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Antibody Binding Is a Dominant Determinant of the Efficiency of Human Immunodeficiency Virus Type 1 Neutralization▽

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Primary and laboratory-adapted variants of human immunodeficiency virus type 1 (HIV-1) exhibit a wide range of sensitivities to neutralization by antibodies directed against the viral envelope glycoproteins. An antibody directed against an artificial FLAG epitope inserted into the envelope glycoproteins of three HIV-1 isolates with vastly different neutralization sensitivities inhibited all three viruses equivalently. Thus, naturally occurring HIV-1 isolates that are neutralization resistant are not necessarily more impervious to the inhibitory consequences of bound antibody. Moreover, the binding affinity of the anti-FLAG antibody correlated with neutralizing potency, underscoring the dominant impact on neutralization of antibody binding to the envelope glycoproteins.

Human immunodeficiency virus type 1 (HIV-1) is the etiological agent of AIDS. HIV-1 establishes persistent infections in humans and has evolved to be relatively resistant to antibodies generated during natural infection (1, 2, 5, 8, 13, 26, 27, 34, 39, 42). Primary (clinical) HIV-1 strains exhibit a range of sensitivities to antibody-mediated neutralization, but they are generally more resistant than the T-cell line-adapted isolates that have been cultured extensively in vitro (9, 20, 24, 30, 44).

The viral targets of neutralizing antibodies are the gp120 exterior and gp41 transmembrane envelope glycoproteins (Envs), which are assembled into trimers on the virion surface (40). During virus entry, gp120 binds host CD4 and chemokine receptors, whereas gp41 mediates the fusion of the viral and target cell membranes. The binding of a single antibody molecule to the HIV-1 envelope glycoprotein trimer is sufficient to inactivate its function, independent of the HIV-1 strain from which the Envs are derived or the particular gp120 or gp41 epitope recognized by the monoclonal antibody (MAb) (36, 41). Even an unrelated antibody, the M2 anti-FLAG antibody, can effectively neutralize HIV-1 virions that carry an exogenous FLAG epitope in the gp120 V4 variable region (33). The V4 region has no known structural or functional roles in viral entry, consistent with the large amount of sequence diversity in this region for different HIV-1 isolates (14, 19, 21, 22, 40). These results suggest the hypothesis that the binding of an antibody anywhere on the HIV-1 envelope glycoprotein spike leads to neutralization and that the infectious trimers on the surface of primary HIV-1 virions resist such antibody binding.

Despite the appeal of the above model, the establishment of antibody-binding assays that reliably predict the neutralization sensitivity of a given HIV-1 isolate has proven to be elusive. To date, no antibody-binding assay using recombinant HIV-1 glycoproteins as binding targets perfectly predicts the HIV-1-neutralizing activity of an antibody, probably due to the failure of the recombinant forms to perfectly imitate the Env spikes on HIV-1 virions (29). Virion-binding assays, in which the ability of an anti-HIV-1-Env antibody to bind virus particles in vitro is examined, are not exact prognostic indicators for neutralization potency either (7, 16, 32, 42). Potential reasons for such difficulty include (i) the existence, often in vastly overwhelming proportions, of nonfunctional (including uncleaved) HIV-1 Env trimers in viral stocks (16, 32); (ii) the replication defectiveness of the vast majority (greater than 99.9%) of HIV-1 virions (4, 23); (iii) the small and varying number of intact Env trimers per HIV-1 virion (10, 15, 23, 43); and (iv) spontaneous and/or ligand-induced dissociation ("shedding") of gp120 from the Env spikes (28, 31, 35). Thus, the precise measurement of MAb binding to functionally relevant HIV-1 Env spikes remains an elusive goal. Consequently, our understanding of the mechanistic basis of HIV-1 resistance to neutralization by antibodies is still incomplete. Here, we study antibody-mediated neutralization in a controlled context by introducing FLAG artificial epitopes into the gp120 V4 region of HIV-1 viruses with dramatically different sensitivities to neutralization; our approach overcomes some of the above difficulties by focusing on the functional portion of HIV-1 Env spikes on virions.

HIV-1_{YU2} is a primary isolate that is extremely resistant to neutralization (24, 25). HIV-1_{JR-FL} is another primary HIV-1 isolate, but it exhibits intermediate sensitivity to neutralization (12). Both HIV-1_{YU2} and HIV-1_{JR-FL} use CCR5 as a second coreceptor. HIV-1_{HXBc2} is a T-cell line-adapted HIV-1 that uses CXCR4 as a coreceptor and is very susceptible to neutralizing antibodies (44). Recombinant HIV-1 encoding firefly luciferase was pseudotyped with the wild-type Envs of HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2}. Viruses produced by transfection of 293T cells were used to measure infectivity and neutralization sensitivity, as described previously (33, 38). The

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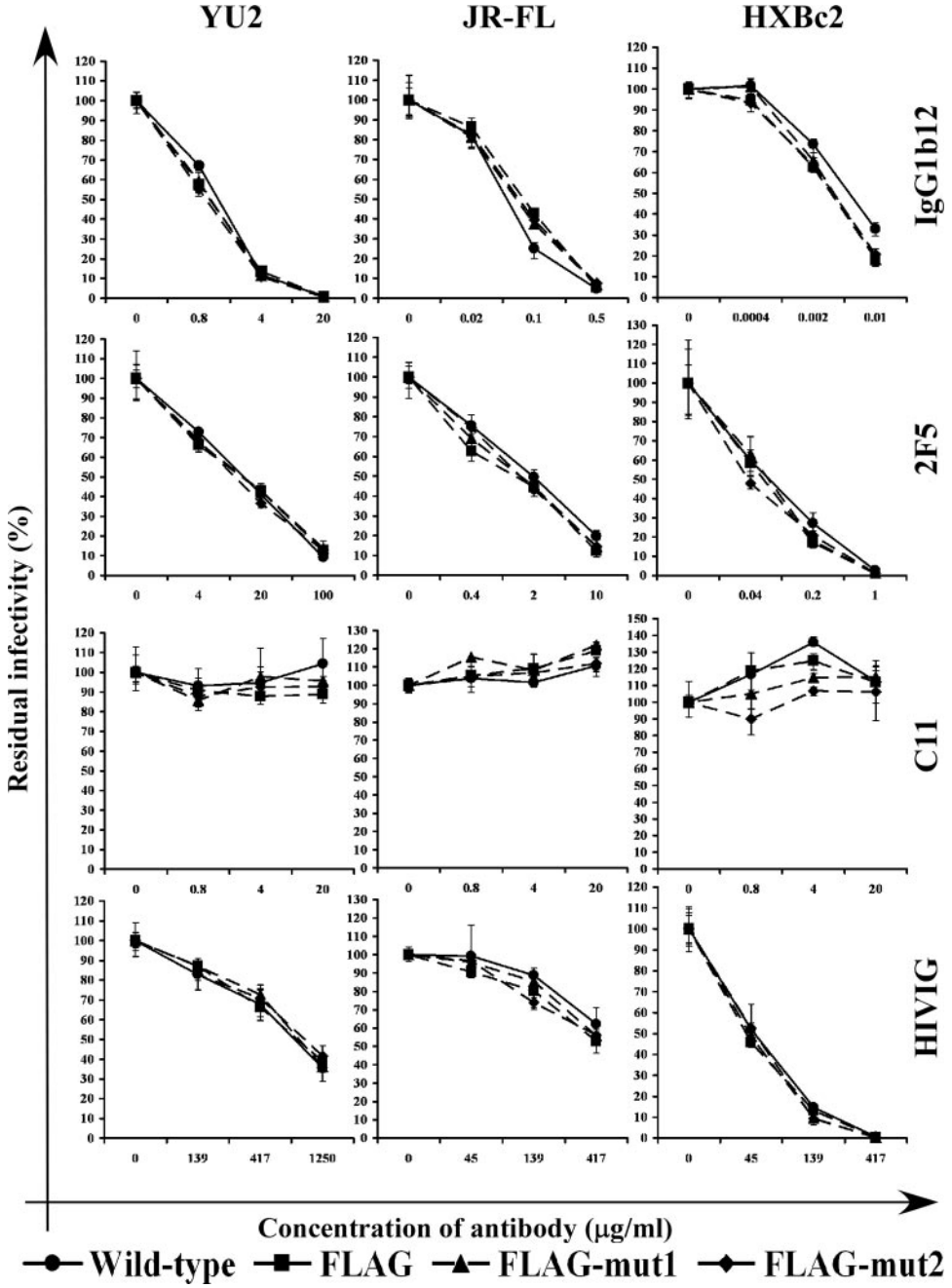

FIG. 1. Envelope glycoproteins from distinct HIV-1 strains exhibit very different sensitivities to neutralization by anti-HIV-1-Env antibodies. Recombinant luciferase-expressing reporter viruses containing the indicated HIV-1 Envs were produced as described previously (17). Viruses with Envs originating from one HIV-1 strain were produced and analyzed for neutralization sensitivity as a set. Within each set, the same amounts of viruses were incubated for 4 h at 37°C with the indicated concentrations of a given antibody in growth medium containing 1 μM Polybrene. Residual infectivities were measured in a single-round entry assay using appropriate target cells (Cf2Th-CD4/CCR5 for HIV-1_{YU2} and HIV-1_{JR-FL}; Cf2Th-CD4/CXCR4 for HIV-1_{HXBc2}). The infectivities after virus/antibody incubation were normalized to that of the same virus without antibody incubation, which was set at 100%. The means and ranges of variation from three parallel measurements are shown. All experiments were repeated at least once, and the results of a typical experiment are shown.

infectivity of recombinant viruses with HIV-1_{YU2} and HIV-1_{JR-FL} Envs was measured by incubating the viruses with Cf2Th-CD4/CCR5 cells, and the infectivity of viruses with HIV-1_{HXBc2} Envs was measured by using Cf2Th-CD4/CXCR4 target cells. For neutralization assays, viruses were incubated with antibodies and 1 μM of Polybrene at 37°C for 4 h prior to exposure to the target cells.

The sensitivity of the viruses with wild-type HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} Envs to neutralization by three relatively potent human MAbs (IgG1b12, 2G12, and 2F5) was as-

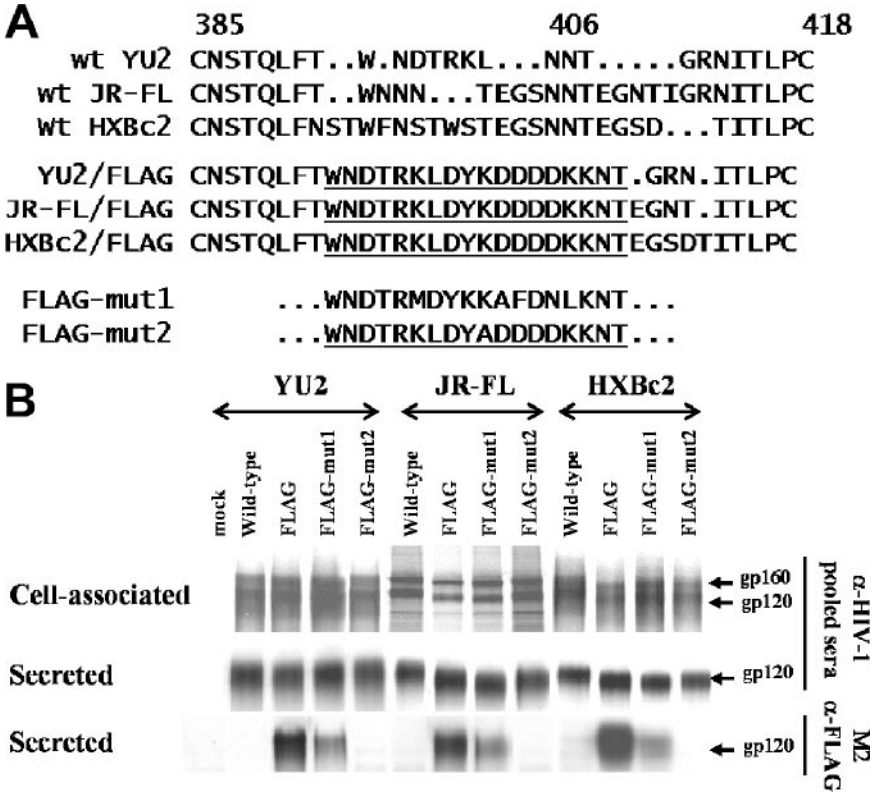

FIG. 2. Design and expression of HIV-1 Envs carrying FLAG artificial epitopes. (A) The amino acid sequences of the wild-type (wt) V4 region from the three studied HIV-1 gp120 Envs are aligned in the top panel. The amino acid numbering corresponds to that of HIV-1_{HXBc2}, per current convention (18). The YU2/FLAG construct contains an insertion of the FLAG epitope tag "DYKDDDDK" and an N406K change to remove a glycosylation site in the HIV-1_{YU2} gp120 V4 region. The underlined YU2/FLAG sequences were used to replace the corresponding sequences in the HIV-1_{JR-FL} and HIV-1_{HXBc2} Envs to create the JR-FL/FLAG and HXBc2/FLAG Envs, respectively. To generate different FLAG derivatives with reduced affinities for the M2 anti-FLAG antibody, the wild-type FLAG sequence was replaced with "AMDYKAFDNL" and "DYADDDDK" to generate the FLAG-mut1 and FLAG-mut2 constructs, respectively. The FLAG-mut1 and FLAG-mut2 constructs were generated by using the Envs from the three studied HIV-1 isolates. (B) HIV-1 Envs were transiently expressed in 293T cells and labeled with [^{35}S]methionine/cysteine. The cell-associated and supernatant Envs were precipitated by pooled sera from HIV-1-infected individuals or by the M2 anti-FLAG antibody. The precipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The duration of exposure of the autoradiographs differs for the Envs derived from each strain of HIV-1.

sessed. Viruses with wild-type HIV-1_{JR-FL} Envs were neutralized by IgG1b12, 2F5, and 2G12 MAbs at 10- to 100-fold-lower concentrations than those required to neutralize the HIV-1_{YU2} viruses (Fig. 1 and data not shown). Viruses with the HIV-1_{HXBc2} Envs were neutralized by IgG1b12 and 2F5 MAbs at concentrations 100- to 1,000-fold lower than the HIV-1_{YU2} viruses (Fig. 1). Sensitivity to neutralization by a polyclonal immunoglobulin preparation (HIVIG) from HIV-1-infected individuals exhibited the following order: HXBc2 > JR-FL > YU2 (Fig. 1). Thus, the Envs from these three HIV-1 strains specify vastly different sensitivities to neutralization by natural anti-HIV-1 Env MAbs.

HIV-1 isolates that differ in neutralization sensitivity levels could hypothetically differ in the binding levels of antibodies to the Envs or in the consequences of antibody binding with respect to virus infectivity. To examine these possibilities, we inserted an artificial epitope, the FLAG tag or its variants, into the V4 region of the HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} gp120 glycoproteins (Fig. 2A). The FLAG-mut1 and FLAG-mut2 variants contain sequence changes designed to decrease the affinity of the M2 anti-FLAG MAb for the epitope. Insertion of the artificial epitopes had no significant effect on the expression levels, proteolytic maturation, or subunit association of the Envs (Fig. 2B). Precipitation of the secreted gp120 glycoproteins by the M2 MAb revealed that the FLAG-mut1 gp120 glycoproteins bound M2 at reduced levels compared to the FLAG gp120 glycoproteins; the FLAG-mut2 gp120 glycoproteins did not detectably bind M2 under these conditions. The lower affinities of the FLAG-mut1 and FLAG-mut2 gp120 glycoproteins of the HIV-1_{YU2} strain for the M2 MAb, compared with the HIV-1_{YU2} FLAG gp120, were confirmed by surface plasmon resonance analysis (data not shown). Thus, we have created epitope-tagged HIV-1 gp120 variants that bind the M2 MAb in the following order: FLAG > FLAG-mut1 > FLAG-mut2.

The infectivity of recombinant luciferase reporter viruses carrying these HIV-1 Envs was tested by a standard single-round entry assay (33). Viruses carrying the artificial epitope tags entered the appropriate target cells at levels within threefold of those of the wild-type glycoproteins (data not shown). Therefore, the insertion of these FLAG variants into the gp120 V4 region exerted little detrimental effect on the basal ability

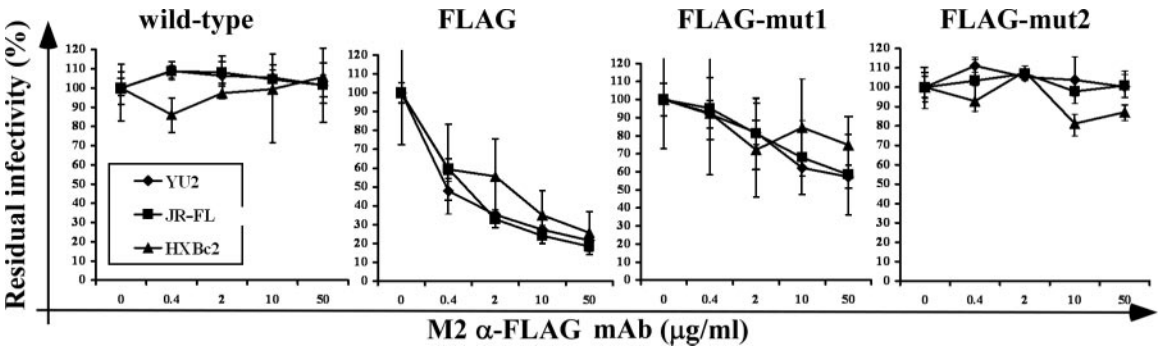

FIG. 3. Sensitivity of recombinant viruses to neutralization by the M2 anti-FLAG antibody. Recombinant luciferase-expressing reporter viruses were produced and tested for sensitivity to neutralization by the M2 anti-FLAG antibody (Sigma). The same amount of viruses in each set (i.e., with Envs originating from one HIV-1 strain) was incubated for 4 h at 37°C with the indicated concentrations of the M2 antibody and 1 μM Polybrene. The residual infectivities after incubation with the M2 antibody were normalized to the infectivity observed in the absence of antibody treatment, which was set at 100%. For viruses with wild-type and FLAG-mut2 HIV-1 Envs, the means and ranges of variation from three parallel measurements from a typical experiment are shown. For viruses with Envs carrying the FLAG and FLAG-mut1 artificial epitopes, data from 10 parallel measurements made in three independent experiments were pooled, and the means and standard deviations are shown.

of HIV-1 Envs to support virus entry. We used a standard neutralization assay to evaluate the effect of the inserted sequences on the general neutralization sensitivity of viruses with the modified Envs. Viruses bearing Envs with the inserted FLAG epitopes were indistinguishable from the viruses bearing the parental glycoproteins in terms of sensitivity to neutralization by neutralizing MAbs IgG1b12 and 2F5 as well as by a nonneutralizing MAb, C11 (Fig. 1, top three panels). Additionally, viruses with the FLAG-tagged and wild-type Envs were neutralized equivalently by HIVIG (Fig. 1, bottom panel). In summary, insertion of the artificial epitope tags had no deleterious effect on the general properties of the HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} Envs with respect to protein expression, processing, trimer stability, entry function, and general neutralization sensitivity.

Next, we compared the neutralization efficiency of the M2 MAb against the viruses. Incubation with 50 μg/ml or less of the M2 MAb did not significantly inhibit infection by viruses carrying the wild-type glycoproteins of HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} (Fig. 3, left panel). In the same concentration range, the M2 MAb equivalently neutralized viruses with the FLAG-tagged HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} Envs (Fig. 3, second panel from left). Therefore, even though the HIV-1_{YU2}, HIV-1_{JR-FL}, and HIV-1_{HXBc2} Envs specify large differences in sensitivity to neutralization by many antibodies, the consequences of antibody binding with respect to inactivation of infectivity are similar for these Env variants.

For all three HIV-1 strains, the M2 MAb neutralized the viruses with the FLAG epitope more efficiently than those with the FLAG-mut1 epitope (compare the middle two panels of Fig. 3). The M2 MAb did not appreciably neutralize the viruses with the FLAG-mut2 epitope (Fig. 3, right panel). Thus, the sensitivities of the viruses with FLAG epitope variants to neutralization by the M2 MAb directly reflected the M2-binding affinities of the viral Envs.

In summary, our results demonstrate that the Envs of neutralization-resistant, primary HIV-1 are not intrinsically resistant to the inactivating consequences of antibody binding. Furthermore, we were able to demonstrate a clear relationship between neutralization potency and the affinity of the antibody for its cognate epitope. Our results are consistent with a large body of evidence suggesting that antibody binding to the HIV-1 Env trimer is necessary and sufficient for at least some level of neutralization (6). Thus, limiting the access of antibodies to epitopes, particularly those that are well-conserved among HIV-1 strains, on the functional Env trimer is essential for the ability of HIV-1 to resist neutralization by antibodies. Multiple determinants in the gp120 variable loops and gp41 ectodomain can contribute to the high level of neutralization resistance of certain HIV-1 strains (3, 11, 37, 38). An understanding of the structure of the HIV-1 Env trimers will reveal the mechanistic basis of these complex interactions and thereby guide interventional strategies.

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