# Human Immunodeficiency Virus Type 1 Neutralization Is Determined by Epitope Exposure on the gp120 Oligomer

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## Summary

The major target of the neutralizing antibody response to infection by the human immunodeficiency virus type 1 (HIV-1) is the outer envelope glycoprotein, gp120. The spectrum of HIV-1 neutralization specificity is currently represented by monoclonal antibodies (mAbs) that can be divided broadly into five groups. We have studied the binding of these mAbs to functional oligomeric and soluble monomeric gp120 derived from the molecular clone of a cell line–adapted isolate of HIV-1, and compared these binding properties with virus neutralization. Binding of all mAbs except those reactive with the V3 loop was much weaker to oligomeric than to monomeric gp120. This reduction in binding to oligomeric gp120 was determined mostly by a slower relative rate of association, although the dissociation rate also had some influence on relative variation in mAb affinity. Virus neutralization correlated broadly with mAb binding to the oligomeric rather than to the monomeric form of gp120, and neutralization potency was related to the estimated association rate. Thus, with the exception of the hypervariable V3 loop, regions of HIV-1 gp120 with the potential to induce a neutralization response are likely to be poorly presented for antibody recognition on the surface of cell line–adapted virions.

The surface envelope glycoprotein (gp120) of the human immunodeficiency virus HIV-1 is essential for receptor binding and viral entry into permissive cells (1, 2). In addition, it is the major target for neutralizing antibodies in vitro and in vivo and thus is an important element in vaccine development (3). Neutralizing antisera and mAbs have been raised against gp120 in the form of recombinant or purified glycoprotein or synthetic peptides, or during natural infection with the virus (4–27). For many mAbs, their epitopes have been mapped by analysis of their binding to synthetic peptides (5–16, 18, 21, 22, 24), recombinant glycoproteins containing site-directed mutations (25–27), or a combination of both strategies (19, 20, 23). Such analyses have allowed a partial understanding of the exposure and spatial relationship of these mAb epitopes on gp120 (19, 20, 22–28).

To date, five different clusters of mAbs that define dominant neutralizing epitopes on gp120 have been described. The V3 loop has been considered for some time as the immunodominant neutralization domain, and V3-reactive mAbs or antisera can potently neutralize HIV-1 (4–16). In general, neutralization via this domain is highly type specific, although some recent reports demonstrate that a broader neutralizing response can be obtained (15, 29–32). Several neutralizing mAbs reactive with the second variable domain (V2) of gp120 have been described (17, 18, 20), and their epitopes have been defined in detail (19, 20, 33). The fourth conserved domain of gp120 (C4) has been implicated as contributing to the binding surface for CD4 (34–36). In accord with this proposal is the finding that mAbs reactive with this region inhibit the interaction between recombinant gp120 and CD4 (22) and neutralize in a group-specific manner (21–23, 34). Another group of neutralizing mAbs of human origin overlaps broadly with the CD4 binding domain of gp120 (24–26, 37–40). These mAbs map to conformationally sensitive, discontinuous epitopes as indicated by extensive mutational analyses (25, 26). Like the C4-reactive mAbs, these mAbs inhibit the interaction of CD4 with monomeric gp120 (24) or virus with $CD4^+$ cells (38). Other neutralization domains that also have discontinuous, conformational epitopes but that do not interfere with virus–receptor binding have recently been defined by human mAbs (41); indeed, these mAbs bind better to soluble CD4 (sCD4)[1]–gp120 complexes than to gp120 alone (27).

The external envelope glycoprotein is expressed on the virion surface in an oligomeric form, most probably a tetramer, in association with the transmembrane glycoprotein gp41

[1] *Abbreviations used in this paper:* sCD4, soluble CD4; TBS, Tris-buffered saline; WB, PBS/1% FCS; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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(42–45). It is likely, therefore, that some epitopes that are accessible on the monomeric form of gp120 will be masked or presented in a different conformation on the functional, virion-associated form. Since it is the availability of an epitope on the virion surface that will influence antibody neutralization activity, it is important to study antibody–gp120 interactions in a system where the viral envelope glycoproteins are presented in their native, functional form. Immunoelectron microscopic analysis of the surface of certain virus-infected cell lines such as H9 shows that the majority of viral envelope glycoprotein present on cell membranes is in the form of adherent virus particles (46 and Sattentau, Q., unpublished data). Moreover, such virus-infected cells readily fuse with $CD4^+$ cells to form syncytia (1, 47). The envelope glycoproteins on the surface of HIV-1–infected cells are therefore likely to be representative of those on virions, both in terms of function and antigenic presentation. For this reason we have chosen to study gp120 on the surface of cells infected with the HIV-1 molecular clone Hx10 derived from the LAI isolate.

The data that we present here demonstrate that neutralizing mAb epitopes, with the exception of the V3 loop, are relatively poorly presented on the surface of functional HIV-1 gp120 oligomers, and that mAb affinity for these epitopes relates directly to their ability to neutralize virus infection.

## Materials and Methods

*Antibodies.* The anti-gp120/V3 mAbs were obtained from the following sources: 110.I (28) from F. Traincard (Hybridolab, Pasteur Institute, Paris, France); 110.5 (9) from Genetic Systems (Seattle, WA); 9284 (8) from DuPont de Nemours (Les Ulis, France); BAT123 (4, 12) from Tanox Biosystems Inc. (Houston, TX) and D. Ho (Aaron Diamond AIDS Research Center, New York); and 8/38c (16) from C. Dean and C. Shotton (Institute for Cancer Research, Surrey, UK). The anti-gp120/V2 mAbs BAT085, G3-136 (18), and G3-4 (17), and anti-C4 mAbs G3-42, G3-519, G3-299, and G3-536 (21, 23) were also from Tanox Biosystems Inc. and D. Ho. The human mAbs 15e, 21h (24), 48d, and 17b (27, 41) were from J. Robinson (University of Connecticut, Storrs, CT). Antisera were from the following sources: human anti-HIV QC1, QC2, and QC5 (48) from P. Clapham (Chester Beatty Laboratories, London, UK); infected laboratory worker serum from W. Blattner (National Cancer Institute, Frederick, MD) (49); rabbit anti–recombinant, soluble gp120 antiserum PB26 (50) from Genentech Inc. (South San Francisco, CA); and rabbit anti–recombinant, soluble gp120 antiserum R1/87 from R. S. Daniels (National Institute for Medical Research, London, UK).

*Cell Culture and Virus Infection.* H9 cells (from R. Gallo, National Institutes of Health, Bethesda, MD) were cultured in growth medium, RPMI 1640/10% FCS, in the presence of 5% $CO_2$. Infection of H9 cells with supernatant containing infectious virus of the Hx10 clone of HIV-1 (from B. Hahn, University of Alabama, Birmingham, AL; 51) was as follows: One million cells in 1 ml were exposed to $10^4$ 50% tissue culture infectious doses of virus-containing supernatant for 2 h at 37°C. After washing, the cells were resuspended in growth medium and cultured for 6–8 d. At this time 100% of the cells expressed large amounts of viral envelope glycoproteins but no CD4 as detected by indirect immunofluorescent staining with mAbs to domain 1 and domain 4 of CD4 and by flow cytometric analysis.

*Analysis of Antibody Binding by Flow Cytometry.* H9 cells infected with Hx10 virus as described above were washed twice in RPMI 1640/5% FCS and were resuspended at a concentration of $2 \times 10^6$ cells/ml. 50 μl of mAb previously diluted in PBS/1% FCS (wash buffer [WB]) were added to 50 μl of cell suspension in a U-bottomed, 96-well microtiter plate and incubated with agitation at 37°C for 2 h. The cells were washed three times in WB and then fixed in WB plus 2% formaldehyde overnight at 4°C. After two further washes in WB, an appropriate dilution of fluorochrome-conjugated second layer antibody was added: anti–human PE or anti–mouse PE (obtained from Immunotech Inc., Marseille, France), or anti–rabbit PE (Sigma Chemical Co., St. Louis, MO). The cells were washed twice as before and then analyzed by flow cytometry using a FACScan® with Consort 30 software (Becton Dickinson and Co., San Jose, CA).

*Kinetic Analysis of mAb Binding.* The kinetic analysis of mAb binding to cell surface antigens using indirect immunofluorescence and flow cytometric analysis has been previously described (52). We have adopted a similar strategy to estimate the rates of mAb association and dissociation and their relative affinities. To estimate the rate of association, we determined the $t_{1/2}$ required for the mAbs to reach equilibrium binding conditions. Although this is not a formal measurement of the mAb association constant, it serves to give an indication of the on-rate. mAbs were diluted in RPMI 1640/5% FCS, added to $10^6$ H9 cells/well of a U-bottomed 96-well microtiter plate to give final concentrations of 10, 1, 0.1, or 0.01 μg/ml in a total volume of 100 μl, and incubated with agitation at 37°C. At the times indicated, 10 μl ($10^5$ cells) were diluted 20-fold in ice-cold WB, centrifuged to remove excess mAb, and then resuspended in WB plus 2% formaldehyde. After overnight fixation, the cells were washed twice as before and then resuspended in the appropriate dilution of anti–species IgG–PE conjugate. Cells were agitated for 2 h at 4°C and then washed and analyzed by flow cytometry as described above. To estimate the rate of dissociation, mAbs at 50 μg/ml were incubated with $10^6$ cells in 100 μl RPMI 1640/5% FCS for 2 h at 37°C with agitation to achieve maximum binding, and then washed twice and resuspended in 100 μl of RPMI 1640/5% FCS and incubated with agitation at 37°C. At the time points shown in Fig. 5, 10 μl of cell suspension was removed and fixed in a 20-fold volume of ice-cold WB/2% formaldehyde overnight at 4°C. The following day the cells were washed, stained with PE conjugate, and analyzed as described above.

*ELISA Assays for mAb Binding and Measurement of mAb-induced gp120 Dissociation.* mAb binding assays were carried out using purified recombinant gp120 molecules derived from the BH10 molecular clone of the HIV-1 LAI isolate. The BH10 glycoprotein expressed in Chinese hamster ovary cells was batch 42 prepared by Celltech Ltd. (Slough, UK) (48) and obtained from the UK Medical Research Council AIDS Directed Programme. BH10 gp120 is of the same primary sequence as gp120 in the infectious molecular clone Hx10. The detection of mAb binding in an ELISA format was essentially as described elsewhere (23). Briefly, recombinant gp120 was captured onto a solid phase via its COOH terminus using sheep polyclonal antibody D7324. The gp120 was added at 25 ng/ml in Tris-buffered saline (TBS) containing 10% FCS. mAbs were added to the gp120 in TBS containing 2% nonfat milk powder and 20% sheep serum. Bound mAb was detected as previously described (23). No detergent was used at any stage to ensure that the native folding of the glycoprotein was not perturbed. Release of gp120 from the surface of Hx10-infected, mAb-treated cells was determined as follows: Three hundred thousand cells in 30 μl, previously incubated at 37°C for 4 h with prebound mAb as described above for the mAb dissociation studies, were pelleted. The super-

natants were harvested and spun at 12,000 rpm for 2 min in microfilters (Spin-X; Costar, Brumath, France) to remove remaining cells and cell debris, and 3 μl of a 10% solution of NP-40 was added. To prevent interference with the gp120 detection, the samples were denatured to eliminate prebound mAb. To do this, SDS and DTT were added to 20 μl of supernatant to yield final concentrations of 1% and 50 mM, respectively. After boiling, the treated supernatants were diluted in 180 μl of TBS/FCS (10%)/NP-40 (1%). 100 μl was then added to duplicate wells of an ELISA plate precoated with the gp120 capture antibody D7324. The detection and calibration of denatured gp120 in the supernatant was carried out as previously described using the murine mAb B13 (from G. Lewis, University of Maryland Medical School, Baltimore, MD), which reacts preferentially with denatured gp120 (28). B13 recognizes a continuous epitope in the gp120 C2 domain (28).

*HIV Neutralization Assays.* To compare mAbs for neutralization activity, we used an assay based on infection of HeLa cells expressing human CD4 and the HIV LTR gene fused to lacZ (53). Infection of these cells with HIV leads to the production of the viral TAT protein, which transactivates the transfected LTR and activates the lacZ gene; addition of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Gibco BRL, Gaithersburg, MD) then results in blue coloration of the infected cells. The advantage of this assay system is that infection of the cells can be detected within 16 h of infection (one cycle of replication), and the infected cells can be easily quantified by visual inspection. 20 μl of a previously titrated suspension of HIV containing ~200 infectious U for the HeLa-CD4-LTR LacZ cells was preincubated with dilutions of the mAbs in DME/5% FCS for 2 h at 37°C in a total volume of 40 μl before addition of 10 μl of HeLa cells at a concentration of $5 \times 10^6$ cells/ml. After 1 h incubation at 37°C with agitation, the cells were washed in PBS/10 mM EDTA and incubated with EDTA-trypsin (Gibco BRL) for 15 min at 37°C. After washing in DME/5% CS, the cells were cultured overnight in flat-bottomed 96-well microtiter plates. The following day the cells were fixed with 1% formaldehyde for 10 min and then stained in a solution of X-Gal for 4 h at 37°C. Cells were then washed once in WB and fixed overnight in WB/2% formaldehyde. Blue-stained cells were counted by eye at a magnification of 100.

## Results

*Binding of Neutralizing Antisera to HIV-infected Cells and Recombinant gp120.* HIV-reactive polyclonal antisera obtained either from seropositive individuals or from animals immunized with recombinant gp120 are neutralizing for HIV-1 in vitro (54). The reactivity of immune sera with gp120 is generally measured using recombinant or purified gp120 in a monomeric form by techniques such as ELISA or Western blotting. Since our aim was to measure the binding of neutralizing antibodies to functional, virion-associated gp120, it seemed appropriate to compare antiserum reactivity with monomeric gp120 in ELISA with that with infected, cell-associated gp120. To do this we stained the H9 T cell line infected with the LAI molecular clone Hx10 with the different antisera listed in Table 1 and detected antibody binding by indirect immunofluorescence and flow cytometric analysis as previously described (55, 56). The binding of a panel of antisera of human or rabbit origin either to infected cells or to recombinant BH10 gp120 in ELISA are shown in Fig. 1, *A* and *B*, respectively. In ELISA, saturation binding for the human sera was achieved at dilutions of between 1/100 and 1/300, and, for the rabbit sera, at ~1/100. By contrast, saturation was not achieved with these sera in the infected cell system, and the binding curves were clearly displaced towards the right, suggesting lower avidity of the anti-HIV-1 antibodies for membrane-bound gp120 than for recombinant gp120. Most striking was the very poor binding of the two rabbit antisera to the infected cells compared with the recombinant glycoprotein. Although the two assays used are not easily comparable in terms of absolute numbers of gp120 molecules expressed, it seemed unlikely that quantitative differences in the amount of gp120 present in each system could explain the large differences in the serum binding. Although these data suggest that there are differences in the binding of anti-gp120 antibodies to monomeric gp120 as compared

**Table 1.** *Antibodies Used in This Study*

Antibodies

Antisera

| Name | Antigen* | Species | Reference |
|---|---|---|---|
| QC2, QC5, QC6 | Virion | Human | 50 |
| LWS | Virion | Human | 51 |
| R1/87 | rgp120 | Rabbit | |
| PB26 | rgp120 | Rabbit | 52 |

mAbs of murine origin

| Location | Name | Antigen | Epitope‡ | References |
|---|---|---|---|---|
| V2 | BAT085 | pgp120 | 169-180 | 18, 19 |
| | G3-136 | pgp120 | 169-183 | 18, 19 |
| | G3-4 | pgp120 | 170-180 | 17, 19 |
| V3 | 8/38c | rgp120 | 300-315 | 16 |
| | 9284 | Virion | 301-312 | 8 |
| | BAT123 | pgp120 | 308-322 | 4, 12 |
| | 110.5 | pgp120 | 310-317 | 9 |
| | 110.1 | rgp120 | 316-322 | 28 |
| C4 | Group | pgp120 | 429-438 | 21, 23 |

mAbs of human origin

| Location | Name | Antigen | Epitope | References |
|---|---|---|---|---|
| CD4 B/S§ | 15e | Virion | Discontinuous | 24, 25 |
| | 21h | | | 26 |
| Complex‖ | 48d | Virion | Discontinuous | 27 |
| | 17b | | | 27 |

* Immunizing antigens are defined as follows: virion, serum or mAb obtained from an HIV-seropositive individual; rgp120, recombinant, soluble gp120; pgp120, gp120 purified from virions.

‡ Epitope refers to the sequence numbering of the HXB2 molecular clone taken from the Los Alamos database 1992.

§ *CD4 B/S*, a discontinuous epitope that overlaps but is not identical to the CD4 binding site on gp120.

‖ *Complex*, a complex, discontinuous epitope, binding of mAbs to which does not interfere with CD4 binding.

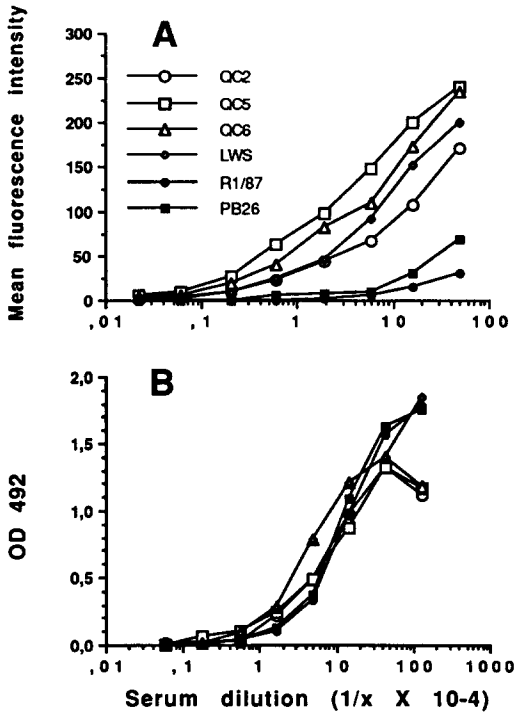


**Figure 1.** Binding of neutralizing antisera to recombinant and cell surface gp120. For cell surface staining (*A*), HIV-1–infected cells were incubated with dilutions of the antisera for 2 h at 37°C, washed, and then stained with the appropriate antibody–fluorochrome conjugate for 1 h at 4°C. After washing and fixation in formaldehyde, the samples were analyzed by flow cytometry using Consort 30 software. Each point represents the mean fluorescence intensity for 10,000 accumulated events. Analysis of mAb binding to recombinant gp120 (BH10) captured by an mAb reactive with the COOH-terminal residues onto the solid phase was done by ELISA (*B*). Results are expressed as the optical density at 492 nm. The symbols represent the same antisera in both *A* and *B*.

with functional, multimeric gp120, there are factors that complicate their interpretation. Polyclonal antisera derived from infected individuals contain antibodies reactive with several viral proteins, including gp41 and the HIV core proteins p18 and p24, all of which can be detected on the surface of infected cells (57, 58). Such antisera also contain cell surface–specific reactivity (for review, see reference 59), although this was found to be a minor component of the signal since uninfected H9 cells stained very weakly or undetectably with these sera (data not shown). Antiserum reactivity with molecules other than gp120 on the cell surface would increase the overall level of staining but dilute the gp120-specific signal, thus decreasing the apparent difference between cell surface and recombinant gp120. We therefore decided to dissect out the various components of the gp120 neutralizing humoral immune response present in the sera using mAbs of defined specificity.

*Binding of Neutralizing mAbs to HIV-infected Cells and Recombinant gp120.* The spectrum of neutralizing epitopes on HIV-1 gp120 is broadly represented by the five clusters of mAbs listed in Table 1. These mAbs are of murine, rat, or human origin and were selected as representative of a variety of different epitopes within each cluster. Each of the mAbs listed has been previously characterized in terms of soluble gp120 binding, epitope specificity, and to some extent HIV-1 neutralization activity. Little is known, however, about their ability to interact with natively folded, gp41-associated, multimeric gp120. Here we have compared the binding of these mAbs to recombinant gp120 in ELISA with their binding to multimeric gp120 present on HIV-infected cells (Fig. 2). Saturation binding to monomeric gp120 in ELISA was achieved with all mAbs tested within a concentration range of 1–10 nM. By contrast, saturation binding to cell surface gp120 was observed only with the V3 mAbs at concentrations between 1 and 3 nM, and with certain human mAbs reactive with complex and CD4/B5 epitopes at concentrations of 30–100 nM. None of the mAbs in the V2 or C4 groups achieved equilibrium binding under the experimental conditions used. The ability of V3 mAbs to saturate gp120 molecules expressed at the cell surface at concentrations comparable to those required to saturate monomeric gp120 implies that the variation in mAb binding between monomeric and cell surface gp120 is unlikely to simply reflect different numbers of gp120 molecules in the two systems; there is clearly a substantial difference between certain of the mAb groups in the strength of their interactions with the two forms of gp120. We tested concentrations of up to 1,334 nM (200 μg/ml) yet were still unable to achieve saturation binding with the V2 and C4 mAbs (data not shown). The poor affinity of the non-V3 mAbs for membrane-associated gp120 is clear from the results presented in Table 2. To obtain an estimate of the relative affinities of the mAbs, we calculated the half-maximal binding values for each mAb for which saturation was achieved and estimated the minimal values for those with which we were unable to obtain saturation. The difference in these values between monomeric and oligomeric gp120 mAb binding varies between none (V3 mAb 110.I) and >350-fold (V2 mAb G3-4).

*mAb Binding Kinetics.* It is unclear from the data presented in Fig. 2 whether the poor mAb binding to multimeric gp120 results from (*a*) a slow association rate; (*b*) a rapid dissociation rate; (*c*) a combination of the two factors; or (*d*) mAb binding inducing a conformational change in gp120 leading to gp120–gp41 dissociation. This latter possibility would give rise to the impression that mAb affinity was artificially low, as has been previously demonstrated for sCD4 binding to virion gp120 (2). The measurement of mAb binding kinetics to cell membrane–anchored molecules by indirect immunofluorescent staining and flow cytometric analysis has been previously described (52). Using similar methodology, we have estimated the relative rates of association and dissociation of each of the mAbs for multimeric, virion-associated gp120. Because we were unable to accurately measure the initial rates of mAb association using this system, we have determined the $t_{1/2}$ for each mAb to reach equilibrium binding. Although this does not yield a formal measurement of the mAb association constant, it serves to give an indication of the on-rate, assuming that the dissociation rate is similar for all mAbs. Fig. 3 shows the binding of mAbs from each of the epitope groups to cell surface gp120 at different times after addition. There are obvious differences in the cell binding

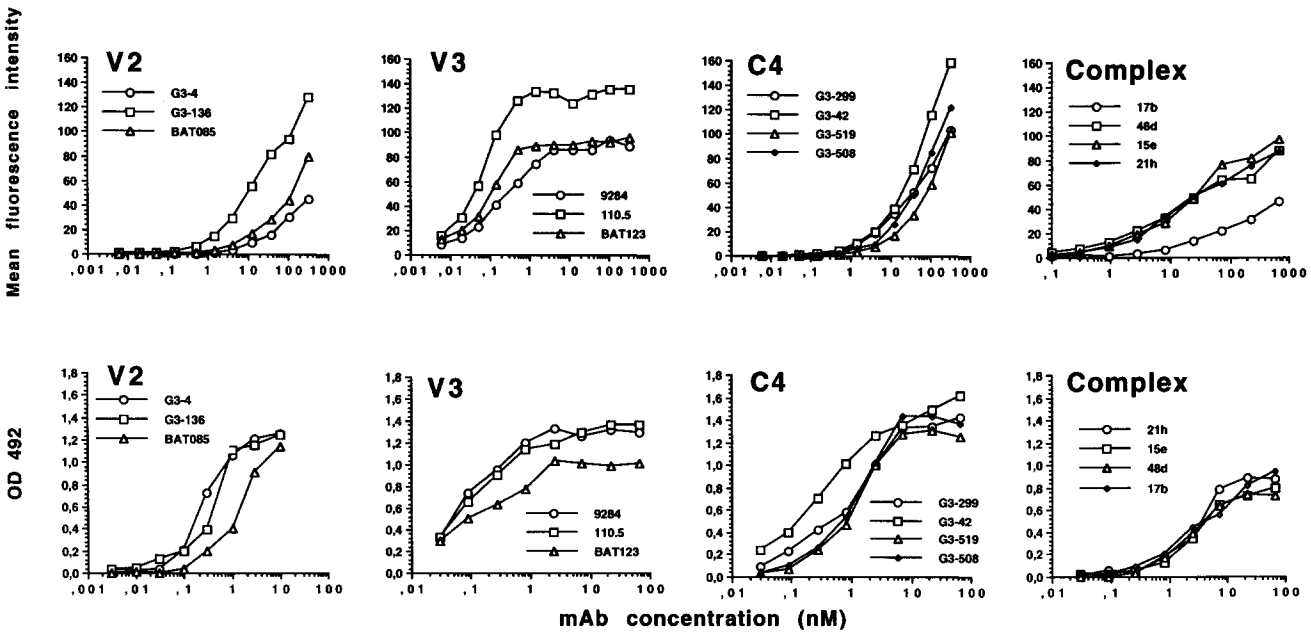


**Figure 2.** Binding of neutralizing mAbs to recombinant and cell surface gp120. (*Top*) HIV-infected cells were stained for mAb binding by indirect immunofluorescence and analyzed by flow cytometry as described in Fig. 1. (*Bottom*) mAb binding to recombinant gp120 in ELISA was carried out as described in Fig. 1.

**Table 2.** *mAb Binding and Neutralization Parameters*

| Region | mAb | Half-maximal binding | | Neutralization | Association | Dissociation |
|---|---|---|---|---|---|---|
| | | Monomer* | Oligomer‡ | | | |
| | | *nM* | | *$ID_{50}$ in nM* | *$t_{1/2}$ (min)* | *$t_{1/2}$ (min)* |
| V2 | G3-136 | 0.4 | >20§ | 7.7 | 40 | >240‖ |
| | G3-4 | 0.2 | >70 | 44.4 | >60¶ | >240 |
| | BAT085 | 1.0 | >70 | 133.4 | >60 | 200 |
| V3 | 110.5 | 0.07 | 0.05 | 0.2 | <5 | 240 |
| | 9284 | 0.07 | 0.12 | 0.93 | <5 | 240 |
| | BAT123 | 0.08 | 0.08 | 0.13 | <5 | 240 |
| | 110.I | 0.45 | 0.5 | 1.3 | 10 | 240 |
| | 8/38c | 10 | 66.7 | 66.7 | 30 | 100 |
| C4 | G3-299 | 1.0 | >50 | 4.1 | 40 | 240 |
| | G3-519 | 1.0 | >50 | 98.4 | >60 | >240 |
| | G3-536 | 1.2 | >50 | 266.8 | >60 | 120 |
| | G3-508 | 1.2 | >50 | 186 | >60 | 240 |
| | G3-42 | 0.3 | >50 | ND | >60 | 240 |
| CD4/BS | 15e | 2.5 | 20 | 4.8 | 20 | >240 |
| | 21h | 2.5 | 20 | 6.0 | 30 | >240 |
| Complex | 48d | 2.5 | 5.0 | 1.3 | 20 | 120 |
| | 17b | 2.0 | >80 | 50 | >60 | 240 |

* Monomeric, recombinant gp120 was captured onto the solid phase and screened for mAb binding by ELISA.
‡ Multimeric gp120 was in the form of the HIV envelope glycoproteins expressed at the surface of infected cells.
§ Minimum half-maximal binding values could not be calculated for mAbs with which saturation staining was not achieved; results are the mean of at least two separate experiments.
‖ Minimum rates of dissociation could not be calculated for mAbs that did not reach 50% dissociation within the time of the experiment.
¶ Minimum and maximum rates of association could not be accurately determined for mAbs that reached equilibrium much faster than we were able to measure or that did not reach equilibrium within the time of the experiment, respectively.

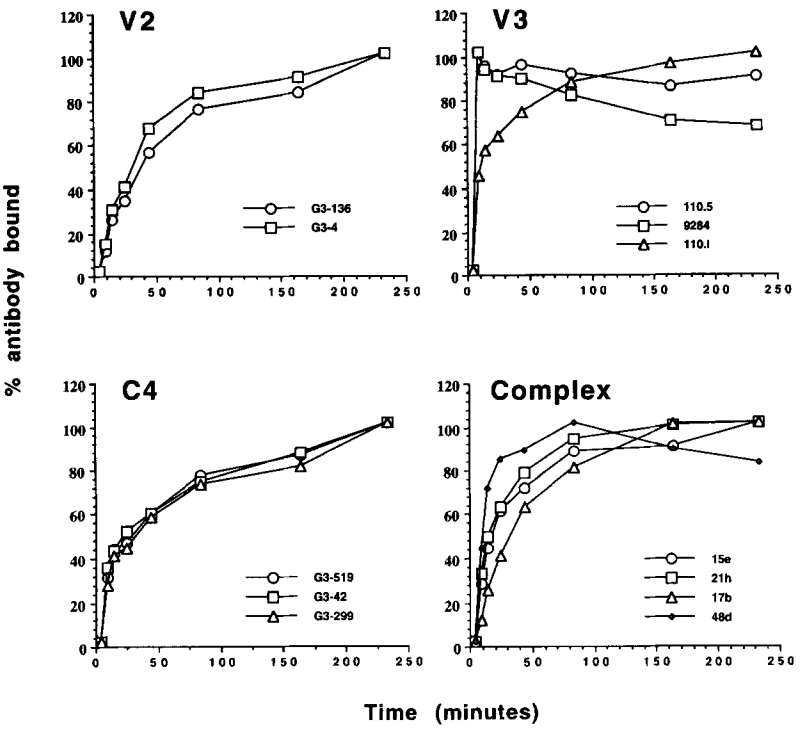


**Figure 3.** mAb association rates. HIV-infected cells were incubated with mAbs at 37°C for different times before washing and fixation. Cells were then stained with the appropriate anti-species Ig coupled to PE before analysis by flow cytometry as described in Fig. 1. The mAb binding curves shown in this figure were carried out using a concentration of 10 μg/ml.

kinetics of the mAb clusters; the majority of V3 loop mAbs reach equilibrium binding in <5 min (the first measurable time point), whereas the other mAbs require 1–2 h or longer. The $t_{1/2}$ values for each mAb to reach equilibrium binding are shown in Table 2. There is a spectrum of rates ranging from <5 min to >1 h. A comparison of the values for the half-maximal binding and the $t_{1/2}$ to saturation for each mAb confirms that there is a broad relationship; those mAbs with the highest relative affinity for oligomeric gp120 (V3, CD4 binding site mAbs, and mAb 48d) have the fastest estimated on-rates, whereas those with the lowest relative affinity (V2, C4, and mAb 17b) have the slowest on-rates. By contrast, there was no overall relationship between the rates of dissociation and the relative affinity of each mAb for oligomeric gp120. Fig. 4 shows the dissociation curves for each group of mAbs; all mAbs had similar dissociation kinetics with the exception of four: BAT085 (V2), 8/38c (V3), G3-536 (C4), and 48d (complex). It should be noted that, since the dissociation rate is higher than for the other mAbs tested, the estimated relative $t_{1/2}$ for the association of mAbs BAT085, 8/38c, G3-536, and 48d will be underestimated. The half-maximal dissociation rates are presented in Table 2. A few of the mAbs with very low affinity for the monomer, such as 8/38c and BAT085, have relatively rapid dissociation kinetics, suggesting that the rate of dissociation from oligomeric gp120 may influence the relative affinity of a minority of mAbs.

The apparent mAb affinity will be influenced by loss of gp120 from the cell surface; gp120 and gp41 from cell line–passaged HIV-1 dissociate spontaneously at 37°C (for review see reference 2), and soluble CD4 binding increases this dissociation (55, 56; for review see reference 2). Since we were interested only in the relative differences between mAbs, the influence of gp120 loss on absolute rates of mAb association and dissociation was discounted. It was necessary, however, to determine whether the binding of particular mAbs to gp120 influenced the rate of gp120–gp41 dissociation, since this would alter the apparent rates of mAb association/dissociation. Fig. 5 shows the quantity of soluble gp120 released after 4 h of incubation of mAb-precoated, HIV-infected cells. Gp120 dissociation was modified compared to the basal level in the presence of several of the mAbs; the effect in this experiment was relatively subtle, however, in that the greatest mAb-induced changes in gp120 dissociation were an increase of 1.3-fold in the presence of mAb 110.I, and a decrease of 1.5-fold in the presence of mAb G3-4. Thus, although the binding of certain mAbs apparently modulates gp120 dissociation, these changes appear to be relatively small and do not influence the overall interpretation of the results.

*Neutralizing Activity of gp120 mAbs.* Although all of the mAbs described here have previously been characterized as virus neutralizing, there has not been a systematic study in which the same assay system has been used to compare the HIV-1 neutralization ability of mAbs from different clusters.

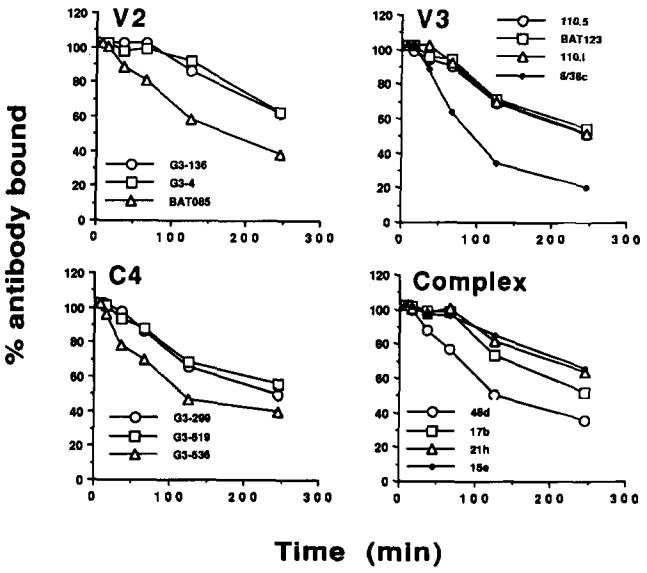


**Figure 4.** mAb dissociation rates. HIV-infected cells were preincubated with mAbs at 50 $\mu$g/ml at 37°C for 2 h before washing and reincubation at 37°C. At the times shown, aliquots of cell suspension were removed, washed, and fixed before washing, staining, and analysis as described for Fig. 3.

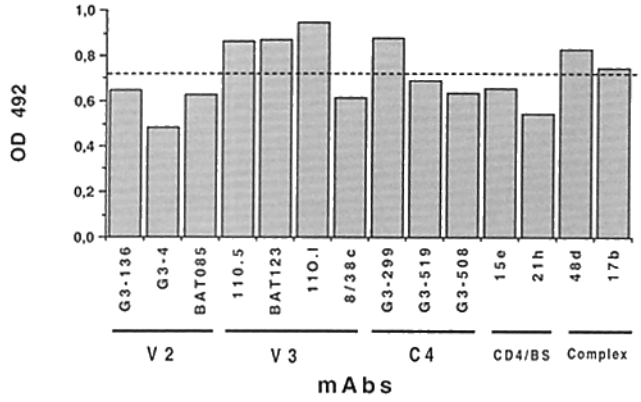


**Figure 5.** mAb-induced gp120 dissociation. Cells preincubated with mAbs as described for Fig. 4 were washed and reincubated at 37°C for 4 h. The cells were pelleted, and the supernatant was spun through 0.22-$\mu$m filters to remove cell debris and treated with NP-40, 1% final concentration. Gp120–mAb complexes in the supernatants were then denatured to prevent interference of gp120 detection by bound Ig, and gp120 concentration was measured by ELISA. Each bar represents the mean signal obtained from duplicate ELISA wells, and the dotted line represents the level of spontaneous gp120 dissociation.

Moreover, their binding to oligomeric gp120 has not been compared with their neutralizing capacity. We have therefore chosen a rapid and quantitative assay of HIV neutralization based on the detection of HIV TAT after infection of $CD4^+$ HeLa cells containing the HIV LTR fused to the LacZ operon (53). Using this assay system, HIV neutralization can be detected within one cycle of virus infection. Virus neutralization was measured by preincubation of a pretitered HIV stock with various concentrations of the different mAbs before addition to the indicator cells. Fig. 6 shows the neutralization obtained with a selection of the mAbs from the five different clusters. All mAbs neutralized virus within approximately a three-decade range (0.1–300 nM), confirming (with the exception of the V3 mAb 8/38c, which had been described as nonneutralizing in the absence of sCD4; 16) their previously described neutralization activity. The V3 mAbs were clearly the most potent, with all but one mAb giving $ID_{50}$ values ranging from 0.13 to 1.3 nM (Table 2). Neutralization was variable within the other groups; V2, C4, and conformational clusters all contained mAbs that gave $ID_{50}$ values of 1–10 nM but also contained mAbs with much weaker neutralization activity (50–267 nM). A comparison of the data in Table 2 suggests that there may be a limited correlation between mAb binding to monomeric gp120 and neutralization, in that the V3 mAbs that bind with the highest relative affinity have the highest neutralization potency. Outside of this group of mAbs, however, there is no clear relationship. By contrast, there appears to be a general correlation between mAb neutralization capacity, relative affinity, and estimated rate of association for oligomeric gp120. As with monomeric gp120, mAbs from the V3 loop cluster bound with the highest relative affinity to oligomeric gp120 and had the strongest neutralizing activity. Outside of this group the pattern was also consistent, in that those mAbs with the highest relative affinities and the fastest rates of association were the best neutralizers, and those with lower relative affinities and slower rates of association were the poorest neutralizers.

## Discussion

In this study we report that antibody binding to monomeric gp120 does not necessarily reflect the level of antibody binding to multimeric, gp41-associated virion gp120, and that the efficiency of mAb binding to oligomeric gp120 is a good indicator of mAb potency as a neutralizing agent. Several points arising from these data have implications for vaccine design and testing.

Our results indicate that, whereas immune antisera raised against live virus during infection with HIV-1 bind well to recombinant gp120, they bind considerably less well to oligomeric, gp41-associated gp120. This implies that a large proportion of the antibody response to HIV infection is against regions of gp120 that are poorly exposed on the oligomer, i.e., most regions other than the V3 loop. This is in agreement with data previously published by our laboratories demonstrating that the antibody specificities most prevalent in human antisera are those to discontinuous cross-reactive epitopes, in particular the CD4 binding site (60). Moreover, we and others have recently demonstrated that certain nonneutralizing mAb epitopes that are present on recombinant, soluble gp120 are completely or partially obscured on oligomeric gp120 (28, 61). It seems likely that this explains at least in part the poor reactivity for oligomeric gp120 of the rabbit antisera raised against recombinant gp120; such antisera contain a large proportion of antibodies that will react preferentially with the monomeric glycoprotein. The reason for the loss of epitopes on native, oligomeric gp120 may be the result

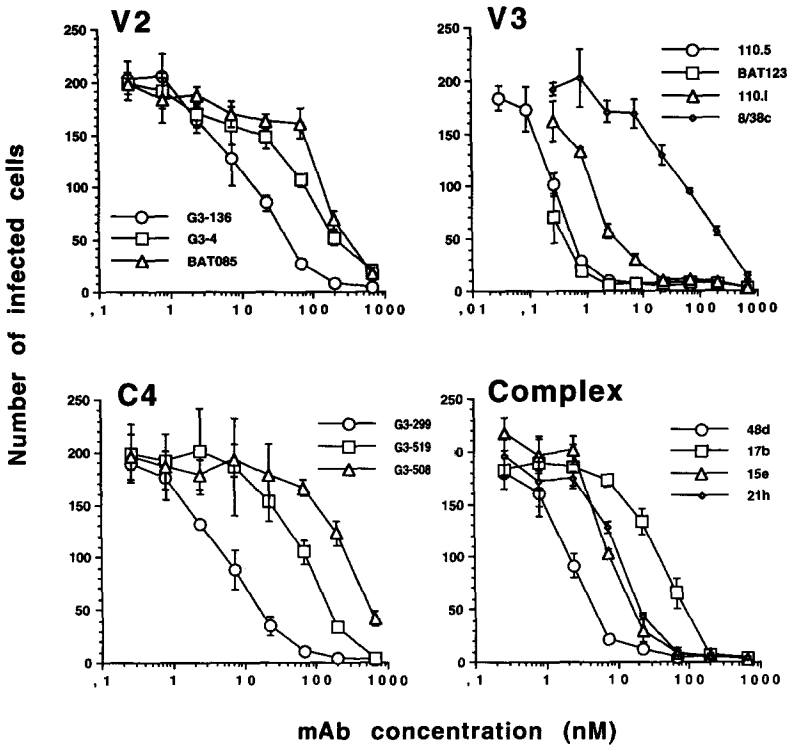


**Figure 6.** mAb neutralization of HIV-1. A stock of virus previously titered on HeLa-CD4-LTR LacZ cells and known to give 200 infected cells/well in 20 μl was incubated for 2 h with mAbs at the concentrations shown in the figure before washing and trypsinization to remove noninternalized virions. The cells were subsequently cultured for 24 h at 37°C before fixation and staining with X-Gal for 4 h at 37°C. The cells were then fixed overnight, and the blue foci were counted by eye under magnification. Each point represents the mean of triplicate wells, and the vertical bars indicate ±1 SD.

of one or several of the following factors: (*a*) epitope masking by gp120–gp41 association; (*b*) epitope masking by gp120–gp120 association; (*c*) condensation of the folded protein leading to epitope masking by carbohydrate groups; or (*d*) alteration of epitope conformation after oligomeric folding and gp41 association. Whatever the molecular basis of the difference in epitope exposure, these data suggest that the use of recombinant, soluble, monomeric glycoprotein for vaccine immunization is likely to shift the immune focus away from neutralizing epitopes present on the oligomer to less relevant epitopes present on the monomer. The effect of this will be to induce a large proportion of the antibody response to epitopes that are poorly represented on the virion surface. The sole exception to this may be the V3 loop, which is well exposed on both the oligomeric and monomeric forms of the T cell line–adapted virus that we have studied. However, one recent study (61) presented evidence that immunization of mice with soluble, gp41-associated oligomeric gp120 resulted in a low frequency of V3 loop reactivity, suggesting that the V3 loop may not be an immunodominant domain in oligomeric gp120. This conclusion is in accord with the finding that the V3 loop of a monocytotropic HIV-1 primary isolate appears to be cryptic on the virion surface (62). Such masking of V3 epitopes on non–cell line–passaged virus may explain our recent observation that HIV-1 primary isolates are relatively poorly neutralized by V3 mAbs, which are potent neutralizers of T cell line–adapted viruses such as the Hx10 clone used in the present study (63, 64).

Antibody affinity is determined by the ratio between the association and dissociation rate constants. It had until recently been considered that the dissociation rate was the major determinant for almost all mAbs, although this assumption was based on the study of antibodies with small synthetic haptens (52). More recent work, in which antibody binding to more complex antigens such as membrane-anchored molecules has been analyzed, suggests that the association rate may play an equal or greater role in determining affinity (52). Our study is in accord with this latter conclusion, since the estimated association rate of mAbs for oligomeric gp120 appears to be the major factor influencing affinity. Interestingly, another group has demonstrated that the major determinant in mAb binding to the V3 loop of soluble, recombinant gp120 is the dissociation rate (65). Thus, antigens may react very differently with antibodies depending on their presentation; in this case soluble monomeric forms compared with the more physiologically relevant, membrane-anchored, oligomeric forms. One interpretation of these differences is that, on the complex, oligomeric form of the viral envelope glycoprotein, many epitopes that are potentially capable of high affinity interactions with an antibody are shielded from that antibody. Thus, the rate at which an antibody can gain access to these sites will dominate the parameters of the binding and neutralization reaction. On monomeric forms of these proteins or on peptide fragments, epitope accessibility is often a less important factor, and the dominant influence on the antibody–antigen affinity is, as is commonly found, the dissociation rate. We have not, however, measured mAb binding kinetics for the monomeric form of gp120 in the present study and are thus unable to make a direct comparison of the two forms of the glycoprotein with the panel of mAbs used. A confounding factor in the measurement and interpretation of mAb binding to gp41-associated gp120 is the phenomenon of gp120–gp41 dissociation (55, 56). We have determined the dissociation of gp120 from infected cells in the presence

and absence of prebound mAbs and note that the binding of certain mAbs does appear to influence dissociation (Sattentau, Q., unpublished data, and see Fig. 5). While this observation does not invalidate our conclusions regarding mAb binding to oligomeric gp120, it suggests that mAb binding may induce conformational changes in gp120 that are akin to those induced by sCD4 (55, 56). Documenting this phenomenon more precisely and determining its contribution to virus neutralization are subjects of our further investigation.

The rate of antibody association with a viral glycoprotein will clearly influence the ability of that antibody to neutralize virus in vivo as well as in vitro. The release of an HIV virion from a cell will be followed by the binding of that virion to its receptor on another cell. If the on-rate of a neutralizing antibody is in the order of seconds, then there is a good chance that the virus will be successfully neutralized. If, however, the $t_{1/2}$ for equilibrium binding is of the order of hours or even minutes, as is the case for many of the mAbs studied here, then the virus will have a strong possibility of escaping neutralization by infecting a target cell. This scenario is particularly relevant in the case of HIV-1, where virus is released in large quantities into the lymphoid tissue where $CD4^+$ T cells are densely packed (59). Any reduction in the on-rate of an antibody–antigen interaction could yield a profound selective advantage to HIV replication in vivo (63).

Our demonstration of a broad correlation between mAb binding to oligomeric gp120 and HIV-1 neutralization implies that mAb affinity may be the primary neutralization determinant, regardless of the mechanism of viral inactivation. Exceptions to this relationship may exist, however; Stern et al. (66) have recently shown that neutralization sensitivity of a mutant compared to the wild-type clone of HIV-1 did not correlate with mAb binding to oligomeric gp120. This suggests that in certain cases mAb binding affinity may be secondary to other factors in determining neutralization efficiency. The nature of such factors is currently obscure.

The binding and neutralization data that we report here have all been carried out on a single virus clone derived from the prototype LAI isolate of HIV-1 (51). This virus was isolated, and has been passaged extensively, in immortalized T cell lines. HIV-1 passage in cell lines as opposed to primary lymphocyte cultures has been shown to alter the biological properties of HIV-1 virions; they become more fusogenic, have a higher affinity for CD4, and are more easily neutralized (2, 59, 63). Preliminary evidence from our laboratory indicates that the relative resistance of primary lymphocyte–passaged HIV to neutralization may be due to an even more profound masking of epitopes on the oligomeric form of gp120 (Sattentau, Q., unpublished data). Study of mAb binding to oligomeric forms of the envelope glycoproteins of these and other viruses will yield valuable information on mechanisms of neutralization.

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