Neutralization Efficiency Is Greatly Enhanced by Bivalent Binding of an Antibody to Epitopes in the V4 Region and the Membrane-Proximal External Region within One Trimer of Human Immunodeficiency Virus Type 1 Glycoproteins[▽]

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Most antibodies are multivalent, with the potential to bind with high avidity. However, neutralizing antibodies commonly bind to virions monovalently. Bivalent binding of a monoclonal antibody (MAb) to a virion has been documented only in a single case. Thus, the role of high avidity in antibody-mediated neutralization of viruses has not been defined clearly. In this study, we demonstrated that when an artificial 2F5 epitope was inserted in the gp120 V4 region so that an HIV-1 envelope glycoprotein (Env) trimer contains a natural 2F5 epitope in the gp41 membrane-proximal envelope region (MPER) and an artificially engineered 2F5 epitope in the gp120 V4 region, bivalent 2F5 IgG achieved greatly enhanced neutralization efficiency, with a 50% inhibitory concentration (IC₅₀) decrease over a 2-log scale. In contrast, the monovalent 2F5 Fab fragment did not exhibit any appreciable change in neutralization efficiency in the same context. These results demonstrate that bivalent binding of 2F5 IgG to a single HIV-1 Env trimer results in dramatic enhancement of neutralization, probably through an increase in binding avidity. Furthermore, we demonstrated that bivalent binding of MAb 2F5 to the V4 region and MPER of an HIV-1 Env trimer can be achieved only in a specific configuration, providing an important insight into the structure of a native/infectious HIV-1 Env trimer. This specific binding configuration also establishes a useful standard that can be applied to evaluate the biological relevance of structural information on the HIV-1 Env trimer.

Immunoglobulin molecules have multiple binding paratopes for antigens; for example, those for IgG1 are bivalent and those for IgM are dodecavalent. It is obvious that multivalent binding is required for the distinct mechanism of neutralization by cross-linking multiple virions to form virus aggregates (reviewed in references 7 and 67). Despite the potential of antibodies for multivalent binding, structural evidence indicates that neutralizing antibodies often bind to an individual virion in a monovalent fashion (19, 20, 27, 29, 50, 53; reviewed in references 12 and 22). Bivalent binding of an antibody to a virion has been documented with clear structural evidence in only one case, in which monoclonal antibodies (MAbs) 17-IA and 8F5 bind to virions of human rhinovirus 14 (HRV14) and HRV2 (19, 43). Even in this unique case, binding bivalency appears to contribute to the neutralization potency of 17-IA but not to that of 8F5 (19, 42, 43). Moreover, these MAbs bind to two hydrophobic canyon structures formed by viral proteins VP1 and VP2 and not to antigenic epitopes within individual viral capsid protomers; thus, this case may represent an exception to the common form of antibody/antigen interactions in which the antibodies bind to individual antigens. Therefore, it is not clear what role antibody-binding multivalency plays in

antibody-mediated neutralization of viruses at the level of interaction between antibody molecules and individual virions.

The binding affinity of an antibody to its target is defined by intrinsic affinity and avidity (reviewed in reference 16). Intrinsic affinity is the force of monovalent binding between an antibody paratope and an antigenic epitope, often measured by binding a Fab fragment to an antigen. Avidity is the additive or synergistic force of engaging multiple antibody paratope/antigen epitope pairs between one antibody and one antigen. In other words, avidity is a functional consequence of antibody-binding multivalency. The effect of avidity on affinity is readily demonstrated in biochemical reactions such as enzyme-linked immunosorbent assay (ELISA) and surface plasmon resonance (SPR), in which high-density antigenic sites are available without distinct spatial restrictions. It is commonly assumed that both affinity and avidity have functional consequences in antibody-mediated neutralization of viruses (reviewed in references 7 and 67). At the level of individual virions, the contribution of antibody-binding avidity to neutralization efficiency is often based on two types of experiments. In one, results from a side-by-side comparison between an antibody and its Fab fragment are often reported as evidence supporting a role of antibody-binding multivalency in virus neutralization. However, the interpretation of this type of experiment is complicated by the size difference between an antibody and a Fab fragment, since steric hindrance is a major mechanism of neutralization (reviewed in references 6 and 23). In a second type of experiment, a correlation between neutralization efficiency and the ability of the antibody/virus complex to resist chemical

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stress without dissociation in the presence of a high concentration of salt in solution is interpreted to support a contributing effect from antibody-binding avidity to neutralization efficiency (2, 21, 36, 49, 51). Data from this type of experiment are limited mostly to measuring binding affinity that is below the affinity required for virus neutralization. Furthermore, these studies often do not distinguish between avidity effects caused by an antibody binding to two (or more) epitopes on one antigen or to multiple epitopes from different molecules on the virion. Therefore, like the situation with antibody-binding multivalency, it remains unclear whether binding avidity contributes to antibody-mediated neutralization of viruses at the level of individual virions.

The envelope glycoproteins (Envs) of human immunodeficiency virus type 1 (HIV-1) exist on the virion or cell surface as trimers of gp120 and gp41 heterodimers (13, 30, 62, 65). High-resolution structural information for a native HIV-1 Env trimer is critically important for understanding the function of HIV-1 Envs as well as for guiding the development of an effective immunogen to elicit broad and potent neutralizing antibody responses. X-ray crystal structures of the gp41 ectodomain fragments in the postfusion conformation have been resolved; however, a high-resolution structure of gp41 in the prefusion conformation is still unavailable and likely will be more informative for understanding the function of HIV-1 Env trimers (9, 47, 52). Two X-ray crystal structures of the gp120 core in both the CD4-liganded and unliganded conformations have been solved, but the biological meanings of these structures, especially how they are related to the native, functional Env trimer, are still being debated (10, 26). Several low-resolution structures of the Env trimers from HIV-1 or the closely related simian immunodeficiency virus (SIV) have been determined using cryoelectron microscopy (cryo-EM) tomography (4, 30, 62, 64, 65, 66). The predicted structures for the Env trimer are in general quite different between the two studies, and the difference is particularly dramatic around the gp41 membrane-proximal external region (MPER). A high-resolution structure of the native HIV-1 Env trimer is needed to resolve these differences. In the meantime, a distinctive standard needs to be developed for evaluating the biological relevance of structural information of an HIV-1 Env trimer.

Our previous studies of the stoichiometry of antibody-mediated neutralization of HIV-1 Env indicated that MAbs b12, 2G12, and 2F5 neutralize by a stoichiometry designated $T=1$, i.e., one antibody binds to and neutralizes one HIV-1 Env trimer (57). Furthermore, when an artificial epitope (FLAG) was inserted in the V4 region of HIV-1 gp120, an epitope-specific anti-FLAG MAb achieved neutralization by the mechanism of steric hindrance (37, 61). Using the well-defined 2F5 neutralizing epitope as a model system (35, 39, 45), we constructed HIV-1 Env proteins carrying one 2F5 epitope in the gp120 V4 region and another 2F5 epitope in the gp41 MPER. Here, we investigated whether binding bivalency leads to enhancement in neutralization efficiency. By studying the detailed requirement for binding bivalency, we also probed the structure of the native, functional HIV-1 Env trimer, aiming to establish a standard that can be employed to evaluate the biological relevance of structural information on the HIV-1 Env trimer.

MATERIALS AND METHODS

Plasmid constructs expressing HIV-1 Env variants. The HIV-1_{YU2} gp160 was expressed from the pSVIIIenv vector (46). The 2F5 epitope was inserted and additional mutations were introduced by site-directed mutagenesis using the QuikChange procedure (Stratagene) (32). The desired mutations and the absence of unintended changes were confirmed by sequencing the entire *env* gene. All plasmids used in one experimental session were prepared using a QIAFilter kit (Qiagen), quantified, and used as a set.

Transient expression and immunoprecipitation of HIV-1 Env variants. To evaluate expression and gp120/gp41 processing of mutant Env proteins, Env-expressing plasmids were cotransfected with a plasmid expressing HIV-1 Tat protein into 293T cells using the Effectene reagent (Qiagen). The HIV-1 Tat protein was included to activate the transcription of the *env* gene in the pSVIIIenv vector. The Env proteins were then labeled with 0.2 mCi [³⁵S]methionine/cysteine for 24 h and detected by immunoprecipitation. For detection of the processed and secreted gp120, culture medium was collected and removed of cell debris by centrifugation at 20,000 × *g* for 15 min. For detection of expression of the gp160 precursor and proteolytic processing of gp120/gp41, the cells were washed and then lysed in a lysis buffer (1% Triton X-100, 0.1% NP-40, 50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and a cocktail of protease inhibitors [Pharmacia]), and the cell lysates were cleared of large, nonsoluble debris by centrifugation at 20,000 × *g* for 30 min at 4°C. Env proteins in the culture medium or the cell lysate were immunoprecipitated with 3 μl of pooled sera from HIV-1-infected patients or 1 μg of MAb 2F5 (Polymun Scientific, Vienna, Austria), respectively. After extensive washing, the precipitated Env proteins were resolved on 8% SDS-PAGE gels and visualized by autoradiography.

Evaluation of apparent affinity of HIV-1 Env trimers expressed on the cell surface by FACS. At 48 h posttransfection, as described above, cells were washed with 1× phosphate-buffered saline (PBS) and released from culture plates by 10 mM EDTA in 1× PBS. The cells were washed again with 1× PBS containing 1% bovine serum albumin (1% BSA/PBS) twice and then blocked with 1% BSA/PBS for 30 min on ice. The 2F5 MAb at different concentrations in 1% BSA/PBS was added to the cells for 1 h on ice and removed from the cells by washing with 1% BSA/PBS twice. After incubating with rhodophyta-phycoerythrin-conjugated anti-human IgG (Sigma Aldrich) at a 1:100 dilution for 1 h on ice, the cells were washed twice again and fixed in 500 μl of 4% formaldehyde solution. A single-channel fluorescence-activated cell sorter (FACS) analysis was conducted to quantify the amount of MAb 2F5 bound to the Env proteins on the cell surface, and the geometric mean of fluorescence intensity of MAb 2F5 staining was calculated to measure the apparent affinity of binding. An aliquot of the same cells was stained with pooled sera from HIV-1-infected patients at a 1:500 dilution as the primary antibody. The level of staining with patient sera was used as the standard to normalize Env expression for the measurement of MAb 2F5 binding.

Production of pseudotyped HIV-1 luciferase reporter viruses, single-round infection assay, and neutralization assay. Recombinant HIV-1 containing firefly luciferase was produced as described previously (24). In brief, 293T cells were cotransfected with the pHIV-1-Luc vector plasmid, the pc-PACK packaging plasmid, and the pSVIIIenv plasmid expressing the Env variants at a ratio of 3:1:1. For transfection of 293T cells in 100-mm tissue culture dishes, 10 or 40 μg of total DNA was used when the Lipofectamine reagent (Invitrogen) or calcium phosphate method was employed, respectively. Viruses were harvested 2 days posttransfection, aliquoted, and stored at -80°C. In some experiments, chimeric viruses containing two different Env variants were produced. 293T cells were cotransfected as described above except that the pSVIIIenv plasmids expressing the desired Env variants were mixed at certain ratios while the total amount of DNA of Env-expressing plasmids was kept constant.

Infectivity of the recombinant viruses was measured by a single-round infection assay using the Cf2Th-CD4/CCR5 cells as target cells, as described previously (24). In brief, the Cf2Th-CD4/CCR5 cells were seeded in 96-well tissue culture Isoplates (EG&G Wallace) at a density of 6,000 cells per well and cultured overnight. After the medium was removed thoroughly, 100 μl of virus suspension in fresh culture medium was added into each well. After 2 days, virus infectivity was quantified by measuring luciferase activity within each infected well using a luciferase detection kit (Pharmingen). Duplicate or triplicate wells of the target cells were infected in parallel for each sample of virus, and the mean value for luciferase activity was obtained as the infectivity of a reporter virus.

MAbs 2F5 and b12 were from Polymun Scientific. The Fab fragment of 2F5 (Fab 2F5) was a gift from Peter Kwong (Vaccine Research Center, NIH). To assess the neutralizing sensitivity of the recombinant viruses to MAb b12 or 2F5, viruses were incubated with the antibodies at various concentrations at 37°C for 2 h. The residual viral infectivity in the antibody/virus mixture was then measured

using the single-round entry assay, as described above. Neutralization sensitivity was measured by the residual infectivity of viruses treated with antibody as a percentage of the infectivity of the same viruses mock treated with culture medium, set as 100%. Each experiment was repeated at least three times, and consistent results were obtained. A typical set of data from one experimental session was reported.

RESULTS

Neutralization efficiency is greatly enhanced by the presence of 2F5 epitopes in both the gp120 V4 and gp41 MPERs of HIV-1 Envs. HIV-1_{YU2} gp160 was chosen as the model Env protein because it is a primary strain. It uses the CCR-5 coreceptor, it is efficient in entry and relatively resistant to neutralization by antibodies, and it has been studied extensively; a high-resolution structure is available (25, 28). We constructed variants of this model Env as illustrated in Fig. 1A. Natural HIV-1_{YU2} gp160 is neutralizable but relatively insensitive to MAb 2F5, because it has a variant 2F5 epitope (-A⁶⁶²LDKWAS-; hyphens represent natural sequences to either side of the core sequence) instead of the canonical sequence (-E⁶⁶²LDKWAS-) (32, 57). The A662E change was introduced to create a canonical 2F5 epitope in MPER, termed MPER-2F5. This change increases its sensitivity to neutralization by 2F5, as previously shown (57). The changes of D664K/K665E were introduced to eliminate the natural 2F5 epitope, named NO-2F5 (68). HIV-1 gp120 contains five variable regions. The V4 region has unique features, including easy accessibility, flexibility, no high-order structure, no known function, and a location far from known functional domains (44, 54; reviewed in reference 55). An exogenous FLAG epitope can be inserted into the gp120 V4 region of various HIV-1 strains without significantly affecting the structural and functional integrity of the carrier Env protein (37, 60). Significantly, the M2 anti-FLAG MAb could neutralize these Env variants from several HIV-1 isolates that have dramatically different neutralization sensitivities to anti-Env antibodies. Our evidence suggests that neutralization via the V4-inserted FLAG epitope is likely through the mechanism of steric hindrance (37). Analogous to the V4 FLAG epitope, a canonical 2F5 epitope was introduced into the HIV-1_{YU2} gp160 V4 region by inserting the “-GLELDKWASLG-” sequence in front of residue N407 and by making a N407A change to remove a glycosylation site immediately adjacent to the inserted epitope. The V4 2F5 insertion was introduced into the NO-2F5 construct, resulting in a variant carrying a 2F5 epitope in the gp120 V4 region and no 2F5 epitope in the gp41 MPER, designated V4-2F5. Finally, the V4 2F5 insertion was introduced into the MPER-2F5 construct, resulting in a variant carrying canonical 2F5 epitopes in both the gp120 V4 region and gp41 MPER, named 2x2F5.

To assess the expression and the gp120/gp41 proteolytic processing of the mutant Envs, these Env variants were transiently expressed and radiolabeled in 293T cells. The overall cell-associated Env proteins and the secreted gp120 were immunoprecipitated by pooled sera from HIV-1-infected patients from cell lysates and culture media, respectively. For all Env variants, the total amounts of gp160 and gp120 species precipitated by patient sera were similar in cell lysates and in culture media, indicating that the constructed changes had no detectable effect on the expression, proteolytic processing, or structural stability of the carrier Env protein (Fig. 1B, upper panel).

When pseudotyped on the HIV-1 luciferase reporter viruses, these Env variants exhibited comparable infectivity levels in a single-round entry assay, with <3-fold differences (Fig. 1C). To evaluate further the structural and functional integrity of these Env variants, the luciferase reporter viruses were subjected to neutralization by MAb b12. MAb b12 binds to the CD4-binding site with high sensitivity to the conformational integrity of an HIV-1 Env trimer, and its epitope is not related to the MPER and V4 epitopes (5, 63). All four Env variants were neutralized similarly by MAb b12 (Fig. 1D). Taken together, these results demonstrate that both the insertion of a 2F5 epitope in the gp120 V4 region and the changes in the MPER 2F5 epitope have no deleterious effect on the expression, gp120/gp41 proteolytic processing, trimer stability, entry function, or neutralization sensitivity of HIV-1 Envs.

To assess whether the engineered 2F5 epitopes were intact and accessible to MAb 2F5, 1 μ g of MAb 2F5 was used to immunoprecipitate radiolabeled Envs from cell lysates and culture media. No NO-2F5 Env proteins were precipitated by MAb 2F5 from either the cell lysates or the culture media, indicating that the D664K/K665E changes eliminated the 2F5 epitope (Fig. 1B, lower panel). The gp160 and gp120 forms were detected at similar levels in V4-2F5 and 2x2F5, indicating that the 2F5 epitope in the gp120 V4 region was functionally intact and accessible to antibody binding. As expected for MPER-2F5, only gp160 and not the gp120 form was precipitated by MAb 2F5. Interestingly, MAb 2F5 precipitated V4-2F5 gp160 more efficiently than MPER-2F5 gp160, suggesting that in the context of soluble monomeric gp160, the 2F5 epitope in the V4 region has higher affinity for MAb 2F5 than the original 2F5 epitope at MPER. It is possible that the lower affinity of MAb 2F5 for the 2F5 epitope in the MPER of soluble gp160 is due to the lack of lipid, since the structure and function of the natural gp41 MPER 2F5 epitope could be affected by the presence of lipid from viral or cellular membrane in close proximity (17, 34).

To compare the apparent affinity of MAb 2F5 for the 2F5 epitopes at different locations in the context of intact HIV-1 Env trimers as well as in the presence of lipid in close proximity, FACS analysis was conducted to measure the binding affinity of MAb 2F5 to Env trimers expressed on the surface of 293T cells. To normalize protein expression levels, the same cells were also stained with a saturating concentration (1:500) of pooled sera from HIV-1-infected patients in parallel. After normalizing to the staining level of patient sera, the geometric mean of fluorescence intensity was used to compare the apparent affinity of MAb 2F5 for individual Env variants. Consistent with previous studies, cells expressing the MPER-2F5 Env were stained somewhat weakly by MAb 2F5, while both V4-2F5 and 2x2F5 Env variants bound to MAb 2F5 with high affinity (Fig. 1E). These results indicated that MAb 2F5 binding to the 2F5 epitope in the V4 region was stronger than that in the MPER, consistent with the results from immunoprecipitations. Contrary to the notion that the presence of lipid in proximity is required for efficient binding of 2F5 to its epitope, the lack of lipid close to the V4 2F5 epitope did not have a detrimental effect on MAb 2F5 binding in our experiment. The higher apparent affinity of MAb 2F5 for the 2F5 epitope in the V4 region relative to the 2F5 epitope located in the MPER could be due to its physical location allowing easy access by

FIG. 1. Insertion of 2F5 epitopes in the V4 region of HIV-1_{YU2} gp160. (A) Amino acid sequences in the V4 region and MPER of the 2F5 variants of HIV-1_{YU2} gp160 are aligned, with wild-type (wt) gp160 shown on top. The modified residues are underlined. (B) HIV-1 Envs were transiently expressed in 293T cells and labeled with [³⁵S]methionine/cysteine. The Envs in the cell lysate and culture medium were immunoprecipitated with 3 μl of pooled sera from HIV-1-infected patients or 1 μg of MAb 2F5. The precipitates were analyzed with 8% SDS-PAGE gels and visualized by autoradiography. Mock, mock transfection. (C) HIV-1 Env variants were pseudotyped on luciferase reporter viruses by transient transfection of 293T cells. Wells containing the Cf2Th-CD4/CCR5 target cells were infected with 6.6 μl of each virus stock in a single-round entry assay. Means and ranges of variation of luciferase signals from triplicate wells are presented to quantify the entry function of individual Env proteins. (D). Twenty microliters of each viral stock was neutralized by the given concentrations of MAb b12. The residual infectivity of antibody-treated viruses was measured using a single-round entry assay and is presented as a percentage of the infectivity of the same virus mock-treated with culture medium, which is set as 100%. One additional virus included in this panel was an Env chimeric virus with the V4-2F5 and MPER-2F5 gp160s at a 1:1 ratio (MPER-2F5+V4-2F5). (E) Apparent binding affinity of MAb 2F5 to the Env variants expressed on the surface of 293T cells. HIV-1 Envs transiently expressed on the surface of 293T cells were stained with the given concentrations of MAb 2F5 or a 1:500 dilution of pooled sera from HIV-1-infected patients. The binding affinity of MAb 2F5 to each Env variant was then measured by FACS in the form of the geometric mean of fluorescence intensity, after being normalized to staining of pooled sera from HIV-1-infected patients. Due to large numbers of staining samples, the experiment was performed three times, each of which covered all data points in comparison. For the purpose of quantitative comparisons summarizing different experimental sessions, the fluorescence intensity of MAb 2F5 staining was normalized to the highest staining level obtained in a given experimental session, which is set as 100%. Means and ranges of variation from three experiments are presented. In some experiments, the cells were stained with up to 200 μg/ml MAb 2F5, and staining with 20 to 40 μg/ml of MAb 2F5 sometimes resulted in reduced levels of fluorescence. Therefore, only staining with 10 μg/ml of MAb 2F5 or lower produced consistent, informative data. For consistency, results from multiple experiments with an upper limit of 10 μg/ml of MAb 2F5 are presented. This level of staining represented the highest concentration of antibody that produced informative data, which might be below the saturating amount of antibody for binding. To test whether the difference in staining levels among the groups (2X2F5, V4-2F5, and MPER-2F5) is statistically significant, a two-way ANOVA analysis was employed to conduct a pair-wise comparison including two of the three groups in each analysis, and the analyses used all of the primary data from three independent experiments, excluding data from all mock stainings. The analysis was performed using the “proc GLM 2-way ANOVA” procedure (SAS version 9.1; SAS, NC). *F* and *P* (statistical probability of difference) values were produced. For pair-wise comparisons, the degree of freedom (*df*) for statistics is 1. For the comparison between 2x2F5 and V4-2F5, *F* is 20.91 and *P* is <0.0001. For the comparison between 2x2F5 and MPER-2F5, *F* is 34.12 and *P* is <0.0001. For the comparison between V4-2F5 and MPER-2F5, *F* is 18.23 and *P* is 0.0002. Thus, differences in apparent affinity among groups visualized in the figure are all statistically significant.

FIG. 2. Bivalency of antibody binding enhances neutralization efficiency. Recombinant HIV-1 luciferase reporter viruses were neutralized by the given concentrations of bivalent MAb 2F5 (A) or monovalent Fab 2F5 (B). To facilitate comparisons, a dashed line is drawn at the level of 50% residual infectivity to visualize the IC_{50} s. The red and green arrows mark the IC_{50} s of MAb 2F5 in neutralizing viruses carrying the MPER-2F5 and 2x2F5 Envs, respectively.

antibodies (30, 65). 2x2F5 exhibited modestly stronger staining than V4-2F5 by the same concentrations of MAb 2F5. Although the observed difference in staining levels between V4-2F5 and 2x2F5 was modest in scale, this difference was consistent in three sets of independent experiments and was statistically significant in a two-way analysis of variance (ANOVA; $P < 0.0001$). These data suggest that while the 2F5 epitope in the V4 region dominates the binding of MAb 2F5 to the 2x2F5 Env trimer, the 2F5 epitope in the MPER of 2x2F5 also contributes to the overall affinity for MAb 2F5 binding, i.e., avidity. In summary, the 2F5 epitope inserted in the gp120 V4 region is functional for MAb 2F5 binding, and the 2x2F5 Env has high avidity for MAb 2F5 via binding to the 2F5 epitopes in both the V4 region and the MPER.

To determine the functional consequence of carrying two 2F5 epitopes in the V4 location and the MPER, we compared neutralization sensitivities of 2x2F5 and other Env variants using a standard neutralization assay based on the single-round entry of HIV-1 luciferase reporter viruses. Removal of the natural 2F5 epitope (NO-2F5) rendered the resultant virus resistant to MAb 2F5 neutralization (Fig. 2A). Surprisingly, the 2F5 epitope in the V4 region (V4-2F5) led to detectable but limited neutralization (50% inhibitory concentration [IC_{50}], $>10 \mu\text{g/ml}$), even though it had high apparent affinities for MAb 2F5 in both immunoprecipitation and FACS assays (Fig. 2A and Fig. 1B and E). In contrast, the MPER-2F5 virus was efficiently

neutralized, especially at higher concentrations of MAb 2F5, even though MPER-2F5 had limited binding affinity to MAb 2F5 in both immunoprecipitation and FACS assays (Fig. 2A and Fig. 1B and E). These contradictory results could be explained by potential differences in neutralization mechanisms involved in neutralizing these two types of virus. While steric hindrance is the most likely explanation for neutralization by antibody binding to the V4 region, it has been reported that MAb 2F5 binding to the MPER could cause a blockage after the engagement of both the CD4 receptor and the CCR5 coreceptor (3, 33). Results here would support the idea that steric hindrance is a relatively inefficient mechanism for antibody-mediated neutralization. This interpretation is consistent with that of similar recent studies of several viruses (35, 37, 39). Strikingly, viruses pseudotyped with the 2x2F5 Env were neutralized by MAb 2F5 with exceedingly high levels of efficiency (Fig. 2A). The IC_{50} for 2x2F5 ($0.0036 \mu\text{g/ml}$) was reduced by over 2 logs compared with that for MPER-2F5 ($0.38 \mu\text{g/ml}$) and even more dramatically compared with that for V4-2F5 ($>10 \mu\text{g/ml}$).

To test whether bivalent binding of IgG molecules to 2F5 epitopes contributes to the enhancement of neutralization of 2x2F5 by MAb 2F5, the same viruses were incubated with monovalent Fab 2F5. Fab 2F5 neutralized the V4-2F5 virus poorly (Fig. 2B). Interestingly, monovalent Fab 2F5 and bivalent MAb 2F5 neutralized the MPER-2F5 virus with similar levels of efficiency (Fig. 2A and B). These data support the idea that MAb 2F5 does not neutralize natural HIV-1 Envs with bivalency, consistent with the 1:1 stoichiometry of antibody-mediated neutralization of HIV-1 Envs demonstrated by our previous study (57). Importantly, monovalent Fab 2F5 neutralized the MPER-2F5 and 2x2F5 viruses with almost identical efficiency (Fig. 2B). Therefore, the increase in the total number of 2F5-binding moieties in the 2x2F5 virus, relative to that of the MPER-2F5 or V4-2F5 virus, is not sufficient to explain the observed enhancement in its neutralization by MAb 2F5 IgG (Fig. 2A).

In summary, the combined data from neutralization by MAb 2F5 and Fab 2F5 indicate that the enhanced neutralization of 2x2F5 by MAb 2F5 is attributable to the bivalency of an IgG molecule. In theory, within one trimer of the 2x2F5 Env protein, one MAb 2F5 molecule can bind simultaneously to the V4 2F5 epitope of one gp120/gp41 subunit and the MPER 2F5 epitope of another subunit, designated the *trans*-configuration. Or, the MAb 2F5 molecule can bind to the V4 and MPER epitopes of a single gp120/gp41 subunit in one 2x2F5 Env trimer, designated the *cis*-configuration.

Enhancement in neutralization can be achieved by bivalent binding of the V4 and MPER 2F5 epitopes from two separate gp120/gp41 subunits within an HIV-1 Env trimer. To test whether the *trans*-configuration can be achieved for bivalent binding of an antibody to an HIV-1 Env trimer, we developed a *trans*-complementation assay of two different Env species. This assay took advantage of the fact that when two closely related Env species are coexpressed in 293T cells, the two kinds of Env protomers or gp120/gp41 subunits mix randomly to form Env trimers with quantitative accuracy (18, 41, 57, 58). A chimeric virus was produced by cotransfecting plasmids expressing the V4-2F5 and MPER-2F5 gp160s at a 1:1 ratio. According to random association of Env protomers as es-

established in our previous studies (57, 58), the resultant virus population should theoretically contain four different types of Env trimers, with the predicted frequencies in parentheses: (V4-2F5)₃ (12.5%), (MPER-2F5)₃ (12.5%), (V4-2F5)₂(MPER-2F5) (37.5%), and (V4-2F5)(MPER-2F5)₂ (37.5%). The (V4-2F5)₃ and (MPER-2F5)₃ trimers will not support bivalent binding in the *trans*-configuration. In contrast, the (V4-2F5)₂(MPER-2F5) and (V4-2F5)(MPER-2F5)₂ trimers could bind MAb 2F5 bivalently in the *trans*-configuration, i.e., binding the V4 2F5 epitope from one gp120/gp41 subunit and the MPER 2F5 epitope from another subunit. If the *trans*-configuration of bivalent binding can be achieved and binding bivalency leads to enhancement in neutralization efficiency, the (V4-2F5)₂(MPER-2F5) and (V4-2F5)(MPER-2F5)₂ trimers should have higher neutralization sensitivity than either the (V4-2F5)₃ or the (MPER-2F5)₃ Env trimer. According to the predicted frequencies, the (V4-2F5)₂(MPER-2F5) and (V4-2F5)(MPER-2F5)₂ trimers should comprise 75% of all Env trimers in the viral stock, accounting for the dominant portion of total viral infectivity of such a chimeric virus. Therefore, if the *trans*-configuration binding bivalency can be achieved and binding bivalency results in enhancement in neutralization, the chimeric virus made of both the V4-2F5 and MPER-2F5 gp160s is predicted to have higher neutralization sensitivity to MAb 2F5 than the viruses containing the V4-2F5 or MPER-2F5 Envs separately.

Our data confirmed that the chimeric virus containing both the V4-2F5 and MPER-2F5 gp160s (MPER-2F5+V4-2F5) was neutralized more efficiently by MAb 2F5 than the viruses made of either the V4-2F5 or MPER-2F5 Env alone, with a 5-fold reduction in IC₅₀ relative to that of the MPER-2F5 virus and an even greater reduction relative to that of the V4-2F5 virus (Fig. 3A). The MPER-2F5+V4-2F5 chimeric virus was neutralized by MAb b12 in a manner similar to that of the viruses carrying either the V4-2F5 or MPER-2F5 Env individually, indicating that chimerism did not affect the structural and functional integrity of the (V4-2F5)₂(MPER-2F5) and (V4-2F5)(MPER-2F5)₂ Env heterotrimers (Fig. 1D). These viruses were also neutralized by monovalent Fab 2F5. Fab 2F5 neutralized the MPER-2F5+V4-2F5 chimeric virus less efficiently than the MPER-2F5 virus, with an IC₅₀ difference of about 3-fold (Fig. 3B). This difference was probably due to the fact that compared with the MPER-2F5 virus, the MPER-2F5+V4-2F5 chimeric virus contained only half of the amount of the MPER 2F5 epitope that is more neutralization sensitive while carrying the V4 2F5 epitope that is less neutralization sensitive. Combining these differences in IC₅₀ in the opposite directions (i.e., both a reduction and an increase in IC₅₀), the *trans*-configuration of bivalent binding within an HIV-1 Env trimer resulted in enhancement of neutralization efficiency by a 15-fold reduction of IC₅₀. Furthermore, the possible permutations of bivalent binding configurations in the (V4-2F5)₂(MPER-2F5) and (V4-2F5)(MPER-2F5)₂ Env heterotrimers are reduced to two compared with six in the 2x2F5 Env homotrimer, and the MPER-2F5+V4-2F5 chimeric virus also contains the (V4-2F5)₃ and (MPER-2F5)₃ homotrimers that will not support bivalent binding and are less neutralization sensitive. These two factors could result in an underestimation of the effect of binding bivalency on neutralization efficiency in this assay. Therefore, the results from the *trans*-complementa-

FIG. 3. Bivalency of antibody binding to an HIV-1 Env trimer can be achieved by binding in the *trans*-configuration. Recombinant HIV-1 luciferase reporter viruses were produced to carry the V4-2F5 or MPER-2F5 Envs individually, and a chimeric virus was also produced by cotransfecting the V4-2F5 and MPER-2F5 gp160s at a 1:1 ratio, termed MPER-2F5+V4-2F5. The reporter viruses were produced as a set and neutralized by the given concentrations of bivalent MAb 2F5 (A) or monovalent Fab 2F5 (B). The red and black arrows mark the IC₅₀s of MAb 2F5 or Fab 2F5 in neutralizing viruses carrying the MPER-2F5 gp160 or the MPER-2F5+V4-2F5 chimeric Envs, respectively.

tion assay largely recapitulated the enhancement in neutralization by binding bivalency observed in the experiment using the 2x2F5 construct.

Bivalent binding of the V4 and MPER 2F5 epitopes from a single gp120/gp41 subunit within an Env trimer is not achieved for the enhancement of neutralization. To test if the *cis*-configuration could support bivalent binding of MAb 2F5 to an HIV-1 Env trimer and neutralization, we used a complementation assay developed on the basis of subunit stoichiometry of HIV-1 Env trimer during virus entry (59). Previously, we have demonstrated that when a fusion-competent Env species is coexpressed with a fusion-incompetent Env species, only those Env trimers containing two or three fusion-competent subunits can mediate virus entry. The L520E change introduces a strongly charged residue into the gp41 fusion peptide that is normally very hydrophobic, resulting in total inactivation of the fusion/entry function. We have shown that among a panel of candidate mutant Envs, the L520E mutation does not affect protein expression and processing, and more importantly, the L520E mutant Env mixes efficiently with related Env proteins to form a heterotrimer with quantitative accuracy (59). The L520E mutation was introduced into the four 2F5 gp160 variants. The radiolabeled Env proteins of the L520E derivative of the four 2F5 Env variants were immunoprecipi-

tated by pooled sera from HIV-1-infected patients and MAb 2F5, similar to the procedures described in the legend to Fig. 1B. As expected, the L520E mutation did not affect protein expression, gp120/gp41 processing, and stability of Env trimers (data not shown).

We generated chimeric viruses containing 20% of the fusion-competent/non-2F5-binding gp160 (NO-2F5) and 80% of one of the fusion-incompetent/2F5-binding gp160s (NO-2F5-L520E, V4-2F5-L520E, MPER-2F5-L520E, or 2x2F5-L520E). Using the 2x2F5-L520E gp160 as an example, random mixing of Env subunits from 20% of NO-2F5 and 80% of 2x2F5-L520E would result in the following four kinds of Env trimers, with their predicted frequencies in parentheses: (NO-2F5)₃ (0.8%), (NO-2F5)₂(2x2F5-L520E) (9.6%), (NO-2F5)(2x2F5-L520E)₂ (38.4%), and (2x2F5-L520E)₃ (51.2%). Since the latter two kinds of Env trimers were incompetent for virus entry due to the presence of two or three 2x2F5-L520E subunits, the infectivity of such a viral stock is supported only by the first two types of Env trimers, i.e., (NO-2F5)₃ (0.8%) and (NO-2F5)₂(2x2F5-L520E) (9.6%). Since we have previously shown that the (NO-2F5)₃ and (NO-2F5)₂(2x2F5-L520E) trimers are similarly infectious (59), the infectivity of such a viral stock reflects the function of the predominant (NO-2F5)₂(2x2F5-L520E) heterotrimer, accounting for 92.3% of the total infectivity [(NO-2F5)₂(2x2F5-L520E) (9.6%) divided by [(NO-2F5)₃ (0.8%) + (NO-2F5)₂(2x2F5-L520E) (9.6%)], or in short, $9.6\% / (0.8\% + 9.6\%) = 92.3\%$]. Therefore, if bivalent binding in the *cis*-transfection can be achieved, one would predict that the virus containing 20% NO-2F5/80% 2x2F5-L520E should be more sensitive to MAb 2F5 neutralization than viruses containing 20% NO-2F5/80% MPER-2F5-L520E or 20% NO-2F5/80% V4-2F5-L520E.

Luciferase reporter viruses were produced by cotransfecting 20% of the NO-2F5 plasmid and 80% of the Env-expressing plasmid of one of the four L520E 2F5 variants. As predicted by the subunit stoichiometry requirement, these chimeric viruses exhibited >10-fold-reduced infectivity compared with that of the control virus carrying only the NO-2F5 gp160; and all viruses carrying the L520E mutant Envs were noninfectious (data not shown). The four chimeric viruses were neutralized by MAb b12 equivalently, confirming that the introduction of the L520E mutation did not change the functional and structural integrity of the Env heterotrimers (Fig. 4A). Taken together, the data indicate that the L520E 2F5 variant Envs form functional Env heterotrimers efficiently with the NO-2F5 gp160.

The NO-2F5-L520E:NO-2F5 chimeric virus was resistant to neutralization by MAb 2F5 since there was no functional 2F5 epitope in either Env (Fig. 4B). The V4-2F5-L520E:NO-2F5 chimeric virus was largely resistant to neutralization by MAb 2F5, the exception being a slight reduction of infectivity at the highest concentration of MAb 2F5 (20 μ g/ml). Importantly, the chimeric viruses of MPER-2F5-L520E:NO-2F5 and 2x2F5-L520E:NO-2F5 were neutralized similarly by MAb 2F5 (Fig. 4B). At higher concentrations of MAb 2F5 (≥ 4 μ g/ml), the 2x2F5-L520E:NO-2F5 chimeric virus was actually neutralized slightly less efficiently than was the MPER-2F5-L520E:NO-2F5 virus. There was a nonneutralized fraction of infectivity in both the MPER-2F5-L520E:NO-2F5 and 2x2F5-L520E:NO-2F5 viruses, which could have resulted from the presence of

FIG. 4. Bivalency of antibody binding to an HIV-1 Env trimer is not achieved by binding in the *cis*-configuration. Recombinant HIV-1 luciferase reporter viruses were produced to carry chimeric Envs by cotransfecting the NO-2F5 gp160 plasmid and the NO-2F5-L520E, MPER-2F5-L520E, V4-2F5-L520E, or 2x2F5-L520E gp160 plasmid at a 1:4 ratio. The reporter viruses were produced as a set and neutralized by the given concentrations of MAb b12 (A) or MAb 2F5 (B).

the (NO-2F5)₃ Env trimer and/or the possibility that the heterotrimer formation between two partner Env proteins was at efficiencies below what is expected for total randomness. Nevertheless, the (NO-2F5)₂(2x2F5-L520E) and (NO-2F5)₂(MPER-2F5-L520E) Env trimers, representing the neutralization-sensitive portion of Env trimers in their respective viral stocks, appeared to have similar sensitivities to neutralization by MAb 2F5. These results are consistent with the notion that the presence of both V4 and MPER 2F5 epitopes within one 2x2F5 gp120/gp41 subunit confers no advantage in neutralization compared to that of the MPER 2F5 alone. Therefore, the *cis*-configuration of bivalent binding by MAb 2F5 to an HIV-1 Env trimer is not achieved in this experimental system.

DISCUSSION

An immunoglobulin molecule rarely binds to a virion surface bivalently, despite the fact that antibody molecules are mostly multivalent (reviewed in references 12 and 22). It is widely assumed that antibody-binding avidity contributes to antibody-mediated neutralization of viruses, although it has not been characterized adequately at the molecular level (see the introduction). In a well-characterized case in which MAbs 17-1A and 8F5 bind bivalently to the virion surface of HRV14 and HRV2, the binding epitope is a canyon structure composed of elements from both the Vp1 and Vp2 proteins in a 2-fold symmetry (19, 42). Bivalent binding of an antibody molecule to

the virion surface may be difficult due to environmental features on virion surfaces as well as the structural nature of IgG. Theoretically, factors on the virion surface restricting bivalent binding by an antibody may include the following. (i) Viral spikes may have limited mobility on virion surfaces. The viral capsid in nonenveloped viruses is a part of the icosahedral structure that is rigid, as suggested by successes in generating the cryo-EM images of many such viruses (reviewed in reference 11). The Env spikes on enveloped viruses can be immobilized by association with the viral matrix protein, such as in HIV-1 (15, 31, 56), or by general crowdedness of Env spikes in viruses with high Env contents, such as vesicular stomatitis virus (VSV) and influenza virus (1, 48). (ii) The distance between two viral spikes is just right or is close to the wing span of the two Fab arms of an antibody in the case of IgG1. (iii) The cognition of a specific viral epitope and its antibody paratope defines the angle of projection for the rest of the antibody molecule, restricting the paratope on the other Fab arm from reaching another epitope. (iv) Nonantigenic elements of both viral and cellular origins on the surface of enveloped viruses can cause steric interference with unpredictability, such as major histocompatibility complex (MHC) and ICAM-1 molecules on HIV-1 virions (8, 14, 38, 40). Characteristics of the physical structure of antibody could also restrict binding in bivalency. First, an IgG molecule, or even a Fab fragment, is rather large compared with the binding epitope on the target viral protein. Also, IgG molecules have very limited structural flexibility (reviewed in references 6 and 23). The antibody hinge region may allow a small degree of flexibility for spatial maneuvering, but it is often short, and the short junction between the CH1 and V domains has only limited flexibility (reviewed in references 6 and 16). Multiple binding-restrictive factors from virions and antibody molecules may function synergistically to form prohibitive barriers for an antibody to bind a virion bivalently. When the binding target of a neutralizing antibody is a single viral spike, the effect from such binding-restrictive factors could be magnified due to the extreme spatial restriction posed by the small size of a viral spike. These reasons could explain why it is rare for a neutralizing antibody to bind to individual virions bivalently. The rarity of natural bivalent binding of an antibody to a virion has so far limited the delineation of the role of binding bivalency/avidity in antibody-mediated neutralization of viruses.

In this study, we have developed an artificial system in which the model antibody can bind to an HIV-1 Env trimer bivalently in the V4 region and the MPER at the same time. In one experimental setting, an additional 2F5 epitope was inserted into the gp120 V4 region so that the HIV-1_{YU2} gp160 carries the 2F5 epitopes in both the V4 region and the MPER. In this case, binding bivalency of MAb 2F5 results in greatly enhanced neutralization efficiency, with an IC_{50} reduction of over 2 logs. In another setting, complementation between one gp160 carrying a V4 2F5 epitope and another gp160 carrying an MPER 2F5 epitope forms heterotrimers that permit MAb 2F5 to bind bivalently across two gp120/gp41 subunits within one Env trimer. In this case, the binding bivalency also resulted in significant enhancement of neutralization efficiency, with an IC_{50} reduction of about 15-fold. In both experimental systems, the monovalent Fab 2F5 controls confirmed the importance of binding bivalency for the enhancement of neutralization effi-

ciency. Therefore, we conclude that in this model system, binding bivalency of a neutralizing antibody to a target Env spike contributes to the efficiency of antibody-mediated neutralization of viruses.

The affinity of binding an antibody to its target is determined by intrinsic affinity and avidity (reviewed in reference 16). Intrinsic affinity is the force of monovalent binding between a single antibody paratope and a single antigenic epitope. Avidity is the overall force of engaging multiple antibody paratope/antigen epitope pairs. It is generally assumed that high valency of binding results in more-stable binding, i.e., higher general affinity or avidity results in more-efficient neutralization. We detected an only limited but appreciable increase in avidity of binding MAb 2F5 to the 2x2F5 Env relative to the V4-2F5 Env and a more-pronounced difference relative to the MPER-2F5 Env. It is possible that measurement of affinity and/or avidity by FACS may not be truly quantitative and that the affinity difference between the 2x2F5 Env and the V4-2F5 Env in binding to MAb 2F5 is thus underestimated in this study. Unfortunately, there is no satisfying form of recombinant HIV-1 Env trimer that would permit quantitative measurements and comparisons of the binding affinity of MAb 2F5 to these 2F5 Env variants in a trimeric form. In general, our results support the idea that antibody-binding bivalency leads to higher binding avidity and higher neutralization efficiency.

This conclusion should be tempered by two imbalances in our experimental system. One is the large difference in binding affinities between two binding epitopes, i.e., the V4 2F5 epitope has significantly higher binding affinity than that of the MPER 2F5 epitope when measured by either FACS or immunoprecipitation. Another is the potential differences in neutralization mechanisms employed by the binding to the two types of epitopes. While binding to the V4 epitope is likely to achieve neutralization by steric hindrance, binding to the MPER 2F5 epitope might cause a neutralizing effect at a step after receptor engagement (3, 33, 37). It has been reported that 2F5 binds to a β -turn structure better than to a α -helix conformation of the MPER domain. Since the transition from the β -turn structure to the α -helix conformation is generally believed to be important for driving the fusion process, 2F5 binding to the MPER might interfere with this suggested conformational transition, thus preventing the fusion reaction and causing neutralization of virus infection (3). Furthermore, neutralization via the MPER 2F5 epitope appears to be significantly more efficient than neutralization via the V4 epitope. The efficient neutralization by bivalent binding in our experimental system is probably a result of the synergistic effect of higher binding affinity and two different neutralization mechanisms, i.e., higher-affinity binding to the V4 epitope and a more-efficient neutralizing mechanism of blocking fusion via the MPER. With this confounding, we cannot definitively conclude with the data obtained here that antibody-binding bivalency contributes to neutralization efficiency by an increase in binding avidity. Nevertheless, our results clearly demonstrate that antibody-binding bivalency contributes to virus neutralization, providing an important framework in which to dissect the mechanisms of antibody-mediated neutralization of viruses. Considered in combination with the fact that bivalency is rare in nature (reviewed in references 12 and 22), this finding encourages us to consider antibody-mediated neutralization of

viruses in the realm of monovalent binding of an antibody molecule to one viral spike.

Our data clearly indicate that if high valency of binding can be achieved, an antibody can block the biological function of the target molecule with greatly enhanced efficiency, in this case, by using virus neutralization as a model. Our findings suggest that in principle, one may be able to design bivalent and/or bispecific protein ligands as inhibitors against viruses or other pathological targets in order to achieve significant increases in blocking potency and/or specificity.

Our data demonstrate that a 2F5 molecule can bind bivalently, bridging the V4 region and the MPER in two gp120/gp41 subunits within one HIV-1 Env trimer, i.e., in the *trans*-configuration. The bivalency of binding cannot be achieved by binding to the two sites within a single gp120/gp41 subunit, i.e., in the *cis*-transfiguration. This finding dictates the following two distinct parameters for the structure of a native, infectious HIV-1 Env trimer: (i) the distance between the V4 region in one gp120/gp41 subunit and the MPER in another gp120/gp41 subunit should be close to the span of two paratopes of a 2F5 molecule, and (ii) the binding of paratopes to these sites should be spatially compatible with the antibody structure, considering the projections of two Fab arms after binding to the Env trimer. Once a high-resolution structure of an HIV-1 Env trimer is available, the ability to dock the 2F5 structure onto the Env trimer structure for bivalent binding in the *trans*-configuration and not in the *cis*-transfiguration will provide a critical test of the biological relevance of such a structure.

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