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Epitope Specificities of Broadly Neutralizing Plasmas from HIV-1 Infected Subjects

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Abstract

The characterization of the cross-reactive, or heterologous, neutralizing antibody (NAb) responses developed during HIV-1-infection is relevant to the development of an HIV vaccine. Here we summarize our recently published study where we characterized the cross-reactive NAb responses in two cohorts of anti-retroviral naïve, healthy, acute and chronically HIV-infected subjects (N=39) and present new information regarding epitope specificities. We determined that anti-CD4-binding site antibodies are responsible for the exceptionally broad cross-neutralizing antibody responses recorded only in rare plasma samples. Plasma from three subjects with moderate to high breadth of heterologous neutralizing antibody responses contained antibodies targeting the 4E10-epitope within the gp41 transmembrane subunit, and plasma from one these 3 subjects also contained antibodies against the 2F5 epitope. However, In most subjects with moderate to high breadth of cross-neutralization, the specificities of their NAb responses remain unknown. Overall, our study indicates that more than one pathway leads to the development of broad cross-reactive NABs during HIV-infection.

Keywords

HIV-1; neutralizing antibodies; broadly cross-neutralizing plasma; epitope specificities

1. Introduction

To date, HIV-1 Env-based immunogens have been unable to elicit a neutralizing antibody (NAb) response that displays broad activity against a wide array of isolates (as reviewed in [1]). Our current inability to elicit by immunization a broad anti-HIV neutralizing antibody response is a major challenge that hinders the development of an effective HIV-1 vaccine. Although cross-reactive NAb responses have not yet been elicited by a vaccine, such responses are present in sera from HIV-1-infected subjects [2-4]. Defining the frequency and epitope-specificities of cross-reactive neutralizing antibody responses developed during natural HIV-1 infection and identifying potential factors that are linked with the development of such

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responses may assist the design of more effective immunogens and the development of more appropriate immunization protocols.

Most subjects infected with HIV-1 mount, within a week or two after infection, an antibody response against the viral Envelope glycoprotein (Env) (the target of NAbs), but this response fails to neutralize the virus [5]. However, during the first month after infection, antibodies that can neutralize the autologous virus emerge [6]. These 'early' neutralizing antibody responses are not effective against heterologous viruses (i.e., viruses isolated from other patients). It is known, however, that sera collected during the chronic phase of infection display varying degrees of cross-neutralizing activities [2-4]. How prevalent these cross-reactive neutralizing antibody responses are during natural HIV-1 infection is unknown. It is also unknown whether the potential of HIV+ sera to neutralize diverse HIV-1 isolates is due to an antibody response that targets a single, or a few, conserved epitopes on the HIV Env, or due to polyspecific antibody responses that may include specificities against variable regions of Env.

The creation of large cohorts of HIV-1-infected subjects and the recent establishment of high-throughput neutralization assays and novel reagents has allowed the screening of large numbers of HIV+ plasmas for cross-neutralizing antibody activities and the mapping of their epitope-specificities. Here we summarize our recently published work on cross-neutralizing antibody responses in HIV-1 infected subjects [7] and provide additional, new information on the epitope-specificities of cross-reactive NAbs present in HIV+ plasmas.

2. Materials and Methods

2.1 Cohorts

The breadth of cross neutralizing antibody responses in HIV+ plasmas was determined in two cohorts of HIV+ subjects. The Vanderbilt cohort (VC) consisted of 53 plasma samples from 21 subjects and the CFAR/UW cohort (CC) consisted of 43 plasma samples from 18 subjects. Three subjects in the VC cohort were females and eighteen were males. The time of sero-conversion in the VC was known. Several members of the VC were also infected with Hepatitis A or B. In contrast, time of sero-conversion was unknown for the CC and no demographic information was available for the subjects. All subjects were anti-retroviral naïve with CD4+ T cell counts ≥ 250 cells per microliter, and with no AIDS-defining illness during the period of observation. A minimum of two temporally spaced plasma samples were obtained for each subject.

2.2. Neutralization Assays

Neutralization assays were performed using Env pseudovirus isolates in the TZM-bl cell based assay, as previously described [7-10]. Briefly, diluted plasmas were incubated with pseudovirus for one hour to allow binding. The plasma/pseudovirus mixture was added to TZM-bl target cells and incubated for 72 hours at 37C. The media was removed, SteadyLite (Perkin Elmer) luciferase reagent was added, and the cell associated luciferase activity was read on a Fluoroscan Luminometer (Thermo). Each plasma sample was first heat inactivated and tested against a panel of well characterized clade B (N:17) and clade A (N:2) pseudo-typed isolates [11]. For subject VC10042, additional clade A isolates (N:3) and clade C isolates (N: 3) were tested. The IC₅₀, the dilution at which a plasma inhibited infectivity by 50%, was calculated and scored. We defined the breadth of cross-neutralizing antibody response as the percentage (0% to 100%) of isolates neutralized by that sample.

2.3. ELISA Assay

ELISAs were carried out using the following peptides: 4E10 (NWFDITNWLWYIRKKK), 4E10 scramble (FTNDWINWLWYIRKKK), and alanine variants of 4E10 (alanine was

substituted into the 4E10 sequence in the positions marked with an arrow in figure 2e). 50 ng of peptide antigen were adsorbed onto each well of 96 well Immulon 2HB ELISA plates (Thermo) overnight in 0.2M NaHCO₃, pH 9.4 at room temperature. After blocking the plate in PBS, 10% dry milk, 0.3% Tween-20, plasma samples diluted in 10% dry milk, 0.03% Tween-20 in PBS in triplicate were titrated and incubated for 1 hour at 37C. Plates were washed in a plate washer and bound antibodies were detected at 37C for one hour with goat-anti-human-IgG-HRP (Biorad), diluted 1:3000. Plates were developed with 50µl TMB-ELISA solution (Pierce), stopped with 50µl 1N H₂SO₄, and the absorption at 450nm was read on Versamaxx microplate reader (Molecular Devices).

2.4. Differential adsorptions of plasma with gp120 variants

Plasma anti-HIV Env antibodies were adsorbed on beads coated with HIV Env as previously described [7,12]. Two different SF162 gp120 proteins were used: the WT, and a CD4-BS mutant that differs from WT by a single amino acid substitution (D368R, based on the HXB2 Env numbering). This substitution abrogates the binding of soluble CD4 and anti-CD4-BS antibodies to gp120 while retaining the ability to bind other MAbs. The SF162 gp120 protein variants were coupled to MyOne Dynabeads Tosylactivated (Invitrogen) following the manufacturer's instructions by reacting 40 mg of magnetic beads with 2 mg protein ligand overnight at 37C with gentle rotation. After collecting the beads on a magnet, the supernatant was removed and the beads were incubated overnight at 37C in PBS, 0.5% BSA, 0.05% Tween 20. The magnetic beads were washed and stored at 4C in PBS, 0.1% BSA, 0.05% Tween-20, 0.02% Sodium Azide. 600µl of plasma diluted 1:20 in DMEM/10%FBS were incubated with 300µl Env protein-coupled beads at room temperature for 120 min with gentle rotation. The samples were placed on a magnet and the beads were isolated. Up to five serial adsorptions were performed for each plasma sample. Bound antibodies were eluted as previously described. The depleted plasmas and the antibodies that were eluted from gp120 were used for ELISA and neutralization assays as described above and summarized in figure 3. Both BSA-coupled beads and blank beads were used as negative controls in all assays.

3. RESULTS

3.1. Frequency of cross-neutralizing breadth in two HIV-1+ cohorts

Cross-neutralizing breadth varied widely among the subjects in both cohorts. Approximately 23% of the subjects we studied exhibited high breadth, capable of neutralizing at least 75% of the isolates tested (figure 1a). Plasma from one subject, VC10042, exhibited exceptional breadth and potency, and was able to neutralize every isolate tested from across clades A, B, and C (figure 1b and [7]). 32% of subjects exhibited moderate breadth, able to neutralize between 30-70% of isolates tested. The remaining 45% of subjects exhibited very limited breadth, neutralizing less than 30% of the isolates tested. Several recent studies have reported similar levels of neutralizing breadth in HIV+ plasmas from chronically infected subjects [13-15].

Our analysis of temporally spaced plasma samples allowed us to assess if cross-neutralizing breadth evolves over time. Plasma samples collected longitudinally from certain subjects displayed different neutralization profiles, both gaining and losing the ability to neutralize certain isolates. Thus, it appears that neutralizing breadth and the antibody repertoire continually evolve over time. Additionally, we evaluated the relationship between breadth and several host factors. Duration of infection ($p=0.001$), plasma antibody binding avidity ($p<0.001$), and plasma viral load ($p=0.002$) were all positively associated with the development of broad cross-neutralizing antibody responses, highlighting the need for prolonged antigenic stimulation and antibody maturation to achieve cross-neutralizing breadth.

3.2. Defining the contribution of antibodies against the variable loops or the MPER in the overall cross-neutralizing potential of HIV+ plasmas

Peptide competition neutralization assays [9] were used to detect the presence in HIV+ plasmas of cross-reactive NAb against the variable regions V1, V2 and V3 of gp120 and of the membrane proximal external region (MPER) of gp41. We found no evidence that NAb targeting linear epitopes within these variable loop regions of the HIV-1 Envelope were responsible for the cross-reactive neutralizing activity we observed in many subjects, despite the fact that all of the plasmas tested, regardless of breadth, had high titers of binding anti-V3 antibodies [7]. Several recent studies have reported similar findings [12,14-18]. It appears therefore that although robust anti-V3 loop antibody responses are developed by most HIV-1-infected subjects, these antibodies are, for the most part, not cross-neutralizing because their target is inaccessible within the virion-associated trimeric HIV-1 Env.

The MPER contains the epitopes of three cross-reactive NAb: 2F5, Z13 and 4E10 [19-21]. MAb 4E10 is the broadest cross-neutralizing antibody currently available [22]. Using ELISA methodology we were able to detect the presence of anti-4E10 antibodies in three subjects, VC10028, VC20013, and CC1782 (figure 2). So far we showed that the anti-4E10-like antibodies in VC10028 display cross-neutralizing activity [7]. We have not yet determined whether the 4E10-like antibodies in the other two subjects also display cross-neutralizing activities. Interestingly, the earliest plasma collected from VC20013 (~1 year after infection) did not contain 4E10-like antibodies while plasma collected ~2 years after infection did contain such antibodies. The above mentioned results are in general agreement with those recently reported by others that, although rare, 4E10-like antibodies are generated during natural HIV-1 infection and contribute to the overall cross-neutralizing activities of HIV+ plasmas [14,15, 23].

We performed alanine scanning mutagenesis of the 4E10 peptide to better define the exact amino acids that are required for the binding of the 4E10-like antibodies in these three subjects and to compare their epitopes to those of the known MAb 4E10. Our data suggest that the amino acid residues that are required for the 4E10-like reactivity in VC10028 plasma are very similar to those required for MAb 4E10 binding (Fig 2). The binding of both VC10028 and MAb 4E10 was abrogated by all but a single alanine substitution (W676A), indicating that they target a similar epitope. It appears, however, that the 4E10-like antibodies in VC20013 and CC1782 recognize the 4E10 epitope differently than MAb 4E10. The substitution of alanine for the tryptophan residue at position 672 (W672A) increased the binding of the VC1782 4E10-like antibodies, in contrast to the complete abrogation of binding of MAb 4E10 to this mutant. Further, the amino acid substitution W678A did not dramatically reduce MAb 4E10 binding, but completely abrogated the binding of CC1782 plasma.

In addition to binding the 4E10 epitope, plasma from subject VC20013 also exhibited modest reactivity to the 2F5 epitope-bearing peptide. The antibody response in this subject most likely targets an MPER domain that overlaps that 2F5 and 4E10 epitopes. Recent reports have identified similar antibody specificities in two subjects using HIV-1/HIV-2 chimeras [14], fine mapping the neutralizing activity to an epitope similar to that of MAb Z31e1 [19]. However, the anti-MPER antibodies in those subjects contributed only minimally to their overall neutralization potential [14].

3.3. Not all antibodies against the CD4 binding site on the gp120 are cross-neutralizing

It is known that anti-CD4-BS antibodies are generated during HIV-1 infection [24] and several anti-CD4-BS MAbs have been isolated over the years [25,26]. So far, however, only one anti-CD4-BS MAb, b12, displays broad cross-neutralizing activity [22]. Two groups recently

reported that the exceptionally broad cross-neutralizing antibody responses of rare HIV-1-infected subjects are due to anti-CD4-BS antibodies [12,18].

To define the contribution of anti-CD4-BS antibodies in the overall cross-neutralizing potential of the plasma samples from our cohorts, we performed adsorption experiments using a variant of gp120 containing the D368R mutation, as previously described by Li et al [12]. The D368R mutation in the CD4-BS prevents the binding of most anti-CD4-BS antibodies, as well as soluble CD4. Adsorption of plasmas with the WT gp120 removes antibodies that bind to gp120, while adsorption with the gp120 CD4-BS mutant leaves anti-CD4-BS antibodies in the flow through fraction (figure 3). A sampling of plasmas representing a range of cross-neutralizing breadths were incubated with either WT gp120 or the D368R mutant gp120 coupled to magnetic beads and the flow throughs and eluates were collected for testing. Interestingly, all of the plasma samples we tested contained very similar titers of binding anti-CD4-BS, regardless of their breadth of cross-neutralization. Thus, the CD4-BS on the HIV Env appears to be immunogenic in most HIV+ subjects.

We next examined whether the anti-CD4-BS antibodies present in these subjects displayed cross-neutralizing activities. The results from this analysis were recently published [7] and are summarized here. In agreement with others [12,18], we observed that the exceptionally broad cross-neutralizing activity of the plasmas from subject VC10042 was due to anti-CD4-BS antibodies. This was not, however, true for the other subjects. In some cases the anti-CD4-BS antibodies did not display any cross-neutralizing activity, while in other cases their cross-neutralizing activity was of narrow breadth. In general, in subjects with low or moderate cross-neutralizing breadth, the anti-CD4-BS antibody fraction did not contribute significantly to cross-neutralizing potential. Thus, the presence of anti-CD4-BS antibodies does not guarantee that the plasma will possess cross-neutralizing potential. It is possible that different epitopes within the CD4-BS are targeted by subjects with broad responses versus those with narrow responses. Mapping the precise epitope within the CD4-BS that defines these exceptionally broad neutralizing responses could provide valuable guidance in designing an immunogen capable of eliciting anti-CD4-BS NAb.

3.4. Currently unknown epitopes targeted by cross-reactive NAb

Our epitope mapping studies yielded a range of results in subjects with moderate and low cross-neutralizing breadth. In one case, the neutralizing potential was adsorbed by both the WT gp120 and the D368R CD4-BS mutant, indicating that the cross-neutralizing antibody response targets epitopes on the gp120 monomer outside the CD4-BS. In other cases we were unable to adsorb the cross-neutralizing activity with either the WT gp120 or the D368R CD4-BS mutant, indicating that the cross-neutralizing antibody response targets epitopes that are not present on the gp120 subunit in its monomeric form. Recent studies have reported similar findings, observing that the cross-neutralizing potential of many subjects did not map to known epitopes on the monomeric gp120 subunit or to the MPER on the gp41 subunit [14,15].

Potentially, the unmapped cross-neutralizing antibody responses target epitopes that are present only on the trimeric form of the HIV Envelope spike, and not on the monomeric gp120 subunit. There are several possibilities as to the makeup of these complex conformational epitopes. It is possible that all three protomers that form a functional Env trimer are involved in forming a quaternary epitope recognized by a cross-neutralizing antibody. It is also possible that 2 of the 3 protomers forming a functional Env trimer form a quaternary epitope recognized by a NAb. Potentially, multiple regions of a single protomer align to produce the complex epitope, but only when the protomer is trimer-associated. It is also possible that sugar molecules are involved in the recognition of a quaternary epitope by a cross-reactive NAb. Identifying these new complex conformational neutralizing epitopes remains a key priority for vaccine research.

4. Conclusion

Our results, and those of others, indicate that there is more than one way that cross-neutralizing breadth may be developed. Rare individuals exist in which cross-neutralization is mediated by a single antibody specificity, as has been described for the CD4-BS of the gp120 subunit of the HIV-1 Env [7,12,14,18]. In most subjects, broad neutralizing activity is likely mediated by a polyclonal response consisting of multiple antibody specificities. Recent studies aimed at dissecting these polyclonal specificities were able to identify several known neutralizing epitopes that partially mediate cross-neutralizing breadth [7,14,15,23]. However, in most subjects tested to date, the majority of the epitope targets of the cross-reactive neutralizing activities in HIV-1+ plasmas have not yet been identified. Developing appropriate reagents that will allow us to define the presence of NABs in HIV+ plasmas that bind to quaternary epitopes and to precisely map these epitopes will improve our understanding of the underlying mechanisms of HIV-1 cross-neutralization, and potentially assist the design of more effective Env-based immunogens for the elicitation of protective antibody responses.

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a

b

VC10042 (22.51yrs)

Figure 1.

(a) Frequency of broadly cross-reactive NAb responses in two HIV+ cohorts. Percent breadth is calculated as the number of isolates out of the total isolates tested that each plasma neutralized. (b) Cross-clade neutralization of subject VC10042 against selected clade A, B, and C viruses. Red line indicates the 50% reduction in neutralization (IC50).

Figure 2.

Characterization of MPER-directed antibodies in plasmas (a) VC10028, (b) CC1782, and (c) VC20013, as well as mAb 4E10 (d). Plasmas were tested in an ELISA format with 4E10 peptide variants as the antigen. The sequence of the 4E10 epitope is noted in panel (e), and arrows indicate amino acid positions that were individually changed to alanine for epitope analysis.

Figure 3.
Schematic of the differential adsorption of HIV+ plasma with gp120wt or gp120bs - coupled beads.