, 2, 4–10). However, due to its sequence heterogeneity among isolates, the V3 loop induces type-specific neutralizing antibodies (1, 4–10), which do not account for the broad virus-neutralizing activity detected in the sera of most infected persons (2, 11, 12). Haigwood *et al.* (13) have shown that such broadly neutralizing human antibodies are directed against discontinuous epitopes on gp120. Indeed, we have recently described a human monoclonal antibody (mAb) (I5e) that recognizes a discontinuous epitope on gp120 important in CD4 binding and in antibody neutralization of divergent HIV-1 isolates (14). This finding is consistent with the report by Olshevsky *et al.* (15), demonstrating that the CD4-binding site is composed of several discontinuous regions of gp120, including the third and fourth conserved domains. We now present data on a mouse mAb (G3-4) that identifies another discontinuous epitope on gp120 involved in neutralization of HIV-1.

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METHODS

Purified glycoprotein gp120 from the III_B isolate (16) was used to immunize male BALB/c mice, and mAb production and screening were done as described (17, 18). mAb G3-4 reactivity with gp120 was determined by a radioimmunoprecipitation assay (2, 3, 14, 18) and by an immunofluorescence test using HIV-1-infected H9 cells and flow cytometry (19). The neutralizing activity of mAb G3-4 against laboratory strains of HIV-1 (III_B, RF, and MN) was determined by using our standard assay (2, 3, 14, 18). Antibody neutralizing activity against a panel of primary HIV-1 isolates was measured by a described assay (14, 20). A solid-phase gp120 immunoassay (14) based on the methods of Moore *et al.* (21, 22) was used to determine the competition between mAb G3-4 (as well as other mAbs) and soluble CD4 (sCD4) (23). The same assay was used to examine the competition between mAb G3-4 and other anti-gp120 mAbs, including I5e (14), G3-519 (18), and BAT123 (17). Epitope-characterization studies using dithiothreitol reduction or tunicamycin treatment were conducted as described (14). Deglycosylation with Endo H was done according to the protocol of McDougal *et al.* (24).

RESULTS

A mAb termed G3-4 (IgG2b, κ light chain) was found to react in a screening immunoassay with the gp120 purified from H9 cells infected with the III_B isolate. As shown in Fig. 1*A*, mAb G3-4 also immunoprecipitated the gp120/gp160 of the III_B isolate, as well as those of a heterologous isolate (RF). In addition, mAb G3-4 specifically stained the surface of live H9 cells chronically infected with III_B or RF but not uninfected H9 cells (Fig. 1*B*). mAb G3-4 did not react with the envelope glycoproteins of the MN isolate by either test (data not shown).

mAb G3-4 was also found to efficiently neutralize the infection of H9 cells *in vitro* by III_B and RF with 90% inhibitory doses (ID_{90}) of ≈ 3 μg/ml and 0.6 μg/ml, respectively, whereas the MN isolate was resistant to neutralization (Fig. 2*A*). It is interesting to note that mAb G3-4 neutralized a heterologous isolate (RF) more efficiently than the homologous virus (III_B). Of the nine primary HIV-1 isolates tested, mAb G3-4 neutralized four (PR, LS, AC, and JR-CSF) with ID_{90} values ranging from 0.3 μg/ml to 10 μg/ml (Fig. 2*B*). Greater than 50% neutralization could not be achieved

Abbreviations: HIV-1, human immunodeficiency virus type 1; V3, third variable region; mAb, monoclonal antibody; sCD4, soluble CD4.

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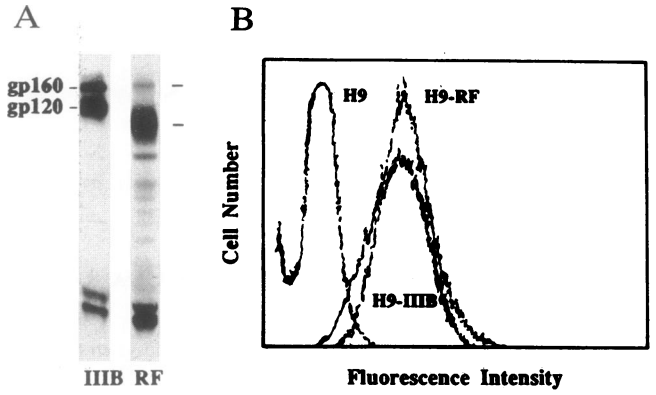

FIG. 1. Reactivity of mAb G3-4 with two HIV-1 isolates (III_B and RF) by radioimmunoprecipitation assay (*A*) and immunofluorescence and analyzed by flow cytometry (*B*).

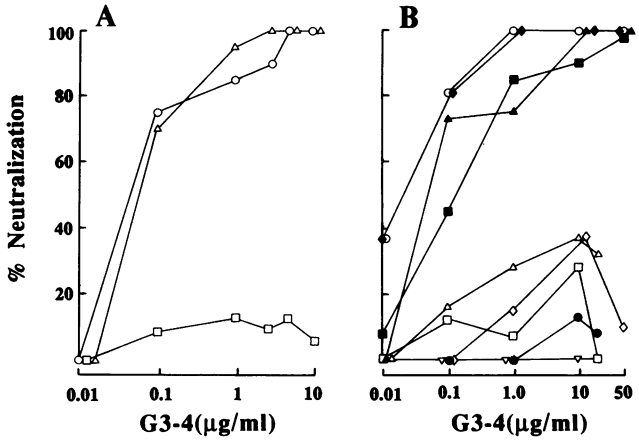

FIG. 2. (*A*) Neutralizing activity of mAb G3-4 against laboratory isolates: III_B (○), RF (△), and MN (□). (*B*) Neutralizing activity of mAb G3-4 against primary HIV-1 isolates: LS (○), PR (◆), AC (■), JR-CSF (▲), MU (△), L (◇), CO (□), JR-FL (▽), and B (●).

against five other primary isolates with up to 20 μg/ml of mAb G3-4.

To pursue one possible mechanism of neutralization, the capacity of mAb G3-4 and sCD4 to inhibit one another in binding to gp120 was examined in a competition immunoassay (14). Fig. 3*A* shows that sCD4 partially blocked the binding of biotinylated G3-4 to gp120 captured on solid phase. In comparison, the binding of biotinylated I5e (14) was most dramatically inhibited by sCD4, whereas that of biotinylated BAT123 (ref. 17; anti-V3 loop antibody) was unaffected. The binding of biotinylated G3-519 [ref. 18; a mouse mAb directed against the putative CD4-binding site in the fourth conserved region of gp120 initially described by Lasky *et al.* (25)] was also partially inhibited by sCD4. Fig. 3*B* shows that mAb G3-4 blocked the binding of biotinylated sCD4 to gp120 in a dose-dependent manner. In comparison, mAb I5e was a more efficient blocker than mAb G3-4, whereas mAb G3-519 blocked slightly less efficiently. As expected, mAb BAT123 did not affect sCD4–gp120 binding.

mAb G3-4 epitope-mapping studies were done by using a panel of synthetic and unglycosylated recombinant envelope peptides as described (14). No mAb G3-4 reactivity was detected in immunoassays with any of the peptides (data not shown), including a full-length envelope polypeptide of the III_B isolate expressed in *Escherichia coli*. These findings suggested that the mAb G3-4 epitope may be conformation-dependent, or discontinuous. Fig. 4*A* shows that mAb G3-4 reactivity with gp120 in a radioimmunoprecipitation assay was lost when the viral lysate was first denatured by dithiothreitol (0.1 M). A similar result was obtained using a slot-blot immunoassay (Fig. 4*D*). In addition, the importance of glycosylation to mAb G3-4–gp120 reactivity was demonstrated by the studies shown in Fig. 4*B*. A metabolically labeled HIV-1 lysate was prepared in the presence of tunicamycin. The unglycosylated envelope precursor polypeptide of 90 kDa was not recognized by mAb G3-4. Furthermore, when a labeled HIV-1 lysate was subjected to Endo H digestion, the partially deglycosylated products of 90–110 kDa were no longer recognized by mAb G3-4 (Fig. 4*C*). Taken together, the above findings suggest that the mAb G3-4 epitope is discontinuous and is significantly affected by oligosaccharide moieties on gp120.

Further attempts to define the mAb G3-4 epitope were made by testing previously characterized anti-gp120 mAbs for their ability to compete with biotinylated mAb G3-4 for gp120 binding. Fig. 5 shows that mAbs BAT123, G3-519 and I5e did not compete with mAb G3-4 for gp120 binding. In contrast, unlabeled mAb G3-4 blocked biotinylated G3-4 binding very efficiently, as expected. These observations suggest that the mAb G3-4 epitope is distinct from the V3

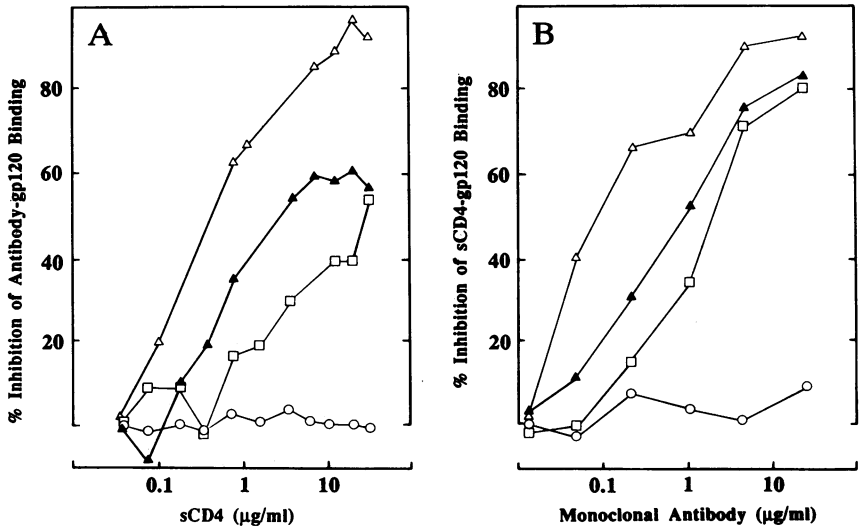

FIG. 3. (*A*) Inhibitory activity of sCD4 on antibody–gp120 binding in immunoassays. (*B*) Inhibitory activity of antibodies on sCD4–gp120 binding. mAbs were G3-4 (▲), I5e (△), G3-519 (□), and BAT123 (○).

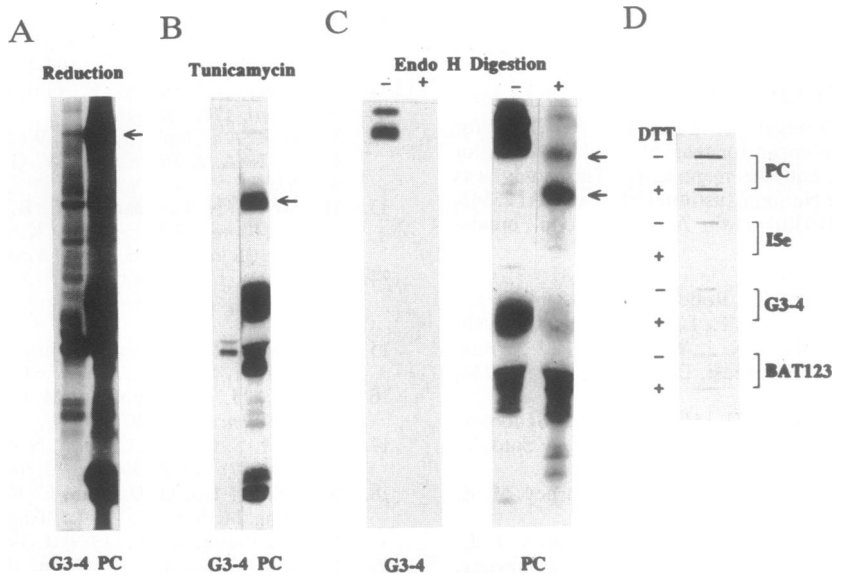

FIG. 4. Radioimmunoprecipitation assay of mAb G3-4 reactivity with HIV-1 lysate reduced with dithiothreitol (*A*), prepared with tunicamycin treatment (*B*), and subjected to endoglycosidase H (Endo H) digestion (*C*). (*D*) Slot-blot immunoassays of mAbs G3-4, I5e, and BAT123 reactivity with the III_B gp120 in the presence of (+) or the absence of (−) dithiothreitol (DTT) reduction. PC, positive-control human serum.

loop, a region of the fourth conserved domain (amino acids 418–445), and the mAb I5e epitope.

DISCUSSION

mAb G3-4 is an efficient neutralizing antibody for 50% of the HIV-1 isolates tested (Fig. 2). This neutralizing epitope appears discontinuous and sensitive to changes in glycosylation (Fig. 4). However, it cannot be composed entirely of oligosaccharide moieties because this antibody does not react with fully glycosylated but reduced gp120 (Fig. 4 *A* and *D*). In addition, the mAb G3-4 epitope is distinct from the mAb I5e epitope (Fig. 5), which is the first reported discontinuous neutralization epitope for HIV-1 (14). Therefore, mAb G3-4 has identified a second discontinuous neutralization epitope, which, in turn, represents an additional target to consider in the development of an AIDS vaccine. As illustrated in Fig. 6, we now have defined three nonoverlapping sets of HIV-1-neutralizing mAbs that can block gp120–CD4 interaction: mAbs I5e (14), G3-519 (18), and G3-4. Each set is distinct from antibodies directed against the V3 loop [e.g., mAb BAT123 (17)]. Among these antibodies, mAb I5e has the greatest potency in inhibiting gp120–CD4 binding (Fig. 3), suggesting that the mAb I5e epitope has the greatest overlap with the conserved CD4-binding site (Fig. 6). We have already suggested that mAb I5e-like antibodies may account for much of the broadly neutralizing activity of serum from infected persons (14). It is yet unknown whether G3-4-like antibodies are present in the serum of AIDS patients.

mAb G3-519 and other similar mAbs (18) recognize a continuous epitope in the fourth conserved domain initially described by Lasky *et al.* (25) as the CD4-binding site. Given the detailed study by Olshevsky *et al.* (15) and our finding that mAb G3-519 and related antibodies have variable neutralizing activity (18) and relatively lower binding inhibitory activity (Fig. 3), it is becoming clear that this fourth conserved

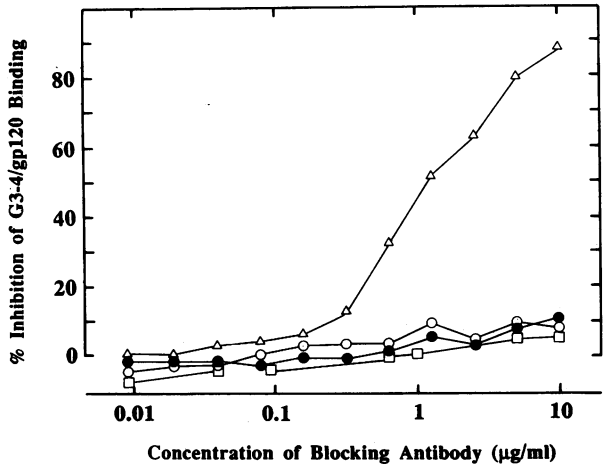

FIG. 5. Inhibitory activity of mAbs G3-4 (▲), BAT123 (○), G3-519 (●), and I5e (□) on the binding of biotinylated mAb G3-4 to captured III_B gp120.

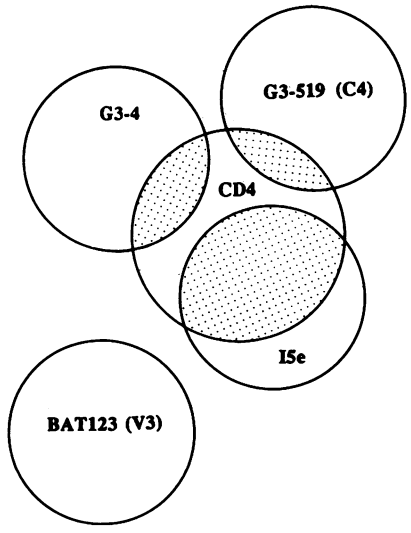

FIG. 6. Schematic representation of the epitopes for mAbs G3-4, G3-519, I5e, and BAT123 on gp120 and their spatial relationship with one another and with the CD4-binding site.

domain (amino acids 418–445) contributes only a limited number of amino acids to the more complex, discontinuous CD4-binding site of gp120.

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