Neutralizing antibodies to the HIV-1 envelope glycoproteins
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Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary HIV-1

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Anti-HIV-1 antibodies whose binding to gp120 is enhanced by CD4 binding (CD4i antibodies) are generally considered non-neutralizing for primary HIV-1 isolates. However, a novel CD4i-specific Fab fragment, X5, has recently been found to neutralize a wide range of primary isolates. To investigate the precise nature of the extraordinary neutralizing ability of Fab X5, we tested different forms (IgG, Fab and single-chain Fv) of X5 and other CD4i monoclonal antibodies for their ability to neutralize a range of primary HIV-1 isolates. Our results show that, for a number of viruses, the size of the neutralizing agent is inversely correlated with its ability to neutralize. Thus, the poor ability of CD4i-specific antibodies to neutralize primary isolates is due, at least in part, to steric factors that limit antibody access to the gp120 epitopes. Studies using temperature-regulated neutralization or fusion-arrested intermediates suggest that the steric effects are important in limiting the binding of IgG to the viral envelope glycoproteins after HIV-1 has engaged CD4 on the target cell membrane. The results identify hurdles in employing the CD4i epitopes as targets for antibody-mediated neutralization in vaccine design, but indicate that the CD4i regions could be efficiently targeted by small molecule entry inhibitors.

Introduction

Human immunodeficiency virus type 1 (HIV-1) entry into host-cells is initiated by the binding of the gp120 subunit of the viral envelope glycoprotein (Env) complex to the host-cell receptor (CD4) (8, 20). This interaction induces conformational changes in gp120 resulting in the exposure of a conserved high-affinity binding site for the co-receptor (the chemokine receptors CCR5 or CXCR4) (46, 47, 54, 56, 59). A second obligatory binding-step between the gp120-CD4 complex and the coreceptor then is thought to induce additional conformational changes that ultimately result in the fusion of viral and host-cell membranes (9, 18).

Neutralizing antibodies (Abs) are believed to act, at least in part, by binding to the exposed Env surface and obstructing the initial interaction between a trimeric array of gp120 molecules on the virion surface and receptor molecules on the target cell (37, 57). In response, HIV-1 has evolved a number of strategies to evade recognition by neutralizing antibodies, particularly those directed to the conserved CD4 and coreceptor binding sites of Env. The extent of protection of these sites from Ab recognition is limited by the necessity to preserve the accessibility for receptor interaction. In the case of the CD4bs this has led to the following structural features: 1) it is partially obscured from antibody recognition by the V1/V2 loop and associated carbohydrate structures, 2) the flanking residues are variable and modified by glycosylation, 3) it is recessed to an extent that limits direct access by an antibody variable region, 4) clusters of residues within the CD4bs that do not directly interact with CD4 are subject to variation among virus strains, 5) many gp120 residues interact with CD4 via main-chain atoms, allowing for variability in the corresponding amino acid side chains (26), and 6) there is considerable conformational flexibility within the CD4-unbound state of gp120 and antibody binding therefore requires relatively large entropic decreases, thus ‘conformationally masking’ the conserved CD4bs (23, 33).

The coreceptor binding site on gp120 is thought to be composed of a highly conserved element on the b19 strand and parts of the V3 loop (41, 42, 61). These elements are masked by the V1/V2 variable loops in the CD4-unbound state (55, 59). Upon CD4 binding, conformational changes are induced, which include displacement of the V1/V2 stem-loop structure and consequent exposure of the coreceptor binding site (31, 47, 60). Binding studies with variable loop-deleted mutants suggest that CD4 induces additional rearrangement or stabilization of the gp120 bridging sheet near the b19 strand to form the final coreceptor-binding surface (59). As the binding to CD4 occurs at the virus:cell interface, the exposed coreceptor binding site is optimally positioned for interaction with the coreceptor.

A highly conserved discontinuous structure on gp120 is recognized by monoclonal (m)Abs that bind better to gp120 upon ligation with CD4. These so-called CD4-induced (CD4i) antibodies, such as 17b and 48d (54, 60), recognize a cluster of gp120 epitopes that are centered on the b19 strand and partially overlap the coreceptor binding site (41, 42, 55, 56). Although such CD4i mAbs can neutralize some T cell line-adapted (TCLA) HIV-1 strains, they are generally poorly neutralizing for primary isolates (40). However, we recently reported the isolation of an antibody Fab fragment, X5, from a phage display library, that is directed to a CD4i epitope and does neutralize a wide variety of primary isolates (32). Here we investigated the differences between Fab X5 and other CD4i mAbs at a molecular level. We provide evidence that size is the determining factor for the inability of CD4i mAbs to neutralize many primary HIV-1 isolates.

Material and methods

Materials. The following materials were obtained from the National Institute of Health AIDS Research and Reference Reagent Program (ARRRP); molecular clones of HIV-1 89.6, HXB2, JR-FL, JR-CSF, ADA; sCD4 (amino acids 1-370; contributed by N. Schuette), recombinant gp120, sCD4, and CD4-IgG (kindly provided by Paul Maddon and William Olson; Progenics, Tarrytown, New York).

Construction and purification of IgG1 X5. Fab X5 was isolated from a phage display library constructed from the bone marrow of an HIV-1 TCLA HIV-1 strains, they are generally poorly neutralizing for primary isolates (40). However, we recently reported the isolation of an antibody Fab fragment, X5, from a phage display library, that is directed to a CD4i epitope and does neutralize a wide variety of primary isolates (32). Here we investigated the differences between Fab X5 and other CD4i mAbs at a molecular level. We provide evidence that size is the determining factor for the inability of CD4i mAbs to neutralize many primary HIV-1 isolates.
ELISA using cell culture supernatant, was chosen for scale-up in the CellCube system (Corning, NY) and purified by affinity chromatography with protein A (Pharmacia, Uppsala, Sweden). Purified IgG1 X5 was coated on microtiter plates and used as an antibody against human antibodies. Purification was determined by SDS-PAGE and the concentration was measured by A_{280}.

Production and purification of antibody fragments. Antibody Fab fragments were produced by papain digestion as described previously (24). Single chain Fv (scFv) X5 was engineered into the pComb3X vector using antibody specific primers (1), produced in E. coli, and purified by nickel chelate chromatography (Qiagen, Valencia, CA) according to the manufacturer's instructions. Purified protein was PBS-dialyzed and stored at -80°C until use. Purity was determined by SDS-PAGE and the concentration was measured by A_{280}. The 17b single chain construct was a gift from Dr. Wayne Marasco (Dana-Farber Cancer Institute, Boston, MA). In short, the 17b scFv was produced by PCR amplification of the heavy and light chain variable region fragments from the 17b hybridoma cell line and cloned into the prokaryotic expression vector, pPHEN (16). From this construct the 17b heavy chain, the (Glu) linker, the light chain variable sequences and a His epitope tag were PCR amplified and subcloned into the inducible expression vector pMT7 (17). Downstream of the Drosophila metallothionein promoter and in frame with the 6xHis-tissue plasmidogen activator leader sequence of the Drosophila metallothionein promoter and in-frame with the 6xHis-tissue plasmidogen activator leader sequence. A Stable S2 Drosophila cell line was established by co-transfection with a hygromycin resistance plasmid pC0-hygro and selection through hygromycin (300 µg/ml). Production of the 17b single chain was induced by the addition of 750 µM CuSO_{4} to the cells at a density of 10^{6} cells/ml in 10% fetal bovine serum media containing 0.1% pluronic (BASF, Mount Olive, NJ). Purification of the single chain was performed in a single step by direct passage over a nickel chelating column (Pharmacia). The 33 kD protein was eluted by 50 mM EDTA, dialyzed, and quantified by A_{280} and SDS gel analysis.

Pseudovirion production. Plasmids containing the env genes of HIV-1 strains HxB2 (35), JR-CSF (65, AD5, (53), ADA-V1Y2 (21) and SOS-JRFL (2) were constructed as described previously. Similarly the env genes of HIV-1 strains JR-FL, HXB2 and 89.6 were cloned into pSVIIIenv (14) and SF162, 62H/T84, JR-CSF, JR-FL and AD into pSV7d (38). Additionally the env genes of anthrophic murine leukemia virus (A-MLV) was cloned into pSV7a (38). Recombinant pseudovirions were produced as described previously (2, 28, 36, 65).

Neutralization assay. Neutralization was measured in various luciferase reporter gene assays as described previously (2, 28, 36, 65).

i) Standard neutralization assay A. A pseudovirus inoculum, previously determined to yield -10,000,000 RU, was incubated with a serial dilution of mAb for 1 h at 37°C and added to 2×10^{5} U87.CD4.CCR5 or U87.CD4.CXCR4 cells (ARRRP). The virus inoculum was normalized for p24 content by ELISA before incubation with the cells. After 24 hrs of incubation fresh medium was added and incubated for an additional 3 days (37°C, 5% CO_{2}). Luciferase activity was measured using the luciferase assay system (Promega, Madison, Wisconsin) according to the manufacturer's instructions.

ii) Standard neutralization assay B. A pseudovirus inoculum was incubated with a serial dilution of mAb for 18 hrs at 37°C and added to U87.CD4 expressing both CCR5 and CXCR4 cells (ARRRP). Luciferase activity was measured using the luciferase assay system (Promega, Madison, Wisconsin) according to the manufacturer's instructions.

iii) SOS neutralization assays. For standard neutralization, a pseudovirus inoculum normalized for p24 content by ELISA was incubated with a serial dilution of mAb for 1 h at 37°C and added to 2×10^{6} U87.CD4.CCR5 cells for 2 h. Unbound virus was removed by changing medium and the culture was incubated for a further 1 h. Alternatively, to measure postattachment neutralization, a pseudovirus inoculum was incubated with 2×10^{6} U87.CD4.CCR5 cells for 2 h to form SOS-attached intermediates (SAI). After replacement of medium, a serial dilution of mAb was added for 1 h. Cells were subsequently treated with 5 mM DTT for 10 min to activate the fusion reaction and the medium was replaced. After an additional 3-day incubation (37°C, 5% CO_{2}), luciferase activity was measured as described above.

iv) Temperature-regulated neutralization assay. A pseudovirus inoculum, previously determined to yield -10,000 RT units, was incubated with a serial dilution of mAb for 1 h at 37°C and added to 5×10^{6} C877 cells expressing CD4 and CCR5 (22). Alternatively, the virus inoculum was incubated for 4.5 hours at 4°C with the cells prior to washing. Serial dilutions of antibodies were added, and the temperature was raised to 37°C. After a 24 hr incubation at 37°C (5% CO_{2}), fresh medium was added and the cells were incubated for an additional three days. Luciferase activity was measured as described above.

ELISA. Microtiter plates (Costar, Corning, New York) were coated overnight at 4°C with 5 µg/ml of anti-gp120 antibody DT274 (Alta BioReagents Ltd., Dublin, Ireland) in PBS (pH 7.5). Plates were blocked with 3% BSA for 1 hr at RT and washed with PBS/0.05% Tween-20 (PBST). Labeled antibodies (1% rabbit, Sigma) were added, diluted in PBST/1% BSA and incubated for 4 hrs at RT, in the presence or absence of scD4 (2 µg/ml). Next, serial dilutions of mAb were incubated with the captured proteins and bound mAb was detected with horseradish peroxidase (HRP)-labeled goat-anti-human IgG F(ab')2 (Pierce, Rockford, Illinois) and tetra-methyl-benzidine (TMB) substrate (BioRad). The color reaction was stopped after 20 min by the addition of 2M H_{2}SO_{4} and absorbance at 450nm was measured.

Modeling. The positions of four-domain CD4 were generated by superimposing the four-domain CD4 structures (pdb accession numbers: 1W10, 1W1Q, 1W1Q, residue 1-363, (58)) onto the trimeric model of the gp120-D1D2 complex (27) using the main-chain atoms of CD4 D1D2 (residue 1-178). RMS deviations for the superpositions are ~1.0 Å for all superpositions. A model having an additional 35° rotation of D3D4 towards the target cell membrane was constructed using the graphics program "0" (19), by altering the backbone angle between CD4 residues 176 and 177 in 1W1P from a q of -96° and 168° for residue 176 to -96° and 162° and a q of -72° and 154° for residue 177 to 153° and 154°. The nine amino acid linker between the last crystallographically ordered residue and the transmembrane spanning region contains 2 prolines. Its length was estimated by using calculations of polymer dimensions (48), where an Ala polymer has an average N-terminal, C-terminal distance of 18 Å and a Pro polymer a distance of 21 Å. All superpositions were carried out with the program LSQKAB, which is in the CCP4 suite of programs (8).

Results

Antibody Fab and scFv fragments of CD41 mAbs are more effective in neutralization of HIV-1JR-CSF than whole IgG1 antibodies.

We recently reported the isolation of a novel CD4i Fab fragment, designated X5, that differed from previously isolated CD4i antibodies, such as 17b or 48d, in that it potently neutralized an array of primary HIV-1 isolates (32). Whole antibody molecules are generally more effective in neutralization than their corresponding Fab fragments (57). We therefore converted Fab X5 to a whole immunoglobulin G1 (IgG1) molecule, expressed it in Chinese hamster ovary (CHO) cells and purified it by affinity chromatography. IgG X5 was tested against CCR5-dependent (R5) HIV-1JR-CSF in a neutralization assay. Surprisingly, the whole antibody neutralized approximately 5-fold less effectively than the Fab fragment (Fig. 6.1A; Table 6.1).

To assess whether the observed phenomenon was a general characteristic of CD4i mAbs, we tested whole antibody and antibody Fab fragments of other CD4i mAbs in neutralization against HIV-1JR-CSF. The Fab fragments of mAbs 17b and 48d were made by papain digestion. Both Fab fragments were more effective in neutralization compared to their corresponding whole antibody molecules (Fig. 6.1B; C: Table 6.1). Additionally, single-chain (sc)Fv variants of X5 and 17b were constructed and tested in neutralization. The smaller
scFvs were even more effective than the corresponding Fab fragments (Fig. 6.1A,B).

Lack of HIV-1JR-CSF neutralization by IgG is not due to decreased functionality

To assess if the inability of the whole antibody molecules to neutralize HIV-1JR-CSF was caused by diminished recognition of gp120JR-CSF, we compared the binding of IgG with Fab fragments to monomeric gp120JR-CSF in ELISA in the presence or absence of sCD4. In contrast to the neutralization results, the IgG molecules of X5 and 17b had a ~2-3-fold higher apparent affinity, probably due to the bivalency of the whole IgG molecule (Fig. 6.2). Fab X5 obtained by papain digestion of IgG X5 had a similar apparent affinity to *E. coli* expressed Fab X5 (data not shown). No difference in apparent binding affinities was observed between Fab and IgG 48d. All antibodies displayed a ~10-fold increase in apparent affinity for gp120 in the presence of sCD4. Although affinity to monomeric gp120 does not directly correlate with neutralization, it does demonstrate that the lack of neutralizing activity was not due to decreased functionality of the whole antibody molecules.

Antibody fragment size-dependent neutralization is a function of viral isolate

To determine whether the observed phenomenon was virus isolate-specific, we assessed a panel of HIV-1 isolates for sensitivity to CD4i antibody (fragment)-mediated neutralization (Table 6.1). HIV-1JR-FL and HIV-1ADA, both R5 primary isolates, showed a broadly similar tendency to be better neutralized by smaller antibody fragments, with the exceptions that the 17b Fab and scFv fragments were equally effective against HIV-1JR-FL. HIV-1ADA was overall somewhat less sensitive to CD4i mAb neutralization than HIV-1JR-CSF. The relatively neutralization-resistant isolate HIV-1YU2 (29, 52) was weakly neutralized by scFv CD4i mAbs but infectivity was enhanced by CD4i whole IgG1 molecules (data not shown).

Table 6.1A: Neutralization (IC<sub>50</sub> in nM) of HIV-1 by CD4i antibodies and antibody fragments (standard neutralization assay A)

<table>
<thead>
<tr>
<th>mAb</th>
<th>Format</th>
<th>HIV-1 Isolate</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>JR-CSF R5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>X5</td>
<td>IgG</td>
<td>1799</td>
</tr>
<tr>
<td></td>
<td>Fab</td>
<td>458</td>
</tr>
<tr>
<td></td>
<td>scFv</td>
<td>224</td>
</tr>
<tr>
<td>17b</td>
<td>IgG</td>
<td>&gt;2000 (22)</td>
</tr>
<tr>
<td></td>
<td>Fab</td>
<td>912</td>
</tr>
<tr>
<td></td>
<td>scFv</td>
<td>158</td>
</tr>
<tr>
<td>48d</td>
<td>IgG</td>
<td>&gt;2000 (41)</td>
</tr>
<tr>
<td></td>
<td>Fab</td>
<td>1698</td>
</tr>
</tbody>
</table>

<sup>a</sup> Neutralization was assessed on U87.CD4 cells expressing either the CCR5 (R5) or the CXCR4 (X4) coreceptor. The neutralization of the dualtropic isolate 89.6 was studied on U87.CD4 cells expressing CXCR4.

<sup>b</sup> Where 50% neutralization was not achieved, the percentage of inhibition at the highest antibody concentration is given in parenthesis.

n.t. not tested
CHAPTER 6

Neutralization (IC<sub>50</sub> in nM) of HIV-1 by CD4i antibodies and antibody fragments (standard neutralization assay B)

<table>
<thead>
<tr>
<th>mAb</th>
<th>Format</th>
<th>HIV-1 Isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SF162</td>
</tr>
<tr>
<td>X5</td>
<td>IgG</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Fab</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>scFv</td>
<td>1.7</td>
</tr>
<tr>
<td>17b</td>
<td>IgG</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>scFv</td>
<td>3.9</td>
</tr>
<tr>
<td>b12</td>
<td>IgG</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>scFv</td>
<td>0.53</td>
</tr>
</tbody>
</table>

<sup>a</sup> Neutralization was assessed on U87.CD4 cells expressing both CCR5 and CXCR4 coreceptors. Coreceptor tropism of the viral isolates is indicated (R5, X4 or R5X4).

<sup>b</sup> As negative control pseudovirions expressing the amphotropic murine leukemia virus (A-MLV) env gene were included.

<sup>c</sup> Where 50% neutralization was not achieved, the percentage of inhibition at the highest antibody concentration is given in parenthesis.

than the Fab fragment, whereas for 17b the two fragments were approximately equally effective (Table 6.1).

Two dual-tropic (R5X4) primary isolates were evaluated, HIV-1<sub>98.6</sub> and a dual-tropic variant of HIV-1<sub>R</sub>, designated HIV-1<sub>1a</sub> (Poignard et al, submitted). When HIV-1<sub>98.6</sub> neutralization by X5 was evaluated on CXCR4-expressing U87 cells, whole IgG and antibody fragments displayed similar potency (Fig. 6.3B, Table 6.1). For HIV-1<sub>1a</sub> evaluated on CXCR4-expressing U87 cells, X5 scFv was clearly more effective than the Fab fragment, whereas for 17b the two fragments neutralization potency displayed by the CD4i mAb X5 and IgG and Fab neutralization on CXCR4-expressing U87 cells, with whole IgG and antibody fragments displayed similar potency, comparable to whole IgG and Fab neutralization on CXCR4-expressing U87 cells (Fig. 6.3C). Thus, the precise pattern of neutralization potency displayed by the CD4i mAb X5 and antibody fragments is dependent upon isolate and coreceptor usage.

In a separate study, using a similar reporter gene neutralization assay in a different laboratory, a panel of HIV-1 isolates was assessed for sensitivity to CD4i antibody (fragment)-mediated neutralization (Table 6.2). In these experiments whole IgGs of CD4bs-specific mAbs were included for reference. Additionally, pseudovirions expressing the amphotropic murine leukemia virus (A-MLV) env gene were included as negative control. As for the experiments described in Table 6.1, the R5 primary isolates HIV-1<sub>R</sub>, HIV-1<sub>R</sub>-CSF and HIV-1<sub>TADa</sub>, were sensitive to neutralization by both X5 and 17b scFv fragments and relatively resistant to Fab X5, IgG X5 and IgG 17b. Another R5 primary isolate, HIV-1<sub>98.6</sub>, was sensitive to CD4i mAbs and antibody fragments, but this isolate was also sensitive to IgG b6, a generally weakly neutralizing anti-CD4bs mAb. Primary dual-tropic R5X4 isolate HIV-1<sub>98.6</sub> was neutralized by mAb X5 and Fab and scFv antibody fragments and by 17b scFv fragment but was resistant to 17b whole IgG (Table 6.2).

CD4i antibody fragments neutralize HIV-1 subsequent to CD4 binding

The CD4-induced conformational changes in gp120 include displacement of the V1/V2 variable loops, thus exposing the coreceptor binding site and the CD4i epitopes (31, 47, 60). To investigate the role of the V1/V2 variable loops in CD4i antibody and antibody fragment-mediated neutralization, we compared the neutralizing activity of different 17b antibody forms against wild-type ADA (WT) (Fig. 6.4A) and V1/V2-deleted ADA (ΔV1V2) (Fig. 6.4B). Removal of the V1/2 loops increased sensitivity to neutralization by mAb 17b, as has been previously reported (5, 21). However, 17b scFv and Fab fragments neutralized the ΔV1V2 virus more...
Figure 6.3: Neutralization of dual-tropic HIV-1 by mAb X5 whole IgG and antibody fragments. Whole antibody (●), Fab fragment (○) and scFv fragment (■) were titrated against HIV-1_AVR (A+B) and HIV-1_AV (C+D) in a pseudotyped luciferase-based neutralization assay using U87.CD4 cells expressing either CCR5 (A+C) or CXCR4 (B+D). Data points are the means of triplicates ± SEM.

Efficiently than the whole antibody, suggesting that some steric constraints on mAb 17b neutralization operate even in the absence of the V1/V2 loops.

Membrane fusion mediated by the HIV-1 envelope glycoproteins is a temperature-dependent process (12, 13). To investigate the sequence of events for mAb 17b-mediated neutralization, we tested the ΔV1V2 virus in a temperature-dependent neutralization assay. Virus and target cells were pre-incubated at 4°C, washed and 17b scFv, Fab or whole IgG added. The fusion reaction was resumed when the temperature was raised to 37°C. Under these conditions, compared with the standard neutralization assay (Fig. 6.4B), the neutralizing efficiency of the scFv was only minimally changed or slightly improved, the efficiency of the Fab fragment was slightly reduced and the whole IgG molecule was markedly less efficient (Fig. 6.4C). This suggests that the antibody fragments can neutralize virus after it has attached to the target cell, whereas whole antibody molecule is much less effective in this context. In other words, it seems likely that neutralization is most efficiently mediated by CD4i antibody binding to its epitope after CD4 engagement and that this is sterically restricted for the whole antibody but not for the fragments.

Finally, we used an activatable fusion intermediate system to look further at the role of CD4i Abs and fragments. Introduction of an intermolecular disulfide bond between gp120 and gp41 subunits has been shown to stabilize oligomeric Env complexes (designated SOS Env) (3). Incorporation of SOS Env into pseudovirions produces particles that bind to CD4 on target cells but do not fuse (2). Addition of a reducing agent activates the fusion reaction. Initially, the neutralization of reduced SOS pseudovirions of HIV-1 JR-FL (SOSJR-FL) was compared to wild-type HIV-1 JR-FL (WTJR-FL) pseudovirions using standard assay conditions. With the exception of whole IgG 17b, all CD4i antibody fragments could neutralize both SOS and WT virus to a similar extent (Fig. 6.5A,B,C,D). Additionally, whole IgG 12b and IgG 2F5, which were included as control antibodies, could efficiently neutralize both viruses under standard conditions (Fig. 6.5D,F). We next used SOSJR-FL to examine the ability of CD4i antibody fragments to neutralize under post-attachment conditions. SOSJR-FL pseudovirions were incubated with cells, unbound virus was washed away and antibody fragments were added to cells with adsorbed SOSJR-FL.

Figure 6.4: Pre- and post attachment neutralization of HIV-1_AVR and HIV-1_AVΔV1V2 by mAb 17b whole antibody and antibody fragments. Whole antibody (●), Fab fragment (○) and scFv fragment (■) were titrated against HIV-1_AVR (A) and HIV-1_AVΔV1V2 (B+C) in a pseudotyped luciferase-based neutralization assay using C8166 cells expressing CD4 and CCR5. In these settings pre- and post-attachment neutralization were measured simultaneously (A+B) by preincubating virus and antibody fragments at 37°C prior to addition to cells. Post-attachment neutralization was measured exclusively (C) by preincubating virus and cells at 4°C, washing the cells, and adding antibody fragments before raising the temperature to 37°C.
pseudovirions. All CD4i antibody fragments could still efficiently neutralize SOSJR-FL under these conditions, in contrast to whole IgG 17b. Whereas neutralization of SOSJR-FL by whole IgG b12 was much weaker under post-attachment conditions, neutralization by whole IgG 2F5 was equally effective, consistent with the concept that b12 neutralizes by inhibiting virus attachment to cells and 2F5 neutralizes in a post-attachment event (2, 36). This provides further evidence that smaller antibody fragments but not whole antibody can gain access to virions attached to target cells.

A previous study has suggested that SOS pseudovirions interact with coreceptor following binding of CD4, based on the observation that SOS pseudovirions incubated with sCD4 bind CCR5+CD4- cells, albeit poorly (2). Similar neutralization of SOS pseudovirions under standard and post-attachment conditions (Fig. 6.5B,C,D), however, suggest that the CD4i epitope is equally available in both assay formats. These results appear contradictory with our previous observation as the CD4i epitope partially overlaps with the coreceptor binding site. It should be noted however that neutralization does not require occupancy of all available sites (36, 49). The remaining sites not utilized for virus-cell binding may thus be sufficient to effect virus neutralization.

Discussion

Both CD4 and coreceptor binding sites on gp120 form potential targets for antibody-mediated intervention. The primary isolate-neutralizing antibodies identified so far that are specific for gp120 are directed to epitopes that appear to be present on the oligomeric Env complex before contact with CD4. They are believed to act, at least in part, by binding to the virion surface and sterically obstructing the interaction between virus and target cell (37, 57). For these antibodies, the larger whole antibody molecules are more effective than the corresponding Fab fragments at neutralization due to greater steric obstruction, but probably also because of increased avidity (bivalency)(57). Antibodies to the CD4i epitopes generally do not display primary isolate neutralizing activity at relevant concentrations.

The major finding of our study is that, for some HIV-1 isolates, the size of CD4i-specific (antibody-derived) neutralizing agents is inversely correlated with neutralization efficiency. Thus, antibody fragments are more effective than whole antibody molecules in neutralization. Further, scFv (25 kD) are generally more effective than Fab (50 kD) fragments. The temperature-regulated (Fig. 6.4) and SOS-arrested (Fig. 6.5) neutralization data presented here are consistent with a model in which CD4i scFvs or Fab, but not IgGs, can neutralize after attachment of the virus to CD4 (43). Because the difference in size is expected to have limited effects on the diffusion rates of the CD4i antibody fragments, these results strongly suggest that the restriction against CD4i antibody neutralization is steric, not temporal. Therefore, the likeliest explanation for the observed sensitivity of neutralization to antibody size is that, after CD4 binding to the virus, the available space between virus and target-cell surface is not enough to accommodate a whole antibody molecule, but is sufficient for antibody fragments. This is consistent with the estimated size of the antibody fragments and predictions of the distance between the CD4i epitope on the CD4-bound envelope glycoprotein trimer and the target-cell membrane (Fig. 6.6). Recent findings demonstrating the inaccessibility of the 17b epitope at the fusing cell interface are in agreement with this hypothesis (10).

The higher sensitivity to CD4i mAbs observed for some isolates is most likely a reflection of the exposure of the CD4i epitope on the oligomer prior to CD4 binding, as suggested previously (37). Additionally, our data suggests that when the CD4i epitope is exposed, any advantage of Fab or scFv fragments disappears (Table 6.1+6.2 and Fig. 6.3A,B,C). It is however noteworthy that exposure of the CD4i epitope, through depletion of the V1/V2 stem-loop structure, not only facilitates virus neutralization by the whole IgG molecule but also increases the potency of the Fab and scFv fragments (Fig. 6.4). Comparison of the relative neutralization potencies of 17b antibody and antibody fragments under different assay conditions (Fig.
6.4A,C) implies that the V1/V2 variable loops continue to play a role in obstructing antibody binding even after CD4 attachment. Thus, our results suggest that CD4I mAb-mediated neutralization is bi-phasic, with 1) a pre-attachment-phase that may not be antibody fragment size dependent, but is dependent on the accessibility of the CD4I epitope on the resting oligomer, primarily governed by the V1/V2 variable loops, and 2) a post-attachment-phase that is antibody fragment size dependent due to steric restrictions imposed by the cellular membrane and the V1/V2 variable loops. The fact that we observe a unique increase in potency for scFv X5 in neutralization of HIV-1IR (Fig. 6.3D) and the fact that scFv X5 but not scFv 17b was more effective than the corresponding Fab fragments in neutralization of HIV-1IR (Table 6.1), however, indicate that there are additional variables influencing these mechanisms. One could speculate that the precise orientation of the epitope may influence the susceptibility of the antibody fragment to steric constraints by the surrounding protein structures at the virus:cell interface.

Attempts to improve Env immunogens include strategies to better expose the coreceptor binding site and the overlapping CD4I epitopes (11, 15, 30, 44). It is argued that increasing CD4I epitope exposure may elicit more potent CD4I mAbs or higher serum antibody titers. Our data, however, suggest that the inability of CD4I mAbs to neutralize primary isolates is not due to lack of potency per se, but due to spatial constraints. As non-syncytium inducing (NSI), R5 HIV-1 variants establish primary infection in humans (39, 50, 51, 63, 64), the inability of CD4I-specific whole Abs to neutralize the majority of R5 viruses used in this study raises concerns as to whether the CD4I epitopes would be useful targets for antibody neutralization in vaccine design.

In conclusion, we show that CD4I-specific mAbs do not neutralize some primary isolates due to steric constraints. We propose that HIV-1 can exclude whole antibody molecules from the CD4I epitopes due to the close physical proximity of the cellular membrane and obstruction by the V1/V2 variable loops. The constraints were especially apparent for the primary R5 isolates tested. This raises questions about the utility of CD4I epitopes as targets for antibody-mediated neutralization in vaccine design. Understanding these viral defenses may suggest new strategies to circumvent them. The fact that
the smaller antibody fragments were able to neutralize does however suggest that the CD4i epitopes could be used as targets for small molecule entry inhibitors.

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References


Figure 2.1: A model for the structure of monomeric and oligomeric gp120. (For legend see page 21).

A

B

C

Figure 8.10: Cartoon model of the HIV-1 putative trimeric envelope spike. (For legend see page 92).