CD4 binding site broadly neutralizing antibody selection of HIV-1 escape mutants

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All human immunodeficiency virus type-1 (HIV-1) viruses use CD4 to enter cells. Consequently, the viral envelope CD4-binding site (CD4bs) is relatively conserved, making it a logical neutralizing antibody target. It is important to understand how CD4-binding site variation allows for escape from neutralizing antibodies. Alanine scanning mutagenesis identifies residues in antigenic sites, whereas escape mutant selection identifies viable mutants. We selected HIV-1 to escape CD4bs neutralizing mAbs b12, A12 and HJ16. Viruses that escape from A12 and b12 remained susceptible to HJ16, VRC01 and J3, whilst six different viruses that escape HJ16 remained sensitive to A12, b12 and J3. In contrast, their sensitivity to VRC01 was variable. Triple HJ16/A12/b12-resistant virus proved that HIV-1 could escape multiple broadly neutralizing monoclonal antibodies, but still retain sensitivity to VRC01 and the llama-derived J3 nanobody. This antigenic variability may reflect that occurring in circulating viruses, so studies like this can predict immunologically relevant antigenic forms of the CD4bs for inclusion in HIV-1 vaccines.

A successful human immunodeficiency virus type 1 (HIV-1) vaccine is expected to need to induce robust CD4+ and CD8+ cellular responses, in concert with a strong and broadly neutralizing antibody response. Designing immunogens that trigger such responses is challenging (reviewed by Haynes & Montefiori, 2006; McCoy & Weiss, 2013), partly due to the diversity (Gaschen et al., 2002) of the viral envelope glycoprotein (Env) which interacts with cell receptors including CD4. Hence, the CD4-binding site (CD4bs) is functionally conserved and is therefore a logical vaccine target to elicit neutralizing Abs. A number of highly effective anti-CD4bs broadly neutralizing monoclonal antibodies (BNMAbs) with distinct neutralization profiles have been generated from HIV-1-infected individuals (Burton et al., 1991, 1994; Corti et al., 2010; Falkowska et al., 2012; Walker et al., 2009; Wu et al., 2010; Zhou et al., 2010) and vaccinated llamas (Forsman et al., 2002; McCoy et al., 2010). A full understanding of the nature of these antibodies would provide clues to the antigenic landscape of the CD4bs, which may be important in developing an inclusive HIV-1 vaccine. In addition, to structurally define current BNMAbs and their corresponding CD4bs footprints, the ability of replication competent viruses to escape such antibodies and to determine whether the mutants remain susceptible to alternative anti-CD4bs BNMAbs will be valuable.

We used the three different anti-CD4bs BNMAbs, b12, A12 and HJ16, to select for escape mutants. B12 was the first human BNMAb to map to the CD4bs and competes for binding to soluble CD4 (sCD4) (Barbas et al., 1992; Burton et al., 1991; Burton et al., 1994; Roben et al., 1994; Zhou et al., 2010). The llama-derived single-chain Ab A12 also competes with b12 and sCD4 (Forsman et al., 2008), as does HJ16 (Corti et al., 2010). Alanine scanning has determined that HJ16 belongs to a class of antibodies that bind to a region distinct from the classic CD4bs (aa 474–476) (Pietzsch et al., 2010). Recently, Balla-Jhagioorsingh et al. (2013) identified a glycosylation site (N276) critical for HJ16-induced escape of a primary HIV-1 strain in an in vivo model.

We used the well-described HIV-1 replication competent clone HXB2 (Ratner et al., 1985) as it is unlike primary isolates highly sensitive to many anti-CD4bs BNMAbs. This is probably because HXB2 was highly passaged in vitro in the absence of humoral responses. Escape viruses were selected in C8166 CD4+ T cells (Salahuddin et al., 1983) in gradually increasing concentrations of BNMAbs (from 50 ng NMAb ml–1). Cell-free supernatants were harvested from cells of cytopathic appearance and added to target cells for a second round of infection, this time with a doubling of the BNMAb concentration. After 2–4 weeks, resistant viruses emerged that could replicate in the presence of high concentrations of each BNMAb (10 μg A12 or HJ16 ml–1 and 20 μg b12 ml–1). Neutralization assays were carried out and proviral full-length env from cells infected with resistant viruses was PCR amplified and sequenced (Dreja et al., 2010).

Selection of HXB2 with b12 resulted in a virus with the single dominant aa change (G366E), within the envelope
CD4bs (Figs 1 and 2). This change is located three aas upstream of the proline to lysine mutation seen in a previous escape study (Mo et al., 1997), where additionally two mutations were observed in the V2 region. Interestingly, we did not identify any additional changes. This lack of other mutations within the V2 could be explained by the ease of neutralization of HXB2, suggesting that supplementary V2 compensatory mutations are not required for resistance. It may be that the G366E mutation did not hamper viral infectivity. Glycine 366 has previously been implicated in b12 binding (Li et al., 2011; Saphire et al., 2001, Zhou et al., 2007). The site is mapped to a model of the crystal structure of the trimeric HIV-1 Env spike (Fig. 2). The HIV-1 viral env expression vector psvIII-HXB2 (Gao et al., 1996) was engineered by site-directed mutagenesis (SDM) to carry the G366E mutation. SDM-pseudotyped virions carrying the luciferase reporter gene were produced as previously described (Dreja et al., 2010). The resulting SDM(b12) confirmed the b12-resistant phenotype (Fig. 3a). The mutation had no effect on viral susceptibility to A12 and HJ16, even though both these BNMAbs compete for binding with b12, with each other and with sCD4. Moreover, the sensitivity of SDM(b12) to VRC01 (Wu et al., 2010; Zhou et al., 2010) remained the same (Fig. 3b). The recently described llama-derived anti-CD4 BNMAb J3 (McCoy et al., 2012) neutralized SDM(b12) as efficiently as wild-type (WT)-HXB2 (Fig. 3b). These results suggest that the aa change responsible for b12 escape is antigenically distinct from those of the human BNMAbs, HJ16 and VRC01, and the llama BNMAbs, A12 and J3. The viral escape from b12 had little effect on the ability of virus to replicate in vitro (data not shown) but was measurably less sensitive to CD4-IgG2 inhibition (IC50 increased from 5 ng ml\(^{-1}\) to 50 ng ml\(^{-1}\) and, for the pseudotype SDM(b12), from 10 ng ml\(^{-1}\) to 100 ng ml\(^{-1}\) Fig. 3b).

The A12-selected escape virus was resistant to A12 and carried the S375N mutation adjacent to the CD4bs (Figs 1 and 2). SDM(A12) confirmed that A12 resistance is conferred entirely by this mutation (Fig. 3a). SDM(A12) remained sensitive to both b12 and HJ16, supporting the notion that the epitopes of A12 and b12 or HJ16 are distinct. The J3 llama antibody neutralized SDM(A12) and there was a small reduction in sensitivity to VRC01 for SDM(A12). There was, however, no apparent effect on the sensitivity of the mutation in SDM(A12) to CD4-IgG2.

In contrast to the b12 and A12 selection, where dominant genotypes were generated, selection with HJ16 yielded a viral swarm containing several different mutant viruses. It could be that there are further options for escape routes
Fig. 3. HXB2- (white circles), SDM(A12)-, SDM(b12)- and HJ16-resistant pseudotyped HIV-1 virions (black circles) were assessed for neutralization resistance against (a) A12, b12 and HJ16 and (b) VRC01, J3 and CD4-IgG2. The percentage neutralization is shown on the y-axis in the presence of BNMAbs at different concentrations (x-axis: ng ml$^{-1}$).
with this single-chain antibody compared with bivalent antibodies that may have more steric hindrance. The proviral env sequences amplified from HJ16 viral-selected cultures [from nucleotide 127 (KpnI) to 2251 (BamHI)] were inserted into the env-expression vector psvIII-HXB2 and six different infectious, resistant clones were identified. Interestingly, and in keeping with the observation that glycosylation may be associated with HJ16 resistance in
primary cell cultures (Balla-Jhagjhoorsingh et al., 2013), we also observed that four mutations of a potential N-linked glycosylation site in the V5 loop affected sensitivity to HJ16 neutralization (N463S, S465F, S465P and S465Y) (Figs 1 and 2). Gray et al. (2011) demonstrated a relatively high degree of sequence variation within the V5 loop in a large, independent panel of Envs, which may affect the accessibility to the CD4bs. Remarkably, none of these four substitutions significantly affected CD4-IgG2 inhibition (Fig. 3b). The mutations were 11 and 9 aa upstream of the core region (474–476), identified as a HJ16 target by Pietzsch et al. (2010). Curiously, of the three HJ16 resistant pseudoviruses with substitutions at position 465, two [psHJ16(S465F) and psHJ16(S465P)] gained sensitivity to VCR01 (Fig. 3a). This is concurrent with to HJ16 activity. Similarly, psHJ16(N463S) maintained wild-type sensitivity to VRC01. Overall, our results suggest that the V5 region is involved in HJ16 and VRC01 binding, as changes in this domain affect neutralization to both BNMAbs. The fifth HJ16 resistant virus had a glycine to aspartic acid change at position 459 [psHJ16(D459D)], resulting in a virus that was marginally more resistant to VRC01. This mutation was identified in HIV-1 (JRCSF)-infected humanized mice treated with 45–46°C W, a BNMA belonging to the HJ16 family (Klein et al., 2012). The G459D mutation is only four aa upstream of the glycosylation site in the V5 loop, and exhibits a similar neutralization profile to psHJ16(N463S). By contrast, similar to S465F and S465P, the HJ16-resistant clone E409R also became more neutralization sensitive to VRC01 (Fig. 3b).

All HJ16-resistant pseudotyped viruses retained sensitivity to J3, b12, CD4-IgG2 and, in four cases, to A12. Interestingly, G459D and E409R appeared more sensitive to A12 neutralization at lower concentrations (<100 ng ml⁻¹) compared with HXB2 and the other pseudoviruses.

In summary, HJ16 and VCR01 share overlapping footprints but have distinct antigenic landscapes. Both target sites were distinct from b12, A12 and J3 with respect to either molecular footprint or antigenic landscape. Importantly, our results suggest that antibodies such as HJ16 and VCR01 could potentially co-operate in vivo. Escape from HJ16-like antibodies would be more difficult in the presence of antibodies such as VCR01, as many escape routes will lead to greater sensitivity to this antibody. Our proof-of-concept study suggests that careful monitoring and analysis of the antigenic landscape determined by BNMAbs revealed by different viral escape routes may be informative in the design of vaccine candidates. As neutralization escape from HJ16, A12 and b12 can be achieved by mutagenesis at different aa positions, we set out to determine whether virus could become resistant to HJ16, A12 and b12 BNMAbs simultaneously. Using the previously successful culturing procedure, we failed to establish a triple selection by providing all the BNMAbs together (four attempts). This implies that there is a limit to how much selective pressure can be sustained in the CD4-binding domain. However, by challenging the virus with one BNMAb at a time, and subsequently adding another selecting BNMAb, we succeeded in developing a triply resistant virus indicating that, at least in theory, it is possible for HIV-1 to escape all three neutralizing mAbs. We used the HJ16 escape virus as our initial virus for selection as it remained sensitive to b12 and A12 neutralization. Thus we further selected this virus population with A12 followed by b12, resulting in a multi-resistant virus. The dominant proviral genotype had the previously identified aa changes corresponding to individual escape mutations described above for all three BNMAbs (Fig. 1): the A12-induced S375N mutation, the HJ16-triggered N463S change and the G366E change provided b12 resistance. The contribution to neutralization resistance of each mutation was confirmed by genetic engineering and the resulting SDM(HJ16/A12/b12) was assessed for neutralization. As expected, SDM(HJ16/A12/b12) was completely resistant to the three BNMAbs, when tested individually, but also partly to VRC01 (IC50 from 150 to >1000 ng ml⁻¹). This partial VRC01 resistance is in accordance with the findings from the individual aa changes, where the N463S and G366E mutations were neutral whereas S375N rendered the virus less sensitive to VRC01 neutralization (Fig. 3b). SDM(HJ16/A12/b12) was marginally less sensitive to J3 (IC50 from 3 to 25 ng ml⁻¹), as predicted by the lower sensitivity of the S375N and G366E mutations (Fig. 3b). As expected, the sensitivity to CD4-IgG was partly lost, which is likely to be due to the G366E mutation described above (IC50 from 2 to 20 ng ml⁻¹). Nevertheless, it is intriguing that albeit marginally resistant to the BNMAbs VRC01 and J3, the triply selected virus retains some sensitivity to these BNMAbs. This suggests that if one succeeds in inducing a range of different anti-CD4bs Abs by vaccination, neutralization control of HIV-1 may be achieved. Also, the three BNMAbs, provided at the same time, never enabled the emergence of a resistant virus, cautiously suggesting that there is a limit to the amount of pressure the virus can withstand. Pre-exposure prophylaxis using modified BNMAb is considered as an option to prevent HIV-1 acquisition (Pace et al., 2013). Our findings would support such an approach and suggest that multiple BNMAbs targeting the CD4bs structure should be considered.

Studies of the antigenic landscape of BNMAbs and the escape routes that viruses master can help us to target important immunogenic epitopes for HIV-1 vaccines, but also to consider the inclusion of anticipated escape structures.

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References


