A comparative immunogenicity study of HIV-1 virus-like particles bearing various forms of envelope proteins, particles bearing no envelope and soluble monomeric gp120

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Abstract

To assess the potential of native Envelope glycoprotein (Env) trimers as neutralizing antibody vaccines, we immunized guinea pigs with three types of VLPs and soluble gp120. Particles included “SOS-VLPs” (bearing disulfide-shackled functional trimers), “UNC-VLPs” (bearing uncleaved nonfunctional Env) and “naked VLPs” (bearing no Env). The SOS-VLPs were found to have a density of about 27 native trimers per particle, approximately twice that of live inactivated HIV-1 preparations. As immunogens, UNC- and SOS-VLP rapidly elicited anti-gp120 antibodies focused on the V3 loop and the gp120 coreceptor binding site. Reactivity to the gp120–gp41 association is stabilized, effectively covering this epitope. Gp120-immune sera reacted with the receptor binding sites of gp120 and were less focused on the V3 loop. Some Env-VLP sera neutralized primary isolates at modest titers. The measurement of neutralization was found to be affected by the cell lines used. Depending on the assay particulars, non-Env specific antibodies in VLP sera could enhance infection, or nonspecifically neutralize. However, a neutralization assay using TZM-BL cells was essentially clear of these effects. We also describe a native trimer binding assay to confirm neutralization activity in a manner that completely eliminates nonspecific effects. Overall, our data suggests that Env-VLP sera were primarily focused on nonfunctional forms of Env on VLP surfaces, possibly gp120/ gp41 monomers and not the trimers. Therefore, to make progress toward a more effective VLP-based vaccine, we will need to find ways to refocus the attention of B cells on native trimers.

Keywords: HIV; Vaccine; Antibody; Neutralizing; Neutralization; VLPs; gp120; gp41; Env

Introduction

A vaccine is desperately needed to prevent the spread of human immunodeficiency virus type 1 (HIV-1) (Garber et al., 2004; Koff et al., 2007; Tramont and Johnston, 2003). It is likely that a potent and broadly reactive neutralizing antibody (nAb) response will be a crucial component of effective vaccine immunity to HIV-1. However, despite the considerable
ingenuity of vaccine researchers, there has been scant progress toward an immunogen able to elicit such a response.

NAbs are thought to target envelope glycoprotein (Env) spikes on HIV-1 surfaces, thereby inhibiting entry into susceptible cells. Functional Env spikes consist of noncovalently associated trimers of gp120/gp41 heterodimers (Earl et al., 1990). The gp120 moiety mediates binding to target cell receptors, triggering gp41-mediated fusion and entry. As the presumptive targets of neutralization, logic has fueled the hypothesis that authentic Env trimers might be able to elicit NAb in a vaccine setting (Emini and Koff, 2004; Koff et al., 2007; Tramont and Johnston, 2003). However, a major stumbling block for vaccinologists has been that authentic trimers are technically challenging to produce in a purified form. A number of groups have expressed soluble recombinant trimers that resemble the native structure to varying degrees (Beddows et al., 2005, 2007; Binley et al., 2000; Chakrabarti et al., 2002; Earl et al., 2001; Farzan et al., 1998; Li et al., 2006; Sanders et al., 2002b; Srivastava et al., 2002; VanCott et al., 1997; Yang et al., 2000). Challenges include optimizing the inefficient gp120/gp41 cleavage process (Yamshchikov et al., 1995) and stabilizing the weak inter-subunit associations within trimers (Staropoli et al., 2000).

Gp120/gp41 processing can be enhanced by optimizing the substrate sequence and by co-expressing furin (Binley et al., 1999–2002; McKenna et al., 2003; Moore et al., 2006), gp120/gp41 monomers (Moore et al., 2006), gp120 shedding (Chertova et al., 2002; McKeating et al., 1991; Wagner et al., 1998), and uncleaved gp160 resulting from inefficient processing (Arthur et al., 2001; Hammonds et al., 2005). As a result, it has often been difficult to unequivocally interpret neutralization data in particle structures (Aberle et al., 1999; Beddows et al., 2005; Edinger et al., 2000).

Particulate vaccine candidates for HIV-1 have been described by many groups (Buonaguro et al., 2005; Evans et al., 2001; Grovit-Feras et al., 2000; Haﬁlar et al., 1991; Hammonds et al., 2003; Katz and Moss, 1997; Lifson et al., 2004; McBurney et al., 2007; McKenna et al., 2003; Montefiori et al., 2001; Vzorov et al., 1999; Wagner et al., 1996; Yao et al., 2000), in some cases progressing to clinical trials (Persson et al., 1998; Tramont and Johnston, 2003). Overall, however, the approach has been under-researched, perhaps in part due to a perceived lack of improvement in nAb induction, compared to other vaccine approaches (Daniel et al., 1994; Polacino et al., 1999; Race et al., 1995; Richmond et al., 1998; Verrier et al., 2000). Underpinning this lack of success are several challenges that may need to be overcome before VLPs can realize their full potential as vaccines.

A key technical issue with VLP or other membranous vaccines is their tendency to elicit “anti-cell” antibodies against non-Env membrane proteins. These antibodies can have unpredictable side effects in neutralization assays that vary from neutralization (Arthur et al., 1992; Chan et al., 1992) to enhancement (Giannecehini et al., 2001; Verrier et al., 2000). One possible explanation for these effects is that anti-cell Abs affect the viability of target cells. Making the situation even more complex, it has been suggested that enhancing Abs may even mask the effect of other, neutralizing Abs (Giannecehini et al., 2001; Hammonds et al., 2005). As a result, it has often been difficult to unequivocally interpret neutralization data in particle and cell-based vaccine studies (Buonaguro et al., 2005; Giannecehini et al., 2001; Hammonds et al., 2005; LaCasse et al., 1999; Langlois et al., 1992; Poon et al., 2005a, 2005b).

A second challenge for VLP vaccines is that native trimers may not be the only form of Env present on their surfaces. Possible non-trimeric forms of Env include gp41 stumps arising from gp120 shedding (Chertova et al., 2002; McKeating et al., 1991; Moore et al., 2006), gp120/gp41 monomers (Moore et al., 2006), and uncleaved gp160 resulting from inefficient processing. These forms of Env do not resemble functional trimers and may elicit non-neutralizing antibody responses.

A third potential challenge of particulate vaccines is that many commonly used adjuvants contain detergent surfactants, exemplified by Ribi adjuvant, containing Tween 80. Surfactants stabilize oil-in-water emulsions to enhance the delivery of proteins to the membranes of immune cells over a large surface area. However, they may intercalate with or destabilize the lipid membranes of VLPs, causing Env to be released (Moore et al., 2006), and as a consequence, trimers may partially or completely fall apart.

Despite these challenges, VLPs remain among the few vaccine candidates able to present truly authentic trimers, and therefore deserve further investigation. In this pilot study, we evaluated the immunogenicity of VLPs that had been adjusted to try to overcome some of the abovementioned pitfalls. Env proteins were truncated to improve expression and to help ensure proper gp120/gp41 cleavage. A gp120–gp41 disulfide bond was introduced to prevent gp120 shedding. Furthermore,
we employed various neutralization assay formats to analyze VLP immune sera, to help identify neutralization amid any background nonspecific activity.

Results

Production of VLP immunogens

In this pilot study, we opted for moderate Env expression by plasmid pCAGGS, together with pNL4-3.Luc.R-E- to express VLPs bearing functional Env trimers (Binley et al., 2003). To stabilize gp120–gp41 association and eliminate the potential problem of gp120 shedding that might occur with “WT-VLPs”, we generated “SOS-VLPs” that introduce a gp120–gp41 disulfide bond (Abrahamyan et al., 2003; Binley et al., 2000, 2003), and UNC-VLPs that abolish gp120–gp41 processing (Fig. 1). Collectively, we refer to these as “Env-VLPs, distinct from “naked” VLPs, lacking Env.

We compared full-length and truncated gp160 forms of Env on VLPs. Gp160ΔCTWT was completely dependent on the expression of structural proteins by pNL4-3.Luc.R-E- for secretion into supernatant (Fig. 2A, lanes 1–3), but full-length gp160 was secreted into the supernatant, even when pNL4-3.Luc.R-E- was not co-expressed (not shown), perhaps indicating spontaneous vesicle formation or an increase in cell lysis. In addition, gp160ΔCT exhibited higher expression and gp120/gp41 cleavage than the full-length form, as observed previously (Binley et al., 2003; Yamshchikov et al., 1995; Yao et al., 2000). Gp160ΔCTSOS was also fully cleaved and reducible by DTT (Fig. 2A, lanes 4 and 5). Since gp160ΔCT is also fusogenic (Abrahamyan et al., 2005; Binley et al., 2003; Crooks et al., 2005; Edwards et al., 2002), we reasoned that this is an authentic form of Env suitable for testing as an immunogen. In contrast to WT- and SOS-VLPs, the Env in UNC-VLPs was uncleaved, as expected (data not shown and Moore et al., 2006).

Electron microscopic analysis of 293T cells co-transfected with pCAGGS gp160ΔCTWT and pNL4-3.Luc.R-E- revealed efficient particle budding (Fig. 2B). SDS–PAGE and Western blotting of VLP preparations provided further evidence that gp160ΔCT was associated with particles (Fig. 2C). The Gag to Env ratio appeared to be similar in WT-, SOS- and UNC-VLPs and live activated HIV preparations ADA and MN in Western blots (data not shown). Concentrated VLP preparations (1000×) typically contain 5 μg gp120 per ml (determined by comparison to a known JR-FL gp120 reference in Western blot) and 500 μg total protein per ml, measured spectrophotometrically. Again, the 1000× concentrated live inactivated virus preparations were very similar. High-resolution negative stain electron microscopy revealed trimers on the surfaces of WT-VLPs (Fig. 2D), similar to those observed on inactivated SIV (Zanetti et al., 2006; Zhu et al., 2003, 2006). The average spike density on several VLP electron micrographs was 27 (range 15–37), approximately twice the number previously counted on live inactivated HIV (range 7–14) (Chertova et al., 2002; Zhu et al., 2003, 2006).

Analysis of Env oligomers

Analysis of Env liberated from WT- and SOS-VLPs in BN-PAGE revealed a major band of approximately 400 kDa,
corresponding to authentic trimers (Zanetti et al., 2006; Zhu et al., 2006), as well as a gp120/gp41 monomer band (Fig. 3A; Moore et al., 2006). This contrasts with soluble gp140_{SOS} that readily dissociates into monomers (Schulke et al., 2002), suggesting that expression in membranes lends some stability to Env trimers, as we initially hypothesized. The dominance of trimer band in both WT and SOS-VLPs suggests that gp120 shedding from WT-VLPs is not a major problem (Chertova et al., 2002; Zhu et al., 2003, 2006). However, probing similar native PAGE blots with gp41 antibodies reveals gp41 stumps in WT that are not seen with SOS (Moore et al., 2006), indicating that some gp120 shedding does occur (data not shown). The consistent induction of gp41 immunodominant loop antibodies in natural infection suggests that gp41 stumps are a common antigen that might be best eliminated from immunogens, perhaps by the SOS mutation.

In contrast to WT- and SOS-VLPs, UNC-VLP-derived Env separated as several species that appear to be monomers, dimers, trimers and tetramers (Fig. 3A) (Earl et al., 1990; Moore et al., 2006; Staropoli et al., 2000; Zhang et al., 2001). Denaturing but not reducing conditions caused WT and SOS trimers to dissociate into monomers (Fig. 3B), but UNC oligomers remained largely unchanged (Fig. 3B), as they may be cross-linked by inter-trimer disulfide bonds (Billington et al., 2007). Denaturing and reducing conditions eliminated oligomers in all preparations (Fig. 3C). SDS–PAGE gels run in parallel indicated that little, if any uncleaved Env existed in WT-VLPs (Fig. 3D) and confirmed that reducing conditions disrupted the gp120–gp41 SOS bond (compare Figs. 3D and E). Here, the gp120 from WT- and SOS-VLP appeared as a doublet. We previously proposed that the two forms of gp120 arise from trimers and putative monomers of gp120/gp41 present on virion surfaces (Moore et al., 2006).

**Immunogenicity of VLPs in guinea pigs**

In this pilot study, we wished to devise an immunization algorithm suitable for further development. We evaluated multiple concepts relating to immunogens and the end stage serum analysis. We immunized a total of 17 guinea pigs with recombinant JR-FL gp120, UNC-VLPs, SOS-VLPs and naked VLPs. Animals were immunized 3 times at 6 week intervals with bleeds collected 1 or 2 weeks thereafter (Fig. 4A). Although VLPs are known to induce potent Ab responses, even without adjuvants (Lorin et al., 2004; Wagner et al., 1998), to ensure maximal Ab titers, we included adjuvants in our regimes (Hammonds et al., 2005). One consideration was that emulsion-based adjuvants could disrupt membranes. We therefore selected immunostimulatory DNA CpG as our main adjuvant, which is unlikely to affect VLP membranes. However, the CpG formulations used were optimized for rabbits and mice, and it was not known if they would be effective in guinea pigs, for which a commercially available CpG product was not available. Therefore, in a few animals (P1, P13 and P14), CpG was supplemented with Ribi Ras3c or QS-21, as indicated (Fig. 4).

**Binding titers to gp120 and a gp41 peptide**

Serum gp120 titers were measured over the course of immunizations. Of the 3 gp120-immunized control animals, P1 had the highest titer (Fig. 4A), perhaps owing to the QS21 adjuvant. In the Env-VLP-immunized animals, we observed high anti-Env titers, comparable to the A62 human plasma and gp120-immune sera (Fig. 4A). The kinetics of the responses to Env-VLPs was rapid: high titers were generated even after a single inoculation (compare animals S2 and S11 in Fig. 4B). The animals that received VLPs formulated in Ribi or QS21 as well as CpG generated titers similar to others, suggesting that CpG was sufficient for maximum titers. As expected, naked VLPs did not elicit significant gp120 titers (Figs. 4A, B).

To evaluate the conformation dependency of antibody binding, we measured serum titers against denatured and reduced gp120 (dgp120). In most Env-VLP sera, dgp120 titers were <10% of those against intact gp120 (exceptions were S8 and S11), but in gp120-immunized animals, they were even lower. In contrast, an HIV-1 seropositive donor plasma exhibited similar titers to dgp120 and intact gp120. Taken together, this suggests that Env conformation was well preserved during adjuvant formulation and delivery and that Ribi or QS-21 formulation had similar effects on antigens compared to CpG alone.

To determine the effect of the SOS mutation on gp41 exposure, we measured antibody responses against a peptide derived from the immunodominant epitope of gp41 (the “AVERY” peptide). Most Env-VLP sera had very low titers. Exceptions were animals S7 and S8 that had been immunized with UNC-VLPs. As anticipated, the naked VLP and gp120 sera did not react with the peptide. In contrast, the HIV-1 seropositive plasma exhibited a high titer, in line with the use of similar peptides for HIV-1 diagnosis (Fig. 4A).

**Mapping the specificity of immune sera**

To map serum specificity, we used virus capture competitions and peptide ELISAs.

![Fig. 3. Native PAGE of VLP-derived Env proteins. WT-VLPs, UNC-VLPs and SOS-VLPs were compared in Bis–Tris gels in (A) native (blue-native), (B) denaturing but nonreducing and (C) reducing conditions. Ferritin was used as a molecular weight marker with bands at 439 and 220 kDa, as indicated. The same samples were analyzed by SDS–PAGE in (D) nonreducing and (E) reducing conditions.](image-url)
Inhibition of virus capture by mAbs

To determine the effect of sera on mAb-mediated particle capture (Derby et al., 2006), graded dilutions of sera were mixed with WT-VLPs bearing VSV-G, which were then added to mAb-coated microplates. This assay has the advantage of employing particulate (and therefore native) Env rather than gp120 or other soluble forms of Env. Using two different V3 mAb prototypes, LE311 and 39F, we found that SOS-VLP and UNC-VLP sera contained high titers of anti-V3 Abs (Figs. 5A, B), as did the HIV+ plasma control. Comparison of these titers to the IC50s of mAb self-competition allowed an estimate of the quantity of V3 blocking antibody (Crooks et al., 2005), which ranges from about 5 to 100 μg/ml in these sera. In comparison, the gp120 sera showed only moderate titers of V3 competing Abs. As expected, the naked VLP sera did not exhibit detectable competition.

Focusing next on the CD4 binding site-overlapping mAbs 15e and b12, we found that gp120 serum P1 effectively inhibited virus capture with a potency corresponding to approximately 10–60 μg/ml CD4 binding site-overlapping IgG equivalents. The gp120 serum S1, VLP sera S6, S11 and the A62 plasma moderately inhibited capture (Figs. 5A, B), while most others were weak or completely ineffective. Inhibition of the two CD4 binding site mAbs by each serum was largely consistent.

We next examined inhibition by antibodies that bind to the “CD4-induced” epitope that overlaps the coreceptor binding site. The A62 HIV+ plasma and most of the Env-VLP sera effectively blocked X5 capture. Moderate blocking was observed for sera S6, and P14, and weak blocking was observed with S1, S2 and the naked VLP sera. The very high titer of the HIV+ plasma reflects recent findings that these antibodies are common in natural infection (Decker et al., 2005).

2G12-blocking activity was very low in all guinea pig sera and the HIV+ plasma control, consistent with the rare and unusual nature of this specificity. MAb 7B2 was next used to measure activity to the immunodominant loop of gp41. The control HIV-1 plasma A62 strongly inhibited 7B2, consistent with the AVERY peptide binding in Fig. 4A. Activity was high in sera S7, S8 and S12, but weak in most others. For sera S7 and S8, this result is consistent with reactivity to the immunodominant peptide in Fig. 4A.
UNC oligomers do not have the same exclusivity for binding only nAbs as SOS and WT trimers (Moore et al., 2006). 7B2 blocking by the S12 serum is anomalous and difficult to explain, given that this site is occluded on SOS-VLPs (Moore et al., 2006). Another guinea pig recently immunized with WT-VLPs also developed 7B2 blocking activity, perhaps arising from exposure of gp41 stumps following gp120 shedding (data not shown).

Peptide ELISA

We next assessed serum binding to a panel of overlapping peptides spanning the V1, V2 and V3 loops (Fig. 5C). Some sera had weak activity against the V1 loop, particularly SOS-VLP sera S12, P13 and P15 (not shown). The V1 loop has, in fact, emerged as a common specificity in gp120-immune sera (Derby et al., 2006; Garrity et al., 1997; He et al., 2002; Li et al., 2006). However, none of the sera reacted with the V2 loop (not shown). The A62 plasma did not react effectively with any of these peptides.

A major focus of many ENV-VLP sera appeared to be the N-terminus of the V3 loop, specifically peptides 8838 (TRPNNNTRKSIHI) and 8839 (NNNTRKSIHIGPGRAF) (Fig. 5C). The A62 plasma and several V3 loop-specific mAbs, including LE311 and 39F (not shown), also recognized these peptides. We found that the gp120 sera had relatively low V3 peptide titers, in line with the weaker V3 mAb capture inhibition (compare Figs. 5A and C). The reactivity of the Env-VLP sera to V3 peptides was generally stronger, but quite variable. Interestingly, V3 mAb competition (Fig. 5A) was not always associated with V3 peptide reactivity, suggesting that...
some binding depends on conformations that are not well approximated by peptides.

Collectively, our mapping efforts indicate that SOS-VLPs and UNC-VLPs elicit high titers of V3 loop-specific Abs and often quite high titers of CD4i-overlapping Abs, but weak CD4 binding site-overlapping antibodies. In contrast, gp120 sera exhibited moderate V3 and CD4i titers and moderate to high titers of CD4bs-overlapping Abs. It is possible that gp120 and Env-VLP sera differ in that the gp120 might exist in a native conformation, while forms of Env presented on the VLPs might resemble “triggered” forms of Env, in which the V3 loop and coreceptor binding sites are more exposed, perhaps by virtue of gp120 association with gp41. The SOS mutation did, however, appear to largely prevent exposure of the gp41 immunodominant epitope, as intended. Regarding adjuvants, there was generally insufficient evidence to suggest that QS21 or Ribi added much beyond that achieved by CpGs. A possible exception was that the gp120 response in P1 was stronger and more focused on the CD4 binding site.

Analysis of neutralization activity

Reports on particle immunogens for HIV-1 have variably described neutralization or enhancement of infection by immune sera (Arthur et al., 1992; Buonaguro et al., 2005; Haffar et al., 1991; Hammonds et al., 2005; McBurney et al., 2007; Poon et al., 2005a, 2005b; Verrier et al., 2000). This may be in part related to the generation of antibodies against non-Env membrane proteins that can have unpredictable effects on infection. Indeed, it is often unclear whether “neutralization” is mediated by Abs directed to Env or to other membrane proteins (“anti-cell” antibodies). With this in mind, we evaluated neutralization in several formats, incorporating controls to help distinguish anti-Env activity from nonspecific effects.

We first measured neutralization against the index primary virus, JR-FL, in a luciferase assay using canine CF2 target cells (Fig. 6A). Canine CF2 cell targets were selected in an attempt to minimize the effects of anti-cell antibodies generated against the 293T (human) produced VLPs. Some SOS- and UNC-VLP sera (S6, S11, S12, P14 and P15) enhanced infection by 2–3 fold (Fig. 6A, column 1), even at high dilutions (exemplified by serum P15 in Fig. 7A), while other Env-VLP sera and the gp120 sera had no effect. In contrast, the naked VLP sera exhibited apparent “neutralization”. In the following two sections, we separately address the activity of gp120/Env-VLP sera and naked VLP sera in the CF2 neutralization assay.

Neutralization activity of the gp120 and Env-VLP sera

Enhancement could arise either from antibodies directed to Env that somehow activate fusion or from antibodies directed to membrane components that facilitate virus attachment. To investigate, we tested the effect of sera on SIVmac316 infection (Fig. 6A, column 2). This virus was made by transfection of the same 293T cells as the HIV pseudoviruses and, therefore, would be expected to bear the same set of cellular membrane proteins. Similar enhancement of this virus was noted with many of the sera, as it was for JR-FL, suggesting that the enhancement stemmed from Abs directed to non-Env membrane components of VLPs. In contrast, the control HIV+ plasmas A62 and N308 neutralized JR-FL, but not SIVmac316.

It has been suggested that enhancing Abs may mask neutralization (Giannecchini et al., 2001; Langlois et al., 1992). Indeed, in a previous study, removal of anti-cell antibodies against FIV particle vaccine by adsorption against producer cells was reported to uncover neutralizing activity (Giannecchini et al., 2001). To test this concept, we adsorbed SOS-VLP serum P15 against 293T cells and removed ∼90% of the anti-cell reactivity, as assessed by FACS (data not shown), while retaining strong gp120 titers (diluted only 2–3 fold during adsorption). However, this did not uncover any neutralization (not shown).

To further determine whether enhancing Abs mask neutralization, we spiked the JR-FL-enhancing serum P15 with neutralizing mAb b12. Comparing neutralization by b12 alone to a b12/serum mixture (Fig. 7B) showed that the serum did not reverse b12 neutralization. Thus, infection by a virus that has been neutralized was not rescued by factors that otherwise enhance infection. In further neutralization assays using a VSV-G pseudotyped virus, the Env-VLP sera showed no enhancement (Fig. 6A, column 3), perhaps because VSV-G entry is amphotropic and therefore influenced by different factors compared to HIV and SIV.

Though we did not see convincing neutralization against the index virus, JR-FL, ELISAs had suggested that sera contained potent anti-Env reactivity (Fig. 4). To assess neutralization in alternative conditions, we measured “post-CD4” activity (Crooks et al., 2005) by pre-incubating JR-FL particles with sCD4 and graded dilutions of sera and then measuring infection of CF2 cells expressing only CCR5. We found that all Env-VLP and gp120 sera neutralized effectively in this format (Fig. 6A, column 4), as did both HIV+ plasmas. However, as expected, naked VLP sera did not. Enhancement was not observed in the post-CD4 format, providing further evidence that enhancing effects are negated when the virus is neutralized. With reference to our previous analysis (Crooks et al., 2005), post-CD4 neutralization suggested the presence of Abs that recognize the V3 loop and/or CD4-induced epitope(s), consistent with our mapping data (Fig. 5). The contribution of V3 Abs to neutralization was investigated in “peptide interference” neutralization assays (Beddows et al., 2005; Li et al., 2006; Selvarajah et al., 2005). Here, V3 peptides strongly inhibited post-CD4 neutralization by serum P13 (Fig. 7C), suggesting a major role for V3 Abs in post-CD4 neutralization.

Although we were encouraged by the strong serum activity in the post-CD4 format, it is important to point out that this assay dramatically expands access to epitopes that are normally cryptic in native trimers and become exposed only fleetingly during infection, as evidenced by the generally weak to modest activity of these types of antibodies in traditional neutralization assays (Bou-Habib et al., 1994; Crooks et al., 2005; Deeker et al., 2005). Indeed, most HIV+ plasmas contain high concentrations of CD4i Abs without having strong neutralizing titers. Therefore, the significance of post-CD4 activity should not be over-interpreted.
Given the lack of neutralization against the JR-FL index virus in the standard format, we tested some more sensitive isolates. The P1 serum strongly neutralized HXB2, a highly sensitive X4 isolate (Fig. 6A, column 5). Perhaps underlying this activity, mapping had indicated high levels of CD4bs-overlapping Abs in this serum (Fig. 5A). However, no Env-VLP other serum neutralized HXB2, probably because the predominant V3 Abs (Fig. 5) do not cross-react with this isolate. The gp120 serum P1 weakly neutralized SF162, as did SOS-VLP sera S12 and P15, but no others (not shown). We also observed weak neutralization against ADA and BaL (not shown), but moderate neutralization against the 1196 isolate (Fig. 6A, column 6, and Fig. 7D). Epitope interference assays suggested that the 1196 neutralization was V3-mediated (Fig. 7E), consistent with the greater susceptibility of this virus to V3-directed neutralization (Binley et al., 2004).

Collectively, our neutralization data so far suggested that Env-VLP sera neutralize via anti-V3 Abs and the gp120 serum P1 neutralizes via CD4 binding site-overlapping Abs. The lack of SF162 neutralization via the Env-VLP sera may stem from differences in the V3 loop between JR-FL and SF162. When we substituted a residue in SF162 to match the N-terminus of the V3 to JR-FL, the mutant virus was significantly more sensitive to Env-VLP sera (Fig. 6A, column 7). The SF162 variant was slightly less sensitive to the neutralizing N308 HIV+ plasma, revealing that the mutation did not globally increase neutralization sensitivity.

Analysis of neutralization by naked VLP sera

In light of the absence of JR-FL neutralization by gp120 and Env-VLP sera, it was not immediately clear why naked VLP
sera potently “neutralized” (Fig. 6A, column 1). Evidence that the neutralization was nonspecific came from the observation that the sera also neutralized SIVmac316 (Fig. 6A, column 2). In addition, interference of P18 neutralization of the 1196 isolate using V3 peptides had no effect (not shown), consistent with nonspecific neutralization.

Another method to help distinguish nonspecific activity from genuine neutralization is that the former may not embody the sigmoidal dose–response typical of neutralization. We and others have observed that IC90 titers typically occur at approximately a 10 fold higher Ab concentration than the IC50 (Binley et al., 2004). In contrast, the “neutralization” sera potently “neutralized” (Fig. 6A, column 1). Evidence that the neutralization was nonspecific came from the observation that the sera also neutralized SIVmac316 (Fig. 6A, column 2). In addition, interference of P18 neutralization of the 1196 isolate using V3 peptides had no effect (not shown), consistent with nonspecific neutralization.

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To determine whether nonspecific neutralization influences genuine neutralization, we evaluated the effect of spiking a naked VLP serum (P18) with the b12 mAb at 2.5 μg/ml. Here, b12 neutralization could be distinguished only when its concentration was sufficient to reach an IC90 (compare Figs. 8A, B).

One possible explanation as to why naked VLP sera nonspecifically neutralized while Env-VLP sera generally enhanced might be that the specificity or titer of anti-cell Abs in Env-VLP sera may differ from those in naked VLP sera.

FACS analysis revealed that naked VLP serum P18 bound more potently to 293T cells than serum P12 (median fluorescence intensities at a 1:2000 serum dilution were 717 and 181, respectively). These differences could underlie the differential effects of these sera in neutralization assays, where nonspecific neutralization might occur above a threshold of anti-cell Abs.

It is possible that the cells in which virus was produced and/or the target cells in which virus infection is measured might influence neutralization. We therefore assessed serum binding to various cell lines by FACS. We found that SOS-VLP serum P12 reacted extremely strongly with 293T cells, the same cell...
line used to produce VLPs (Fig. 8C) and almost as strongly against U87 cells (a human glioma cell line). However, its reactivity against canine CF2 cells used in the neutralization assays described above was much lower, though still significantly positive. This suggests that a fraction of the anti-cell activity in sera is cell line-specific.

Alternative neutralization assays

To investigate the effect of differential cell reactivity on neutralization measurement, alternative neutralization assays were performed at a separate site (D.C.M.). In an assay measuring infection of 293T cell-produced virus in TZM-BL target cells (Fig. 6B columns 1–5), the nonspecific neutralizing activity of naked VLP sera was much lower and reactivity against the MuLV control virus was also low (Fig. 6B, column 5). Serum titers against SF162, JR-FL, 1196 or BaL pseudotyped viruses were, in general, higher (Fig. 6B, columns 1–4) than the corresponding titers using CF2 target cells (Fig. 6A and data not shown). Serum P1 was very potent against SF162 (Fig. 6B, column 1), consistent with the presence of CD4 binding site-overlapping antibodies. Neutralizing activity against JR-FL, though higher than before, remained relatively modest (Fig. 6B, column 2). Neutralization of 1196 and BaL was more impressive. Titers against 1196 were, in many cases, more than ten fold higher than in the initial CF2 assay (compare Fig. 6A, column 6 to Fig. 6B, column 3). It is possible that Ribi and QS21 adjuvants given to animals P13 and P14 contributed to especially high titers.

The above results were surprising, considering the greater overall anti-cell reactivity that would be expected against the human HeLa-based TZM-BL target cells. In addition to the cell line, differences in protocol may contribute to this effect. In the TZM-BL assay, but not the CF2 assay, sera, virus and cells are co-incubated during the entire infection period. We have found that this tends to increase neutralization IC50 titers (not shown). Another possibility may relate to coreceptor density. The CF2 cells in the initial assay express high levels of CCR5 to assist in efficient infection. This may decrease the window of opportunity for antibodies that neutralize via binding the V3 or coreceptor binding sites (Crooks et al., 2005). The lower CCR5 density on TZM-BL cells could therefore contribute to the greater neutralization potencies. Finally, in the CF2 assay, we used a short spinoculation step to increase overall infection levels that was not used in the second assay. However, we found that spinoculation does not significantly affect the activity of neutralizing mAbs (not shown).

In further neutralization assays employing PBMC-produced JR-FL primary viruses, also assayed on PBMC, we observed complete neutralization with most VLP sera at a 1:18 dilution, but little or no neutralization by the gp120 sera (Fig. 6B, column 6). We also observed strong activity against SIVmac251 in this format (Fig. 6B, column 7). It appears that, as in the CF2 assay, toxicity adversely affected neutralization measurement. Taken together, these results highlight the unpredictable effects of anti-cell antibodies. Given that the rules governing these effects are far from clear, our results provide a cautionary note, suggesting that particular care should be taken when examining sera generated against membranous antigens.

Measurement of trimer binding in blue native PAGE

In circumstances when neutralization assays are compromised by high background activity, BN-PAGE offers an independent way examine trimer binding. We recently reported that a shift in mobility of a VLP-derived trimer band in BN-PAGE, associated with a depletion of unliganded trimer, correlates with mAb neutralization (Crooks et al., 2005; Moore et al., 2006). Here, we adapted the method to measure neutralization by polyclonal sera and plasmas. We first tested the ability of the guinea pig sera to bind to JR-FL Env trimers in the presence or absence of additional neutralizing mAb b12 (Fig. 9A). We found that Env-VLP sera depleted the unliganded trimer to 43–96% of the control density, suggesting weak neutralization. Tellingly, the naked VLP serum P16 did not deplete unliganded trimers significantly, suggesting that nonspecific effects are inconsequential to this assay. In experiments in which VLP sera and b12 were mixed together, the effect of b12 to deplete unliganded trimers was not affected (Fig. 9A). The shifted trimers were somewhat diffuse compared to when b12 was used alone, perhaps indicating multiple forms of trimer–Ab complexes.

For reference purposes, we analyzed the ability of the neutralizing human HIV+ plasma N308 and the non-neutralizing plasma L503 to shift JR-FL trimers. Like b12, N308 potently depleted unliganded trimers (Fig. 9B). At low dilution, this was accompanied by a smear that resolved into distinct shifted bands at higher dilutions, suggesting differing numbers of neutralizing Abs attached to trimers. In contrast, the non-neutralizing plasma L503 did not detectably deplete unliganded trimers, even at the lowest dilution, confirming that neutralization can be measured unequivocally by BN-PAGE, as defined by a loss of unliganded trimer, accompanied by a gain in higher molecular weight liganded forms.

We next determined whether Env-VLP sera could mediate more effective trimer shifts of isolates they effectively neutralized. In Fig. 9C, mAb b12 and the Env-VLP sera P13, P14 and S12 all shifted SF162 308T/H trimers to varying degrees. P13 was more effective than P14 and S12, as reflected in neutralization assays (Fig. 6A, column 7). On the other hand, the naked VLP serum P16 did not shift trimers, consistent with its lack of neutralization.

Strong reactivity of VLP sera and HIV plasmas to a non-Env component of VLPs

We next examined the reactivity of various antibodies with VLPs. Thus, we compared neutralizing mAbs, VLP sera, HIV patient sera and HIV negative sera as Western blot probes for VLPs in native PAGE with some remarkable results (Fig. 9D). The neutralizing mAb cocktail bound both the strong trimer band, and the weaker monomer band (Moore et al., 2006). In contrast, the SOS-VLP serum bound extremely strongly to a ~150 kDa band and much less to the trimer, suggesting that...
gp120/gp41 monomers are a prime target of B cells. However, surprisingly, the naked VLP serum also bound extremely well to a band of the same size as the monomer. Since the naked VLP sera do not react with Env (Fig. 4), this indicates reactivity to a non-Env component of VLPs. An HIV positive patient serum gave similar results to the SOS-VLP sera: weak trimer binding, but strong reactivity to a band around the size of the monomer. In contrast, an HIV negative serum was essentially unreactive. Probing SIV-VLPs, we again observed strong binding of HIV+ plasma to the ∼150 kDa protein, suggesting that VLPs and HIV infection elicits strong responses to a non-Env protein (Gag or host protein) component of VLPs. Overall, these results suggest that non-trimer components of particles are preferred targets for B cells both in immunizations and natural infection.

**Discussion**

Here we showed that VLPs are a versatile vaccine platform, providing a complete framework to assess VLP-Env trimers as immunogens and a powerful end-stage system for analyzing sera (Crooks et al., 2005; Derby et al., 2006). Our initial vaccine approach emerged from the idea that earlier particulate HIV vaccine candidates may have failed to elicit broadly neutralizing antibodies because the Env trimers are unstable. In particular, gp120 shedding might leave behind depleted trimers or gp41 stumps (Chertova et al., 2002; Grovit-Ferbas et al., 2000; Hammonds et al., 2003; McKeating et al., 1991; Moore et al., 2006). To address this problem, we introduced a disulfide bridge to stabilize gp120−gp41 (SOS). To further optimize VLP immunogens, we truncated the gp41 cytoplasmic tail to increase expression and gp120/gp41 cleavage, with only a mild effect on neutralization sensitivity (Abrahamyan et al., 2005; Binley et al., 2003; Edwards et al., 2002; Gabuzda et al., 1992; Mammano et al., 1997). Since SOS-VLPs retain fusion competency (Abrahamyan et al., 2003; Binley et al., 2003; Crooks et al., 2005; Moore et al., 2006), we reasoned that they were a viable starting point for testing the ability of authentic trimers to elicit neutralizing antibodies (Abrahamyan et al., 2003; Binley et al., 2003).

**Nonfunctional gp120/gp41 monomers may be a prime Ab target on VLPs**

In spite of very high binding titers to monomeric gp120 and neutralization activity against sensitive primary isolates, SOS-VLP sera did not potently neutralize the JR-FL parent virus or most other neutralization-refractive isolates. This mirrors the majority of vaccine studies in which immune sera neutralize only V3-sensitive primary isolates like BaL, SF162 and 1196 (Koff et al., 2007). Indeed, the neutralizing activity of VLP sera appeared to stem at least in part from V3 antibodies. The fact that the V3 loop is cryptic on trimers of neutralization-resistant viruses like JR-FL (Bou-Habib et al., 1994) raises the question: where do these anti-V3 Abs come from? Since our control immunogens, UNC-VLPs also elicited strong responses to the V3 loop, we suggest that, in each case, B cells responded to forms of Env other than functional trimers, perhaps gp120/gp41 monomers (SOS-VLPs) or unprocessed gp120−gp41 (UNC-
VLPs). Thus, although SOS-VLPs eliminate gp120 shedding, the SOS mutation is only a partial solution toward presenting trimERIC Env on VLPs for vaccine purposes.

Previous analyses have suggested that gp120/gp41 monomers, unlike trimers, these are highly accessible to V3 Abs (Burrer et al., 2005; Moore et al., 2006; Nyambi et al., 1998; Poignard et al., 2003) and may be a primary target for B cells. We hypothesize that monomers act as decoys, distracting the attention of B cells from the more compact trimers, in what might be considered an “immunogenic hierarchy”. Evidence of such hierarchies in studies of immune responses to HIV-1 has in fact been widely reported (Cleveland et al., 2000; Garrity et al., 1997; Jelonek et al., 1996; Liu et al., 2006). Perhaps at odds with this concept, affinity selection in germinal centers dictates that higher affinity B cell blasts proliferate at the expense of lower affinity ones. Thus, antibody responses to multiple separate proteins might be expected to develop independently and equivalently, as if they were given alone, as for the trivalent measles, mumps and rubella vaccine (Usonis et al., 2005).

However, the situation with VLPs is in fact quite different because trimers and monomers are linked on the same particle and therefore may be considered as a single target. Thus, B cells would be selected against only the most accessible foreign target(s), the V3 loop, of the most accessible structure (the monomer), at the expense of other epitopes, in this case, the trimer.

We previously showed that monomer is only a minor component of particles, compared to the predominant trimer (Fig. 3 and Moore et al., 2006). This raises the question why a minor species would dominate the B cell response. The answer may simply be that form rather than quantity dictates the antibody response. We previously showed that non-neutralizing antibodies capture HIV with high efficiency, even though they do not bind efficiently to trimers, suggesting that monomer binding mediates the capture. BN-PAGE further confirmed the more promiscuous binding properties of monomers (Moore et al., 2006), suggesting that the monomer may dominate as an antigen simply because it is a particularly accessible target.

Of particular significance to the present study, a previous report compared the immunogenicity of UNC and “SOSIP” mutants of a soluble gp140 glycoprotein and gp120 monomer in rabbits (Beddows et al., 2007). Sera generated against SOSIP stabilized trimers potently neutralized the parental JR-FL isolate. This activity was not due to V3 Abs, but its precise specificity remains unclear. Although the neutralization was type-specific, because the JR-FL isolate is refractive to neutralization, these results suggest the SOSIP may be a possible step in the right direction for HIV vaccine research.

Both SOSIP gp140 and SOS-VLPs vaccine approaches emerged from the early observation that SOS gp140 readily dissociates into monomers and the consequent need to stabilize them as trimers (Schulke et al., 2002). The relative success of SOSIP gp140 raises questions about what might underlie the quite different findings of the present study. At odds with the idea that particles present native Env, and therefore may be particularly effective immunogens, the results of these two studies might be interpreted to suggest that the reverse is, in fact, true. More likely, however, the differences probably arise from the fact that monomeric forms of Env were completely eliminated in SOSIP gp140 immunizations, but not in SOS-VLPs (Moore et al., 2006). However, since VLP trimers are recognized exclusively by neutralizing Abs (Moore et al., 2006), unlike even the most advanced soluble trimers (Dey et al., 2006), VLPs may still have an advantage for presenting truly native trimers, if the right formulation can be found.

**Factors affecting the quality of the immune response**

We were cautious that commonly used adjuvants such as alum and Ribi might disrupt particle membranes, perhaps affecting Env conformation. Therefore, we selected immunostimulatory CpG as our primary adjuvant. Our mapping data indicate, however, that sera from the few animals that received VLPs in emulsion adjuvants differed little from those who received CpG alone. There was an indication, however, that the additional adjuvants led to higher neutralization titers against certain viruses (exemplified by the activities of sera P13 and P14 in Fig. 6). Therefore, it will be worth investigating further how adjuvants can help in getting the most out of these immunogens.

The route of immunization could affect the stability of VLPs after immunization, perhaps also affecting the quality of the antibody response. Intranasal administration, for example, has been shown to be effective (McBurney et al., 2007). However, since the nAb titers in other studies do not appear to dramatically better, the more fundamental problem of nonfunctional forms of Env may need to be solved before the extent of the advantages offered by varying route and adjuvant can be fully appreciated. Another, perhaps more significant factor is the choice of model species for vaccination trials. It has been suggested that some species may simply not have the IgG repertoire to generate nAbs (Koff et al., 2007). In line with this idea, we have recently begun VLP immunizations in macaques that suggest a greater focus on the CD4bs and improved neutralization activity.

**The effects of “anti-cell” antibodies**

A drawback of membranous immunogens is that they induce Abs against non-Env components, exemplified by the strong reactivity of VLP and HIV patient sera to a non-Env VLP band in BN-PAGE (Fig. 9D). Antibodies against non-Env VLP surface components can enhance or nonspecifically “neutralize” infection in a largely unpredictable manner (Arthur et al., 1992). The cleanest method to assess neutralization was the native trimer shift assay (Figs. 9A–C). This assay should in future help in assessing the progress of any membranous HIV vaccine. On the other hand, in traditional neutralization assays, we were able to incorporate controls at various levels to identify and eliminate any nonspecific activity.

**The future of HIV-VLP immunogens**

In natural infection, the delay in development of nAbs (Deeks et al., 2006), despite the early development of gp120
binding antibodies, suggests that nonfunctional forms of Env provide the virus with a valuable fitness advantage by diverting antibody responses from functional targets. To make progress, we may need to get beyond these defenses (Burton and Parren, 2000). It may be possible to refocus Ab responses against trimers (Moore et al., 2006) by modifying or blocking epitopes on nonfunctional Env (Cole et al., 2004; Garrity et al., 1997; Keller and Arora, 1999; Selvarajah et al., 2005; Srivastava et al., 2003). However, it could be argued that trimers are inherently poor immunogens and that removal of irrelevant forms of Env may not change that fact. On the other hand, the development of cross-neutralizing activity in a subset of infected HIV patient sera, suggests that B cells can in fact eventually respond to trimers. The neutralizing activity of SOSIP gp140 sera provides evidence that trimers are not inherently poor antigens. Thus, if we can find a way to steer B cells away from irrelevant targets, we may in future be able to elicit responses against native trimers.

Materials and methods

Plasmids and mutagenesis

The plasmid pCAGGS was used to express membrane-bound forms of the primary R5 isolate, JR-FL (Binley et al., 2003; Moore et al., 2006). We expressed full-length gp160 and gp160ΔCT, truncated at amino acid 709, leaving 3 amino acids of the gp41 cytoplasmic tail. The SOS and UNC mutants to stabilize gp120–gp41 association have been described previously (Binley et al., 2000). Gp160-expressing plasmids of isolates SF162, BaL, HXB2, ADA and 1196 were obtained from Drs. Leo Stamatatos, David Montefiori and the NIH AIDS Repository. A mutant of SF162 Env in which the threonine residue at 308 of the V3 loop was exchanged for a histidine was created by Quikchange mutagenesis. Truncated gp160ΔCT versions of 1196, SF162, SF162 308T/H and SIVmac316 EnvS were cloned into pCAGGS in a similar manner to that described for JR-FL (Binley et al., 2003). The plasmid pVSV-G was obtained from Dr. Nathaniel Landau. Sub-genomic plasmids pNL4-3.Luc.R-E- and pSG3ΔEnv have been described previously (Binley et al., 2002; Li et al., 2005).

MAbs, soluble CD4, recombinant gp120 and HIV+ donor plasmas

Anti-gp120 monoclonal antibodies (mAbs) included b12 and 15e, directed to epitopes that overlap the CD4 binding site (CD4bs) (Burton et al., 1994); 2G12, directed to a unique glycan-dependent epitope on gp120 (Sanders et al., 2002a; Scanlan et al., 2002); X5 directed to a CD4-inducible (CD4i) epitope (Lerbrín et al., 2003); LE311, 39F and PA1 directed to the V3 loop (Crooks et al., 2005; Schulke et al., 2002); and B12, directed to an epitope in the C2 domain of gp120 that is preferentially exposed on denatured forms of the molecule (Moore et al., 2006). Anti-gp41 mAb 7B2 is directed to the cluster I region (Binley et al., 2000).

MAb 2G12 was provided by Dr. H. Katinger (Polymun Scientific Inc., Vienna, Austria). MAbs 39F, LE311 and 7B2 were provided by J. Robinson (Tulane University). MAb B12 was provided by Dr. George Lewis (Institute of Human Virology, Baltimore, MD). MAb PA1, four-domain soluble CD4 (sCD4) and JR-FL gp120 were provided by Progenics Pharmaceuticals Inc (Tarrytown, NY). MAb b12 and 2G12 are broadly neutralizing (Binley et al., 2004); mAb X5 neutralizes primary isolates in the presence of soluble CD4 (Crooks et al., 2005; Moulder et al., 2002); the V3 loop-specific Abs neutralize a subset of primary isolates (Binley et al., 2004); mAb 7B2 is non-neutralizing. The HIV-1-infected donor plasmas A62, L503 and N308 were described previously (Crooks et al., 2005). The anti-p24 serum was obtained from the NIH AIDS Reagent Program.

Peptides

Eleven residue-overlapping 15-mer peptides of the V3 loop of JR-FL were obtained from the NIH AIDS Reagent Program. Five residue-overlapping 15-mer peptides of the V1/V2 loop of JR-FL were synthesized at The Torrey Pines Institute for Molecular Studies and purified to >90%. A peptide of the gp41 immunodominant region, sequence RVLAVERYLKDGQQLL-GIWGC5GKLIC, termed the “avery” peptide was purchased from AnaSpec (San Jose, CA).

Production of VLPs

VLPs were produced by transient transfection of 293T cells with plasmids pNL4-3.Luc.R-E- and a pCAGGS-based Env-expressing plasmid by calcium phosphate precipitation. Two days later, supernatants were collected, preclarified by low speed centrifugation, filtered through a 0.45 μm filter and pelleted at 50,000× g in a Sorvall SS34 rotor. To remove residual medium, pellets were diluted with 1 ml of PBS then recentrifuged in a microfuge at 15,000 rpm. VLPs were then resuspended in PBS at 1000× the original concentration. Particles were inactivated using aldrithiol (AT-2) (Russo et al., 1998), after which they were re-centrifuged and washed with PBS.

Immunoprecipitation

Supernatants of 293T cells were metabolically labeled and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), as previously described (Binley et al., 2000). Reduced and nonreduced samples were prepared by boiling for 5 min in Laemmli sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) in the presence or absence of 20 mM dithiothreitol (DTT).

SDS–PAGE, native PAGE and Western blot

VLP preparations were separated by SDS–PAGE, and Western blots were probed with mAbs PA1 and B12 diluted to 1 μg/ml in PBS containing 2% nonfat milk, detected with a goat anti-mouse alkaline phosphatase conjugate (Jackson) and
developed using BCIP/NBT colorimetric reagents (Sigma-Aldrich). VLP-derived Env was also analyzed by blue native PAGE (BN-PAGE), as described previously (Moore et al., 2006).

Electron microscopy

Transfected 293T cells producing VLPs were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h. Samples were then washed in 0.1 M sodium cacodylate, fixed in 1% osmium tetroxide and incubated in 0.5% tannic acid in 0.05 M cacodylate for 30 min. Following a 10 min wash with 1% sodium sulfate/0.1 M cacodylate buffer and further rinsing in 0.1 M cacodylate, the cells were dehydrated in a graded ethanol series, cleared in propylene oxide and embedded in EMbed-812/Araldite (Electron Microscopy Sciences, Fort Washington PA). Ultra thin sections were cut and mounted on parlodion-coated copper grids and stained with uranyl acetate and lead citrate. The sections were then examined on a Philips CM-100 electron microscope (FEI, Hillsborough, OR) and documented on Kodak SO-163 photographic film.

In high magnification negative stain electron microscopy, aliquots (25 μl) of virus particle preparations were washed in 140 μl of PBS and pelleted by an Airfuge centrifuge (Beckman Coulter) at 100,000×g for 15 min. The virus particles were then resuspended and fixed for 30 min in 20 μl of 2.5% glutaraldehyde/PBS at 4 °C. The fixed virus particles were washed in PBS and pelleted in Airfuge centrifuge again and finally resuspended in 15 μl of saline. 4.5 μl of resuspended sample was placed on a carbon-covered grid for 30 s. Excess virus particles and buffer were removed by filter paper blotting, and 5 μl of 1% uranyl formate stain was immediately added and incubated for 1 min. Excess stain was then removed, and the samples were allowed to air dry. Sample grids were screened at ×30,000, and electron micrographs were recorded at a nominal magnification of ×100,000 at 100 kV on a JEOL JEM 1200EX electron microscope.

Immunogenicity of VLPs in guinea pigs

Dunkin Hartley guinea pigs were immunized with SOS-VLPs, UNC-VLPs, “naked” VLPs or gp120, at the Scripps animal facility or Pocono Rabbit Farm (denoted by the animal identifier prefixes “S” and “P”). Five microgram Env equivalents of VLPs were used for each dose, as determined by Western blot, comparing to the known monomeric JR-FL gp120 standard. Naked VLPs were normalized for equivalent p24 to the other VLP preparations in Western blot. Recombinant monomeric gp120 was initially used at 5 μg dose (immunizations 1 and 2) consistent with the dose of Env in Env-VLP vaccinations. However, due to weak responses, the third and final dose was increased to 50 μg. Most immunogens were formulated in an equal mixture with the CpG-based adjuvant Immuneasy (Qiagen) optimized for rabbits and mice. In some immunizations, the saponin-based QS21 or Ribi Rnas3c adjuvants were used. All immunizations were administered by a combination intradermal and intramuscular routes in two locations each, at days 0, 43 and 97. Sera were collected 1 or 2 weeks after each immunization.

Neutralization assays

Sera were heat-inactivated at 56 °C for 30 min then analyzed for neutralization. Each assay was performed in duplicate and repeated at least 2 times. Representative data are shown. HIV+ plasmas A62 and N308 were used as reference controls. Neutralization assays were performed using either Env-pseudotyped viruses produced by transfection of 293T cells or PBMC-grown viruses.

Neutralization assays using CF2 cell targets

Neutralization assays using CF2 cells were described previously (Crooks et al., 2005; Kolehinsky et al., 2001). Briefly, virus was incubated with graded dilutions of Ab for 1 h at 37 °C. The mixture was then added to CF2 cells, spinoculated then incubated for 2 h at 37 °C, after which the medium was changed. The cells were cultured a further 3 days and then luciferase activity was measured. Post-CD4 binding neutralization was assayed by treating VLPs with scCD4 and graded dilutions of sera and using CF2 cells bearing only CCR5 as targets (Crooks et al., 2005). In “peptide interference” neutralization experiments, fixed concentrations of V3 peptides were added to virus–Ab mixture to adsorb V3-specific nAbs.

Neutralization assays using TZM-BL cell targets

A neutralization assay using TZM-BL cells has been described previously (Li et al., 2005), in which trypsinized TZM-BL cells were added to virus–serum mixtures and left for 3 days without a change of medium or a “wash out” format in which virus–Ab mixtures were added to wells that had been pre-seeded with TZM-BL cells, and the medium changed after a 2 h incubation, followed by 2 days of culture.

Neutralization assays using PBMC targets

Live virus (JR-FL or SIVmac251) was cultured in activated human PBMC. Virus was mixed with graded concentrations of Ab for 1 h at 37 °C. Mixtures were then added to fresh activated PBMC and cultured for 3 days. The structural proteins p24 or p27 were detected by ELISA, as described previously (Hammonds et al., 2005).

Trimer binding in native PAGE

We measured the ability of antibodies to bind VLP-derived Env trimers, based on the premise that trimer–Ab complexes migrate more slowly than their unliganded counterparts in BN-PAGE (Crooks et al., 2005; Moore et al., 2006). Specifically, JR-FL 140T SOS-VLPs were incubated with HIV+ plasmas, VLP sera and/or mAb b12 Fab for 15 min, followed by a wash. Trimers were then resolved by BN-PAGE and Western blot. Since the migration properties of trimer–Ab complexes can vary, binding was measured by depletion of the unliganded trimer. Using NIH Image software, a box was drawn around the unliganded trimer band and the density was measured in arbitrary units. The density of a control bands with no ligand...
added was used as a reference for measuring the % residual unliganded trimer in lanes in which ligands were added.

**Adsorption of sera against 293T cells**

Sera were adsorbed against 293T cells to remove reactivity against membrane-associated proteins (Giannecchini et al., 2001; Langlois et al., 1992). Briefly, pellets containing 10^8 293T cells were mixed with 200 μl of serum and mixed overnight at 4 °C. Cells were then pelleted and the serum retrieved. The process was repeated 2 more times. The supernatant serum was completely cleared of cells and then assayed by ELISA to detect anti-gp120 Abs compared to unabsorbed sera to determine whether anti-gp120 titers were affected by adsorption.

**Flow cytometry**

Guinea pig serum binding to 293T, CF2 and HOS cells was assessed. Cells were incubated with sera at graded dilutions for 1 h at RT. An FITC conjugate (Jackson, West Grove, PA) was used to detect guinea pig IgG binding by FACS.

**Analysis of serum binding titer and specificity by ELISA**

We measured anti-gp120 titers of guinea pig sera by ELISA, as described previously (Binley et al., 1997). A goat anti-guinea pig or human alkaline phosphatase conjugate (Accurate) was used to detect bound Ab using the AMPAK system (Dako). Reactivity against epitopes exposed on denatured gp120 was measured against captured gp120 that had been treated with 1% SDS and 50 mM DTT and boiled for 2 min and then diluted in PBS before captured on D7324-coated plates. Peptide ELISAs were performed in a similar manner.

**Serum mapping by inhibition of mAb-virus capture**

To determine the specificity of sera, we examined their ability to inhibit JR-FL WT-VLP capture by a panel of mAbs, as described previously (Derby et al., 2006). VSV-G was co-expressed on particle surfaces to enhance detection of captured virus and also to provide a read-out of capture that is essentially unaffected by possible serum neutralization (Moore et al., 2005). Briefly, mAbs were coated on ELISA microwells overnight at 5 μg/ml. Wells were then washed and blocked with 3% bovine serum albumin (BSA) in PBS. Graded dilutions of sera were incubated with JR-FL WT-VLPs bearing VSV-G for 30 min. For X5 mAb capture, a fixed concentration of 5 μg/ml scCD4 was added throughout. Mixtures were then added to mAb-coated ELISA wells for 3 h followed by washing with PBS (Poignard et al., 2003). CF2.CD4.CCR5 cells (Kolchinsky et al., 2001) were overlaid and 2 days later, infection was measured by assaying luciferase.

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