JOURNAL OF VIROLOGY, Feb. 1996, p. 753–762
0022-538X/96/$04.00+0

Vol. 70, No. 2

Humoral Response to Oligomeric Human Immunodeficiency Virus Type 1 Envelope Protein

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Received 1 June 1995/Accepted 18 October 1995

The humoral immune response to human immunodeficiency virus type 1 (HIV-1) is often studied by using monomeric or denatured envelope proteins (Env). However, native HIV-1 Env complexes that maintain quaternary structure elicit immune responses that are qualitatively distinct from those seen with monomeric or denatured Env. To more accurately assess the levels and types of antibodies elicited by HIV-1 infection, we developed an antigen capture enzyme-linked immunosorbent assay using a soluble, oligomeric form of HIV-1_{IIIB} Env (gp140) that contains gp120 and the gp41 ectodomain. The gp140, captured by various monoclonal antibodies (MAbs), retained its native oligomeric structure: it bound CD4 and was recognized by MAbs to conformational epitopes in gp120 and gp41, including oligomer-specific epitopes in gp41. We compared the reactivities of clade B and clade E serum samples to captured Env preparations and found that while both reacted equally well with oligomeric gp140, clade B seras reacted more strongly with monomeric gp120 than did clade E samples. However, these differences were minimized when gp120 was captured by a V3 loop MAb, which may lead to increased exposure of the CD4 binding site. We also measured the ability of serum samples to block binding of MAbs to epitopes in gp120 and gp41. Clade B serum samples consistently blocked binding of oligomer-dependent MAbs to gp41 and, to a slightly lesser extent, MAbs to the CD4 binding site in gp120. Clade E serum samples showed equivalent or greater blocking of oligomer-dependent gp41 antibodies and considerably less blocking of CD4-binding-site MAbs. Finally, we found that <5% of the antibodies in clade B sera bound to epitopes present only in monomeric gp120, 30% bound to epitopes present in both monomeric gp120 and oligomeric gp140, and 70% bound to epitopes present in oligomeric gp140, which includes gp41. Thus, captured oligomeric Env closely reflects the antigenic characteristics of Env protein on the surface of virions and infected cells, retains highly conserved epitopes that are recognized by antibodies raised against different clades, and makes it possible to detect a much greater fraction of total anti-HIV-1 Env activity in sera than does native monomeric gp120.

The envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1), gp120 and gp41, are targets of neutralizing antibodies in infected individuals (25, 38, 46). Much of the effort to define and harness the humoral immune response has focused on the surface glycoprotein, gp120 (1, 3, 37, 44, 52). Indeed, a number of potent, broadly neutralizing human monoclonal antibodies (MAbs) and recombinant human Fab fragments directed to epitopes in gp120 have been described (2, 6, 17, 20, 21, 41, 45, 54, 56). However, antibodies that effectively neutralize primary isolates of HIV-1 have proven difficult to elicit by immunization with Env protein subunits (19, 27, 29). One possible explanation for the failure of candidate Env subunit vaccines to elicit antibodies that neutralize primary isolates is that they have not preserved conformational structures on the envelope protein to which broadly cross-reactive antibodies bind. That Env protein conformation is an important determinant for the humoral response is indicated by the findings that antibodies to conformational epitopes are prevalent in human sera and that most broadly cross-reactive neutralizing antibodies characterized to date recognize discontinuous epitopes in gp120 (37, 52). Thus, identifying the factors that contribute to native Env protein conformation will be important for vaccine development and for characterizing the humoral response to infection and the mechanisms of neutralization.

Recent studies have shown that the oligomeric nature of the HIV-1 envelope protein may strongly influence its antigenic structure (4, 11, 30, 34). Like most other viral membrane proteins (10), HIV-1 Env forms an oligomeric complex shortly after synthesis, with both dimers and higher-order structures having been reported (12, 15, 40, 50, 55, 58). Although assembly initially involves the gp160 precursor, the oligomeric nature of Env is retained following cleavage into gp120 and gp41 subunits. As a consequence, the Env protein exists as an oligomer both on the surface of infected cells and in virus particles, with each oligomer containing several gp120 and associated gp41 subunits. We have shown that immunization with soluble, native, oligomeric HIV-1 Env results in the efficient production of broadly cross-reactive antibodies to conformational determinants in both gp120 and gp41 (4, 10a, 11). Of particular note was the finding that large numbers of MAbs to epitopes in the gp41 ectodomain react exclusively or preferentially with oligomeric protein (4). The strong influence of quaternary interactions on gp41 antigenic structure is consistent with its role in subunit-subunit interactions and Env protein assembly (12, 14, 42, 55). By contrast, a significant number of MAbs to epitopes in gp120 reacted more strongly with mono-

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meric protein, suggesting that some regions of gp120 are masked or altered by interactions with adjoining gp120 or gp41 subunits (34). These findings may have important consequences: Sattentau and Moore have found that antibody binding to monomeric gp120 does not necessarily predict strong binding to oligomeric gp120 on virions and that the efficiency of antibody binding to oligomeric Env correlates well with neutralization activity (48). This may be particularly important for primary clinical isolates, for which Moore et al. have proposed that access to neutralizing epitopes around the CD4 binding site are restricted in the Env protein oligomer relative to monomeric gp120 (30). As a consequence, it will be important to identify and characterize neutralizing antibodies that bind strongly to epitopes that are better represented on gp120 and gp41 oligomers than on monomeric protein.

To study the humoral response to HIV-1 infection in the context of oligomeric HIV-1 Env protein, we have developed an antigen-capture enzyme-linked immunosorbent assay (ELISA) that utilizes soluble, oligomeric Env protein. This protein, termed gp140, contains all of gp120 and the entire gp41 ectodomain. The gp140, expressed by a recombinant vaccinia virus, is secreted from cells largely as dimers and higher-order forms (11). The oligomeric gp140 ELISA allows (i) rapid and quantitative assessment of the levels of antibodies to linear and conformational determinants present in human sera to oligomeric Env; (ii) detection of a much greater fraction of total anti-Env activity in sera than does native monomeric gp120; (iii) determination of the extent that oligomerization contributes to the antigenicity of Env protein during HIV-1 infection; and (iv) simplified screening for MAbs that recognize epitopes that are better represented on oligomeric Env.

MATERIALS AND METHODS

Sera and plasma. One set of 10 human sera and one set of 15 human plasmas obtained from HIV-1-positive individuals were used. Serum and plasma samples were inactivated at 56°C for 1 h. Each sample was then mixed 1:1 with phosphate-buffered saline (PBS)–0.5% Triton X-100, aliquoted, and stored at −85°C until use. At that time, they were diluted to a 5% solution in PBS with 0.05% NaN_3, stored at 4°C, and never refrozen. Mixed HIV-positive serum was obtained by mixing equal volumes of 10 sera or 10 plasmas. Plasma and peripheral blood mononuclear cells (PBMC) were obtained from clinically healthy HIV-1-infected young men in the United States and Thailand as previously described (26, 27). All plasma samples were reactive to HIV-1 by commercial ELISA and Western blotting (immunoblotting). The corresponding virus genetic subtype of each plasma sample was determined from primary PBMC or PBMC derived after coculture, by DNA sequence of the *env* gene as previously described (26), or by heteroduplex mobility assay (8). Locally (Philadelphia) acquired serum samples are presumed to be clade B.

MAbs. The MAbs used in this study were raised against soluble monomeric or oligomeric forms of HIV-1_{IIIB} Env (4, 11). MAbs used included D47 (to the V3 loop), D20 and D60 (conformation-dependent MAbs to the CD4 binding site in gp120), T4 and D12 (conformation- and oligomer-dependent MAbs to gp41), and D43, D50, and D61 (MAbs to linear epitopes in gp41). Operationally, MAbs that react with fully reduced and denatured Env by Western blot analysis are defined as binding to linear epitopes, while those that do not are defined as binding to conformational determinants. MAbs were purified from hybridoma supernatants by protein G affinity chromatography, and their concentrations were determined by UV absorbance at 280 nm. MAbs were diluted to 40 μg/ml, stored at −85°C, and thawed only once. For biotinylation, purified MAbs were extensively dialyzed against sodium bicarbonate (50 mM, pH 8.5) prior to addition of a 20-fold molar excess of biotin (Pierce ImmunoPure NHS-SS-Biotin, catalog no. 21331) made fresh as a 2-mg/ml solution in double-distilled H_2O immediately before use. After 1 h at room temperature, the MAb-biotin mixture was dialyzed against PBS without calcium and magnesium for 12 h, and the concentrations of the biotinylated MAbs were determined again by UV absorption at 280 nm. The MAbs were diluted to 40 μg/ml, frozen at −80°C, and thawed only once.

Antigens. All Env protein constructs were derived from HIV-1_{IIIB} and expressed by recombinant vaccinia virus vectors. Viruses used included vPE8, which expresses gp120 (13), and vPE12B (11), which expresses a secreted form of Env containing gp120, the gp41 ectodomain, and a small deletion at the gp120-gp41 junction to prevent cleavage. Finally, viruses that do not express Env protein (WR and vSC8) were used as controls. To obtain secreted forms of Env protein, B-SC-1 (simian epithelial) cells were infected with the appropriate virus at a multiplicity of infection of 1 or more and placed in serum-free medium 2 to 4 h postinfection, and the medium was collected 24 h postinfection. The Env-containing medium, clarified by centrifugation, was used directly for the ELISAs unless otherwise noted. The amount of medium added to each well was determined empirically for each protein preparation and was generally on the order of 5 to 20 μl per well for vPE12B and 12.5-50 μl per well for vPE8.

Velocity gradient centrifugation. gp140 harvested from infected cell supernatants as described above was concentrated approximately 10-fold in an Amicon filtration cell and placed atop a 5 to 20% (wt/vol) continuous sucrose gradient (12). Samples were centrifuged in an SW40 rotor at 40,000 rpm for 20 h at 4°C. Fractions were collected from the bottom, and aliquots from each were analyzed by ELISA (as described below) or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12).

ELISA. ELISAs were performed by using 96-well plates from Costar (catalog no. 9018). Unless otherwise noted, all incubations were done at room temperature, using a volume of 100 μl per well for at least 1 h followed by three washes (175 μl per well) with PBS containing 0.05% Tween 20 (wash solution). All experimental conditions were done at least in triplicate. gp120 and gp140 were collected as described above and captured to 96-well plates in one of two ways: directly to plastic, or by use of a capture antibody. For adsorption to plastic, Env protein or capture antibody (approximately 80 ng per well) was diluted into a pH 8.5 solution containing 20 mM Tris-HCl, 100 mM NaCl, and 0.05% NaN_3, added to 96-well plates, and incubated overnight at 4°C. Following adsorption to plastic, a 200-μl-per-well blocking step was performed with wash solution containing 0.5% gelatin and 0.05% NaN_3 (blocking solution) for ≥5 min with shaking. After adsorption of the capture antibody, the appropriate antigen was diluted in blocking solution and incubated in antibody-containing wells at room temperature for at least 1 h. Negative control wells were not exposed to antigen but otherwise treated identically.

Serum and plasma samples were diluted into blocking solution prior to use. When many samples were compared in a single experiment, incubations of 2 h or longer were used. Biotinylated detection antibodies were used, unless otherwise noted, at a molar excess concentration of 0.2 μg per well diluted into blocking solution. Horseradish peroxidase (HRP) conjugates were diluted into wash solution, added to wells, and incubated for 30 min or longer. The conjugates used include avidin-HRP (Pierce) and goat anti-human immunoglobulin G (IgG)-HRP. Following conjugate incubation, a single 2× PBS–0.05% Tween 20 high-salt wash (200 μl per well) was performed to lower background binding (24); this procedure was followed by three washes with normal wash solution. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt tablet (Pierce Chemical Co.)-based substrate was used in the manner suggested by Pierce. The color reaction was stopped by the addition of 100 μl of 1% SDS to each well. Optical densities (ODs) were determined at a wavelength of 405 nm.

Competition ELISAs. To determine if different serum samples contain antibodies that block binding of various detection MAbs to gp140, we captured gp140 to 96-well plates, using a capture antibody to gp41 (D50) or to the V3 loop (D47). After washing, captured Env was incubated with the indicated concentrations of pooled or individual serum or plasma samples for 1 h, washed three times, and then incubated with a biotinylated detection MAb as described above. Data were normalized to the amount of reactivity seen in the absence of serum.

To determine the proportion of IgG in HIV-1 clade B plasma that recognizes epitopes unique to monomeric gp120 or oligomeric gp140, we incubated plasma with medium containing gp120 or gp140 overnight at 4°C. Aliquots of the plasma-Env mixture were then analyzed by ELISA to determine residual binding activity against plastic-captured gp120 or gp140. The amount of gp120-containing medium required to completely block subsequent binding to plastic-captured gp120 and the amount of gp140-containing medium required to block subsequent binding to captured gp140 by ELISA were determined. Generally, between 1.5 and 2.25 ml of concentrated (10-fold) gp120- or gp140-containing medium was required to completely block binding to the homologous protein by ELISA. Serial twofold dilutions of plasma incubated overnight with saturating amounts of gp120- or gp140-containing medium (or medium from cells infected with a control virus) were then incubated with plastic-captured gp120 or gp140 or control medium, and absorbance was determined as usual.

RESULTS

Development of an ELISA using soluble, oligomeric Env. A significant fraction of neutralizing activity present in HIV-1-positive human serum cannot be accounted for by antibodies that bind to native, monomeric gp120 (52). This study and other recent studies indicate that Env protein quaternary structure can have significant antigenic consequences (4, 11, 30, 40, 43). Thus, it will be important to use HIV-1 Env protein complexes that retain native oligomeric structure in order to fully evaluate the humoral response to HIV-1 infection. To accomplish this quickly and quantitatively, we have developed an antigen capture ELISA that uses soluble, oligomeric Env. This

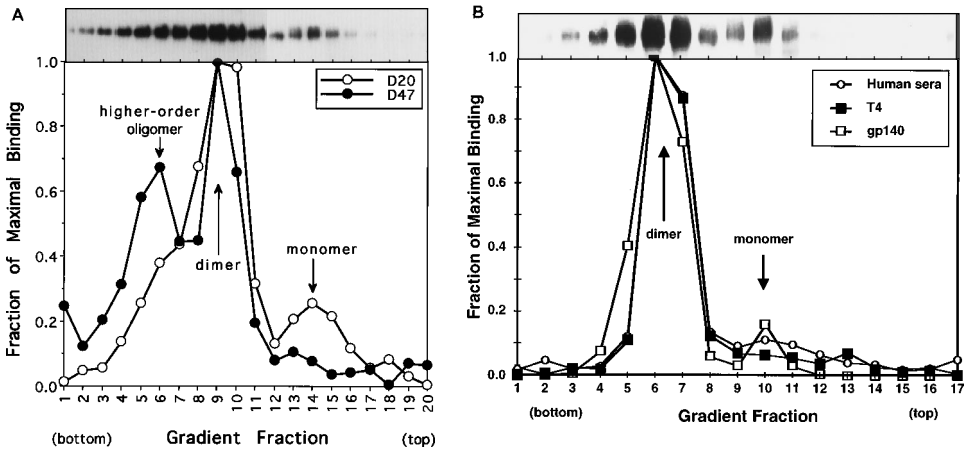

FIG. 1. (A) Detection of monomeric and oligomeric gp140 by Western blotting and ELISA. gp140 was subjected to sucrose velocity gradient centrifugation to resolve monomeric and oligomeric species. Aliquots of gradient fractions were analyzed by SDS-PAGE and Western blotting to monitor the distribution of gp140 across the gradient (top). Additional aliquots were incubated in 96-well plates containing a capture MAb directed against the V3 loop (D47). Captured gp140 was detected with biotinylated MAbs to the CD4 binding site (D20) or to the V3 loop (D47), and ODs are expressed as fractions of maximal binding for each MAb. Peaks corresponding to monomer, dimer, and higher-order oligomer are indicated. (B) The experiment was performed identically to that in panel A but with a different gp140 preparation. The distribution of gp140 across the gradient was determined by Western blotting and scanning densitometry (open squares). Aliquots of gradient fractions were then incubated in 96-well plates containing a capture MAb directed against the V3 loop (D47). Captured gp140 was detected with a biotinylated MAb that recognizes only oligomeric Env (T4) or with pooled HIV-1-positive human serum.

Env protein, expressed by recombinant vaccinia virus vPE12B, contains gp120 and the entire gp41 ectodomain (11). In addition, the cleavage site between gp120 and gp41 has been deleted to prevent loss of gp120 during purification or antibody binding. The uncleaved protein, termed gp140, was collected from the medium of cells 24 h after infection with vPE12B and subjected to sucrose velocity gradient centrifugation to resolve monomeric and oligomeric species. Aliquots from each gradient fraction were analyzed by SDS-PAGE and Western blotting to determine the distribution of gp140 across the gradient. As described previously (11) and shown in the top panels of Fig. 1A and B, gp140 sedimented largely in oligomeric form, with dimeric protein constituting the major oligomeric species. Small amounts of monomeric (fraction 14) and higher-order oligomeric forms of gp140 were also recovered. Chemical cross-linking confirmed the monomeric and oligomeric nature of gp140 across the gradient (data not shown) as we have shown previously (11, 12, 15).

In order for gp140 to be useful in an ELISA format, it must retain its native, oligomeric structure throughout the assay. To test this, aliquots of each gradient fraction were incubated in 96-well plates containing a capture MAb (D47) directed to the V3 loop. Captured gp140 was then detected by using a biotinylated MAb to the CD4 binding site (D20) or to the V3 loop (D47). As shown in the bottom portion of Fig. 1A, the distribution of gp140 across the gradient was faithfully reflected by D20 reactivity, which revealed both the dimeric and monomeric gp140 peaks (fractions 9 and 14, respectively). A similar profile was obtained with other CD4-binding-site detection MAbs, with a polyclonal serum directed against gp120 (not shown), and with HIV-1-positive human sera (Fig. 1B). When the V3 loop MAb D47 was used to detect gp140, the monomer peak was not detected since the single V3 loop in monomeric gp140 was occupied by the D47 capture MAb. By contrast, dimeric Env was detected by D47, indicating that the captured protein could simultaneously bind two copies of D47. This finding provided immunological confirmation that the captured gp140 remained oligomeric. In addition, D47 exhibited enhanced reactivity with higher-order oligomers (centered on fraction 6) compared with other detection MAbs. However, this did not occur when MAbs other than D47 were used to capture gp140. Enhanced binding of biotinylated D47 to higher-order oligomers captured by D47 may simply reflect a greater proportion of available V3 loop epitopes in these molecules. At the minimum, half of the V3 loops in dimeric gp140 must be occupied by the capture antibody, while only 25% must be occupied to capture tetrameric protein. Binding of one MAb to a V3 loop may also induce a conformational change that makes other V3 loops in the oligomer more accessible. Further confirmation that captured gp140 retained its native oligomeric structure came from analyzing gradient fractions with a MAb that recognizes only oligomeric Env (12). As shown in Fig. 1B, the oligomer-specific MAb T4 reacted with oligomeric gp140 but not with monomeric protein.

To further assess the conformational integrity of the gp140 and to study the effects of different capture procedures, we tested seven MAbs for the ability to detect gp140 captured by a MAb to the V3 loop (D47) or to a linear epitope in gp41 (D50) or captured directly to plastic (without drying). Because the bulk of gp140 secreted from cells is oligomeric (Fig. 1A and B) and because human serum does not react preferentially with the small amount of contaminating monomer (Fig. 1B), we used gp140 collected directly from infected cell supernatants for this and all subsequent experiments. To more easily compare the effects of different capture techniques on the ability of different MAbs to detect gp140, several variables must be taken into account. First, different amounts of Env are captured to the plate by the three techniques, and differences are also observed between experiments. To correct for this, the data were normalized to the reactivity observed with a MAb to a conformation-independent epitope in gp41 (D61) that reacts strongly with Env under all capture conditions. Second, the rates and absolute levels of substrate coloration associated with different biotinylated detection MAbs can vary markedly even under optimal conditions for reasons that may include differences in antibody affinities, biotinylation efficiency, and epitope exposure. To account for this, data for each detection MAb were normalized to the OD obtained when gp140 was adsorbed directly to plastic. Thus, reactivity for each MAb to plastic-captured gp140 is 1.0, and deviation from a normalized

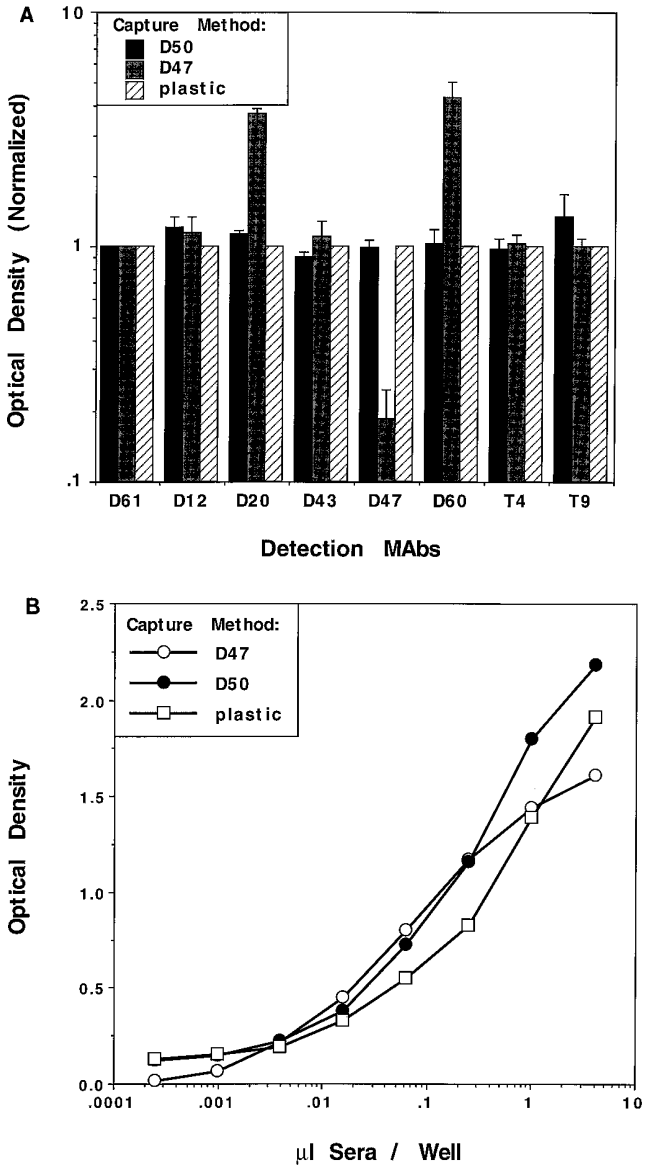

FIG. 2. Effects of the capture technique on gp140 detection. (A) gp140 collected directly from the medium of cells infected with vPE12B was captured by a MAb to a linear epitope in gp41 (D50) or a MAb to the V3 loop (D47) or captured directly to plastic. A panel of biotinylated MAbs was then used to detect bound gp140. The data were normalized as described in Materials and Methods to simplify comparisons between different MAbs and capture techniques and to make it possible to average results from different experiments. (B) gp140 was captured by D47 or D50 or directly to plastic. Pooled HIV-positive serum was titrated against gp140 and detected by using a goat anti-human IgG-HRP conjugate. Background ODs were subtracted from absolute ODs for each volume of serum added.

OD of 1.0 when gp140 is captured by a MAb indicates either a greater (>1.0) or lesser (<1.0) affinity for gp140.

As shown in Fig. 2A, gp140 was recognized by the entire panel of detection MAbs, including MAbs to conformational determinants in gp120 (D20 and D60) and gp41 (T4 and D12). The reactivity with T4 and D12 was particularly important since T4 reacts exclusively and D12 preferentially with oligomeric Env (4). The means by which gp140 was captured to the plate had little effect on antibody reactivity, with three exceptions. Both D20 and D60, which bind to conformational determinants associated with the CD4 binding site in gp120, reacted far more strongly with gp140 captured by the V3 loop MAb D47. Enhanced binding of soluble CD4 and antibodies to the CD4 binding site has been described as a consequence of antibody binding to the V3 loop, both with recombinant gp160 (57) and with Env expressed on the cell surface (53). These findings are consistent with V3 loop antibody-induced conformational changes that increase exposure of CD4-binding-site epitopes and may explain the synergistic neutralization sometimes observed with antibodies directed against these sites (28, 53, 57). Our data show that oligomeric gp140 captured by V3 loop antibodies exhibits this dynamic property. Finally, the V3 loop MAb D47 bound equally well to gp140 captured either directly to plastic or with an antibody to gp41 (D50). It bound significantly less well to gp140 captured by D47, as expected. At the minimum, half of the V3 loop epitopes in dimeric gp140 must be masked by the capture antibody. Since binding was reduced by greater than 50% when gp140 was captured by D47, either the remaining free V3 loop is not as accessible or a population of gp140 dimers are bound to the plate by two D47 MAbs. Both explanations likely contribute to this result since reduced concentrations of D47 capture MAb, which should increase the fraction of gp140 molecules captured by a single binding event, resulted in slightly enhanced detection by D47 (not shown).

To test the reactivity of human serum with gp140 and to determine if the method of capture strongly influences serum antibody binding, we incubated gp140 captured by D47 (V3 loop), by D50 (linear gp41), or directly to plastic with serial dilutions of pooled HIV-1-positive human serum. As shown in Fig. 2B, antibody binding to gp140 could be readily detected to dilutions of 1:10,000 (equivalent to 0.01 μl serum per well in the figure). Further, the binding profile was not strongly influenced by the capture method. Thus, gp140 provides a convenient source of oligomeric protein that retains its native structure in an ELISA format: it binds a panel of conformationally sensitive MAbs to epitopes in gp41 and gp120 (Fig. 2A); it binds soluble CD4 (not shown); antibody reactivity is largely independent of capture technique (Fig. 2A); and the dynamic interaction between the V3 loop and CD4 binding site is retained (Fig. 2A). In addition, the gp140 ELISA provides a sensitive and quantitative approach to detect and characterize antibody reactivity to Env present in HIV-1-positive human serum (Fig. 2B).

Reactivities of plasma samples from individuals infected with clade B or E isolates with oligomeric gp140. A large number of MAbs raised against native oligomeric IIIB Env protein recognize broadly conserved epitopes that are present in most clade B isolates. In addition, a significant fraction of these MAbs, particularly those against gp41, exhibit cross-clade reactivity (10a). Thus, oligomeric gp140 may be more likely than monomeric gp120 to retain highly conserved, conformational determinants to which antibodies generated against diverse HIV-1 strains cross-react. To test this hypothesis, pooled and individual serum samples from individuals known to be infected with either clade B or clade E HIV-1 strains were examined for the ability to recognize oligomeric gp140 or monomeric gp120 by ELISA. These proteins were captured directly to plastic (Fig. 3A) or by a MAb to the V3 loop (D47; Fig. 3B). Whether captured directly to plastic or by the V3 loop MAb D47, the pooled clade B serum samples reacted only slightly better (approximately 1.2- to 1.3-fold) with oligomeric gp140 than did the clade E serum samples. When individual serum samples were tested, differences in reactivity to gp140 were neither large enough nor consistent enough to enable us to distinguish clade B from E samples on

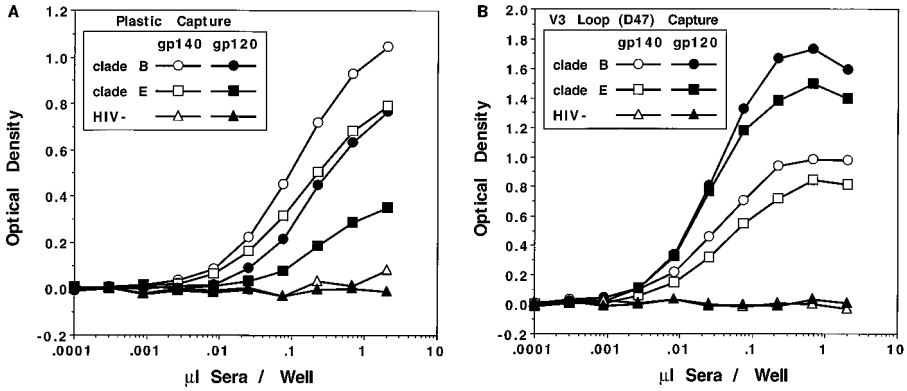

FIG. 3. Reactivities of clade B and E seras with gp140 and gp120. gp140 or gp120 was captured directly to plastic (A) or by MAb D47 to the V3 loop (B). Captured antigen was incubated with pooled clade B plasma, pooled clade E plasma, or HIV-negative (HIV−) serum. Bound antibody was detected by using goat anti-human IgG-HRP. Reactivity observed in the absence of antigen at each serum dilution was defined as background and subtracted. All datum points were performed in triplicate.

a reproducible basis (not shown). Thus, oligomeric gp140 retains broadly conserved epitopes to which antibodies generated against divergent HIV-1 strains cross-react.

In contrast to gp140, gp120 captured directly to plastic was recognized by pooled clade B sera approximately 2.2-fold more strongly than pooled clade E serum samples. This finding is consistent with the results of Moore et al. (32), who found that clade E gp120s were antigenically distinct from clade B gp120s in both the V3 loop and epitopes associated with the CD4 binding site, and also with the fact that the ectodomain of gp41 (present in gp140) is more highly conserved than the gp120 subunit (51). However, when gp120 was captured by a V3 loop MAb, we found that clade E samples were indistinguishable from clade B samples (Fig. 3B). Since the clade B sera used here did not react with the IIIB V3 loop (Fig. 4), it is unlikely that this result is due to the capture antibody blocking a significant fraction of clade B V3 loop-directed reactivity, thereby diminishing the differences in gp120 recognition by sera from the two clades. Rather, this finding suggests that binding of an antibody to the V3 loop induces structural changes in gp120 that expose more highly conserved determinants. While this may involve the CD4-binding-site region, other portions of gp120 may be affected as well. Thus, attempts to serotype HIV-1 strains on the basis of serum reactivity must carefully take into account the way in which antigen is presented.

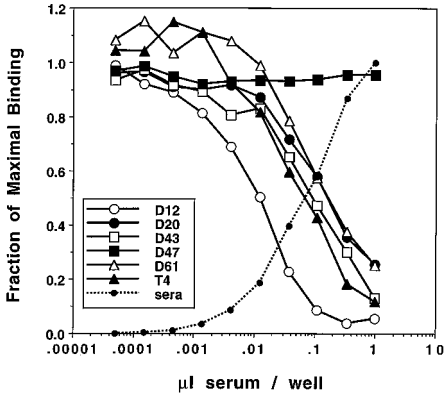

FIG. 4. Ability of pooled HIV-positive serum to block MAb detection of gp140. gp140 was captured by the anti-gp41 MAb D50. Essentially identical results were obtained when gp140 was captured by using the V3 loop MAb D47 (not shown). Pooled HIV-positive serum was added to wells containing captured gp140 at the indicated volumes. Wells were then incubated with goat anti-human IgG to detect bound serum antibodies (dotted line) or incubated with an excess of the indicated biotinylated detection MAb to measure serum blocking activity. Reactivity of each detection MAb seen in the absence of serum was defined as 1.0. Detection MAbs used were D12 (conformation-dependent gp41), D20 (CD4 binding site), D43 (linear gp41), D47 (V3 loop), D61 (linear gp41), and T4 (oligomer-specific gp41).

Ability of HIV-1-positive sera to block MAb binding to gp140. We next examined whether preincubation of captured gp140 with different dilutions of HIV-1-positive serum could block subsequent binding of MAbs to biologically important determinants such as the V3 loop and CD4 binding site or to novel oligomer-dependent determinants in gp41. gp140 was captured with a MAb to gp41 (D50; Fig. 4) or to the V3 loop (not shown), incubated with serial dilutions of pooled HIV-1-positive serum, and then incubated with various biotinylated detection MAbs. Binding of the V3 loop MAb D47 was not blocked by preincubation with serum, consistent with the low prevalence of HIV-1_{IIIB}-like strains circulating in the United States (9). Four MAbs were blocked by serum to similar levels: D20, a conformation-dependent MAb directed against the CD4 binding site; D43, which maps to residues 635 to 678 in gp41; D61, directed against the cluster 1 epitope in gp41 (residues 592 to 608); and T4, a MAb that binds to an oligomer-dependent conformational determinant in gp41. Similar results were obtained with an additional MAb to the CD4 binding site (D60) and to another oligomer-dependent epitope in gp41 (T10) but are not shown to simplify the figure. The similarity with which these MAbs were blocked by serum shows that antibodies to these (or overlapping) epitopes are similarly prevalent in sera. Essentially identical results were obtained when gp140 was captured by a MAb to the V3 loop (data not shown). No blocking was obtained when HIV-negative human serum was used (data not shown and Fig. 3). Finally, an antibody (D12) to a conformation-dependent epitope in gp41 that binds preferentially to oligomeric Env was blocked more strongly than the other MAbs tested (Fig. 4).

Taken together, these results suggest that antibodies to a number of epitopes in gp41, including oligomer-dependent determinants, are as prevalent in HIV-1-positive human serum as are antibodies to the CD4 binding site. However, it is possible that the ability of sera to block binding of gp41 antibodies might be due to indirect effects. For example, it is possible that antibodies to gp120 might sterically hinder subsequent binding of antibodies to determinants in gp41. To investigate this possibility, we preincubated clade B sera with gp120 overnight to

TABLE 1. Percent blocking of MAbs by pooled clade B HIV-1-positive human sera[a]

MAb	Amt of serum (μl)	Adsorption (%)	
		Postmedium	Post-gp120
D43	0.1	45	45
	0.033	33	36
	0.011	14	20
D61	0.1	65	65
	0.033	44	48
	0.011	26	34
T4	0.1	66	65
	0.033	56	55
	0.011	36	38

[a] Pooled clade B serum was preincubated with native monomeric gp120 overnight (post-gp120 adsorption) or with gp120-negative medium (postmedium adsorption). As shown in Fig. 6, preincubation with gp120 blocked subsequent binding to captured gp120 in an ELISA, whereas preincubation with normal medium did not. The serum was then tested for the ability to block subsequent binding of MAbs, including D43 (residues 635 to 678), D61 (cluster 1), and T4 (oligomer dependent), to gp41 in a manner identical to that described in the legend to Fig. 4. Because of the volume of medium required to adsorb out gp120 reactivity, the maximum amount of serum that could be used in a single ELISA well was 0.1 μl. Blocking for three concentrations of serum is shown.

adsorb out antibodies to gp120. We found that preincubation of clade B sera with gp120 blocked subsequent binding to captured gp120 by ELISA by 80 to 100%, depending on the gp120 preparation (see Fig. 6). However, sera preincubated with gp120 still retained the ability to block binding of gp41 MAbs to gp140 (Table 1). Thus, the ability of sera to efficiently block binding of MAbs to gp41 was not due to steric hindrance from antibodies that bind to gp120.

To determine if there is significant variation in the levels of antibodies to gp41 epitopes between different HIV-positive individuals, we compared the abilities of individual clade B (Fig. 5A and B) or E (Fig. 5C) serum or plasma samples to block binding of antibodies to the CD4 binding site and to oligomer-sensitive epitopes in gp41. gp140 was captured with an antibody to the V3 loop (D47), incubated with a single concentration of an individual serum sample, and then probed with a biotinylated detection MAb to the CD4 binding site (D20) or to oligomer-sensitive epitopes in gp41 (T4 and D12) (Fig. 5). While considerable variation was seen in the levels of reactivity between serum samples, the gp41 MAbs were generally blocked more effectively than the MAb to the CD4 binding site. This was particularly true of the clade E samples, though some clade B serum samples blocked the CD4-binding-site MAb more effectively than the oligomer-specific MAb T4. Thus, individual serum samples are often able to block binding of MAbs to novel oligomer-dependent epitopes in gp41 to a greater extent than antibodies to the CD4 binding site.

HIV-1-positive serum contains significant levels of antibodies that bind to oligomeric gp140 but not to native monomeric gp120. The ability of HIV-1-positive human sera to effectively block a number of MAbs to linear and conformational epitopes in oligomeric gp41 suggested that a significant fraction of total anti-Env activity in serum is due to antibodies that bind to epitopes present in oligomeric gp140 but that are absent in native monomeric gp120. To address this question directly, we incubated pooled clade B HIV-positive plasmas with saturating amounts (determined empirically) of either monomeric gp120 or oligomeric gp140 prior to ELISA analysis against captured gp120 or gp140. We found that preincubation of pooled plasma with sufficiently high concentrations of gp120 completely blocked subsequent binding to captured gp120 by

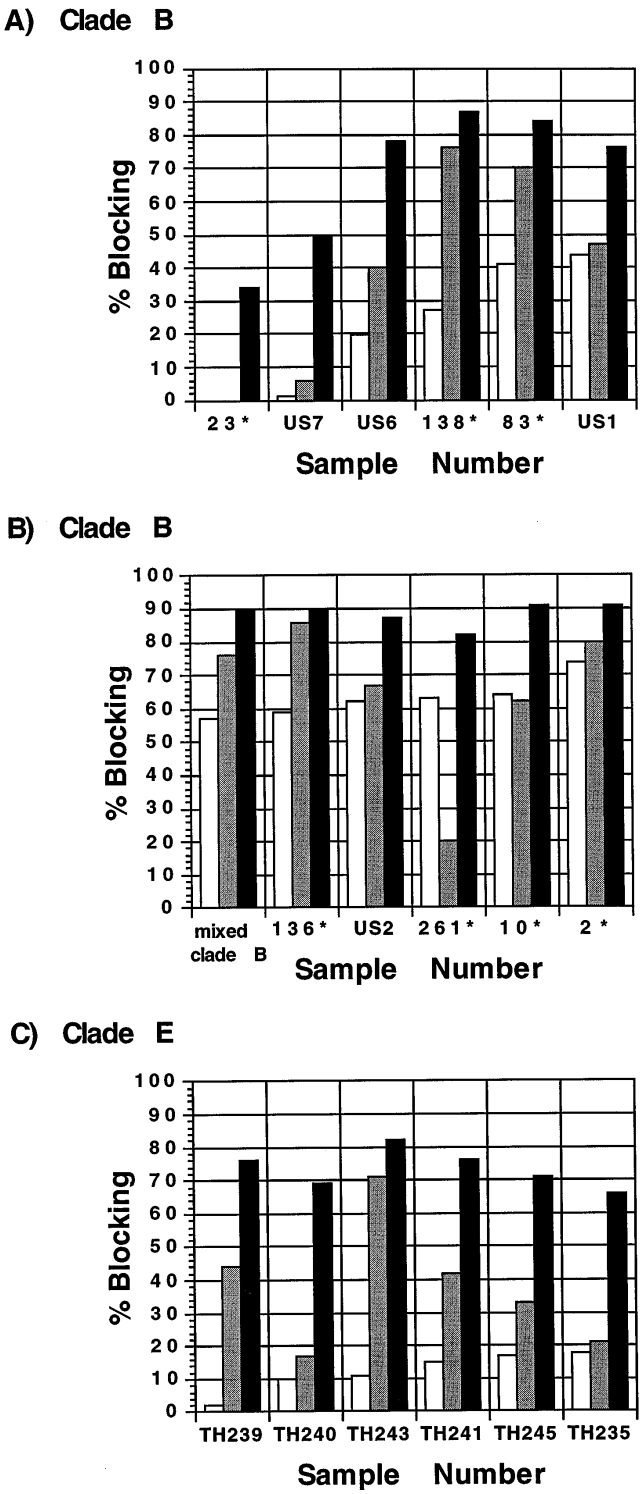

FIG. 5. Abilities of individual HIV-positive sera to block MAb detection of gp140. gp140 was captured by using the V3 loop MAb D47 and incubated with 0.1 μl of individual clade B or clade E sera as indicated. Subsequent binding of three different biotinylated detection MAbs was measured, and the efficiency with which each serum sample blocked binding was determined (reactivity in the absence of serum is defined as 0% blocking). Open, gray, and black bars indicate blocking of the CD4-binding-site anti-gp120 MAb D20, oligomer-specific anti-gp41 MAb T4, and oligomer-sensitive anti-gp41 MAb D12, respectively. Numbered individuals are presented, grouped according to clade: individual locally acquired HIV-positive sera (A and B; designated by a number and *), individual clade B plasmas (designated US), and individual clade E plasmas (C; designated TH).

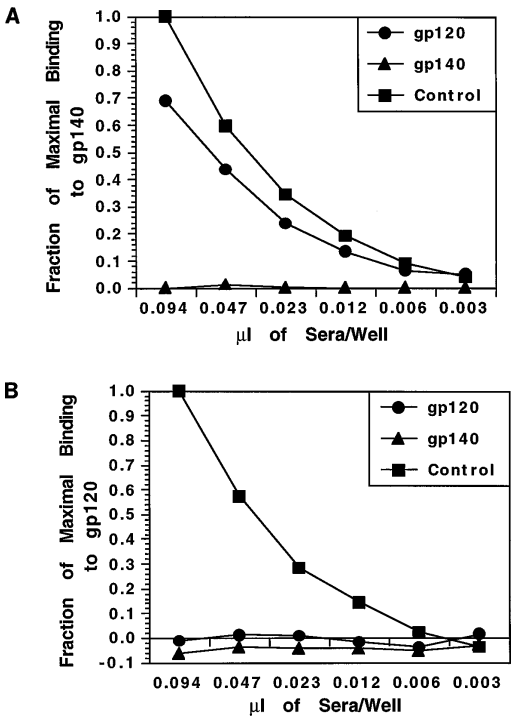

FIG. 6. gp120- and gp140-specific antibodies in clade B sera. (A) Pooled clade B human plasma was preincubated overnight at 4°C with gp120-containing medium from cells secreting gp120 (from infection with vPE8), with gp140-containing medium from cells infected with vPE12B, or with medium from cells infected with a wild-type vaccinia virus (control). Serial twofold dilutions of the plasma-medium mixtures were then incubated with plastic-captured gp140, and the extent of binding was determined. (B) Pooled clade B human plasmas were preincubated with gp120, gp140, or control medium as for panel A, after which serial twofold dilutions of the plasma-medium mixtures were incubated with plastic-captured gp120 and the extent of binding was determined.

ELISA. Likewise, preincubation with sufficiently high concentrations of gp140 completely blocked subsequent binding to captured gp140. As shown in Fig. 6, incomplete blocking was obtained when heterologous proteins were used: preincubation of plasma with gp120 completely blocked subsequent binding to gp120 but reduced subsequent binding to gp140 by only 30% (Fig. 6A). By contrast, preincubation of plasma with gp140 completely blocked subsequent binding to gp140 and blocked binding to gp120 by >95% (Fig. 6B). Thus, of the antibodies capable of binding to HIV-1_{IIIB} Env present in the pooled human serum sample used here, <5% bound to epitopes that were present only in monomeric gp120, 30% bound to epitopes present in both monomeric gp120 and oligomeric gp140, and 70% bound to epitopes present in oligomeric gp140 but not in native monomeric gp120.

DISCUSSION

The HIV-1 Env glycoprotein, like many viral and cellular membrane proteins, forms a noncovalently associated oligomeric complex shortly after synthesis (10, 15). Because proper folding and assembly are required for transport from the endoplasmic reticulum (10, 22), only oligomeric Env is delivered to the cell surface and incorporated into virions. Though a variety of oligomeric structures for Env have been reported, most studies have identified dimers and tetramers as the most prevalent oligomeric species (12, 40, 50, 55). Regardless of the protein's final oligomeric state, an increasing body of evidence suggests that HIV-1 Env protein quaternary structure has implications both for the production of antibodies and for their detection (4, 11, 30, 34, 40, 43, 45). In a recent study, we found that a significant fraction of murine MAbs to gp120 reacted more strongly with monomeric Env, suggesting that some epitopes are altered or masked in the native oligomer (4). This can have important implications for virus neutralization. Moore et al. (30) found that primary isolates, in contrast to laboratory-adapted strains, were relatively resistant to neutralization by a panel of MAbs that all bound strongly to monomeric gp120 from both sets of viruses. Thus, the resistance of primary isolates to neutralization by certain antibodies may be due to partial masking of relevant epitopes in the context of the native, oligomeric protein.

While some antibodies react more strongly with monomeric Env, others react more strongly with the oligomer (4, 40, 43). A recently described antibody that exhibits potent neutralizing activity against diverse HIV-1 strains, for example, recognizes Env oligomers better than monomeric gp120 (45). In fact, this antibody failed to react to soluble monomeric Env glycoproteins from several primary HIV-1 isolates even though it effectively neutralized these virus strains (30, 45). Epitopes in gp41 are particularly sensitive to quaternary interactions, consistent with the role of the gp41 ectodomain in subunit-subunit recognition and assembly (12, 14, 42, 55). We found that 82% of 52 anti-gp41 MAbs raised against soluble, oligomeric Env reacted with conformational determinants and that more than 60% bound preferentially or exclusively to Env oligomers (4, 11). That antibodies to oligomeric gp41 are generated during the course of virus infection has been shown by Pinter and coworkers, who described several human MAbs that react more strongly or specifically with oligomeric determinants in gp41 (40).

The ability of antibodies to bind preferentially or exclusively to monomeric or oligomeric Env indicates that immunization with Env complexes that maintain native quaternary structure may strongly influence the nature of the humoral response. Immunization with native oligomeric protein may increase the fraction of antibodies to conserved, conformation-dependent epitopes as well as antibodies that react exclusively or preferentially with oligomeric Env. By contrast, immunization with monomeric gp120 may result in the production of antibodies that react well with monomeric protein but weakly with the native oligomer. Env quaternary structure can also influence our understanding of the humoral response to HIV-1 infection. Screening for the presence of anti-Env antibodies by using either denatured Env or peptides, while effective in detecting antibodies to linear determinants, would fail to detect antibodies to conformational epitopes, a major shortcoming since antibodies directed to conformational determinants are prevalent in the sera of infected individuals (31). Further, Steimer et al. showed that a significant fraction of broadly neutralizing activity in pooled HIV-1-positive human serum could not be accounted for by antibodies that bind to native monomeric gp120 and suggested that these antibodies might bind to epitopes in gp41 or to oligomer-dependent determinants in gp120 (52). Thus, to more fully understand the humoral response to HIV-1 infection, it will be important to use oligomeric Env as a target in the detection of antibodies and for studying correlations between binding and neutralization activity.

The most accurate way to detect antibodies to native Env in HIV-positive serum would be to use whole virus as a target. An alternative approach is to analyze binding of antibodies to Env protein expressed on the surface of infected cells by fluorescence-activated cell sorting analysis (35, 49). In both cases, the

Env protein is presented in native, oligomeric form. However, to compare antibody reactivities to both monomeric and oligomeric forms of Env under identical conditions and to make it possible to rapidly and quantitatively analyze large numbers of samples, we developed an antigen capture ELISA that uses oligomeric Env. Rather than use whole cell or virus lysates, we have taken advantage of a soluble oligomeric protein (gp140) that in many ways reflects the structure of Env on the surface of infected cells and virions (4, 11). The protein is secreted at high levels by cells infected with vPE12B: a single 10-cm-diameter dish of infected cells easily provides enough antigen for 500 samples. The gp140 remains oligomeric and reactive with CD4 and a large panel of MAbs under a variety of capture conditions, including direct adsorption to plastic (without drying). Because of the propensity of laboratory-adapted strains to shed gp120, we used a protein that contained a deletion at the gp120-gp41 junction to prevent cleavage. While the noncleaved gp140 must differ in some respects from the native, cleaved protein on the surface of virions and infected cells, these differences may not be significant: of 138 MAbs that we raised against gp140, more than 80% reacted strongly with Env on the surface of infected cells, and none were specific for the noncleaved form (11). The use of noncleaved gp140, however, may be necessary only when one is examining laboratory-adapted strains of HIV-1. Primary isolates may not shed gp120 to such an extent, so that fully cleaved gp140 from primary isolates may potentially be used in this assay (18, 33, 39). Another difference between gp140 and cell surface Env is that gp140 is present in both dimeric and higher-order oligomeric forms, while Env protein on the cell surface is likely to be more homogeneous. As a consequence, this assay may not efficiently detect antibodies that react solely with higher order forms of Env.

Using preparations of oligomeric gp140 and monomeric gp120, we found that of the antibodies in clade B serum capable of binding to HIV-1_{IIIB} Env, <5% bound to epitopes present only in monomeric gp120, 30% bound to epitopes present both in gp120 and gp140, and 70% bound to epitopes present in oligomeric gp140 that were absent from monomeric gp120. Since a number of MAbs to gp41, including several that recognize oligomer-dependent epitopes, were blocked by serum more effectively than MAbs to the CD4 binding site in a competition ELISA, it is likely that the bulk of the gp140-specific antibodies present in clade B serum bind to epitopes in the gp41 ectodomain, though some may bind to oligomer-dependent determinants in gp120. Antibodies that bind preferentially or exclusively to monomeric gp120 have already been described (4, 34, 45), though our results suggest that these antibodies comprise a minor fraction of antibodies that recognize gp120 determinants. However, it is important to note that we used HIV-1_{IIIB} Env as a target and that none of the serum samples used in this study contained significant levels of antibodies to the IIIB V3 loop. Thus, it is likely that we are underestimating the proportion of type-specific antibodies present in serum—antibodies that are more likely to bind to epitopes in gp120 and that are more likely to preferentially react with monomeric Env.

Our earlier work with antibodies to oligomeric Env showed that the reactivities of antibodies directed against linear epitopes could also be influenced by Env quaternary structure (4). Previous studies using peptides have shown that high titers of antibodies to the cluster 1 determinant in gp41 (residues 598 to 604) are present in human serum (5, 16, 59) and are 100-fold more prevalent than cluster 2 (residues 644 to 663) antibodies (59). However, using oligomeric Env protein and a competition ELISA, we found that cluster 1 MAbs and a MAb to residues 635 to 678 were blocked by HIV-positive serum to similar extents. This may be due to the presence in serum of conformation-dependent antibodies not detected by earlier studies. Alternatively, the efficiency with which any given MAb is blocked by sera may be due in part to indirect effects: steric hindrance or conformational changes in Env induced by antibodies binding elsewhere in the molecule. Regardless of the exact mechanism, however, these studies demonstrate how the interpretation of the humoral response to HIV infection, and the definition of immunodominance, can be influenced by Env oligomeric structure.

Identification of epitopes to which broadly cross-reactive neutralizing antibodies are directed is critically important for vaccine development, especially in light of the fact that a number of HIV-1 genotypes (clades) have been identified throughout the world. Whether HIV-1 genotypes are antigenically distinct is less clear. While some serum samples from individuals infected with clade E (Thailand) or clade B (United States) viruses exhibit cross-clade neutralizing activity, others do not (23, 26). MAb binding studies using Env proteins from genetically distinct viruses have shown that antigenic subtypes related to viral genotype may be identified, though many MAbs exhibit cross-clade reactivity (32). We have found that approximately 30% of the MAbs raised against oligomeric gp140 react with Env proteins derived from clade E isolates (10a), indicating that gp140 retains highly conserved epitopes. That HIV-positive serum contains antibodies that bind to conserved epitopes in oligomeric gp140 was shown by the fact that pooled clade B and E serum samples reacted almost equally well with HIV-1_{IIIB} gp140. Interestingly, clade B sera reacted more strongly with IIIB gp120 than did clade E serum samples, suggesting that a significant fraction of antibodies to conserved epitopes in clade E serum react with epitopes present in oligomeric Env containing gp41 that are absent in native, monomeric gp120. These results are similar to those of Ichimura et al. (23), who found that cross-clade reactive antibodies to linear determinants were more prevalent to gp41 than to gp120. However, we found that clade B and E serum samples reacted equally well to gp120 when it was captured by an antibody to the V3 loop, in contrast to gp120 captured directly to plastic. These results may be due to increased exposure of conserved epitopes associated with the CD4 binding site. A number of studies have shown that V3 loop antibodies enhance subsequent binding of both soluble CD4 and antibodies to the CD4 binding site and that these reagents in turn enhance exposure of the V3 loop (7, 28, 36, 47, 53, 57). Since oligomeric gp140 captured by a V3 loop antibody retains this dynamic property (Fig. 2A), the ability of clade E serum samples to recognize clade B gp120 may depend in part on structural changes associated with the CD4 binding site.

The antigen capture ELISA described here makes it possible to quickly and quantitatively assess the abilities of different MAbs and serum samples to react with either monomeric or oligomeric forms of Env protein. By preserving epitopes that are dependent at least in part on Env quaternary structure, approximately twice as many antibodies to Env generated during the course of viral infection can be detected. While the levels of broadly cross-reactive antibodies to epitopes dependent on Env oligomeric structure are significant, it must now be determined if the presence or levels of these antibodies have any clinical correlates and if these antibodies possess significant biological activity.

ACKNOWLEDGMENTS

We thank members of the Doms, Bates, and Malim laboratories for helpful discussions and suggestions, and we thank Joanne Berson and

Deborah Long for carefully reading the manuscript. The contributions of Francine McCutchan (Henry M. Jackson Foundation) for assistance in genetic characterization of sera and Donald Burke (WRAIR) for making these sera reagents available are gratefully acknowledged. We thank Stephen Abedon for careful work in the development of the oligomeric Env ELISA. Finally, we thank John Moore and Quentin Sattentau for providing unpublished results on HIV-1 neutralization and epitope exposure on oligomeric Env.

This work was supported by NIH grant R01 AI35383 to R.W.D. and by funds from the NIH AIDS Targeted Antiviral Program to P.L.E.

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