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Soluble CD4 broadens neutralization of V3-directed monoclonal antibodies and guinea pig vaccine sera against HIV-1 subtype B and C reference viruses

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Abstract

To better understand the limits of antigenic reactivity and epitope accessibility of the V3 domain of primary HIV-1 isolates, we evaluated three human anti-V3 monoclonal antibodies (mAbs) and selected guinea pig vaccine sera for neutralization against reference panels of subtype B and C pseudoviruses derived from early stage infections. The mAbs and vaccine sera potently neutralized several prototype viruses, but displayed substantially less neutralization of most reference strains. In the presence of soluble CD4 (sCD4), the breadth of V3-mediated neutralization was increased; up to 80% and 77% of the subtype B and C viruses respectively were sensitive to V3-mediated neutralization. Unlike sCD4, the reaction of CD4-binding site mAbs b12 and F105 with native virus did not lead to full exposure of the V3 domain. These findings confirm that V3 antibodies recognize most primary viral strains, but that the epitope often has limited accessibility in the context of native envelope spike.

Introduction

The trimeric HIV-1 Env spike is composed of the surface unit gp120 and transmembrane gp41 glycoproteins. Viral entry into susceptible cells is mediated by the interaction of gp120 with the cell surface receptor CD4, leading to conformational changes that form and expose the co-receptor binding region of gp120 (Kwong et al., 1998; Salzwedel et al., 2000; Wyatt and Sodroski, 1998; Xiang et al., 2002). The V3 region of gp120 is critical for co-receptor recognition and determines which co-receptor, CCR5 or CXCR4, is used for viral entry (Cormier and Dragic, 2002; Huang et al., 2005; Suphaphiphat et al., 2003). Hence, while relatively variable in linear sequence, the V3 region has some level of functional and structural conservation (Cardozo et al., 2007; Haynes and Montefiori, 2006; Huang et al., 2005; Rosen et al., 2005; Sharon et al., 2003).

During HIV-1 infection, antibodies to the V3 loop are common (Broliden et al., 1992; Gorny et al., 2006; Haynes and Montefiori, 2006; Krachmarov et al., 2001; Kraft et al., 2007;

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Pantophlet and Burton, 2006; Profy et al., 1990; Schreiber et al., 1994; Spenlehauer et al., 1998; Wu et al., 1995; Zolla-Pazner, 2004). However, the V3 region appears to play a limited role in the neutralization of most primary virus isolates (Binley et al., 2004; Burton et al., 2004; Lusso et al., 2005; Stamatos et al., 1998; Vancott et al., 1995). Early vaccine studies using V3 peptides as immunogens showed a highly type-specific neutralizing antibody (NAb) response to V3 (Javaherian et al., 1990), while more recent studies of V3 mAbs and immune sera suggest that the V3 NAb response can be more broadly reactive (Derby et al., 2007; Haynes et al., 2006; Moore et al., 1995b; Wu et al., 2006; Yang et al., 2004; Zolla-Pazner, 2005). Human anti-V3 mAbs from both subtype B and non-subtype B infected individuals can neutralize a subset of subtype B and non-subtype B primary virus strains. Interestingly, the breadth and potency of this neutralizations is maximized when the V3 region is built into an unmasked V3 sensitive Env such as on virus SF162 (Binley et al., 2004; Gorny et al., 2006; Krachmarov et al., 2006; Li et al., 2005; Moore et al., 1995a; Pantophlet et al., 2007; Patel, Hoffman, and Swanstrom, 2008; Zolla-Pazner et al., 2008). These data, along with recently described atomic level structures of V3 mAb liganded to cognate peptides, confirm that there are conserved motifs within the V3 region (Cardozo et al., 2007; Huang et al., 2005; Sharon et al., 2003; Stanfield et al., 2004; Stanfield et al., 2006).

These findings are consistent with our understanding that the V3 region is displayed to varying degrees in the context of the quaternary structure of the native viral spike of individual strains of HIV-1. However after binding to the CD4 receptor, conformational changes in Env result in exposure of specific regions previously inaccessible to antibody. CD4 binding significantly enhances gp120 binding by mAb 17b, which recognizes the co-receptor binding site (Decker et al., 2005; Hoffman et al., 1999; Salzwedel et al., 2000; Sullivan et al., 1998a; Sullivan et al., 1998b; Xiang et al., 2002). Similarly, the V3 loop appears to be accessible to antibody when gp120 is in a CD4-bound state (Krachmarov et al., 2006; Lusso et al., 2005; Mbah et al., 2001; Potts et al., 1993; Sullivan et al., 1998b). We therefore postulated that the limited breadth of neutralization by recently isolated broadly reactive V3 mAbs, and by V3-directed vaccine sera, may be due to poor epitope accessibility rather than antigenic diversity of the V3 region (Bou-Habib et al., 1994; Krachmarov et al., 2005; Stamatos et al., 1998; Vancott et al., 1995).

To study the breadth and potency of anti-V3 antibody mediated virus neutralization, we used recently established reference panels of 12 acute subtype B and 12 acute subtype C Env-pseudoviruses (Li et al., 2005; Li et al., 2006). We evaluated three well-characterized anti-V3 mAbs and five guinea pig (GP) vaccine-induced immune sera known to contain high levels of anti-V3 antibodies. The mAbs and immune sera were assayed in the presence of varying concentrations of sCD4 to test if the resulting conformational change in Env would lead to exposure and recognition of the V3 loop. One anti-V3 mAb, 447-52D, is known to react with the GPGR sequence at the tip of the V3 loop which is conserved among most subtype B isolates (Gorny et al., 1992; Zolla-Pazner et al., 2004). The other two mAbs, 2219 and 3074, were derived from subtype B and the circulating recombinant form (CRF) 02_AG infected individuals respectively, and were selected for their broad profile of V3 reactivity (Gorny et al., 2002; Gorny et al., 2006; Krachmarov et al., 2005; Krachmarov et al., 2006). The GP vaccine sera were the result of plasmid DNA prime, recombinant adenoviruses serotype-5 (rAd5) boost immunization with Env constructs containing a deletion of the V1V2 loop and two small deletions on both arms of the V3 stem. This immunization strategy produced high V3-directed NAb responses (Chakrabarti et al., 2002; Chakrabarti et al., 2005; Wu et al., 2006; Yang et al., 2004). Two GPs were immunized with a subtype B immunogen and three were immunized with a subtype C immunogen.

Results

SCD4 enhances neutralization by V3-directed mAbs

We tested the subtype B-derived anti-V3 mAbs 447-52D and 2219 and the CRF02_AG-derived mAb 3074 against subtype B viruses SF162, BaL.26, ADA, JRFL and YU2. All three mAbs neutralized SF162 and BaL.26 by > 90% at 10 ug/ml (data not shown), but displayed more variable activity against ADA, JRFL and YU2 (Table 1). Of interest, all three mAbs displayed less than 50% neutralizing activity against JRFL even though this virus contain an identical V3 amino acid sequence to BaL.26 (Supplementary Fig. 1). However, pre-incubation with sCD4 substantially increased the sensitivity of JRFL, ADA and YU2 to neutralization by each of the V3 mAbs. In our luciferase-based pseudovirus neutralization assay, viral entry is quantified by relative light units (RLU) and neutralization is calculated as the percent reduction in virus entry caused by antibody compared to baseline viral entry with no antibody present (Fig. 1A). To account for the effect on virus entry by sCD4, antibody-mediated neutralization was calculated using a baseline of viral entry that occurred with the relevant concentration of sCD4 present. Adjusted neutralization (Fig. 1B) depicts the V3 mAb-mediated neutralization that occurred with various concentrations of sCD4 present. As sCD4 concentration increased from zero to 5 ug/ml, 447-52D adjusted neutralization of JRFL increased from 38% to 95%. In these experiments, the mAb was used at a fixed concentration of 10 ug/ml. A similar pattern of JRFL neutralization was observed for mAbs 2219 and 3074 (Fig. 1C). Hence, the weak JRFL neutralization by these V3 mAbs was not due to poor antigenic recognition of the JRFL V3 region, but rather due to limited accessibility of the epitope. SCD4 also enhanced the V3 mAb neutralization of ADA and YU2 (Fig. 1D and 1E). Note that the CRF02_AG-derived mAb 3074 was able to neutralize subtype B viruses JRFL and ADA when sCD4 was present, though not as potently as the two subtype B-derived mAbs. Interestingly, mAb 17b neutralization was only modestly increased in the presence of sCD4, and was never above 50% against JRFL, ADA and YU2 (Fig. 1C, 1D and 1E). This suggests that while the level of sCD4 used in these experiments was enough to inhibit viral entry by 50% – 80%, and was enough to expose the V3 region, it was insufficient to result in full conformational exposure of the co-receptor binding site. The experiments described above were performed with varying concentrations of sCD4 and a fixed concentration of the V3 mAbs. We also determined the effect of a fixed concentration of sCD4 on the potency of mAb 447-52D. The neutralization IC_{50} for 447-52D decreased from >10 ug/ml in the absence of sCD4 to 0.67 ug/ml and 0.025 ug/ml in the presence of 1 and 5 ug/ml of sCD4 respectively, a greater than 400-fold increase in mAb neutralization potency (Fig. 1F).

SCD4 enhances and broadens neutralization by GP vaccine sera

We previously demonstrated that DNA prime, rAd5 boost immunization with Env constructs containing a deletion of the V1V2 loop and two small deletions on both arms of the V3 stem, produced high V3-directed NAb responses (Chakrabarti et al., 2002; Chakrabarti et al., 2005; Wu et al., 2006; Yang et al., 2004). In the present study, we examined two such GP sera from animals immunized with an HIV-1 subtype B-based Env immunogen. GP sera 82-2 and 82-4 had high and moderate NAb levels respectively (Supplementary Fig. 2). Virus neutralization by both sera was largely mediated by antibodies to the V3 loop, as shown by peptide competition studies (Wu et al., 2006).

As we previously described, these sera displayed little neutralization of viruses JRFL, ADA and YU2. However, in the presence of sCD4, a 1:10 dilution of the sera neutralized all three viruses (Fig. 2A). To assess the increase in potency of the sera caused by sCD4, the sera were serially diluted in the presence of 1 ug/ml or 5 ug/ml of sCD4 (Fig. 2B). The neutralization IC_{50} of serum 82-2 against JRFL improved from 1:12 in the absence of sCD4

to 1:100 and >1:1,000 in the presence of 1 and 5 ug/ml of sCD4, respectively. Similarly, the neutralization IC_{50} of 82-4 increased from < 1:10 in the absence of sCD4 to 1:30 and 1:625 in the presence of 1 and 5 ug/ml of sCD4, respectively. The V3 specificity of these neutralization activities was confirmed by peptide competition assays using a V3 peptide that matched the immunogen V3 sequence (Fig. 2C).

To understand the potential breadth of reactivity of these V3 mAbs and V3-directed GP vaccine sera, we extended this analysis to a recently described reference panel of 12 acute subtype B viruses (Li et al., 2005). The neutralization profiles of three mAbs and two GP sera against four selected viruses are graphically shown in Fig. 3. These four viruses demonstrate the spectrum of V3 neutralization observed among the subtype B reference viruses. Virus 6535.3 was highly sensitive to V3-mediated neutralization by two mAbs without sCD4 present, but displayed increased sensitivity to mAb 3074 and GP 82-2 serum after sCD4 was added; virus WITO4160.33 became generally sensitive to mAb and serum V3-mediated neutralization after sCD4 was added to the assay; virus CAAN5342.A2 became sensitive to the GP sera, but not the mAbs and virus THRO4156.18 was resistant to both anti-V3 mAbs and GP sera, even after sCD4 was added. Note that the concentration of sCD4 required to inhibit 50% or 80% viral entry, and hence to expose the V3 loop to antibody, varied among viruses (Fig. 3 and Table 1). As summarized in Table 1, the anti-V3 mAbs at 10 ug/ml, and the GP vaccine sera at a 1:10 dilution, displayed limited neutralization breadth among the 15 viruses tested. It should be noted that this panel of 15 pseudoviruses, which includes JRFL, ADA, YU2 and 12 reference strains, was chosen to exclude viruses such as BaL.26 and SF162 that are highly sensitive to V3-mediated neutralization. We used a value of 50% virus neutralization by 10 ug/ml of mAb, or a 1:10 serum dilution, as measure of positive virus neutralization. Our prior published data suggests that a GP vaccine serum neutralization value of 50% or greater is a readily reproducible measure of antibody-mediated virus neutralization (Shu et al., 2007; Wu et al., 2006; Yang et al., 2004). By this measure, the three mAbs and two GP sera neutralized two to four of the 15 viruses (13–27%). In the presence of sCD4, the mAbs and GP sera both displayed increased neutralization breadth. For example, mAb 2219 neutralized two of 15 viruses without sCD4 and nine of 15 viruses in the presence of sCD4, and serum 82-2 neutralization increased from four to 12 of 15 viruses. Hence, up to 80% of the subtype B viruses tested were sensitive to V3-mediated neutralization when sCD4 was present. This increase in breadth was statistically significant for mAb 2219 and for both GP sera (Table 1). Statistical analysis comparing the percent neutralization by each mAb and serum against all 15 viruses, with and without sCD4, was also performed. The inclusion of sCD4 in the assay resulted in a statistically significant increase in the median neutralization values for all three anti-V3 mAbs and both GP vaccine sera, but not for mAb 17b (Supplementary Fig. 3).

SCD4 broadens neutralization of V3-directed mAbs and subtype C GP vaccine sera against a reference panel of subtype C viruses

We further evaluated the effect of sCD4 on neutralization of a standardized panel of 12 subtype C reference viruses (Li et al., 2006). We used the same three mAbs, including the CRF02_AG-derived 3074, and included sera from three selected GPs that received a subtype C Env immunogen. The subtype C Env immunogen was similar to the subtype B immunogen except that the V3 region was replaced with a subtype C V3 sequence (Wu et al., 2006). All three GP sera neutralized V3 sensitive subtype C viruses (Supplementary Fig. 2) and had modest to high levels of anti-V3 NAbs (Wu et al., 2006). The neutralization profiles of five selected subtype C viruses are graphically shown in Figure 4. TV1.29 was chosen because it was previously shown to be sensitive to V3 neutralization by subtype C GP vaccine sera (Wu et al., 2006); the other four viruses demonstrate the spectrum of V3 neutralization observed among the subtype C reference viruses. The anti-V3 mAbs

displayed little neutralization of the subtype C reference viruses, but the presence of sCD4 resulted in mAb neutralization of some viruses such as ZM233M.PB6 and CAP210.2.00.E8 (Fig. 4). The GP vaccine sera displayed neutralization of some subtype C viruses without sCD4 present, but against other viruses such as Du156.12, neutralization was increased by adding sCD4 to the assay (Fig. 4). Using the same 50% value to denote positive virus neutralization, mAb 3074 neutralization increased from two to five of 13 viruses when sCD4 was present and GP serum 86-6 neutralization increased from five to ten of 13 viruses (Table 2). While these increases in neutralization breadth were not statistically significant (Table 2), comparisons of median percent neutralization values with and without sCD4 indicate significant increases in potency for mAbs 2219, 3074 and GP serum 86-6 (Supplementary Fig. 4). We verified the specificity of the neutralization by GP sera 83-2, 86-2 and 86-6 against virus TV1.29 by V3 peptide competition (Fig. 5). The presence of 5 ug/ml of sCD4 markedly increased the neutralization titers of all three immune sera against TV1.29, and this was inhibited by 30 ug/ml of a V3 peptide that matched the V3 immunogen sequence. These data indicate that similar to subtype B viruses, the subtype C V3 region is recognized by known V3 mAbs and by immune sera, but is poorly accessible to antibody on the functional virion.

Approximately 25% of pseudoviruses in the subtype B and C panels we tested remained resistant to V3-antibody neutralization, even in the presence of sCD4 at IC_{80} or higher concentrations. The explanation of this observation could be that sCD4 did not trigger exposure of V3 loop or that antigenic variation resulted in lack of recognition by any of the antibody reagents tested. This could be partially addressed by testing antibody reactivity to the soluble monomeric g120 captured on an ELISA plate. Of the three subtype B viruses that were resistant to V3-mediated neutralization, the gp120 of SC422661.8 and RHPA4259.7 was strongly bound by mAb 447-52D (data not shown). This suggests that this neutralization resistance was due to a lack of exposure of the V3 loop on the native virus. In contrast, the gp120 from virus THRO4156.18, which has an unusual GPGG sequence at the tip of the V3 loop, did not react with 447-52D. Hence, both mechanisms may be occurring; the masked V3 loop of some viruses are not readily exposed, even when high concentrations of sCD4 are present and amino acid sequence variation may account for the neutralization resistance of other viruses. Although V1V2 length and positioning of glycosylation have been suggested as major factors in masking the V3 epitope (Krachmarov et al., 2006), we did not find significant differences in the length of V1V2 or number of potential N-linked glycosylation sites between viruses that were sensitive to anti-V3 antibodies and those that were resistant.

Anti-CD4-binding site (CD4bs) mAbs b12 and F105 did not affect V3-mediated neutralization

In the hope of finding an antibody that would bind to the CD4bs of the HIV-1 Env and induce conformational changes similar to those induced by sCD4, we tested the anti-CD4bs mAbs b12 and F105 for their effect on neutralization of V3-directed mAb and GP immune serum. Although the presence of sCD4 increased neutralization of JRFL by 447-52D and immune serum 82-4, the presence of b12 and F105 at concentrations sufficient to inhibit JRFL entry by greater than 50% did not induce V3 mediated neutralization (Fig. 6). Similar results were obtained with virus YU2 (data not shown). These data suggest that mAbs b12 and F105 do not induce a conformation alteration in Env that exposes the V3 loop to NAbs.

Discussion

The V3 region of HIV-1 has been considered a potential vaccine target because of its immuno-dominance in eliciting antibody responses. Anti-V3 antibodies are common during natural infection and are readily elicited by numerous peptide- or Env-based vaccine

immunogens (Broliden et al., 1992; Javaherian et al., 1990; Krachmarov et al., 2005; Krachmarov et al., 2001; Kraft et al., 2007; Letvin et al., 2001; Profy et al., 1990; Vancott et al., 1995; Wu et al., 2006; Yang et al., 2004; Zolla-Pazner, 2004; Zolla-Pazner et al., 2008). Early studies examined vaccine sera against T-cell line adapted HIV-1 strains that were highly sensitive to V3-mediated neutralization. Vaccine sera displayed potent neutralization against prototype viruses such as MN, RF and IIIB, but usually failed to neutralize primary virus stains that were not adapted to growth in cell lines (Javaherian et al., 1990; Mascola et al., 1996; Montefiori et al., 1993; Spenlehauer et al., 1998; Wrin et al., 1995; Wrin and Nunberg, 1994). In addition, V3-elicited antibodies were often found to be highly strain specific, suggesting that the antigenic diversity of the V3 loop posed a major obstacle for NAbs to this variable region of Env (Lu, Putney, and Robinson, 1992; Nara et al., 1990; Palker et al., 1988; Park et al., 1998; Zwart et al., 1992). However, our current understanding of the V3 domain suggests a significant degree of functional conservation, likely due to the interaction of V3 with the CCR5 or CXCR4 co-receptor prior to gp41-mediated viral fusion with the target cell membrane (Cormier and Dragic, 2002; Huang et al., 2005; Stanfield et al., 2004; Stanfield et al., 2006). This functional conservation is consistent with some level of antigenic conservation and the observation that human anti-V3 mAbs can be reactive with the V3 loop of numerous strains of HIV-1 (Gorny et al., 2002; Gorny et al., 2006; Gorny et al., 1993; Krachmarov et al., 2005; Krachmarov et al., 2006; Moore et al., 1995b). These more broadly reactive human anti-V3 mAbs are able to neutralize a subset of primary strains of HIV-1, but are ineffective against many other HIV-1 isolates. This likely results because the V3 loop is partially or completely inaccessible to antibody in the context of the native structure of the trimeric gp120-gp41 complex (Binley et al., 2004; Krachmarov et al., 2005; Lusso et al., 2005; Moore et al., 1995a; Pantophlet et al., 2007; Pantophlet and Burton, 2006; Spenlehauer et al., 1998).

Env binding to CD4 induces a conformational stabilization of Env that results in the formation of the co-receptor binding surface, as well as movement of the V1V2 domain and exposure of the V3 loop. Studies with soluble gp120 and Env expressed on the surface of transfected cells have shown increased binding of co-receptor binding site mAbs such as 17b and 48d, and anti-V3 mAbs after Env binds to sCD4 (Krachmarov et al., 2006; Lusso et al., 2005; Mbah et al., 2001; Salzwedel et al., 2000; Sullivan et al., 1998b; Wyatt and Sodroski, 1998; Xiang et al., 2002). These data suggested that sCD4-induced exposure of the V3 loop could maximize V3-mediated neutralization of free virus and would allow us to test whether the lack of anti-V3 neutralization of most viruses was due to poor recognition of the V3 loop or to limited accessibility of the epitope on native virus. To address this question with representative viruses, we used panels of well-characterized subtype B and C Env-pseudoviruses that were derived during the acute phase of HIV-1 infection. We selected three human anti-V3 mAbs, two derived from subtype B and one from a CFR02-AG infected patient, and GP vaccine sera elicited by subtype B or C Env immunogens that were known to contain moderate to high levels of anti-V3 antibodies. Consistent with previous reports, the anti-V3 mAbs and GP vaccine sera strongly neutralized several prototype V3 sensitive viruses, but displayed more variable neutralization against the subtype B and C reference viruses (Binley et al., 2004; Li et al., 2005; Li et al., 2006; Wu et al., 2006; Yang et al., 2004). However, upon pre-incubation with sCD4, 12 of 15 (80%) subtype B viruses and 10 of 13 (77%) subtype C viruses were sensitive to V3-mediated neutralization. These findings suggest that the V3 loop of most HIV-1 isolates is recognized by anti-V3 mAbs and immune sera, and that the low level of V3-mediated neutralization of many primary virus strains is largely a result of inaccessibility of the V3 loop. The level of accessibility of the V3 loop is in part dependent on the antibody tested. For example, we found mAb 447-52D to have weak neutralization activity against virus JRFL, but mAb B4e8, not tested here, is reported to neutralize JRFL with an IC_{50} of 2.4 ug/ml. (Pantophlet et al., 2007).

Of note, the amount of sCD4 required to trigger exposure of the V3 loop varied among viruses. Generally, concentrations of sCD4 that inhibited viral entry by 50% to 80% were sufficient to trigger exposure of the V3 loop. For some viruses this required as little as 1 ug/ml of sCD4 (e.g., JRFL), but the sCD4 IC_{80} value for other viruses was as high as 200 ug/ml. As has been previously described, the CRF02_AG-derived anti-V3 mAb 3074 displayed greater cross-subtype neutralizing activity than the subtype B-derived mAbs 447-52D and 2219. Even in the presence of sCD4, 447-52D and 2219 displayed little or no cross neutralization against the subtype C viruses while 3074 neutralized seven of 15 subtype B viruses. Therefore, a subtype A V3 sequence or a consensus V3 sequence that is representative of multi-subtypes may be a better immunogen than a subtype B V3 sequence. This is borne out by our experience with subtype B and C Env immunogens. The subtype C Env generated anti-V3 antibodies that cross-neutralized sensitive subtype B viruses, whereas the anti-V3 response elicited by the subtype B Env was restricted to subtype B viruses (Wu et al., 2006). Also, priming of immune responses with DNA from a subtype C Env elicited a broader response than did priming with a subtype B Env (Zolla-Pazner et al., 2008).

Overall, these data have implications for V3-based immunogen design. In contrast to epitopes such as the membrane proximal external region and the CD4bs of gp120, which appear to be poorly immunogenic, anti-V3 antibodies can be elicited by current Env-based vaccines. Such anti-V3 Abs display cross-subtype V3 reactivity, but do not neutralize the majority of primary viruses and Env-pseudoviruses against which they have been tested. If it were possible for an antibody to induce conformation changes similar to those of sCD4, anti-V3 antibodies could play a major role in virus neutralization. To evaluate this directly, we assayed two well-characterized anti-CD4bs mAbs to determine if their binding to native virus resulted in exposure of the V3 loop. MAb b12 is the only CD4bs-directed mAb that can neutralize most primary virus strains; mAb F105 neutralization is restricted to a smaller subset of neutralization sensitive viruses. We chose virus JRFL because its V3 loop was highly exposed to mAb 447-52D neutralization by incubation with relatively low concentrations of sCD4 (Fig. 1). Also, JRFL is neutralized by both b12 and F105, although the former is much more potent than the latter. Concentrations of either mAb sufficient to inhibit JRFL entry by 50% - 80% had no effect on 447-52D neutralization of JRFL. This was perhaps expected for b12, which binds to the outer domain of gp120 without causing significant conformational rearrangements (Zhou et al., 2007). In contrast, thermodynamic and structural data show that effective binding of sCD4 requires conformational changes in gp120 (Kwong et al., 2002; Zhou et al., 2007). F105 also induces moderate conformational rearrangement during binding to gp120, but our data suggest that these do not result in full exposure of the V3 loop to antibody. Whether the characteristic of sCD4 binding that results in exposure of the V3 loop is unique to sCD4 remains to be determined. Of note, numerous studies have suggested additive or synergistic interactions between V3 antibodies and antibodies to other regions of Env (Buchbinder et al., 1992; Cavacini et al., 1993; Laal et al., 1994; Li et al., 1997; McKeating et al., 1992; Montefiori et al., 1993; Moore and Sodroski, 1996; Pinter, Honnen, and Tilley, 1993; Potts et al., 1993; Thali et al., 1992; Tilley et al., 1992; Vijh-Warrier et al., 1996). It therefore remains possible that an antibody-mediated rearrangement of Env conformation could lead to increased sensitivity to V3 loop NAbs.

In summary, selected human anti-V3 mAbs and vaccine immune sera can recognize the antigenic structure of V3 domain of the most circulating subtype B and C virus strains, but the V3 loop is often masked, or partially masked, in the context of the native viral spike. Vaccine elicited anti-V3 antibodies may have enough potency to neutralize a subset of V3 sensitive primary viruses, but this antibody specificity will likely need to be augmented by NAbs against other viral epitopes for an antibody-based vaccine to be effective.

Materials and methods

Antibodies, cells and virus stocks

The anti-V3 mAbs 447-52D, 2219 and 3074 were described previously (Conley et al., 1994; Gorny et al., 1992; Gorny et al., 2002; Gorny et al., 2006; Nyambi et al., 1998). The co-receptor binding site antibody 17b was obtained from James Robinson (Tulane University). The CD4bs mAbs b12 was provided by Dennis Burton and Ralph Pantophlet (Scripps Research Institute); F105 was from the laboratory of Marshall Posner (Dana-Farber Cancer Institute). Two domain sCD4 (sCD4-183) was obtained through the NIH AIDS Research and Reference Reagent Program and from Pharmacia. Human embryonic kidney cell line 293T was purchased from the American Type Culture Collection and maintained in DMEM (Gibco, Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum and 100 ug/ml of penicillin/streptomycin. The TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program, as contributed by John Kappes and Xiaoyun Wu. This cell line is a genetically engineered HeLa cell clone that expresses CD4, CXCR4, and CCR5, and contains HIV Tat-responsive reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under regulatory control of an HIV-1 long terminal repeat.

Pseudovirus was prepared by transfecting 293T cells (6×10^6 cells in 50 ml growth medium in a T-175 culture flask) with 10 μg of *rev*/*env* expression plasmid and 30 μg of an *env*-deficient HIV-1 backbone vector (pSG3ΔEnvelope), using Fugene 6 transfection reagents (Invitrogen). Pseudovirus-containing culture supernatants were harvested two days after transfection, filtered (0.45 μm), and stored at −80°C or in the vapor phase of liquid nitrogen. The virus 50% tissue culture infectious dose ($TCID_{50}$) was determined on TZM-bl cells by serial 5-fold dilutions of the pseudovirus stock in quadruplicate wells. At approximately 48 hours, the infection levels were determined by a luciferase assay (Bright Glo, Promega, Madison, WI) using the cell lysate. Infections producing the RLU three times above the mock infection background were scored as positive.

Constructions of plasmid DNA, recombinant adenoviruses and vaccination

The construction of plasmid DNA and rAd5 were described previously (Wu et al., 2006). Selective modifications of the HIV-1 Env included ΔCFI, indicating deletions in the cleavage site, fusogenic domain, and spacing of heptad repeat 1 and 2, and ΔV1V2(V3-1AB), indicating deletions of the V1V2 loop and two small deletions on both arms of the V3 stem (Chakrabarti et al., 2002; Chakrabarti et al., 2005; Wu et al., 2006; Yang et al., 2004). HIV-1 *env* genes encoding BaL gp145ΔCFI, and Hx/BaL gp145ΔCFI were synthesized using human-preferred codons as previously described. In gp145ΔCFIΔV1V2 (ZA012V3-1AB), amino acids 202-388 of BaL V3 were replaced by amino acids 195–382 of subtype C ZA012 V3 sequence.

GP immunizations were performed as previously descried (Wu et al., 2006). Briefly, animals were immunized intramuscularly with 500 ug (in 400 ul PBS) of the gp145 version of plasmid DNA at weeks 0, 2 and 6. At week 14, GPs were boosted with 10^{11} particles (in 400 ul PBS) of rAd5 expressing the corresponding gp140 version of the HIV-1 Env protein. Serum tested in this study was collected two weeks after the rAd5 boost immunization. GP group 82 received a subtype B V3 in a subtype B Env backbone (BaL gp145 ΔCFI ΔV1V2 (V3-1AB)); groups 83 and 86 received a subtype C V3 (ZA012V3-1AB) in a subtype B Env backbone. This backbone was BaL gp145 ΔCFI ΔV1V2 for group 83 and Hx/BaL gp145 ΔCFI ΔV1V2 for group 86.

Neutralization and V3 peptide competition assays

Neutralization was measured using HIV-1 Env-pseudoviruses to infect TZM-bl cells as described previously (Li et al., 2005; Shu et al., 2007). Briefly, 40 ul of virus was incubated for 30 minutes at 37°C with 10 ul of serial dilutions of test antibody or serum samples in duplicate wells of a 96-well flat bottom culture plate. For neutralization assays with sCD4, 5 ul of sCD4 was added to the virus for 30 minutes followed by addition of 5 ul of test mAb or serum sample. To keep assay conditions constant, sham media was used in place of sCD4 or antibody in specified control wells. The mAb and sCD4 concentrations, and serum dilutions, were defined at the point of incubation with virus supernatant. 10,000 TZM-bl cells were then added to each well in a volume of 20 ul, and plates were incubated overnight at 37°C in a 5% CO_2 incubator. One set of eight wells received mock antibody followed by virus and cells as controls for virus entry, and a set of eight wells received cells with mock virus to control for luciferase background. After overnight incubation, 150 ul of fresh medium was added to each well. Infection levels were determined the next day using a quantitative luciferase assay measuring luciferase activity present in cell lysate (Promega). The virus input was set at a multiplicity of infection (Ortiz et al.) of approximately 0.1, which generally results in 100,000 to 400,000 RLU in the luciferase assay. Neutralization curves were fit by non-linear regression using a 4-parameter hill slope equation programmed into JMP statistical software (JMP 5.1, SAS Institute Inc., Cary, NC). The 50% or 80% inhibitory concentrations (IC_{50} and IC_{80}) are reported as the reciprocal serum dilutions or mAb concentrations required to inhibit infection by 50% or 80%.

The V3 peptide competition assays were done in the same assay format as the neutralization assay, except that the control or test peptide was added to antibody or serum 30 min prior to the addition of virus. The concentration of peptide used was 30 ug/ml, and represents the concentration that was present when peptide, serum or antibody and virus were incubated together. The V3 peptide sequences used in this study were based either on BaL.26 (TRPNNNTRKSIHIGPGRAFYTTG) or ZA012 (TRPNNNTRKSMRIGPGQTFYATG), each matching the representative vaccine strain. A scrambled V3 peptide (IGPGRATRPNNNFYTTGTRKSIH) was used as a negative control. These peptides were synthesized by SynPep (Dublin, CA).

Statistical analysis

Comparisons of neutralization breadth in the absence and presence of sCD4 were performed by McNemar's test using the statistical package within R software. Comparisons of neutralization values in the absence and presence of sCD4 were performed by paired Wilcoxon signed-rank test using the statistical package within GraphPad Prism (V5.0) software. P-values ≤ 0.05 are reported as significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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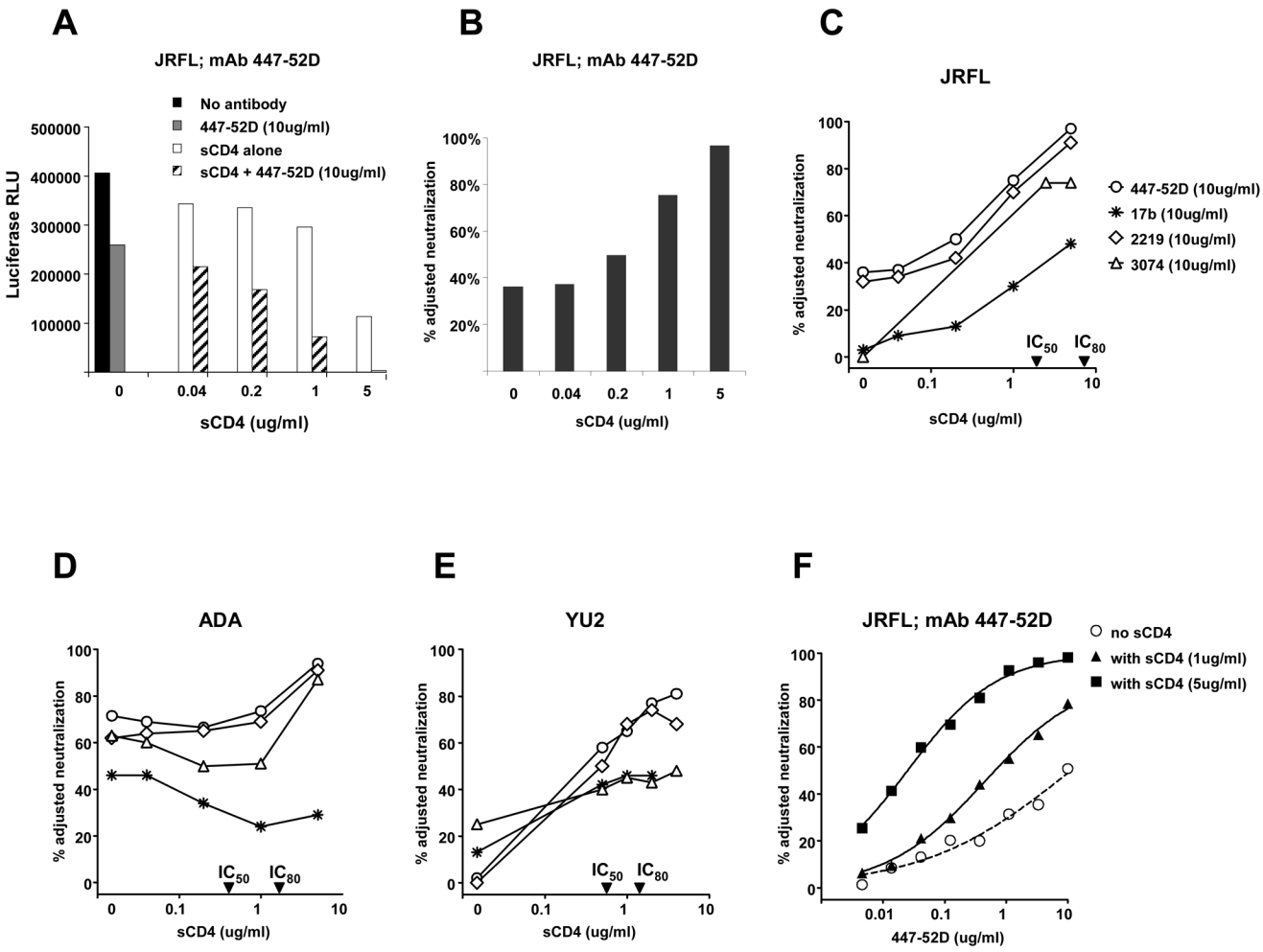

Fig. 1.

Effect of sCD4 on neutralization of JRFL, ADA and YU2 by anti-V3 mAbs 447-52D, 2219, 3074 and anti-co-receptor mAb 17b. (A) Viral entry expressed as luciferase (RLU) of JRFL is shown with varying concentrations of sCD4. Black bar shows viral entry with no mAb and no sCD4 present. Grey bar shows viral entry with 447-52D. White bars show the effect of sCD4 alone on viral entry. Hatched bars show the effect of each concentration of sCD4 with a fixed amount (10 ug/ml) of mAb 447-52D. (B) The percent adjusted neutralization was calculated from (A) using RLU values for each concentration of sCD4 as the baseline for viral entry. The additional effect on viral entry by 10 ug/ml of mAb 447-52D is the percent adjusted neutralization. (C), (D) and (E) show the percent adjusted neutralization by several mAbs against viruses JRFL, ADA and YU2. Legend in (C) applies to (D) and (E). (F) Serial dilutions of mAb 447-52D alone, or with a fixed concentration of sCD4 were tested to calculate the impact of sCD4 on the potency of mAb 447-52D neutralization. Note that for graphs C, D and E, the IC_{50} and IC_{80} effect of sCD4 against each virus are indicated on the X-axis.

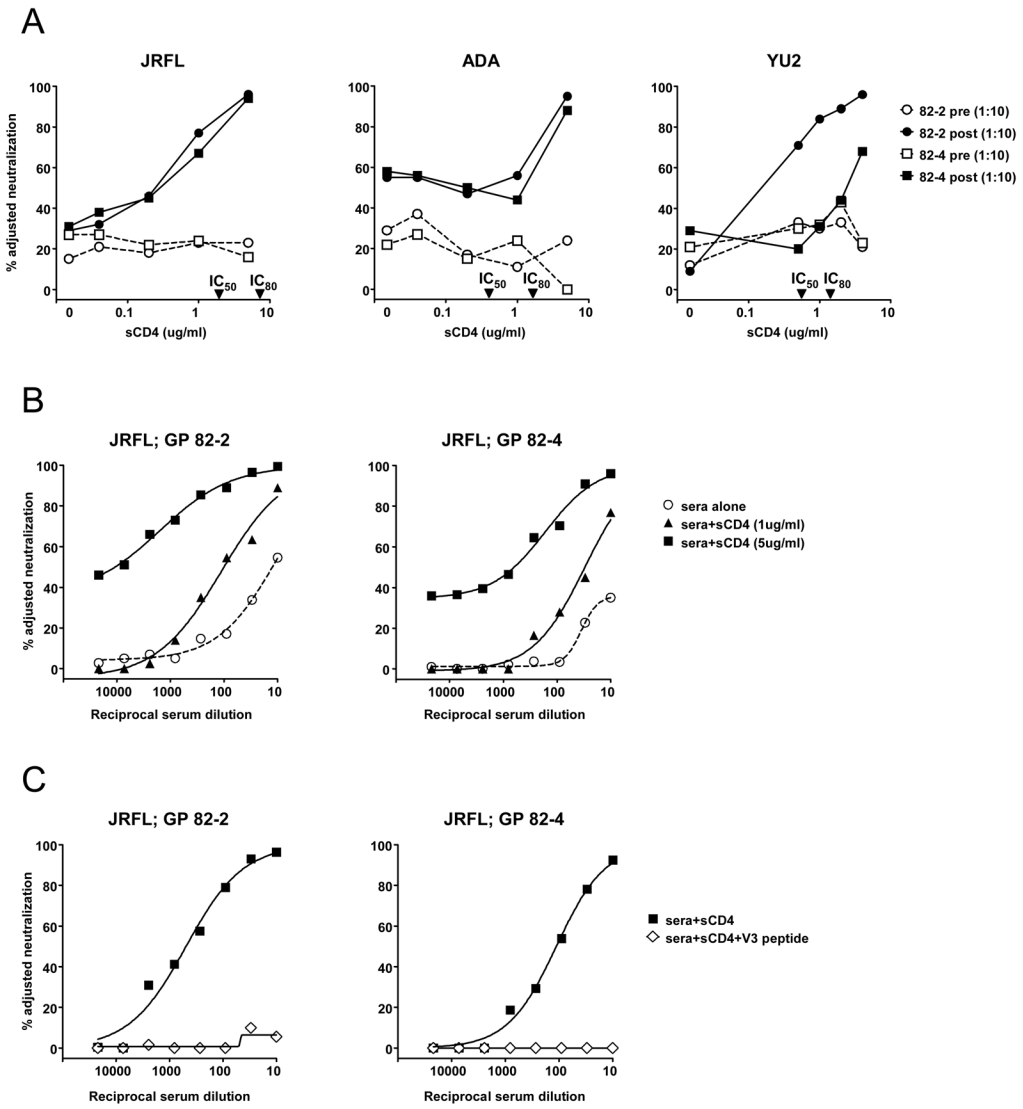

Fig. 2.

Neutralization by GP vaccine sera. (A) The effect of sCD4 on neutralization by GP sera 82-2 and 82-4. Pre-immune and vaccine sera are shown by open and filled symbols respectively. The IC_{50} and IC_{80} of sCD4 against each virus are indicated on the X-axis. (B) Serial dilutions of GP vaccine sera alone, or with a fixed concentration of sCD4 were tested to calculate the impact of sCD4 on the potency of serum neutralization. (C) With 5 ug/ml of sCD4 present, GP vaccine sera neutralize JRFL predominantly via anti-V3 antibodies. A V3 peptide (30 ug/ml) matched to the vaccine immunogen was used to inhibit the V3 mediated neutralization by GP vaccine sera. A scrambled V3 peptide had no inhibitory effect (not shown).

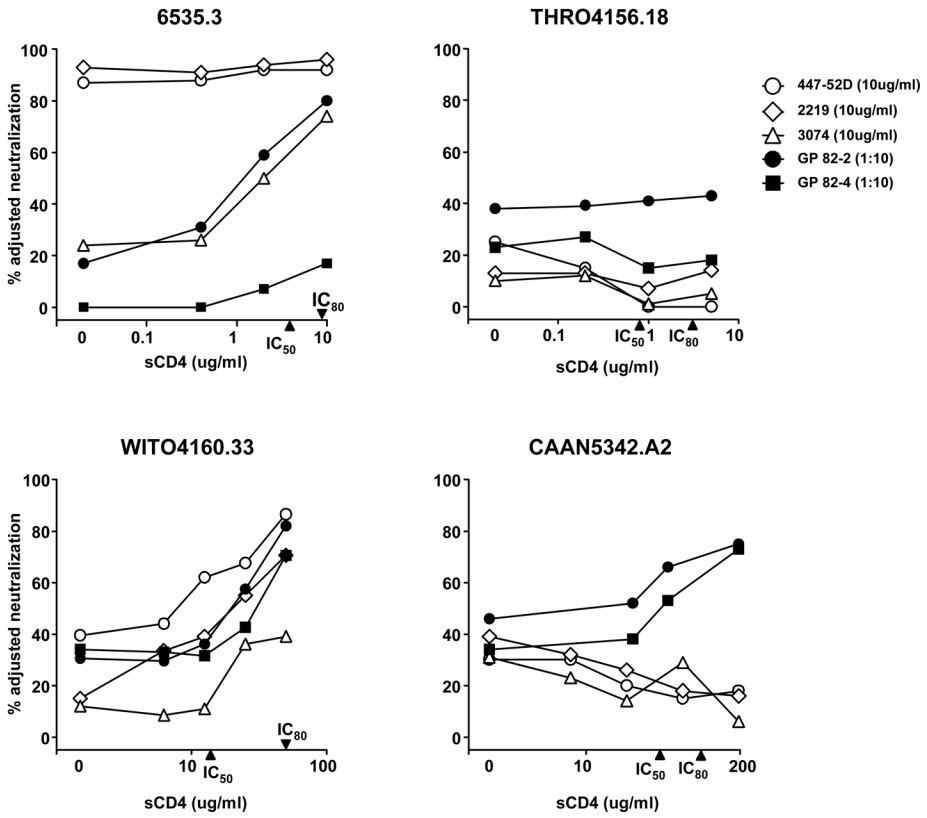

Fig. 3.

The effect of sCD4 on V3-mediated neutralization of four selected subtype B reference viruses. Data for anti-V3 mAbs and subtype B GP vaccine sera are shown against each virus. The IC_{50} and IC_{80} of sCD4 against each virus are indicated on the X-axes. Note that the scale of the X-axes differs among viruses.

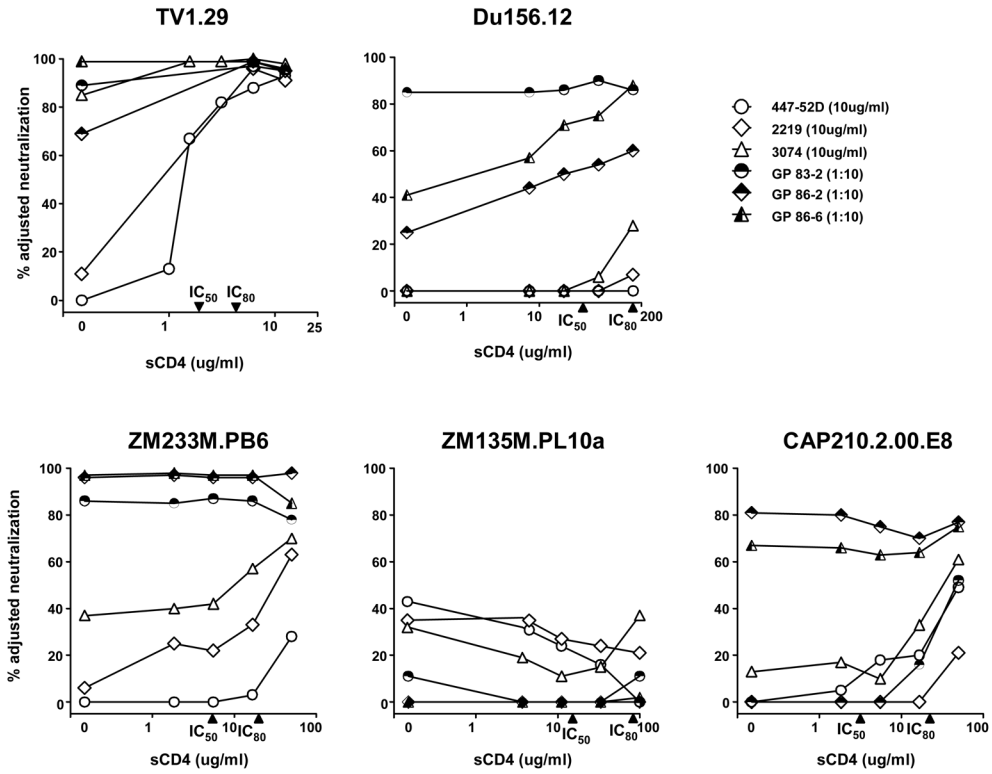

Fig. 4.

The effect of sCD4 on V3-mediated neutralization of TV1.29 and four selected subtype C reference viruses. Data for anti-V3 mAbs and subtype C GP vaccine sera are shown against each virus. The IC_{50} and IC_{80} of sCD4 against each virus are indicated on the X-axes. Note that the scale of the X-axes differs among viruses.

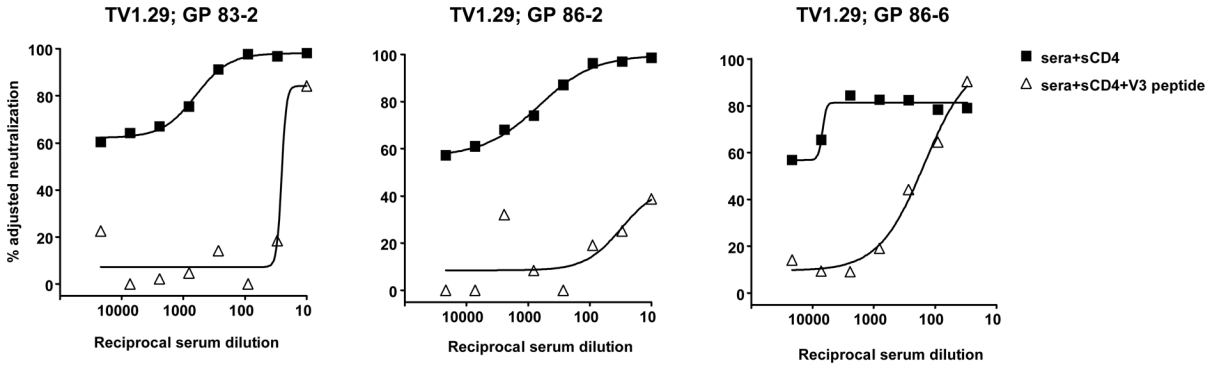

Fig. 5.

In the presence of 5 ug/ml of sCD4, GP vaccine sera neutralized subtype C virus TV1.29 predominantly via anti-V3 antibodies. A V3 peptide (30 ug/ml) matched to the subtype C vaccine immunogen was used to inhibit the V3-mediated neutralization by the sera. A scrambled V3 peptide had no inhibitory effect (not shown).

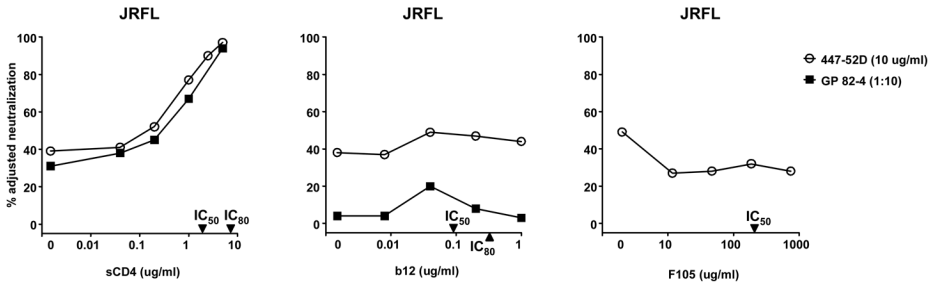

Fig. 6.

Lack of effect of anti-CD4bs mAbs b12 and F105 on neutralization of JRFL by mAb 447-52D and GP vaccine serum 82-4. Unlike sCD4, which increases neutralization sensitivity of JRFL to anti-V3 antibodies, b12 and F105 did not affect neutralization sensitivity of JRFL to anti-V3 antibodies. The IC_{50} and IC_{80} of sCD4, b12 and F105 against JRFL are indicated on the X-axes.

Table 1

Neutralization by the indicated anti-V3 mAbs and GP vaccine sera against subtype B viruses with and without sCD4 present

	sCD4 IC_{80} ug/ml	447-52D 10 ug/ml		2219 10 ug/ml		3074 10 ug/ml		82-2 1:10		82-4 1:10	
		−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4
JRFL	7.2	36%	**99%**	32%	**98%**	0%	**94%**	29%	**100%**	31%	**97%**
ADA	1.7	**72%**	**82%**	**62%**	**78%**	**63%**	**69%**	**55%**	**73%**	**58%**	**67%**
YU2	1.4	2%	**73%**	0%	**64%**	25%	41%	9%	**97%**	29%	40%
6535.3	8.6	**87%**	**93%**	**93%**	**93%**	24%	**74%**	17%	**85%**	4%	16%
QH0692.42	6.4	27%	**51**%	28%	45%	**68%**	**73%**	46%	**66%**	12%	47%
SC422661.8	125.3	0%	16%	18%	25%	18%	18%	41%	0%	37%	22%
PVO.4	46.0	4%	43%	35%	**63%**	39%	**57%**	**56%**	**90%**	44%	**71%**
TRO.11	>200	50%	48%	31%	37%	29%	13%	**70%**	**75%**	**66%**	**67%**
AC10.0.29	80.9	8%	39%	47%	**57%**	13%	12%	**59%**	**80%**	13%	**55%**
RHPA4259.7	21.6	36%	34**%**	29%	37%	34%	45%	15%	12%	9%	11%
THRO4156.18	3.1	25%	0%	14%	12%	10%	8%	38%	39%	23%	21%
REJO4541.67	22.5	31%	21%	47%	**74%**	29%	**55%**	34%	**78%**	6%	41%
TRJO4551.58	17.7	0%	**86%**	0%	**83%**	5%	**65**%	0%	**87%**	14%	**73%**
WITO4160.33	50.0	40%	**76%**	15%	**67%**	12%	41%	31%	**68%**	34%	**55%**
CAAN5342.A2	95.2	30%	26%	39%	26%	32%	17%	46%	**67%**	34%	**59%**
Breadth		2(13%)	7(47%)	2(13%)	9(60%)	2(13%)	7(47%)	4(27%)	12(80%)	2(13%)	8(53%)
P-value		0.0625		0.016		0.0625		0.008		0.031	

Values indicate percent neutralization when no sCD4 was present in the assay (−sCD4) and with sCD4 present (+sCD4) at a concentration sufficient to inhibit viral entry by 80%. The later value is the percent adjusted neutralization and indicates the effect of the mAb or GP sera calculated based on baseline viral entry with sCD4 present. The second column shows the concentration of sCD4 required to inhibit viral entry by 80%. Note that the IC_{80} for sCD4 was derived separately for each virus and the percent adjusted neutralization mediated by mAb or GP sera was calculated using viral entry values at this level of sCD4 (e.g., Fig. 3). Values greater than 50% neutralization are shown in bold and were used to calculate the breadth of neutralization. P-values were computed using the two-sided McNemar's test.

Table 2

Neutralization by the indicated anti-V3 mAbs and GP vaccine sera against subtype C viruses with and without sCD4 present

	sCD4 IC_{80} ug/ml	447-52D 10 ug/ml		2219 10 ug/ml		3074 10 ug/ml		83-2 1:10		86-2 1:10		86-6 1:10	
		−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4	−sCD4	+sCD4
TV1.29	4.3	0%	**100%**	11%	**87%**	**85%**	**96%**	**89%**	**95%**	**69%**	**95%**	**99%**	**99%**
Du156.12	200.0	0%	4%	0%	8%	0%	28%	**85%**	**88%**	25%	**62%**	41%	**85%**
Du172.17	10.2	24%	31%	20%	24%	25%	**66%**	0%	6%	**69%**	**85%**	**66%**	**79%**
Du422.1	100.0	16%	10%	17%	28%	1%	40%	1%	16%	8%	4%	0%	**58%**
ZM197M.PB7	58.1	1%	0%	2%	11%	0%	8%	0%	0%	0%	16%	0%	**59%**
ZM214M.PL15	160.0	24%	25%	9%	3%	0%	16%	0%	31%	0%	34%	0%	**59%**
ZM233M.PB6	19.9	0%	6%	7%	43%	37%	**58%**	**86%**	**87%**	**96%**	**97%**	**97%**	**97%**
ZM249M.PL1	43.9	4%	15%	10%	28%	0%	6%	0%	0%	0%	0%	0%	1%
ZM53M.PB12	45.3	23%	10%	10%	9%	12%	**54%**	0%	0%	5%	26%	0%	**89%**
ZM109F.PB4	2.5	17%	31%	45%	46%	**75%**	**70%**	**56%**	47%	**84%**	**81%**	**87%**	**67%**
ZM135M.PL10a	83.5	44%	10%	36%	22%	33%	31%	1%	0%	11%	0%	0%	0%
CAP45.2.00.G3	93.3	2%	12%	6%	25%	4%	28%	0%	0%	0%	0%	7%	43%
CAP210.2.00.E8	22.7	0%	31%	0%	0%	13%	41%	0%	22%	**82%**	**76%**	**67%**	**69%**
Breadth		0(0%)	1(8%)	0(0%)	1(8%)	2(15%)	5(38%)	4(31%)	3(23%)	5(38%)	6(46%)	5(38%)	10(77%)
P-value		1		1		0.25		1		1		0.0625	

Values indicate percent neutralization when no sCD4 was present in the assay (−sCD4) and with sCD4 present (+sCD4) at a concentration sufficient to inhibit viral entry by 80%. The later value is the percent adjusted neutralization and indicates the effect of the mAb or GP sera calculated based on baseline viral entry with sCD4 present. The second column shows the concentration of sCD4 required to inhibit viral entry by 80%. Note that the IC_{80} for sCD4 was derived separately for each virus and the percent adjusted neutralization mediated by mAb or GP sera was calculated using viral entry values at this level of sCD4 (e.g., Fig. 4). Values greater than 50% neutralization are shown in bold and were used to calculate the breadth of neutralization. P-values were computed using the two-sided McNemar's test.