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Virology 360 (2007) 329–340

VIROLOGY

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A comparative immunogenicity study in rabbits of disulfide-stabilized, proteolytically cleaved, soluble trimeric human immunodeficiency virus type 1 gp140, trimeric cleavage-defective gp140 and monomeric gp120

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Received 27 July 2006; returned to author for revision 22 August 2006; accepted 18 October 2006

Available online 28 November 2006

Abstract

The human immunodeficiency virus type 1 (HIV-1) surface envelope glycoprotein (Env) complex, a homotrimer containing gp120 surface glycoprotein and gp41 transmembrane glycoprotein subunits, mediates the binding and fusion of the virus with susceptible target cells. The Env complex is the target for neutralizing antibodies (NAbs) and is the basis for vaccines intended to induce NAbs. Early generation vaccines based on monomeric gp120 subunits did not confer protection from infection; one alternative approach is therefore to make and evaluate soluble forms of the trimeric Env complex. We have directly compared the immunogenicity in rabbits of two forms of soluble trimeric Env and monomeric gp120 based on the sequence of HIV-$1_{JR\text{-}FL}$. Both protein-only and DNA-prime, protein-boost immunization formats were evaluated, DNA-priming having little or no influence on the outcome. One form of trimeric Env was made by disrupting the gp120–gp41 cleavage site by mutagenesis ($gp140_{UNC}$), the other contains an intramolecular disulfide bond to stabilize the cleaved gp120 and gp41 moieties (SOSIP.R6 gp140). Among the three immunogens, SOSIP.R6 gp140 most frequently elicited neutralizing antibodies against the homologous, neutralization-resistant strain, HIV-$1_{JR\text{-}FL}$. All three proteins induced NAbs against more sensitive strains, but the breadth of activity against heterologous primary isolates was limited. When antibodies able to neutralize HIV-$1_{JR\text{-}FL}$ were detected, antigen depletion studies showed they were not directed at the V3 region but were targeted at other, undefined gp120 and also non-gp120 epitopes.

Keywords: HIV-1; Envelope; Cleavage; Trimers; Immunogen; Neutralizing antibodies

Introduction

The development of an effective vaccine to prevent the spread of human immunodeficiency virus type 1 (HIV-1) is a global health priority (reviewed in Desrosiers, 2004; Garber et al., 2004; Klausner et al., 2003; McMichael, 2006). It is likely that a potent and broadly reactive neutralizing antibody (NAb) response will be a critical constituent of any vaccine-elicited protective immune response against HIV-1. NAbs are targeted to, and raised by, the viral envelope glycoproteins (Env) encoded by the *env* gene. During synthesis of the Env precursor (gp160), the protein is trimerized in the endoplasmic reticulum (ER) via non-covalent interactions between gp41 subunits (Earl et al., 1991). Cellular endoproteases of the furin family then cleave gp160 into gp120–gp41 complexes in a post-ER compartment (probably the *trans*-Golgi network) (Moulard and Decroly, 2000). The functional Env complex is a homotrimer containing

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doi:10.1016/j.virol.2006.10.032

three cleaved heterodimers in which the gp120 (SU) and gp41 (TM) glycoproteins are non-covalently associated. This fully matured Env complex is incorporated into virions, where it serves to mediate receptor binding and the subsequent conformational changes that drive the fusion of the viral and cell membranes (Berger et al., 1999; Gallo et al., 2003; Poignard et al., 2001; Wyatt and Sodroski, 1998).

Recombinant monomeric gp120 subunits have been tested extensively as immunogens, but they have succeeded only in eliciting antibodies capable of recognizing the immunizing antigen and of neutralizing atypically sensitive HIV-1 strains, including ones adapted to replication *in vitro* (Parren et al., 1999). When tested in efficacy trials, the monomeric gp120 vaccines were found to be ineffective (Flynn et al., 2005; Gilbert et al., 2005; Graham and Mascola, 2005). Because of the importance placed upon the induction of an effective NAb response, designing and testing more sophisticated forms of Env-based immunogens is one of the foci of current HIV-1 vaccine research. One approach involves making soluble recombinant proteins that better mimic the form of the native, trimeric Env complex present on the virion surface (Burton et al., 2004). Usually, such proteins are made by truncating the full-length gp160 protein immediately prior to the gp41 transmembrane domain, a procedure that allows a soluble gp140 protein to be secreted and purified. Mutations introduced to disrupt the endoproteolytic cleavage site between gp120 and gp41 fortuitously stabilize the interactions between the gp41 ectodomains ($gp41_{ECTO}$) sufficiently to allow purification of trimeric Env proteins ($gp140_{UNC}$) (Center et al., 2004; Chakrabarti et al., 2002; Srivastava et al., 2002; Yang et al., 2000, 2002; Zhang et al., 2001). Additional trimer-stabilizing modifications can be added to these $gp140_{UNC}$ proteins (Center et al., 2004; Chen et al., 2004; Srivastava et al., 2003; Yang et al., 2000, 2002; Zhang et al., 2001). When tested in small animal models, some $gp140_{UNC}$ proteins are moderately superior to monomeric gp120 proteins for induction of NAbs (Barnett et al., 2001; Bower et al., 2004; Kim et al., 2005; VanCott et al., 1997; Yang et al., 2001).

As an alternative approach, we have engineered a disulfide bond to stabilize the interaction between the gp120 and $gp41_{ECTO}$ subunits; this modification enables the SOS gp140 protein to be cleaved without the rapid dissociation of gp120 from $gp41_{ECTO}$ (Binley et al., 2000; Schulke et al., 2002). In its full-length form, the SOS form of Env is fusion-competent provided the disulfide bond is reduced after receptor binding has occurred (Abrahamyan et al., 2003; Binley et al., 2003). An additional substitution (I559P) introduced into $gp41_{ECTO}$ strengthens gp41–gp41 interactions, allowing the purification of soluble trimers known as SOSIP gp140 proteins (Sanders et al., 2002). Replacement of the natural sequence of the cleavage site between gp120 and gp41 with an hexa-Arg (R6) motif increases the efficiency of cleavage (Binley et al., 2002). In a recent study, NAbs against some primary HIV-1 Env-pseudotyped viruses were induced in a subset of rabbits immunized with SOSIP gp140 trimers (Beddows et al., 2005).

Cleaved and uncleaved forms of gp140 trimers do differ in their structure, as judged by MAb-reactivity profiles (Herrera et al., 2005; Pancera and Wyatt, 2005; Schulke et al., 2002; Si et al., 2003). However, it has been unclear whether one form is superior to the other as an immunogen. Here, we describe a formal comparison of the immunogenicity of SOSIP gp140 trimers with uncleaved gp140 trimers and monomeric gp120 using a DNA-prime, protein-boost immunization format. In sensitive assays based on Env-pseudotyped viruses, antibodies raised to SOSIP gp140 were more frequently active against $HIV\text{-}1_{JR\text{-}FL}$ than antibodies to monomeric gp120 or $gp140_{UNC}$ proteins. However, the breadth of the NAb response to all three immunogens was quite limited. In a sub-study, we compared responses to purified SOSIP gp140 and gp120 proteins administered without DNA priming, the results suggesting that, overall, DNA-priming prior to protein immunization had little impact on the generation of NAbs. Our conclusion is that JR-FL SOSIP gp140 trimers were superior to $gp140_{UNC}$ and gp120 proteins in this study, albeit to a modest extent. Additional development work will be required if SOSIP gp140 proteins are to become practical vaccines.

Results

Design, expression and immunization of monomeric and trimeric Env immunogens

We recently described a study in which we compared different ways to present Env glycoproteins in the DNA priming (soluble vs. membrane-bound Env) and soluble protein boost (soluble, cleaved trimeric SOSIP.R6 gp140 Env vs. bead-immobilized trimeric Env) phases of an immunization regimen in rabbits (Beddows et al., 2005). Here, we have built on that study by now directly comparing the immunogenicity of monomeric gp120, trimeric SOSIP.R6 gp140 and a trimeric, cleavage-defective form of Env ($gp140_{UNC}$), all based on the $HIV\text{-}1_{JR\text{-}FL}$ Env sequence.

The design, expression and characterization of the soluble, cleaved, trimeric SOSIP.R6 gp140 Env protein has been described elsewhere (Beddows et al., 2005; Binley et al., 2000, 2002; Sanders et al., 2002; Schulke et al., 2002). Membrane-bound and soluble forms of cleavage-defective Env ($gp140_{UNC}$) were both made by replacing the sequence of the wild-type (Wt) cleavage sequence, REKR (Dubay et al., 1995), with a non-scissile IEGR motif (Schulke et al., 2002). The soluble, trimeric form of $gp140_{UNC}$ was purified from transfection supernatant using lectin affinity and size exclusion chromatography (SEC) columns (Schulke et al., 2002).

We conducted a prolonged series of immunizations involving priming the rabbits twice with DNA expressing a membrane-bound form of Env, followed by 4 boosts with soluble proteins. The study involved five Arms, each containing 4 rabbits (Fig. 1A). In Arms F, G and H, the animals were primed twice during a 4-week period with DNA expressing membrane-bound proteins Wt gp140 (i.e., gp140 that is potentially cleavable but probably substantially uncleaved), $gp140_{UNC}$ (i.e., the cleavage-defective IEGR mutant) and SOSIP.R6 gp140 (i.e., gp140 intended to be cleaved as efficiently as possible), respectively. Membrane-bound forms of Env (i.e., gp140 cytoplasmic-tail

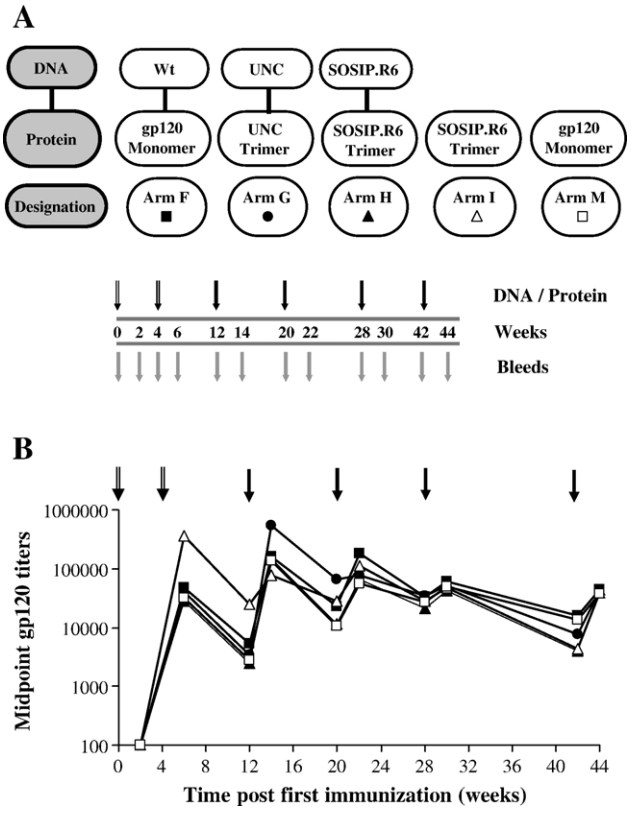

Fig. 1. Design of the immunizations and the induction of anti-gp120 binding antibodies. (A) The schematic for the immunization study highlights the membrane-bound DNA construct (gp140 Wt, $gp140_{UNC}$ or SOSIP.R6 gp140) used for priming (open arrows), the type and timing of each of the protein immunizations (filled arrows) and the serum collection times (grey arrows). (B) Generation of anti-gp120 antibodies in the study. Rabbits were primed (filled symbols) or not (open symbols) with 1 mg of pPPI4 expressing either codon-optimized membrane-bound gp140 Wt Env (square), $gp140_{UNC}$ (circle) or SOSIP.R6 gp140 (triangle) at the times indicated. The anti-gp120 antibody responses were measured by ELISA. Each datum point represents the mean ($n=4$ animals) midpoint anti-gp120 binding titer for each immunization group (Arms F, G, H, I and M).

truncated proteins) were used for DNA priming because our initial study indicated they were superior to soluble proteins for this purpose (Beddows et al., 2005). The protein boosts in Arms F, G and H were the corresponding soluble gp120 or gp140 proteins (30 μg) given over a 44-week period, initially using QS-21 adjuvant, later Ribi adjuvant (see below). No DNA priming was carried out in Arms I and M; these arms directly compared the immunogenicities of soluble SOSIP.R6 gp140 trimers (Arm I) and gp120 monomers (Arm M) (Fig. 1). The animals were bled at the indicated times and their sera were tested for anti-Env binding and neutralizing antibody responses.

Anti-gp120 binding antibody responses

Midpoint gp120 binding Ab titers were determined by ELISA for each animal at each time point and are presented as the mean values for the 4 rabbits in each Arm (Fig. 1B). Immediately after the two DNA immunizations, the mean anti-gp120 binding titers in sera from the animals immunized with gp120 (Arm F, 4.7×10^4), $gp140_{UNC}$ (Arm G, 4.0×10^4) and SOSIP.R6 gp140 (Arm H, 2.8×10^4) were similar. The titers then declined gradually by approximately 10-fold but were elevated by ~100-fold by the first protein boost (week 14 bleed). Additional transient increases occurred in response to subsequent boosts, but the maximum response never went beyond the peak titer established at week 14. Overall, the anti-gp120 binding titers in Arms F–H were always similar at all time-points (±3-fold), although the titers in the SOSIP.R6 immunized animals (Arm H) were usually a little lower than in the animals given gp120 or $gp140_{UNC}$, as observed previously (Beddows et al., 2005).

The midpoint anti-gp120 binding titers in sera from animals immunized with Env proteins without DNA priming (Arms I and M) varied over time in a similar manner to those in the DNA-primed rabbits. Thus, the peak titers were generated early (weeks 6 and 14 for SOSIP.R6 gp140 or gp120 immunized animals, respectively) and were not enhanced by subsequent immunizations.

Of note is that the adjuvant used for the Env protein boosts was switched from the dose-sparing, saponin-derived adjuvant QS-21 to a monophosphoryl lipid A-based adjuvant (Ribi) at week 28. Our previous immunization study suggested that while anti-Env binding titers were not affected by such an adjuvant switch, this strategy might have a modest, beneficial effect on overall neutralization titers (Beddows et al., 2005). In the present study, the change of adjuvant again made no difference to the overall anti-gp120 binding titers (Fig. 1B).

There was no significant difference between the anti-gp120 binding antibody titers in animals immunized with SOSIP.R6 (with or without DNA prime) and in those immunized with either gp120 or $gp140_{UNC}$ at either week 22 ($p=0.36$; Mann–Whitney, one tail) or week 44 ($p=0.42$).

Neutralization of Env-pseudotyped HIV-1

To assess functional antibody responses, we first measured neutralizing activity against HIV-1 pseudotype viruses expressing the JR-FL or MN Env proteins, using U87.CD4 cells bearing the appropriate coreceptor (Beddows et al., 2005). JR-FL Env is derived from the CCR5-using (R5) primary isolate $HIV\text{-}1_{JR\text{-}FL}$ which is moderately neutralization resistant and represents the autologous strain for the immunization studies, while MN Env is derived from a highly passaged, CXCR4-using (X4), neutralization sensitive strain, $HIV\text{-}1_{MN}$. We previously used these Env-pseudotyped viruses to monitor the generation of a baseline level of neutralizing activity ($HIV\text{-}1_{MN}$) and to identify sera containing NAbs active against more resistant primary isolates ($HIV\text{-}1_{JR\text{-}FL}$) (Beddows et al., 2005).

Neutralization of Env-pseudotyped $HIV\text{-}1_{MN}$ was determined for every serum sample available from each animal. Data derived from selected time points are shown in Table 1. The test sera were obtained following the second DNA and the four protein immunizations for the DNA-primed groups (Arms F, G and H), and following the second, third, fourth, fifth and sixth protein immunizations for the protein-only groups (Arms I and M).

DNA priming (week 6) was able to induce NAbs against Env-pseudotyped $HIV\text{-}1_{MN}$, but only to the 50% endpoint and at

Table 1
Neutralization of Env-pseudotyped HIV-1 by gp120-, $gp140_{UNC}$- and SOSIP.R6-immunized rabbits

Arm	DNA	Protein	Animal	Neutralization of Env-pseudotyped HIV-1 MN and JR-FL by rabbit sera[a]							
				Week 6		Week 14		Week 22		Week 30	
				MN	JR-FL	MN	JR-FL	MN	JR-FL	MN	JR-FL
F	Wt	gp120	840	27	ND	>160	–	153	–	>160	–
			841	21	ND	>160	–	>160	–	>160	–
			842	40	ND	>160	–	>160	16	>160	–
			843	95	ND	>160	–	98	–	>160	–
G	UNC	UNC Trimer	844	16	ND	116	–	143	–	>160	–
			856	–	ND	>160	–	>160	–	>160	–
			846	17	ND	40	–	88	17	144	–
			847	–	ND	–	–	60	13	117	–
H	SOSIP.R6	SOSIP.R6 Trimer	848	16	ND	>160	–	123	14	>160	–
			849	18	ND	151	–	105	19	>160	63
			850	–	ND	>160	–	93	17	154	–
			851	27	ND	>160	–	>160	19	>160	–
I	None	SOSIP.R6 Trimer	852	>160	45	>160	19	>160	18	133	–
			853	>160	17	>160	104	>160	102	106	33
			854	57	–	>160	–	>160	–	83	–
			855	130	–	98	–	>160	–	95	–
M	None	gp120	1188	–	–	>160	–	>160	–	124	–
			1189	14	–	148	–	>160	–	88	–
			1190	–	–	98	16	140	17	39	–
			1191	>160	–	>160	–	>160	–	>160	–

[a] The reciprocal of the dilution that resulted in 50% neutralization of Env-pseudotyped HIV-1 on coreceptor-bearing U87.CD4 cells (mean of two experiments). A dash indicates that <50% neutralization was observed at the 1:10 dilution. ND, not done.

modest titers. These titers were then boosted after the immunizations with soluble Env proteins, such that neutralization at the 90% endpoint was routinely observed (data not shown). The maximum 90% titers against $HIV\text{-}1_{MN}$ were attained after one (week 14), two (week 22) or three (week 30) protein immunizations (Arms F, G and H) (data not shown). For the animals given only soluble Env protein (Arms I and M), 90% neutralization of $HIV\text{-}1_{MN}$ was maximal after the second (week 6), third (week 14) or fourth (week 22) immunization. $HIV\text{-}1_{MN}$ neutralization titers (90% endpoint) were not significantly different when the rabbits immunized with gp120 (Arm F, week 30 and Arm M, week 14) and SOSIP.R6 gp140 (Arm H, week 30 and Arm I, week 14) were compared (Mann–Whitney *U* test), irrespective of whether the animals had been primed with DNA before receiving three protein immunizations (data not shown). This pattern of response is broadly consistent with what we observed previously (Beddows et al., 2005).

NAbs against the autologous, but more resistant $HIV\text{-}1_{JR\text{-}FL}$ strain were generated more slowly and their appearance could not be predicted from inspection of the anti-gp120 binding or $HIV\text{-}1_{MN}$ neutralization titers (Table 1). In addition, the titers against $HIV\text{-}1_{JR\text{-}FL}$ were lower and were only detectable in a subset of animals. Among the DNA-primed animals (Arms F, G and H), only those immunized with SOSIP.R6 gp140 had a consistent neutralization response against $HIV\text{-}1_{JR\text{-}FL}$. The 50% neutralization titer was >10 for sera from all 4 animals in Arm H at week 22. However, only animal #849 generated what we consider to be a significant and sustained neutralizing antibody response to $HIV\text{-}1_{JR\text{-}FL}$ (50% titer >40). Two of the four animals immunized with SOSIP.R6 gp140 protein only (#852 and #853) generated NAbs against $HIV\text{-}1_{JR\text{-}FL}$, one of them (#853) to the 90% endpoint (Table 1 and data not shown). In contrast, sera from animals immunized with gp120 or $gp140_{UNC}$ neutralized $HIV\text{-}1_{JR\text{-}FL}$ more weakly and transiently (Table 1). Neutralization of the control MLV Env-pseudotyped virus was not seen, indicating that non-specific inhibitory or toxic factors were not present in the test sera (data not shown).

In contrast to our previous study (Beddows et al., 2005), we found that switching from the adjuvant QS-21 to Ribi from week 28 made no difference to the generation of NAbs in any of the study Arms.

Independent assessment of neutralization activity

The neutralization data described above are consistent with our previous observations that some rabbits immunized with SOSIP.R6 gp140, but not those receiving gp120, can generate antibodies able to neutralize $HIV\text{-}1_{JR\text{-}FL}$ (Beddows et al., 2005). As an independent test of our findings and to determine whether the SOSIP gp140 immunogens could elicit cross-reactive neutralizing antibodies against heterologous HIV-1 strains, selected sera were also tested in the PhenoSense™ HIV Entry Assay (Richman et al., 2003). The test panel comprised 9 HIV-1 Env-pseudotyped viruses derived from sexually transmitted, clonal, geographically diverse, R5 primary isolates that are representative of a broad range of neutralization phenotypes.

Pre-immune sera and sera obtained at weeks 30 and 44 (Arms F, G and H), or at week 14 (Arms I and M), from all four rabbits in each Arm were tested. The neutralizing activities of sera from animals in Arms F, G and H at week 44 were not significantly different from the activities of the corresponding week 30 sera. Hence, for brevity, only the data from week 30 are presented in Table 2. None of the pre-immune sera contained neutralizing activity (<50% inhibition at 1:25 dilution) (data not shown). Regardless of the method of immunization (DNA prime, protein boost or protein only immunizations) or the Env protein used (gp120 monomer, $gp140_{UNC}$ trimer or SOSIP.R6 gp140 trimer), all the immunogens induced antibodies that neutralized the atypically sensitive strains, HIV-1_{SF162} and HIV-$1_{NL4/3}$, to high titers (Table 2). However, only sera from animals immunized with SOSIP.R6 gp140 protein with (#849) or without (#852 and #853) DNA-priming had any neutralizing activity against HIV-1_{JR-FL}. These animals are the same three whose sera could also neutralize HIV-1_{JR-FL} in our in house neutralization assay.

Neutralization curves generated against HIV-$1_{NL4/3}$, HIV-1_{JR-FL} and the murine leukemia virus (MLV) Env-pseudotyped virus control, for one animal immunized with gp120 (#842) and for one given SOSIP.R6 gp140 (#849), are highlighted in Fig. 2. At a 1:10 dilution, the SOSIP.R6 gp140 serum neutralized the HIV-1_{JR-FL} pseudovirus by 100%, the HIV-$1_{NL4/3}$ pseudovirus by 85% but the MLV pseudovirus by <50%. The gp120 serum neutralized the HIV-$1_{NL4/3}$ pseudovirus by 80% at 1:10 dilution but failed to neutralize either the HIV-1_{JR-FL} or the MLV pseudoviruses (<50%). The pattern of progressively increasing neutralization with decreasing serum dilution suggests the infectivity reduction is a specific process. Few of the test sera were able to cross-neutralize the 6 other Env subtype B HIV-1 strains in the panel, although 3 of 8 sera from animals given SOSIP.R6 gp140 and 2 of 4 sera from $gp140_{UNC}$-immunized animals had some activity against HIV-1_{BaL}, compared to 0 of 8 for the gp120-immunized animals. The reference HIV-1 positive plasma, N16, was broadly active against the test strains. None of the rabbit sera neutralized the control MLV Env-pseudotyped virus, indicating that non-specific inhibitory or toxic factors were not present in the test sera (Table 2).

Qualitative assessment of HIV-1 neutralization responses

The neutralization of HIV-1_{JR-FL} by sera from some of the rabbits immunized with SOSIP.R6 gp140 trimers in both the in-house Env-pseudotyped virus neutralization assay and the PhenoSense™ HIV Entry Assay, and the limited cross-reactivity of these sera against other primary viruses, are findings consistent with our earlier study (Beddows et al., 2005). To try to understand why the autologous virus was

Table 2
Assessment of cross-neutralizing activity in the PhenoSense HIV entry assay

Arm	DNA	Protein	Week	Animal	Neutralization of Env-pseudotyped HIV-1 or MLV by rabbit sera[a]									
					SF162	NL4/3	JR-FL	BaL	QH0692	JR-CSF	US93073	92HT593	QH0115	MLV
F	Wt	gp120	30	840	424	101	–	–	–	–	–	–	–	–
				841	289	81	–	–	–	–	–	–	–	–
				842	582	152	–	–	–	–	–	–	–	–
				843	786	31	–	–	–	–	–	–	–	–
G	UNC	UNC Trimer	30	844	1227	110	–	28	–	–	–	–	–	–
				856	461	79	–	–	–	–	–	–	–	–
				846	776	43	–	25	–	–	–	–	–	–
				847	97	72	–	–	–	–	–	–	–	–
H	SOSIP.R6	SOSIP.R6 Trimer	30	848	80	66	–	–	–	–	–	–	–	–
				849	271	89	257	–	25	–	–	–	–	–
				850	839	103	–	–	–	–	–	–	–	–
				851	1050	478	–	42	–	–	–	–	–	–
I	None	SOSIP.R6 Trimer	14	852	975	146	44	32	–	–	–	–	–	–
				853	330	60	156	–	–	–	–	–	–	–
				854	919	64	–	30	–	–	–	–	–	–
				855	191	62	–	–	–	–	–	–	–	–
M	None	gp120	14	1188	237	192	–	–	–	–	–	–	–	–
				1189	128	67	–	–	–	–	–	–	–	–
				1190	109	124	–	–	–	–	–	–	–	–
				1191	168	69	–	–	–	–	–	–	–	–
Reference HIV-1^+ plasma N16				Mean	3945	1071	60	282	66	190	54	79	–	–
				SD	244	166	4	117	7	11	5	43	NA	NA

[a] The reciprocal of the dilution which resulted in 50% neutralization of Env-pseudotyped HIV-1 on coreceptor-bearing U87.CD4 cells. A dash indicates that <50% neutralization was observed at the 1:25 dilution. Plasma (N16) was from an HIV-1-infected individual and was used as a reference standard to control for assay-to-assay variation (n=9 tests). MLV, murine leukemia virus Env-pseudotype; NA, not applicable.

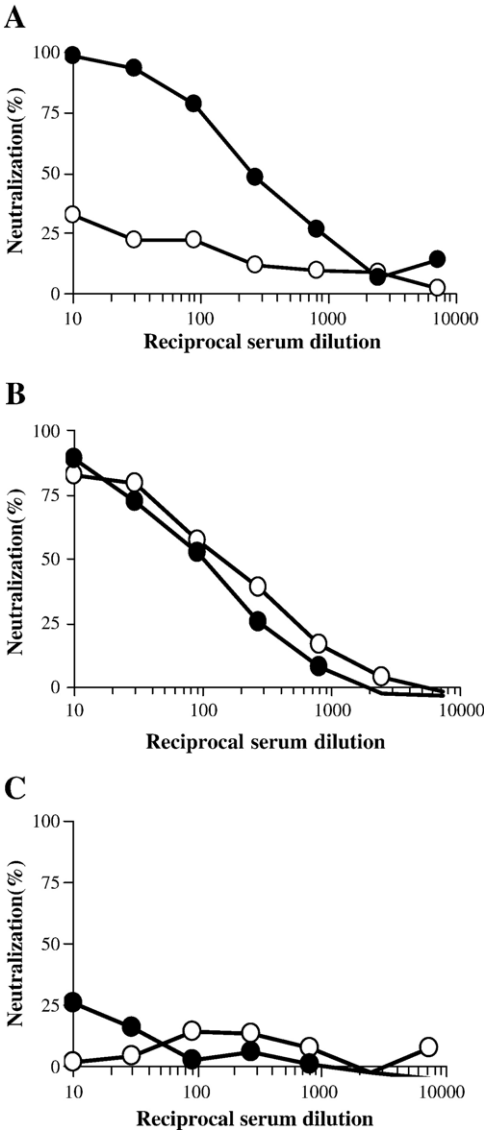

Fig. 2. Neutralization of JR-FL by SOSIP.R6 gp140 but not gp120 immunized animals. A week 30 serum sample from an animal immunized with gp140 Wt DNA/gp120 protein (Arm F, #842, open symbol) was compared with a serum sample from an animal immunized with SOSIP.R6 gp140 DNA/SOSIP.R6 gp140 (Arm H, #849, closed symbol) in the PhenoSense™ HIV Entry Assay. Neutralization curves are shown for each serum against (A) HIV-1_{JR-FL}, (B) HIV-$1_{NL4/3}$ and (C) murine leukemia virus (MLV) Env-pseudotyped viruses.

neutralized, but most heterologous strains were not, we analyzed the antibody specificities present in the most potent serum samples.

To do so, we first purified the immunoglobulin (Ig) fractions from serum samples from two animals primed with DNA then boosted with Env protein (Arm F, #841; Arm H, #849), and from two immunized with protein only (Arm I, #853; Arm M, #1191). Animals #841 and #1191 had received gp120, the other two (#849 and #853) were immunized with SOSIP.R6 gp140. Comparing the unfractionated sera and the purified Ig showed the neutralizing activity against HIV-1_{JR-FL} and HIV-1_{MN} was Ig-mediated (Table 3). It was not due therefore to chemokines or other serum factors that could interfere with infection of the target cells and give a false indication of the presence of NAbs in the test sera.

We next wished to determine whether neutralization of HIV-1_{JR-FL} was caused by antibodies directed to gp120 and, within gp120, to the V3 region. To do this, we incubated selected sera with bead-immobilized BSA (negative control), $gp120_{JR-FL}$ or a cyclic $V3_{JR-FL}$ peptide (Beddows et al., 2005). To validate the depletion procedure, we tested whether the bead-immobilized protein or peptide could remove the anti-$V3_{JR-FL}$ MAb PA1 or the anti-gp120 MAb b12, after these MAbs were spiked into a pool of pre-immune rabbit sera (Figs. 3A and 3B). Both the gp120- and the V3 peptide-beads depleted the PA1 MAb from the test serum by >99%, and the gp120-beads removed a similar proportion of the added b12 MAb. As expected, the V3 peptide-beads did not remove any of the b12 MAb, and neither MAb was depleted by the control BSA-beads.

When sera from selected gp120 (#841 and #1188)- and SOSIP.R6 gp140 (#849 and #853)-immunized rabbits were similarly treated, the gp120-beads lowered the anti-gp120 binding antibody titers by 50-fold (98.3±0.9% depletion) in each case (Fig. 3C). The depletion procedure was therefore capable of removing most of the antigen-induced anti-gp120 antibodies from the sera. Only a minor fraction of the total anti-gp120 antibodies in the sera was directed towards the V3 region of gp120 because the V3 peptide-beads had little effect on the overall anti-gp120 titers (Fig. 3C).

The untreated serum and the BSA-, V3 peptide- and gp120-depleted serum fractions were then tested for neutralizing activity against HIV-1_{JR-FL} and HIV-1_{MN} Env-pseudotyped viruses (Table 4). As expected, the sera from the gp120-immunized rabbits (#841 and #1188) were unable to neutralize HIV-1_{JR-FL}, either before or after the bead-depletion procedure. The HIV-1_{JR-FL} neutralizing activity of sera from the SOSIP.R6 gp140-immunized rabbits (#849 and #853) was slightly reduced (~25%) by incubation with the V3 peptide-beads but was substantially, yet incompletely, reduced (~60%) by the gp120-beads. Neutralization of HIV-1_{MN} was only slightly lowered by treatment of the sera with the V3 peptide-beads but almost completely eliminated by the gp120-beads (Table 4). This observation is consistent with the conclusion that V3 antibodies constitute only a minor fraction of the total anti-gp120 antibodies present in the sera overall (Fig. 3C).

Although the anti-gp120 binding (Fig. 3C) and HIV-1_{MN} neutralizing activities (Table 4) were substantially (~99% and ~90%, respectively) depleted when the sera from SOSIP.R6 gp140-immunized rabbits were incubated with the gp120-beads, this procedure reduced the HIV-1_{JR-FL} neutralizing activities (Table 4) of sera from animals #849 and #853 by only 53% and 64%, respectively. For comparison, two sera from a previous study (Beddows et al., 2005) were also included in this experiment; the depletion profiles obtained were similar to those obtained before (Table 4).

To start to define the neutralizing antibody specificities present within the more active rabbit sera, we investigated whether NAbs directed towards the V1/V2 loops of gp120 or the membrane-proximal external region (MPER) of gp41 had been

Table 3
Neutralization of JR-FL and MN Env-pseudotyped HIV-1 by rabbit immune sera is mediated by Ig

Arm	DNA	Protein	Animal	Neutralization of Env-pseudotyped HIV-1 by serum or Ig [a]							
				JR-FL				MN			
				Serum		Ig		Serum		Ig	
				50%	90%	50%	90%	50%	90%	50%	90%
F	Wt	gp120	841	–	–	–	–	>160	25	112	17
H	SOSIP.R6	SOSIP.R6 Trimer	849	81	16	99	15	>160	41	148	29
I	None	SOSIP.R6 Trimer	853	53	12	38	–	125	32	144	19
M	None	gp120	1191	–	–	–	–	>160	27	>160	15

[a] The reciprocal of the dilution of sera or Ig preparation which resulted in 50% or 90% neutralization of Env-pseudotyped HIV-1 on coreceptor-bearing U87.CD4 cells (representative of two independent experiments). A dash indicates that <50% neutralization was observed at the 1:10 dilution. Sera was taken from bleeds collected at week 14 (Arms I and M) or 30 (Arms F and H).

elicited by our immunization procedures. Sera from both gp120-immunized animals (#841 and #1188) and SOSIP gp140-immunized animals (#849 and #853) were tested. In peptide competition assays, neither a V1 peptide nor a V2 peptide was able to reduce the neutralizing activity of any of these sera against either of the HIV-$1_{JR\text{-}FL}$ and the HIV-1_{MN} Env-pseudotyped viruses; adding both peptides together was also without effect (Fig. 4A, and data not shown). For an assessment of neutralizing activity against the gp41 MPER, we used chimeric SIVmac239 viruses expressing the MPER-located epitopes for the human NAbs 2F5 and 4E10 (Yuste et al., 2004). Neither the chimeric viruses SIV239-2F5 and SIV239-4E10 nor the parental SIVmac239 virus were neutralized by any of the sera tested (Fig. 4B and data not shown). Taken together, these studies suggest that little or no neutralizing antibody activity in these rabbit sera was directed against the V1/V2 region of gp120 or the MPER region of gp41.

Discussion

We previously reported on the immunogenicity of a cleaved, disulfide-stabilized trimeric gp140 (SOSIP.R6) protein derived from the primary virus-like, neutralization-resistant strain, HIV-$1_{JR\text{-}FL}$ (Beddows et al., 2005). In this follow-up study, we have directly compared the immunogenicity of monomeric gp120, cleavage-defective trimeric gp140 ($gp140_{UNC}$) and SOSIP.R6 gp140 proteins, all based on HIV-$1_{JR\text{-}FL}$.

As previously, we compared the immune responses in rabbits that received Env trimers or gp120 monomers, with or without prior priming with a mammalian expression vector encoding the homologous codon-optimized *env* DNA administered through an *in vivo* electroporation procedure (Mathiesen, 1999; Widera et al., 2000). In contrast to our earlier study (Beddows et al., 2005), the DNA priming procedure did not improve the overall gp120 binding or HIV-$1_{JR\text{-}FL}$ Env-pseudotyped virus neutralization titers, though it did again increase the numbers of animals responding, at least initially. In a recent study of a DNA prime, protein boost protocol intended to explore the immunogenicity of gp120 proteins from multiple genetic subtypes, the sensitive strains MN, SF162 and NL4/3 were neutralized in the PhenoSense™ HIV Entry Assay at titers significantly higher than those we describe here. However, as in our own study, the more resistant strains were neutralized only sporadically (Wang et al., 2006). The reasons why the two protocols yielded different outcomes in respect of the neutralization-sensitive viruses are presently under investigation.

All twelve rabbits in the DNA-prime, protein-boost Arms and all eight rabbits in the protein only immunization Arms generated antibodies capable of neutralizing HIV-1_{MN} to >90%. HIV-1_{MN} neutralization titers usually peaked following three protein immunizations (week 30 for Arms F, G and H; week 14 for Arms I and M). Overall, there was no significant difference in neutralization of HIV-1_{MN} by sera from animals immunized with SOSIP.R6 and from ones that were not.

Following two DNA and two protein immunizations (week 22), all four SOSIP.R6 gp140 immunized animals produced antibodies capable of neutralizing HIV-$1_{JR\text{-}FL}$ by >50% in our in-house Env-pseudotype assay, albeit to low titer. In comparison, sera from one and two of the four animals, respectively, that were immunized with monomeric gp120 or $gp140_{UNC}$ under similar conditions, could neutralize HIV-$1_{JR\text{-}FL}$ by >50%. In contrast to animals immunized with gp120 or $gp140_{UNC}$, two of the SOSIP.R6 gp140-immunized animals, one receiving a DNA-prime (Arm H, #849), one given protein-only (Arm I, #853), generated NAb responses against HIV-$1_{JR\text{-}FL}$ that were both strong and sustained.

Sera from all animals were independently tested, under contract, using the PhenoSense™ HIV Entry Assay. This assay confirmed that neutralizing antibodies against HIV-$1_{JR\text{-}FL}$ had been induced in three of the eight animals immunized with SOSIP.R6 gp140 (#849, #852, #853; Table 2). The two sera most strongly active against HIV-$1_{JR\text{-}FL}$ in our in-house Env-pseudotype assay (#849 and #853) were also the most effective against the same virus in the PhenoSense™ HIV Entry Assay. Sera from none of the animals immunized with gp120 or $gp140_{UNC}$ proteins neutralized HIV-$1_{JR\text{-}FL}$ in the PhenoSense™ HIV Entry Assay. In the latter assay, neutralization of the hypersensitive strains HIV-$1_{NL4/3}$ and HIV-1_{SF162} was more consistently achieved, and at high titer, but these responses were seen irrespective of the nature of the immunogen. In contrast,

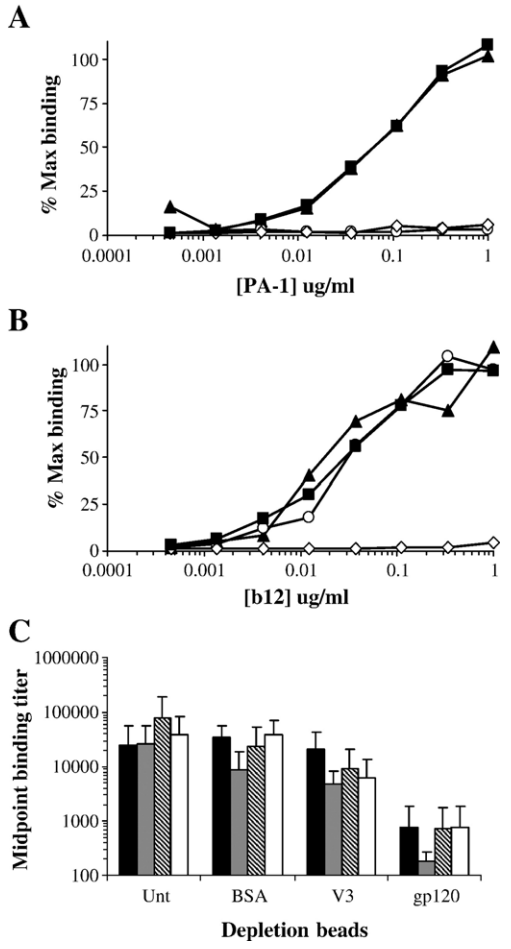

Fig. 3. Depletion of gp120 binding antibodies by gp120 and V3 peptides. CNBr-Sepharose beads with coupled BSA (filled triangles), cyclic $V3_{JR\text{-}FL}$ peptide (open circles) or $gp120_{JR\text{-}FL}$ (open diamonds) were used to deplete a rabbit pre-immune serum pool spiked with (A) the V3-specific MAb PA1, or (B) the anti-gp120 MAb b12, before testing in a gp120 ELISA. The non-depleted serum was also assayed (filled squares). (C) Sera from rabbits in the DNA-primed Arms F (#841; filled bars) and H (#849; grey bars), and the non-primed groups I (#853; hatched bars) and M (#1188; open bars) were left untreated ('Unt') or depleted using BSA-, V3- or gp120-coupled beads before determination of midpoint binding titers against gp120. The data shown are representative of the results of 2–3 individual experiments.

there was little cross-neutralization of the more resistant strains in the test panel, although three sera from SOSIP.R6 gp140-immunized animals and two sera from rabbits given $gp140_{UNC}$ did weakly neutralize $HIV\text{-}1_{BaL}$. Overall, the outcome of the study is qualitatively similar to our earlier one using SOSIP.R6 gp140 (Beddows et al., 2005).

Various depletion experiments showed that anti-gp120 antibodies were almost entirely responsible for neutralization of $HIV\text{-}1_{MN}$ by the test sera, with little contribution from anti-V3 antibodies. In addition, peptide-competition assays suggest that little or no neutralizing activity against $HIV\text{-}1_{JR\text{-}FL}$ or $HIV\text{-}1_{MN}$ could be attributed to antibodies directed against the V1/V2 region of gp120. In contrast, V1-directed antibodies did contribute significantly to the neutralizing activity of sera generated by immunizing guinea pigs with trimeric YU-2 gp140 proteins (Li et al., 2006). The reasons for the differences between this outcome and ours could lie with the Env proteins used, the adjuvants or the test animals. We did observe that a significant fraction of the neutralizing response to $HIV\text{-}1_{JR\text{-}FL}$ in our test sera was attributable to antibodies that were not depleted from the sera by incubation with monomeric gp120. This neutralizing activity could reflect the presence of antibodies against gp120 epitopes that are not well exposed on monomeric gp120 (for example, CD4-induced epitopes), of antibodies to conformational epitopes that are not present on the monomeric form of gp120 (for example, trimer-specific epitopes or gp120 epitopes affected by the presence of gp41), or of antibodies directed at epitopes that are simply not present on any form of gp120 (for example, gp41 epitopes). Of note, of course, is that the SOSIP.R6 gp140 immunogen does contain the gp41 ectodomain. However, we used SIVmac239 chimeric viruses to perform a preliminary investigation of whether neutralizing antibodies directed against the MPER region of gp41 were present (i.e., antibodies with properties similar to human MAbs 2F5 and 4E10) (Yuste et al., 2004). We found no evidence for the presence of such antibodies in the sera we tested. Additional studies of antibody specificity, isotype and avidity will be important to gain a better understanding of how sera from Env-immunized animals does, occasionally, neutralize representative primary viruses at respectable titers (Bower et al., 2006; Burton et al., 2004).

Although we have been able to generate antibodies with activity against the homologous strain using the SOSIP.R6 gp140 protein, at least two significant obstacles remain to be overcome. Firstly, the induction of such antibodies in rabbits is variable; not every animal immunized in an identical manner responds in the same way. This phenomenon has also been reported by others (Grundner et al., 2004) and it may be attributable to individual variation in the ability to induce effective T-helper responses for B-cell maturation (Grundner et al., 2004). Additional research on how best to present Env trimers to the immune system, and how to induce T-helper responses, may yield dividends. The second issue relates to the difficulty in inducing cross-neutralizing responses to representative primary isolates both within and across the genetic subtypes. Even within subtype B, our success rate was limited in this and our previous study (Beddows et al., 2005). A lack of breadth in the neutralizing antibody response is a common observation in studies of the immune response to various $gp140_{UNC}$ proteins (Barnett et al., 2001; Bower et al., 2004; Kim et al., 2005; Yang et al., 2001). Overcoming this significant obstacle may depend on the outcome of further rounds of protein engineering guided by additional information on the structure of the native Env trimer and its sites for the binding of neutralizing antibodies (Burton et al., 2004).

Materials and methods

Design, expression and purification of Env proteins and plasmids used for immunizations

All plasmids and Env glycoproteins were derived from the genetic background of $HIV\text{-}1_{JR\text{-}FL}$ a subtype B, R5 primary

Table 4
Neutralization of Env-pseudotyped HIV-1 following depletion on BSA, V3 peptide or gp120-conjugated beads

Arm	DNA	Protein	Animal	Neutralization of Env-pseudotyped HIV-1 by rabbit antisera[a]							
				JR-FL				MN			
				Untreated	BSA	V3	gp120	Untreated	BSA	V3	gp120
F	Wt	gp120	841	–	–	–	–	144 (23)	145 (29)	120 (46)	31 (33)
H	SOSIP.R6	SOSIP.R6 Trimer	849	104 (26)	93 (21)	78 (16)	49 (10)	158 (5)	150 (21)	141 (36)	20 (7)
I	None	SOSIP.R6 Trimer	853	42 (11)	36 (2)	33 (3)	15 (5)	144 (24)	>160 (NA)	81 (70)	11 (3)
M	None	gp120	1188	–	–	–	–	>160 (NA)	>160 (NA)	106 (58)	18 (8)
	SOS.R6	SOSIP.R6 Trimer	241	88	98	67	12	128	103	37	–
	Wt	gp120	5695-3	–	–	–	–	134	146	63	21

[a] Dilution of rabbit antisera before (untreated) or after depletion on BSA, cyclic V3 peptide or gp120-coupled cyanogen bromide–Sepharose beads which resulted in 50% neutralization of Env-pseudotyped HIV-1 on coreceptor-bearing U87.CD4 cells. Values represent the mean of three to four independent experiments (SD in parentheses) for animals 841, 849, 853 and 1188 or the mean of two experiments for 241 and 5695-3 (sera taken from previous study; Beddows et al., 2005). A dash indicates that <50% neutralization was observed at the 1:10 dilution. NA, not applicable as all values >160.

isolate (Haas et al., 1996). All Env proteins were expressed from the high level mammalian expression vector, pPPI4, using codon-optimized genes, as described previously (Binley et al., 2000, 2002; Sanders et al., 2002; Schulke et al., 2002). The $gp140_{UNC}$ construct was made by introducing two amino acid substitutions (R508I/K510G) into the natural Env cleavage site (Dubay et al., 1995), by site-directed mutagenesis (Stratagene, La Jolla, CA). This alteration, from REKR to IEGR, prevents proteolytic cleavage by cellular endoproteases (Schulke et al., 2002). The sequence integrity of all clones was confirmed prior to use. The expression and purification of the Env constructs used in this study have been described in detail (Beddows et al., 2005).

Immunization protocol

The care, maintenance and immunization of rabbits were carried out by Aldevron LLC (Fargo, ND) under contract, as previously described (Beddows et al., 2005; Mathiesen, 1999; Widera et al., 2000). The rabbits were DNA-immunized using an electroporation procedure at weeks 0 and 4, followed by boosting immunizations with 30 μg of a JR-FL Env protein, formulated in QS-21 adjuvant (Antigenics Inc., New York, NY) (Fig. 1A, Arms F, G and H). For the 'protein only' immunization regimens (Arms I and M), rabbits were immunized with 30 μg of the indicated Env protein at weeks 0 and 4, followed by further protein immunizations at the same times as the DNA-primed animals. Thus, all the rabbits received the same total number of immunizations, administered at the same times, differing only in the mode of application for the first two immunizations (DNA vs. protein). From week 28, instead of QS-21, the various Env proteins were formulated in Ribi adjuvant. For immunization, Env proteins were administered by injection into multiple anatomical sites (50 μl intradermal at six sites, 200 μl intramuscular, in each hind leg and 300 μl subcutaneous in the neck region; a total of 1 ml), as described elsewhere (VanCott et al., 1997). The timing of each bleed and dose and the constructs used for each study animal are outlined in Fig. 1A.

ELISA for antibody binding to monomeric gp120

The binding of rabbit serum antibodies or MAbs to monomeric gp120 immobilized on plastic via the C-terminal antibody D7324 was measured by enzyme-linked immunosorbent assay (ELISA) using the appropriate anti-species alkaline phosphatase conjugate and the AMPAK colorimetric detection system (Dako Diagnostics), as previously described (Moore et al., 1996; Moore and Sodroski, 1996). Midpoint binding titers were estimated by interpolation.

HIV-1 neutralization assays

Env genes expressing full-length gp160 proteins were derived from the HIV-1 isolates JR-FL (obtained from Dr. T. Dragic, Albert Einstein College of Medicine, New York) and MN (Beddows et al., 2005). The generation of Env-pseudotyped virus stocks and the use of the engineered HIV-1 coreceptor bearing cell lines U87.CD4.CCR5 and U87.CD4.CXCR4 for Env-pseudotyped virus infection and neutralization, have been described previously (Beddows et al., 2005; Gordon et al., 1999; Herrera et al., 2003).

The potential for peptides derived from the V1 (KDVNATNTTNDSEGTMERGEIKN) or V2 (NITTSIRDEVQKEYALFYKLDVVPIDNNNT) regions of JR-FL to reduce the neutralizing capacity of selected rabbit sera was assessed by competitive inhibition. Neutralization assays were performed as described above, but with minor modifications at the initial virus-serum incubation stage. Thus, peptides (50 μg/ml) were incubated with a 1:5 dilution of rabbit serum for 30 min at room temperature followed by the addition of $HIV\text{-}1_{JR\text{-}FL}$ or $HIV\text{-}1_{MN}$ Env-pseudotyped luciferase reporter viruses for 1 h at 37 °C. The mixture was then transferred to wells containing the appropriate coreceptor bearing U87.CD4 cells.

The neutralization properties of selected pre- and post-immune sera were also evaluated, under contract, by Monogram Inc. (South San Francisco, CA). The automated PhenoSense™ HIV Entry Assay measures the infectivity of

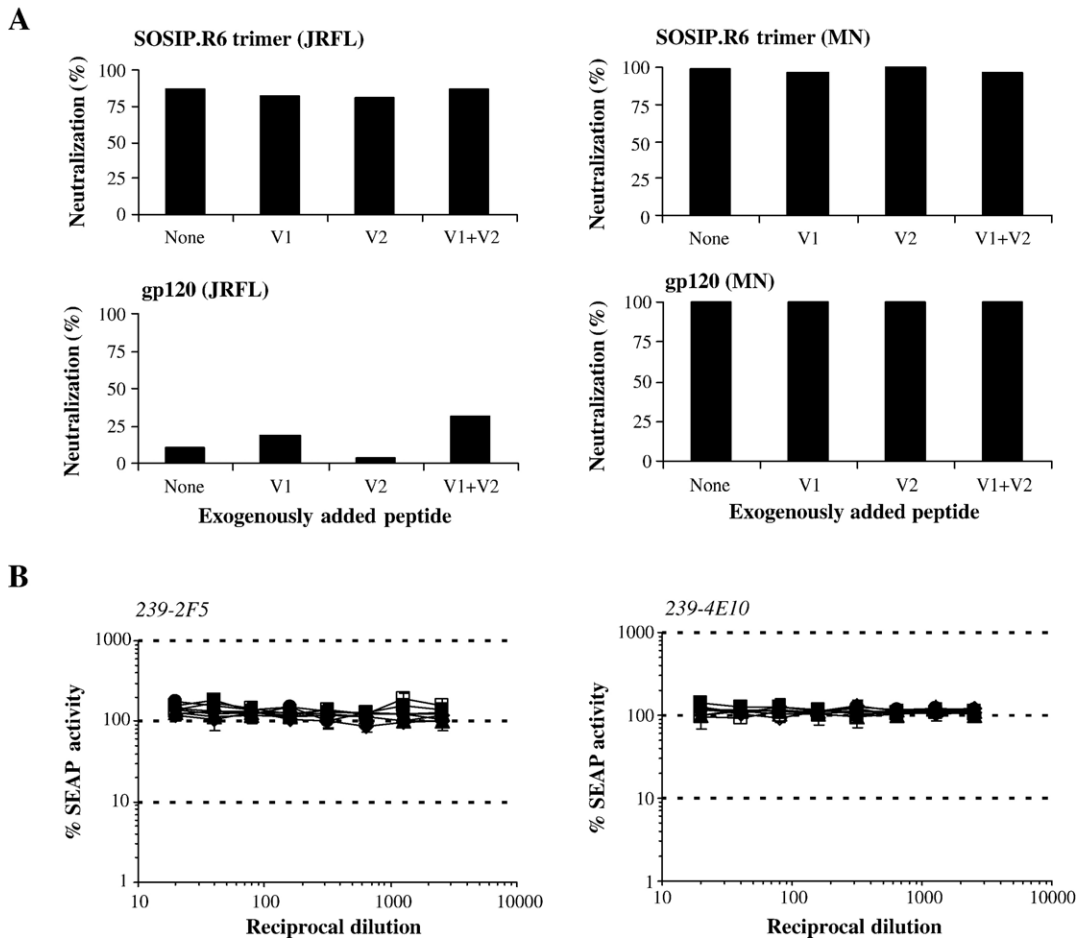

Fig. 4. Exploration of neutralizing antibody specificity to V1V2 and gp41 MPER epitopes. (A) Neutralization of HIV-1_{JR-FL} (left panels) and HIV-1_{MN} (right panels) by selected post-immune rabbit sera in the presence or absence of exogenously added V1 and V2 peptides. Week 14 serum samples were tested from the subset of rabbits that were immunized with either SOSIP.R6 trimer (#853; top panels) or gp120 (#1188; bottom panels), in the absence of DNA priming. (B) Neutralization of SIVmac239 chimeric viruses expressing epitopes for the human NAbs 2F5 (239-2F5) and 4E10 (239-4E10). Pre-immune (filled symbols) and post-immune (open symbols) rabbit sera were tested at the indicated dilutions. The tested samples were pre-immune and week 30 bleeds from rabbits immunized using a DNA-prime, protein boost protocol (gp120, #841, diamond; SOSIP.R6 gp140, #849, triangle) or pre-immune and week 14 bleeds from rabbits immunized in a protein-only procedure (gp120, #1188, square; SOSIP.R6 gp140, #853, circle). The data points represent the mean (±SD) percentage neutralization compared to control (no antibody) and were derived from triplicate determinations within a single experiment.

Env-complemented, luciferase-encoding pseudoviruses in a single-cycle assay using CCR5/CXCR4-expressing U87.CD4 cells (Binley et al., 2004; Richman et al., 2003).

The neutralizing antibody specificities of selected pre- and post-immune sera were also evaluated against SIVmac239 virus engineered to express the epitopes for HIV-1 NAbs 2F5 and 4E10, as described elsewhere (Yuste et al., 2004).

Immunoglobulin (Ig) purification

Total Ig was purified from final bleed rabbit sera using Protein A Sepharose (1 ml column; GE Healthcare Biosciences, Piscataway, NJ), according to the manufacturer's instructions. Briefly, rabbit sera were diluted to a volume of 12 ml in phosphate-buffered saline (PBS) and added to the column at a flow rate of 0.5 ml/min. The column was then washed with twenty column volumes of buffer before elution with 0.1 M glycine. The eluted fractions were immediately neutralized with 1 M Tris (pH 9). The presence of Ig in the fractions was then assessed using reduced SDS gels. The fractions containing Ig were pooled and desalted into PBS using a PD-10 Desalting Column (GE Healthcare Biosciences). Amicon Ultra Centrifugal Filters with a 30-kDa molecular weight cutoff (Millipore, Billerica, MA) were then used to concentrate the eluted material to ~80% of the initial serum volume. Ig concentrations were estimated using a Rabbit IgG Assay Kit (Pierce, Inc., Rockford, IL) and anti-gp120 titers were determined in an ELISA. The purified Ig samples were then further adjusted with PBS to be comparable with the Ig concentration in the unfractionated rabbit serum, to facilitate direct comparisons between the serum and the purified Ig in subsequent neutralization assays.

Antibody depletion from sera by gp120 or V3 peptides

Cyanogen bromide (CNBr)-activated Sepharose 4B beads were used to couple monomeric $gp120_{JR-FL}$, cyclic $V3_{JR-FL}$ peptide Ac-CTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHC-NH2) or

BSA, according to the manufacturer's instructions (Amersham Biosciences). The depletion of rabbit antibodies from immune sera using these beads was performed as previously described (Beddows et al., 2005).

Statistical analyses

Prism (Graphpad) was used for statistical analysis. The titers did not consistently pass the D'Agostino-Pearson omnibus normality test when pooled into groups according to the immunization regimen. Differences were therefore analyzed non-parametrically (Mann–Whitney test).

Acknowledgments

We are grateful to Anthony Villa, Trinity Zang, Irene Vishnefsky (Progenics), Natasha Popli, Juan Zurita and Kathryn David (Weill Medical College) for their excellent technical assistance. The earlier or current contributions of Drs. Mika Vesanen, James Binley, Min Lu, Norbert Schulke and Jason Gardner to the overall development of this project are appreciated. We thank Jessica Harms (Aldevron Inc.) for carrying out the rabbit immunization studies, Dr. Terri Wrin (Monogram Inc.) for the PhenoSense™ HIV Entry Assays, and Dr. Tanya Dragic for the full-length gp160 JR-FL Env plasmid used in this study. We are grateful to Drs. Ralph Pantophlet and Dennis Burton for helpful discussion and in particular the generous donation of V1 and V2 peptides used in this study.

HIV vaccine research at Progenics is supported by NIH contract N01 AI 30030, at the Weill Medical College by NIH grants and contracts AI 30030, AI 36082 and AI 45463 and by the International AIDS Vaccine Initiative. The Department of Microbiology and Immunology gratefully acknowledges the support of the William Randolph Hearst Foundation.

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