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"Unconventional" Neutralizing Activity of Antibodies Against HIV

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Abstract: Neutralizing antibodies are recognized to be one of the essential elements of the adaptive immune response that must be induced by an effective vaccine against HIV. However, only a limited number of antibodies have been identified to neutralize a broad range of primary isolates of HIV-1 and attempts to induce such antibodies by immunization were unsuccessful. The difficulties to generate such antibodies are mainly due to intrinsic properties of HIV-1 envelope spikes, such as high sequence diversity, heavy glycosylation, and inducible and transient nature of certain epitopes. *In vitro* neutralizing antibodies are identified using "conventional" neutralization assay which uses phytohemagglutinin (PHA)-stimulated human PBMCs as target cells. Thus, in essence the assay evaluates HIV-1 replication in $CD4^+$ T cells. Recently, several laboratories including us demonstrated that some monoclonal antibodies and HIV-1-specific polyclonal IgG purified from patient sera, although they do not have neutralizing activity when tested by the "conventional" neutralization assay, do exhibit potent and broad neutralizing activity in "unconventional" ways. The neutralizing activity of these antibodies and IgG fractions is acquired through post-translational modifications, through opsonization of virus particles into macrophages and immature dendritic cells (iDCs), or through expression of antibodies on the surface of HIV-1-susceptible cells. This review will focus on recent findings of this area and point out their potential applications in the development of preventive strategies against HIV.

Key words: Antibody; Neutralizing activity; Human immunodeficiency virus (HIV)

Human immunodeficiency virus type 1 (HIV-1) spike is a trimeric complex of gp120-gp41 heterodimers. Gp120, a surface attachment protein, and gp41, the membrane-spinning protein, are non-covalently linked. They are initially produced as a single glycoprotein precursor *gp*160, which is cleaved by a cellular protease. Gp120 serves as a ligand for virus attachment and for interaction with receptor and co-receptor. By so doing, it determines HIV-1 tropism. Gp41 mediates fusion process between virus membrane and target cell membrane. By so doing, it facilitates the formation of fusion pores in target cell membrane, which in turn serves as a gateway for viral core to be delivered into cytosol of target cells. It has

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been shown that both gp120 and gp41 go through many conformational changes from virus attachment to target cells to the formation of fusion pore (42).

Gp120 and Gp41 not only play an essential role in virus entry, they are also major targets for antibody responses during natural infection and vaccination. Neutralizing antibodies block viral entry by recognizing epitopes on the envelope spike critical for their interaction with receptor and co-receptors, or for the fusion process. Antibodies that neutralize a broad range of primary isolates of HIV-1 have been extremely difficult to generate. Despite almost two decades of effort, only a limited number of such antibodies have been identified. They include two antibodies 2F5 and 4E10 that direct against the membrane proximate region of gp41 (26, 27, 35, 45) and three antibodies 2G12, b12, and 447-52D that direct against gp120 (6-12). The antibody b12 interacts with the epitope that overlaps with CD4 binding site of gp120 (4). The antibody 2G12 recognizes cluster α 1 to 2 linked mannose residues on the distal ends of oligomannose sugar located on the carbohydrate-covered silent face of gp120 (38). The antibody 447-52D recognizes gly-pro-gly-arg (GPGR) motif at the center of V3 loop (12). The GPGR motif is conserved among clade B viruses, but not non-clade B viruses (13). In non-clade B virus the arg residue is replaced by the gln residue (11). Data derived from the crystal structure of envelope-antibody complexes suggest that the neutralizing activity of these antibodies (except for 2G12 and 447-52D) is mediated by an unusually long CDR3 H3 loop that penetrates deeply into the antigen cleft, which is obscured in the heterotrimic envelope (30). Unfortunately, antibodies with such broadly neutralizing activity occur very rarely in patients and attempts to induce such antibody responses by immunization were unsuccessful (30). Thus, so far we still do not know how to induce such antibodies through rational immunogen design.

In contrast, nonneutralizing antibodies are almost always generated during natural infection or vaccination. Some have very high affinity, but fail to block viral entry, suggesting that nonneutralization epitopes are wither buried within the intact envelope spike or exposed but not critical for viral entry. By molecular modeling, Poignard *et al*. described that gp120 monomer exists three faces-neutralizing, nonneutralizing, and silent (31). The neutralizing face corresponds to the surface of gp120 trimer that interacts with its receptor and co-receptors. This face is exposed at the surface of the intact trimeric envelope spike and conserved. The silent face is heavily glycosylated and does not elicit antibody responses, though it is well exposed at the surface of the intact trimeric envelope spike. The non-neutralizing face elicits strong antibody response. However, since this face is buried within the intact trimeric envelope spike, antibodies that bind to this face do not bind to the intact spike on virions and have no neutralizing activity. This model also implies that the nonneutralizing antibodies to gp120 are elicited by either monomeric gp120 shed from the virions or infected cells or by gp160 precursor proteins found in the debris of dying HIV-1-infected cells. Since no similar modeling has been done for gp41, we do not know the structural basis of neutralization versus nonneutralization epitopes in gp41. A few studies have shown that most of the epitopes of gp41 are buried underneath the gp120 trimer in the intact envelope spikes (6, 18,

33, 40, 42), with notable exception of epitopes in cluster I determinant (28) and 2F5 (26, 27) and 4E10 epitopes (35, 45). Interaction of gp120 with CD4 causes conformational changes of the envelope spikes, resulting in exposure of some of these hidden epitopes and making them accessible to antibody binding (34). From an evolution point of view, because non-neutralizing antibodies do not exert selective pressure on virus survival, non-neutralization epitopes are usually more conserved than neutralization epitopes.

Historically, whether an antibody has neutralizing activity is determined using "conventional" neutralization assay *in vitro*. The assay uses phytohemagglutinin (PHA)-stimulated human PBMCs as target cells. Thus, in essence it evaluates inhibitory effect of antibody on HIV-1 replication in $CD4^+$ T cells. However, recently, several laboratories inclu-ding us demonstrated that some antibodies and polyclonal IgG purified from patient sera, although they do not have neutralizing activity when tested by the "conventional" neutralization assay, do exhibit potent neutralizing activity in "unconventional" ways (7, 8, 15-17, 19, 20, 23, 41). The neutralizing activity of these antibodies and purified polyclonal IgG is acquired through post-translational modifications (7, 8, 23), through opsonization of virus particles into macrophages and immature dendritic cells (iDCs) (15-17, 41), or through expression of antibodies on the surface of HIV-1-susceptible cells (19, 20). These findings not only shed new light on our understanding neutralizing activity of antibodies against HIV, but also high-lights potential applications in the development of preventive strategies against HIV. Thus, our following review will focus on some of recent findings of the "unconventional" neutralizing activity of these antibodies and purified polyclonal IgG.

NEUTRALIZING ACTIVITY THROUGH POST-TRANSLATIONAL MODIFICATIONS

Miranda *et al*. (23) recently found that monoclonal antibody F240 that recognizes cluster I determinant, which spans residues 592-604 of gp41, does not neutralize HIV-1 when it is produced by the parental hybridoma. However, when genes encoding the heavy and light chains of antibodies were cloned and transfected into Chinese hamster ovary (CHO)-K1 cells, antibodies produced by CHO-K1 transfectants acquired a strong neutralization activity against a wide range of HIV-1 isolates without a change in immunoreactivity. When the primary sequences of heavy and light chains of antibodies from transfectants and from the parental hybridoma were compared, they were identical. However, when the glycosylation of the heavy and light chains of antibodies from transfectants and from the parental hybridoma was compared, antibody produced by transfectants was glycosylated to a much greater extend than antibody produced by the parental hybridoma. The neutralizing activity was abrogated by the treatment of transfectants with peptide *N*-glycosidase in an HIV-1-isolate-specific manner. Thus, the alteration of glycosylation events outside the antigen-binding domain could turn this non-neutralizing anti-HIV-1 gp41 antibody into a broad neutralizing antibody.

Tyrosine sulfation, another post-translational modification, of human antibodies has recently been shown to contribute to neutralization activity of antibodies. Choe *et al*. (7) demonstrated that a subset of human CD4 inducible antibodies 47e, 412d, CM51, and E51, which direct against CD4-inducible, CCR5-binding

site of gp120, are modified by sulfation. The sulfation occurs at the tyrosine residue of CDR3 of the heavy chain of these antibodies. In human species, the heavy chain CDR3 sequence is derived from one of 23 functional diversity (D) region genes together with one of six joining (J) region genes. The junctions bounding of the D region are also modified by N and P linked addition. Most D region genes encode sequences rich in tyrosines. Nine of 23 D genes encode sequences containing one or more tyrosines immediately adjacent to an aspartic acid, a motif present at most sites of tyrosine sulfation (32). Interestingly, in the presence of soluble CD4 the sulfated antibodies bind more efficiently gp120 than unsulfated antibodies and the binding of gp120-CD4 complex by sulfated antibodies was specifically inhibited by sulfated peptides based on the CCR5 amino terminus. Since the amino terminus of CCR5 (residues10-18), which is known to contain sulfated tyrosine residues, plays an important role for virus entry, these sulfated human antibodies emulate CCR5 by interacting with gp120 proteins derived from R5, but not X4 isolates. By so doing, they neutralize efficiently entry by R5, but not X4 isolates. The mutation of tyrosine residue to phenylalanine in the CDR3 region of antibodies or using small hairpin RNA (shRNA) to interfere with the message of the two known tyrosyl protein sulfotransferases TPST1 and TPST2 (3, 29) resulted in unsulfated antibodies that are less efficient to bind to gp120-CD4 complex and less potent in neutralizing primary isolates than sulfated antibodies. As noted, these sulfated antibodies were derived from two infected individuals with potent anti-HIV-1 gp120 activity in their sera (24, 39) and a tyrosine-sulfated peptide derived from the heavy-chain CDR3 region of the antibody E51 binds gp120 and inhibits HIV-1 infection (8), but whether sulfated antibodies contribute to long-term control of HIV-1 *in vivo* remains to be determined.

Thus, it appears that dependent on post-translational modifications of antibodies, a non-neutralizing antibody could be turned into a broadly neutralizing antibody or a less potent into a more potent neutralizing antibody. It will be extremely interesting to determine the underlying mechanisms why the post-translational modifications could have such effects.

NEUTRALIZING ACTIVITY THROUGH OPSONIZATION

Besides $CD4^+$ T cells, macrophages and iDCs are also cell targets for HIV-1 infection. *In vivo* macrophages are a major reservoir of HIV-1 (1) and iDCs are among the first cells infected by HIV-1 after mucosal transmission (21). To test the neutralizing activity of anti-HIV-1 antibodies in various cells, Holl *et al.* (16) found that some antibodies with no neutralizing activity on HIV-1 replication in $CD4^+$ T cells efficiently inhibit the replication of primary isolates of HIV-1 in macrophages and iDCs or in macrophages alone. The antibodies 257-D IV, 268-D IV, F425B4a1, F425B4e8 and 391-D, which efficiently inhibit the replication of primary isolates of HIV-1 in both macrophages and iDCs, recognize epitopes in the V3 loop; while the antibodies 240D, 246D, 50-69 and F240, which efficiently inhibit the replication of primary isolates of HIV-1 in macrophages alone, recognize epitopes in the gp41. The neutralizing activity by the antibodies 240D, 246D and 50-69 is particularly interesting since they

recognize the cluster I determinant of gp41 (amino acid residues 579-604) and no antibodies to such a determinant have been shown to exhibit potent neutralizing activity before (5, 14, 43).

Several lines of evidence indicate that the mechanism of inhibition of HIV-1 replication in macrophages and iDCs by these antibodies is distinct from neutralizing activity by "conventional" neutralizing antibodies, in which only Fab portion of antibody molecules is sufficient for neutralization of infectivity. Instead, these antibodies required both Fab and Fc portions for neutralization. First, the inhibition of HIV-1 replication in macrophages and iDCs by these antibodies could be reversed by pretreatment of macrophages with anti-Fc$_\gamma$RI (CD64) antibody and iDCs with anti-Fc$_\gamma$RII (CD32) antibody before the virus and antibody mixtures were added to the cells. Second, neutralizing activity by antibody 240D was abolished in macrophages and iDCs when peptide corresponding to the cluster I determinant (amino acid residues 593-616) was added to the virus and antibody mixture 1 hour before being added to cell targets. Third, when neutralizing activity between the Fab fragment and the whole IgG of anti-V3 loop antibody 447-52D was compared, in PHA-stimulated PBMCs, comparable neutralizing activity for infectivity was observed between the Fab fragment and the whole IgG; whereas in macrophages and iDCs neutralizing activity by the whole IgG was 2-to3-log more efficient than by the Fab fragment. Thus, it appears that these anti-V3 loop and cluster I determinant antibodies directed against epitopes distinct from those recognized by "conventional" neutralizing antibodies and will not impair virus entry into cells, but they will link infectious virus particles to the target cells by efficient binding of the Fc portion of IgG to Fc$_\gamma$R and by binding of Fab portion to the HIV-1 envelope. And through the Fc$_\gamma$R-mediated endocytosis pathway, endocytosed virus particles were shown to end up into the tetrapanin-rich compartment (41).

Interestingly, the above mentioned neutralizing activity by specific antibodies directed against V3 loop and cluster I determinant in macrophages and iDCs was also observed by purified HIV-1-specific polyclonal IgG from sera of infected individuals (15, 17, 41). Holl *et al* showed that purified polyclonal IgGs displayed a higher HIV-1 inhibitory capacity on monocyte-derived macrophages and iDCs than on PHA-stimulated PBMCs (15, 17). In addition, they showed that Fc$_\gamma$RI is involved in the mechanism for inhibiting HIV-1 infection of macrophages (15) and Fc$_\gamma$RII is involved in the mechanism for inhibiting HIV-1 infection of iDCs (17) by IgG and no induction of maturation was detected at concentrations of IgG that result in a 90% reduction. Finally, when iDCs were treated with IFN-γ to induce Fc$_\gamma$RI expression, the inhibitory activity by IgG was enhanced.

Wilflingseder *et al* also compared the effect of different opsonization pattern of HIV-1 on infection and provirus formation in iDCs (41). They found that nonopsonized and complement-opsonized HIV-1 productively infect iDCs; whereas the infection by HIV-1 -specific IgG-opsonized or HIV-1-specific IgG and complement-opsonized HIV-1 is significantly impeded and provirus formation is also significantly reduced. The inhibition by HIV-1-specific IgG is not due to "conventional" neutralizing antibodies in the HIV-1- IgG pool because all differently opsonized prepara tions caused a vigorous infection of PHA-stimulated PBMCs. In addition, IgG-opsonized HIV-1 is also impeded in

long-term transfer from DCs to $CD4^+$ T cells.

Taken together, these studies demonstrated that both HIV-1-specific polyclonal IgG from patient sera and some monoclonal antibodies, which do not have neutralizing activity when tested by "conventional" neutralization assay, do exhibit potent neutralizing activity when tested in macrophages and in iDCs. Neutralizing activity requires both Fab and Fc portions of IgG molecules and mediates through $Fc_\gamma Rs$. Thus, the induction of HIV-1-specific polyclonal IgG that are able to efficiently inhibit HIV-1 replication in iDCs and macrophages should be one of the components in rational immunogen design.

NEUTRALIZING ACTIVITY THROUGH MEMBRANE TARGETING

Recently we found that a non-neutralizing human anti-HIV-1 gp41 antibody TG15 (36, 44), when expressed as a soluble protein, does not neutralize viral entry. But when expressed on the surface of HIV-1 susceptible cells, it can not only block virus replication from various clades, but also HIV-1 envelope-mediated cell-cell fusion. The inhibition is at the level of virus entry, HIV-1 envelope specific, independence of virus tropism (20). Thus, a nonneutralizing antibody can also be turned into a broadly neutralizing antibody when expressed onto the surface of HIV-1-susceptible cells. We named this approach a membrane-bound antibody.

Moreover, this membrane-bound antibody not only inhibits virus infection of cell-free HIV-1, but also inhibits the transmission of HIV-1 captured and transferred from DCs to $CD4^+$ T cells (19). "Conventional" soluble neutralizing antibodies are known to be effective in blocking infection by cell-free viruses. But whether they are also effective in blocking infection by cell-associated viruses is questionable. For example, neutralizing activity by DC-captured and transferred viruses was demonstrated only when antibody and virus were preincubated before being added to DCs (9, 10). However, after virus was captured by DCs, antibody no longer prevented the transmission of virus from DCs to $CD4^+$ T cells (10). The failure of "conventional" soluble antibody to neutralizing DC-captured and transferred viruses could be due to the fact that the transmission of HIV-1 from DCs to $CD4^+$ T cells occurs within an infectious synapse. It was reported by McDonald *et al*. (22) that before DCs contact target $CD4^+$ T cells the HIV-1 captured and internalized by DCs were evenly distributed throughout cells. However, soon after DCs contact target cells the majority of the HIV-1 relocated to the initial site of contact to form an infectious synapse. Within the infectious synapse local concentration of HIV-1 as well as receptor and co-receptor are greatly increased, which facilitates transmission from DCs to $CD4^+$ T cells. Thus, DCs may facilitate escape from neutralizing antibodies. Infectious synapse could be too tight to be accessible to conventional soluble antibodies. However, the membrane-bound antibody, due to its presence on the target cell surface, may likely migrate to the infectious synapse. By so doing, it effectively blocks transmission from DCs to $CD4^+$ T cells. Thus, expressing antibody on the surface of HIV-1-susceptible cells can not only turn a non-neutralizing antibody into a broadly neutralizing antibody, but also can block virus transmission under conditions where conventional soluble neutralizing antibody fails to do so.

Although the underlying mechanism of inhibition

by membrane-bound antibody is still unknown, it is likely to have something to do with the epitope that the antibody TG15 recognizes. The epitope that the antibody TG15 recognizes locates in the cluster II determinant (amino acid residues 644 to 663) of the ectodomain of gp41 (43). This determinant resides within the second heptad repeat. HIV-1 gp41-mediated fusion is triggered by interaction between the second and the first heptad repeats, which converts a prehairpin gp41 trimer into a fusogenic three-hairpin bundle (42). A peptide T20 derived from this second heptad repeat acts as a fusion inhibitor (2). We envision two possibilities that are not mutually exclusive as to how the membrane-bound antibody works. The first possibility is that the cluster II determinant is indeed buried within the intact envelope spikes, but exposed when gp120s are shed from the spikes. Since a virion can contain a mixture of intact spikes and so-called dead spikes from which gp120 has been shed, it can be bound by the membrane-bound antibody. When the antibody is produced as a soluble protein, even if it can bind to these dead spikes, it can not prevent the remaining intact envelope spikes from interacting with their cognate receptor and co-receptors. As a result, it does not neutralize viral entry. However, when the antibody is expressed on the cell surface, the membrane-bound antibody directly binds to the dead spikes and traps virions so that they can not move freely. In so doing, it prevents the intact envelope spikes from interacting with their cognate receptor and co-receptors. The membrane-bound antibody may even mediate endocytosis of viruses into the endosome/ lysosome for viral degradation. The second possibility is that the cluster II determinant is totally buried within the envelope spikes of virions. Because of this, the antibody does not neutralize viral entry when produced as a soluble protein. However, the interaction of gp120 to its receptor and co-receptors causes drastic conformational changes of gp41 from a prehairpin gp41 trimer into a fusogenic three-hairpin bundle (42). During this process, the cluster II determinant and other hidden epitopes in gp41 are exposed. The membrane-bound antibody happens to be nearby geographically. The interaction of the membrane-bound antibody to the inducible cluster II determinant blocks the subsequent fusion process. The experiments are on-going to investigate these two possibilities.

In summary, against the conventional wisdom that neutralizing activity of a given antibody is determined by the primary sequences of Fab portion of antibody molecules, recent studies show that the neutralizing activity of antibody molecules is also strongly influenced by post-translational modifications, by opsonization of the whole IgG, or by the accessibility of antibodies to inducible epitopes during the fusion process. Thus, whether an antibody has neutralizing activity not only depend on the primary sequences of the heavy and light chains of antibody molecules, but also depend on how they are post-translational modified, whether target cells express $Fc_{\gamma}Rs$, and whether the antibodies are present at the right time and the right place during the fusion process.

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