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Analysis of the neutralizing antibody response elicited in rabbits by repeated inoculation with trimeric HIV-1 envelope glycoproteins

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Abstract

The elicitation of broadly neutralizing antibodies directed against the human immunodeficiency virus type 1 (HIV-1) envelope glycoproteins, gp120 and gp41, remains a major challenge. Attempts to utilize monomeric gp120 as an immunogen to elicit high titers of neutralizing antibodies have been disappointing. Envelope glycoprotein constructs that better reflect the trimeric structure of the functional envelope spike have exhibited improved immunogenicity compared with monomeric gp120. We have described soluble gp140 ectodomain constructs with a heterologous trimerization motif; these have previously been shown to elicit antibodies in mice that were able to neutralize a number of HIV-1 isolates, among them primary isolate viruses. Recently, solid-phase proteoliposomes retaining the envelope glycoproteins as trimeric spikes in a physiologic membrane setting have been described. Here, we compare the immunogenic properties of these two trimeric envelope glycoprotein formulations and monomeric gp120 in rabbits. Both trimeric envelope glycoprotein preparations generated neutralizing antibodies more effectively than gp120. In contrast to monomeric gp120, the trimeric envelope glycoproteins elicited neutralizing antibodies with some breadth of neutralization. Furthermore, repeated boosting with the soluble trimeric formulations resulted in an increase in potency that allowed neutralization of a subset of neutralization-resistant HIV-1 primary isolates. We demonstrate that the neutralization is concentration-dependent, is mediated by serum IgG and that the major portion of the neutralizing activity is not directed against the gp120 V3 loop. Thus, mimics of the trimeric envelope glycoprotein spike described here elicit HIV-1-neutralizing antibodies that could contribute to a protective immune response and provide platforms for further modifications to improve the efficiency of this process. Published by Elsevier Inc.

Keywords: Antibody; Inoculation; Glycoprotein

Introduction

Neutralizing antibodies are a major line of defense against many viral infections. Protection of vaccinated subjects from viral infection in many cases principally relies on preexisting neutralizing antibodies (Burton et al., 2001). For HIV-1, the elicitation of neutralizing antibodies with the potency to inhibit primary viral isolates and the breadth to recognize different viral strains is inefficient. Vaccination strategies that elicit high levels of broadly reactive neutralizing antibodies would be extremely desirable.

Evidence for a protective role of neutralizing antibodies against HIV-1 infection comes primarily from passive transfer studies. A number of neutralizing antibodies and combinations thereof have been examined for their ability to protect rhesus macaques or chimpanzees from viral challenge. Neutralizing antibodies administered prior to viral

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challenge protected the majority of subject animals from infection with HIV-1, simian immunodeficiency virus (SIV), or a chimeric simian-human immunodeficiency virus (SHIV) (Berman et al., 1990; Mascola et al., 1999, 2000; Parren et al., 2001). In animals that did become infected, peak viremia was diminished, which is a prognostic indicator of slow disease progression. In a number of vaccinated animals, the levels of vaccine-induced neutralizing antibodies correlated with protection and a lower virus load (Berman et al., 1990; Bruck et al., 1994). In some HIV-1-infected long-term nonprogressors, exceptionally high titers of broadly neutralizing antibodies have been detected (Pilgrim et al., 1997).

As a result of HIV-1 infection, a vigorous antiviral immune response occurs. Despite this immune response, progression to acquired immunodeficiency syndrome (AIDS) generally ensues after many years of infection. The selective pressure exerted by the humoral response is highlighted by the difference in neutralization sensitivity that can be observed between T-cell-line adapted (TCLA) isolates and primary isolates (Daar et al., 1990; Mascola et al., 1994; Moore et al., 1992; Wrin et al., 1995; Zhang et al., 1997).

The major targets for HIV-1-neutralizing antibodies are the envelope glycoproteins, gp120 and gp41. The preponderance of evidence indicates that these envelope glycoproteins are organized into trimeric spikes on the viral surface and, as such, are the only viral proteins accessible to the humoral immune system on virions. The complex organization of gp41 and gp120 as trimers of heterodimers has important implications for immune diversion. It has been suggested that in vivo, disassembled envelope glycoprotein spikes or viral debris may be the predominant immunogen (Parren et al., 1997). The interaction of gp41 and gp120 is noncovalent, thought to be labile, and allows gp120 to be shed from the functional spike. Upon disassembly of the glycoprotein complexes, many surfaces that are occluded on the functional trimer are presented to the immune system. Both gp120 and gp41 may also change conformation upon dissociation. As a consequence, many antibodies elicited in vivo are raised against these decoy epitopes and do not bind the functional envelope glycoprotein complex (reviewed in (Wyatt and Sodroski, 1998)). It has also been suggested that monomeric gp120 in its free state is a very flexible molecule (Myszka et al., 2000). HIV-1 gp120 may sample many conformations and present a multitude of different conformations to the humoral immune system, many of which may be irrelevant for virus neutralization.

The anticipation of similar immunogenic properties of the native trimeric HIV-1 envelope glycoprotein complex and monomeric gp120 and the relative ease of production of gp120 led to the utilization of gp120 as an immunogen in many animal and human trials. To date, all gp120-based immunogens have been disappointing in their ability to elicit broadly neutralizing antibodies (Barnett et al., 1997; Belshe et al., 1998; Berman et al., 1990; Connor et al., 1998; Mascola et al., 1996; Wrin et al., 1995). Consistent with this, gp120 has not demonstrated efficacy in eliciting protection against HIV-1 (Vaxgen trial announcement).

An understanding of the mechanistic basis for the poor immunogenic potential of gp120 has led to the design of immunogens based on the trimeric envelope glycoprotein complex. Neutralization by antibodies has in some cases been shown to correlate with binding to envelope glycoprotein complexes, but not to monomeric gp120. This suggests that, conversely, neutralizing antibodies might be better elicited by moieties that mimic the functional HIV-1 envelope glycoprotein trimer (Fouts et al., 1997; Roben et al., 1994; Sattentau and Moore, 1995; Sullivan et al., 1995). Several approaches have been taken to stabilize the labile HIV-1 envelope glycoprotein complexes. A number of constructs consisting of the gp120 and gp41 ectodomains have been generated (Earl et al., 1994; Farzan et al., 1998; Yang et al., 2000a, 2001, 2002). In most of these soluble ectodomain constructs the gp120–gp41 cleavage site was modified to prevent shedding of gp120 from the complex. In some cases, soluble HIV-1 envelope glycoprotein ectodomain constructs have been made with intact gp120–gp41 cleavage sites by introducing disulfide bonds between gp120 and gp41 subunits (Binley et al., 2000). Even with disruption of gp120–gp41 proteolytic cleavage, soluble HIV-1 envelope glycoproteins assemble into several oligomeric states, including dimers and tetramers. The C-terminal introduction of heterologous trimerization domains dramatically improves the stability and homogeneity of soluble HIV-1 envelope glycoprotein trimers (Yang et al., 2000b, 2002). One soluble ectodomain construct contains a heterologous trimerization domain derived from the yeast transcription factor GCN4 (Yang et al., 2000b) appended to the envelope glycoprotein from the highly neutralization-resistant primary isolate YU2 (Li et al., 1991). This soluble ectodomain construct, YU2 gp140 (−/GCN4), has been shown to elicit neutralizing antibodies with some breadth in mice (Yang et al., 2001). The amount of serum obtained in these experiments, however, limited the testing of a broad panel of HIV-1 isolates and characterization of the neutralizing activity in these sera.

In an attempt to present trimeric HIV-1 envelope glycoprotein complexes in a more natural context, we have generated solid-phase proteoliposomes (PLs) containing cleavage-defective HIV-1 envelope glycoproteins derived from the primary isolate YU2 (EnvPLs) (Grundner et al., 2002). The EnvPLs allow for the simultaneous capture and purification of envelope glycoproteins on a solid support and the reconstitution of a lipid membrane. The membrane-anchored HIV-1 envelope glycoproteins in PLs antigenically resemble the envelope glycoproteins expressed on the surface of cells (Grundner et al., 2002). Here, we sought to compare the immunogenic properties of YU2 gp120, YU2 gp140 (−/GCN4), and EnvPLs in rabbits. In this study, the soluble YU2 gp140 (−/GCN4) trimers elicited

neutralizing antibodies that were able to neutralize the YU2 virus and heterologous primary isolates. The EnvPLs elicited broadly neutralizing antibodies better than gp120, although this activity was less potent and broad than that seen in the sera from animals immunized with the YU2 gp140 (−/GCN4) proteins. In this study, we also analyze the neutralizing capacity of the serum IgG from the gp140 (−/GCN4) and EnvPL-immunized rabbits and begin the initial mapping of the neutralization specificity. Interestingly, in most animals, the majority of the neutralizing antibody response elicited by these trimeric immunogens was not directed against the V3 loop of either homologous or heterologous virus. These findings support the hypothesis that trimeric envelope glycoprotein formulations represent an advance over gp120-based immunogens in eliciting neutralizing antibodies.

Results

The immunogenicity of YU2 gp120, gp140 (−/GCN4) and EnvPLs was tested in rabbits. The molecular clone YU2 was chosen for the preparation of EnvPLs for immunization studies because it exhibits one of the most neutralization-resistant phenotypes. This molecular clone was not passaged in tissue culture prior to molecular cloning (Li et al., 1991) and is relatively resistant to neutralization by antibodies and soluble forms of the CD4 receptor. We hypothesized that breadth of neutralization would most likely be exhibited by antibodies that are raised against neutralization-resistant molecular clones, with neutralization extending from there to more neutralization-sensitive clones. This expectation is supported by a previous study in mice, which demonstrated that soluble trimers based on the YU2 envelope glycoprotein were more effective at eliciting neutralizing antibodies against primary HIV-1 isolates than trimers based on the TCLA HXBc2 envelope glycoprotein (Yang et al., 2001).

The immunization protocol consisted of a priming inoculation followed by subsequent inoculations at 2, 6, 10, 22, and 34 weeks after priming (Fig. 1). After the 4th inoculation, sera were collected 7 to 10 days post-injection and were assessed for binding to gp120 by ELISA and subsequently for HIV-1 neutralizing reactivity. All groups except group E generated high anti-gp120 IgG antibody titers with endpoint titers of 12,500–62,500 (see ELISA data in Table 1). The rabbits immunized sequentially with EnvPLs containing envelope glycoproteins from different HIV-1 strains (group E) exhibited only low or no reactivity with YU2 gp120. One of the animals (rabbit 19) in group C also made a poor response against YU2 gp120 (Table 1).

Anti-HIV-1-neutralizing activity of sera after four inoculations

To assess neutralizing activity in the sera, 1:5 dilutions of pre-immune and immune serum obtained after four inoculations were tested in a single-round neutralization assay (Mascola et al., 2002b) against the clade B molecular clones HXBc2, SF162, BaL, 89.6, ADA, and YU2 (Table 1). It is worth noting that although a 1:5 dilution of serum is the lowest serum dilution practically achievable in this assay, it is still 5-fold less than the serum concentration present in vivo. We purposely selected a set of viruses representing a range of neutralization phenotypes, with increasing resistance from the TCLA isolate HXBc2 to the primary isolate YU2. The relative resistance of these isolates is reflected in the necessary amount of the monoclonal antibodies 2F5 and 2G12 or a mixture of IgG purified from sera of HIV-1-infected individuals (HIV-IG) required for virus neutralization (Table 2).

The weakest neutralizing activity was found in the YU2 gp120 group (group A), with only two animals generating antibodies neutralizing >50% of infection with the isolate SF162, the most neutralization-sensitive of the primary isolates tested. No homologous neutralization of the YU2

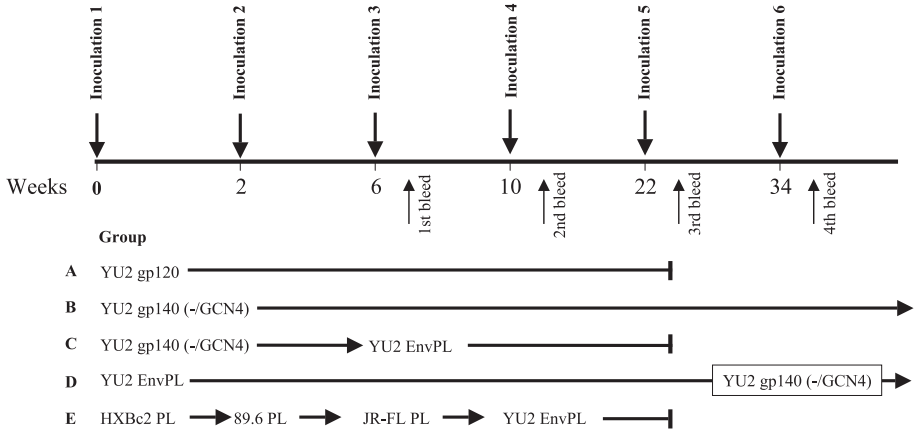


Fig. 1. Schematic diagram of rabbit immunization protocol. The numbers of inoculations and the bleeds are indicated for each of five groups of rabbits (A–E). Within each group designation, the horizontal line(s) indicate repeated administration of the same immunogen. A vertical bar indicates that the group was terminated; an arrowhead indicates that the group will undergo further analysis.

Table 1
Envelope glycoprotein reactivity and neutralizing ability of sera inoculated rabbits[a]

Group	Immunogen	Animal	Endpoint ELISA	% Neutralization					
				Viral isolate					
				HXBc2	SF162	89.6	BaL	ADA	YU2
A	YU2	7	+++	37	52	36	22	10	-3
	gp120	8	+++	44	10	26	-5	-6	-52
		9	++	37	35	-7	-3	-6	-36
		10	++	29	36	2	-3	-1	-14
		11	+++	13	55	-2	-2	11	-32
B	YU2	12	+++	64	72	35	32	14	71
	gp140	14	+++	82	80	13	24	11	90
	(-/GCN4)	15	+++	83	85	32	10	6	65
		16	++	48	28	-9	-20	-8	-27
C	YU2 gp140	17	++	48	17	13	8	-14	31
	(-/GCN4)/	18	++	27	6	0	13	5.3	-51
	YU2 PL	19	+	36	14	-7	-36	-3	-44
		20	+++	57	45	-4	18	4	45
		21	+++	48	64	21	31	6	96
D	YU2 PL	22	+++	68	61	22	-12	10	-32
		23	++	39	75	-2	25	5	78
		24	+++	64	*94	*83	46	3	36
		25	++	62	62	9	-14	3	8
		26	+++	77	61	41	43	-4	-2
E	HXBc2 PL/	27	+	41	39	12	1	5	ND
	89.6 PL/	28	+	48	65	-23	4	10	-62
	JR-FL PL/	29	–	45	37	7	-16	6	-40
	YU2 PL	30	–	69	56	20	19	25	-62
		31	+	21	51	13	6	2	-47

50-80% >80%

[a] HIV-1 single round in vitro neutralization using rabbit serum at a 1:5 dilution against a panel of viral isolates. Serum was collected after four inoculations. Numbers under viral isolates indicate % neutralization, which was determined as follows. The effect of individual pre-immune serum on infection was determined using the reactivity of individual pre-immune sera as baseline neutralization. Color-coded light blue boxes indicate 50–80% neutralization; dark blue, >80% neutralization. Asterisk indicates neutralization observed against unusually high enhancement of infection with pre-immune serum. Endpoint ELISA titers were defined as last reciprocal serum dilution at which a signal of greater than two-fold over the signal of pre-immune serum was detected. The symbols represent endpoint titers: (+++) 62,500, (++) 12,500, (+) 2500, (+/–) 2500–500, and (–) less than 500. ND: not determined. The values reported are from a single representative experiment performed in duplicate.

isolate was observed in this group; in contrast, the immune serum from this group consistently enhanced infectivity of the YU2 isolate (Table 1).

The group B animals inoculated with YU2 gp140 (–/GCN4) exhibited the greatest degree of both homologous and heterologous neutralization (Table 1). Three out of four animals significantly neutralized the isolates, HXBc2, SF162, and YU2, with one of the animals (#14) neutralizing both HXBc2 and YU2 and one animal (#15) HXBc2 and SF162 by more than 80%. By comparison, 1 mg/ml of a mixture of HIV-IG was required in the same experiment to neutralize infection of the HXBc2, SF162, and YU2 viruses by 97% 94%, and 65%, respectively (this represents an approximate 1:10 dilution of normal serum IgG concentrations; Table 2). To achieve neutralization of >90% of the HXBc2 isolate, 0.5 and 5 μg/ml of the potent neutralizing monoclonal antibodies 2F5 and 2G12, respectively, were required. To achieve >90% neutralization of SF162, 50 μg/ml 2F5 were required; 2G12 neutralized the SF162 isolate by only 73% at the highest antibody concentration tested (50 μg/ml) (Table 2). Greater than 50% neutralization of the primary isolates 89.6, BaL, and ADA by rabbit sera was not observed. To overcome the limitations of the amount of protein that could be inoculated into each animal in the EnvPL format, one group of rabbits (group C) received two inoculations of 25 μg of YU2 gp140 (–/GCN4) followed by two inoculations of YU2 EnvPLs. In this group, only two animals displayed neutralizing activity greater than 50% with one and two viruses, respectively (Table 1). Rabbit 21 showed the greatest homologous neutralization of the YU2 isolate of all rabbits with 96% neutralization. This neutralizing activity, however, crossed over only to the SF162 isolate, which was neutralized by 64%. Overall, the neutralizing reactivity in this group was markedly poorer than that in the group inoculated with YU2 gp140 (–/GCN4) glycoproteins alone.

In the group (group D) inoculated with YU2 EnvPLs, all five animals displayed neutralizing activity greater than 50% against at least two viral isolates. Animal 24 may be

Table 2
Effects of selected antibodies on the FACS-based neutralization assay[a]

Ab	Concentration (μg/ml)	% Neutralization					
		Viral isolate					
		HXBc2*	SF162	89.6	BaL	ADA	YU2
HIV-IG	10,000	ND	100	100	99	97	100
	1000	97	94	81	71	75	65
2F5	50	ND	94	93	78	86	95
	5	100	71	53	41	47	63
2G12	50	ND	73	84	81	24	−1
	5	96	60	62	62	8	1

[a] HIV-1 single-round in vitro neutralization assay using HIV-IG and monoclonal anti-envelope glycoprotein antibodies (Ab) against a panel of viral isolates. Numbers indicate % neutralization. Data were obtained in the same experiment as data in Table 2. (*) For the neutralization of HXBc2, 10-fold lower concentrations of HIV-IG and monoclonal antibodies than indicated were used.

the only exception in this regard, since the neutralizing activity of 83% and 94% against isolates 89.6 and SF162, respectively, are likely due to the unusual and unexpected enhancing effect detected in pre-immune serum of this animal (−281% and −225%). This unexplained enhancement may artificially elevate neutralization values that we have reported for this one animal due to the manner in which we calculate % neutralization. That is, we calculate % neutralization relative to the pre-bleed value, which in this case is negative. This adjustment then sets the "zero point" at a relatively low level that could possibly lead to an overestimate of the actual neutralizing activity present and therefore the values reported should be interpreted with caution for this particular serum sample. Only one animal significantly neutralized the YU2 isolate (animal 23 with 78% neutralization), while other animals showed little neutralization (animals 22 and 26). Overall, the neutralization activity of sera from this group, when achieved, was weaker than that observed in the animals inoculated with YU2 gp140 (−/GCN4) glycoproteins.

The sequential inoculation of the group E rabbits with different envelope glycoprotein EnvPLs was designed to focus the immune response on the conserved elements common to the four clade B envelope glycoproteins. This group displayed the lowest anti-gp120 titers as determined by ELISA (Table 1). This may be a consequence of a lower envelope glycoprotein dose in this group due to less efficient JR-FL envelope glycoprotein incorporation into EnvPLs as analyzed by FACS and gels (data not shown). Very limited neutralizing activity was observed in this group, with sera exhibiting enhancement of YU2 infection and only weak neutralization of SF162 (animals 28, 30, 31) and HXBc2 (animal 30). The poorer neutralization activity exhibited by these sera probably reflects the lower overall titers of anti-gp120 binding antibodies present in the sera.

By both inspection (see Fig. 1S, Supplementary Information) and statistical analysis of the neutralizing profiles elicited by monomeric YU2 gp120 as compared to the YU2 gp140 (−/GCN4) trimers, we concluded that the gp140 molecules were a superior immunogen. By inspection of the percentage neutralization of the HXBc2 and SF162 isolates, we also concluded that the EnvPLs elicited better responses than monomeric gp120. Statistical analysis was done comparing neutralization of HXBc2, YU2, and SF162 by the sera from groups A versus B by a two-tailed Student's t test. Statistical significance was achieved comparing HXBc2 neutralization between the two groups (mean for gp120 monomer 32 ± 5; mean for gp140 (−/GCN4) 69 ± 8, $P < 0.005$) and if we assigned a value of 1 to the negative values in the YU2 gp120 group, high statistical significance was also achieved (mean for the gp120 of 1; mean for the gp140 (−/GCN4) of 56.5; $P = 0.01$). For the SF162 comparison, the mean for the gp120 group was 37 ± 8 as compared to 66 ± 13 for the gp140 (−/GCN4) group, but was not statistically significant ($P < 0.09$). Collectively, these data support our conclusions.

HIV-1-neutralizing reactivity in rabbit sera after repeated boosting

To assess if additional boosting could further increase neutralizing antibody titers, animals from groups B (gp140 (−/GCN4) glycoproteins) and D (EnvPLs), which had demonstrated a neutralizing antibody response after four inoculations, were rested for 3 months and additionally boosted. A second 3-month rest and boost followed (Fig. 1, inoculations 5 and 6). Only one animal from the group inoculated with YU2 gp120 was inoculated for a fifth time as a control. This animal had shown the highest overall neutralizing activity among the animals in this group (group A, Table 1). While the fifth inoculation for the animals from groups B and D consisted of YU2 gp140 (−/GCN4) and YU2 EnvPLs as in the previous inoculations, the sixth inoculation consisted of YU2 gp140 (−/GCN4) for all animals in both groups B (nos. 12, 14–16) and D (nos. 22–26). After boosting, the anti-gp120 IgG titers, as determined by ELISA, showed little increase in most sera. The highest increases in titers from boost 3 to boost 5 were observed in sera from rabbit 12 (34%), rabbit 14 (14%), rabbit 22 (27%), and rabbit

25 (18%). For all other animals, the increase in gp120 reactivity was below 10% (data not shown).

A consistent boosting effect could be observed on the neutralizing activity in most sera primed and boosted with the oligomeric glycoproteins (Table 3). When the neutralizing activity against the initial set of clade B viral isolates in the sera after inoculations 4, 5, and 6 were compared, the neutralizing activity in the sera increased from inoculations 4 to 6 in all animals except rabbit 15 (Table 3). In serum from this animal, the neutralizing activity against HXBc2, SF162, and 89.6 was already significant after the 4th inoculation and remained relatively constant after inoculations 5 and 6. An increase in neutralizing activity by over 30% could be readily detected in some animals after inoculations 5 and 6. Notably, neutralization of the 89.6 isolate could now be observed in three of the four animals inoculated with YU2 gp140 (−/GCN4). The animal inoculated with gp120, in contrast, lost all neutralizing activity in the course of the repeated boosting regimen (Table 3). In summary, the neutralizing breadth and potency of the majority of tested sera increased with additional inoculations. With the exception of one virus/serum pair (YU2 and serum from rabbit 15, sixth inoculation), serum at a 1:5 dilution from three out of four animals in the group inoculated with YU2 gp140 (−/GCN4) neutralized all HIV-1 isolates tested in this experiment by more than 50%. For all virus/serum pairs in the group inoculated with YU2 EnvPLs that displayed neutralizing activity greater than 50% after four inoculations, the neutralizing activity increased after two additional inoculations. Three other virus/serum pairs in this group exhibited neutralizing activity after six inoculations, where none greater than 50% had been detected after four inoculations.

Titration of neutralizing sera after six inoculations

To assess the IC_{50} of the sera that exhibited neutralizing activity, sera from rabbits 12, 14, 15, 22, 23, 24, and 26

Table 3
Neutralizing activity of sera from rabbits after additional boosting[a]

	Immunogen	Rabbit	Inoculation	% Neutralization			
				Viral isolate			
				HXBc2	SF162	89.6	YU2
Group A (monomer)	YU2 gp120	7	4	43	34	50	3
			5	5	13	24	-44
Group B (soluble trimers)	YU2 gp140 (-/GCN4)	12	4	57	64	51	40
			5	78	59	70	60
			6	92	73	84	88
		14	4	72	67	20	77
			5	58	67	41	80
			6	87	78	65	84
		15	4	88	88	44	46
			5	89	93	59	55
			6	88	77	52	30
		16	4	34	4	-14	-23
			5	57	41	0	-27
			6	49	22	-11	-24
Group D	YU2 EnvPL	22	4	57	66	1	-36
			5	74	54	20	-48
			6	91	73	37	-28
		23	4	27	36	-21	61
			5	16	29	-12	58
			6	61	42	-16	89
		24	4	53	86	23	7
			5	13	78	11	13
			6	76	88	45	37
		25	4	51	42	22	-19
			5	21	27	18	-27
			6	73	51	65	11
		26	4	76	64	56	-6
			5	41	51	38	-39
			6	77	65	70	-7

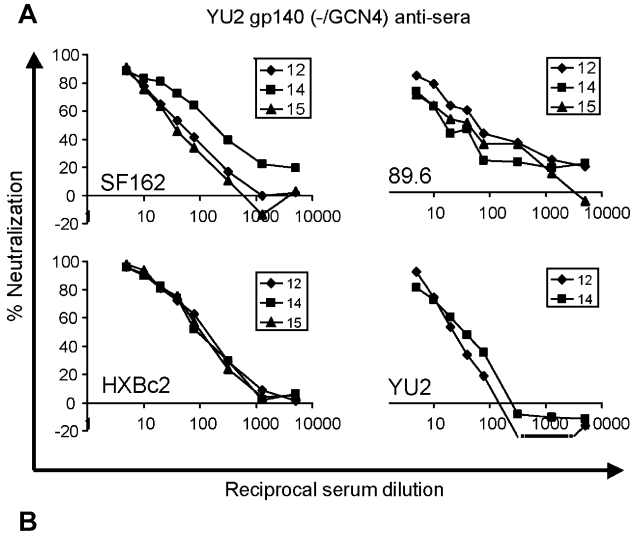
50-80% >80%

[a] HIV-1 single-round in vitro neutralization assay using rabbit serum (4th, 5th, and 6th inoculations) at a 1:5 dilution against a panel of viral isolates. Numbers indicate % neutralization. Serum was collected after each inoculation 4, 5, or 6 for each animal as indicated. Percent neutralization was calculated as described for Table 1. Color-coded light blue boxes indicate 50–80% neutralization; dark blue, >80% neutralization.

were tested at different serum dilutions for HIV-1 neutralizing activity. The sera were only tested against viruses that they could neutralize at a level greater than 50–60% at a 1:5 dilution. Fig. 2A shows a titration of the sera from three of the Group B rabbits against 4 viruses (HXBc2, SF162, 89.6 and YU2). All sera tested showed a clear dose-response of viral neutralization similar to the curves in Fig. 2A. IC_{50} values were derived from these dose-response data (Fig. 2B). Most of the sera tested showed 50% neutralization of the HXBc2, SF162, 89.6, and YU2 viruses at serum dilutions greater than 1:30 (Fig. 2B). The TCLA strain HXBc2 was neutralized at the 50% level by serum dilutions of 1:123, 1:107, and 1:101 for the three animals inoculated with YU2 gp140 (−/GCN4) that had generated neutralizing activity in their sera.

Analysis of the neutralizing activity following serum IgG fractionation

There are often nonspecific effects on viral entry caused by undefined components in animal sera that could enhance or obscure immunoglobulin-mediated viral neutralization. To demonstrate that the dose-dependent neutralizing activity present in the immune sera after six inoculations was mediated by immunoglobulin, the IgG was fractionated from individual sera and tested for neutralization. As described in Materials and methods, neutralization assays were performed in duplicate at an IgG concentration of 10 mg/ml, the normal level of IgG found in sera, to assess the maximal potency and breadth of neutralization of the immune sera against a broader array of clade B primary isolates (Table 4). This concentration of IgG was roughly 5-fold higher than could be obtained by using non-fractionated serum at the lowest dilution possible in the neutralization assay (1:5) and is reflected by the higher IgG neutralization values obtained relative to the serum values presented in Table 3. Pre-immune serum IgG was used as a control to determine the level of non-specific viral neutralization mediated by immunoglobulins at this concentration. At 10 mg/ml, homologous YU2 neutralization exceeding 75% was accomplished by three rabbits inoculated with gp140 (−/GCN4) glycoproteins and by three animals immunized with the EnvPLs (Table 4). Readily detectable heterologous neutralization by IgG from YU2 gp140 (−/GCN4) rabbits 12 and 14 of the primary isolates SF162, 89.6, BaL, JR-CSF, BR07MC at levels of 69–99% was observed (Table 4). The IgG from the sera of the rabbit 14 was able to neutralize ADA at a level of 50%. The remainder of the primary isolates in the panel could not be neutralized at a level that we considered significant. Occasionally, enhancement of viral infection was observed as indicated by the negative values in Table 4, presumably caused by Env-specific IgG at sub-saturating concentrations. Including neutralization of HXBc2 previously observed using sera from animals 12 and 14, the IgG from the two highest neutralizing titer rabbits (12 and 14) was able to neutralize 8 of 13 isolates tested to a readily detectable level (Table 4).



B

viral isolate	Rabbit	YU2gp140GCN4 12	14	15	YU2 EnvPL 22	23	24	26
HXBc2		123	107	101	83	ND	44	49
SF162		50	206	40	65	3	50	ND
89.6		63	20	32	ND	ND	ND	32
YU2		30	35	ND	ND	31	ND	ND

Fig. 2. Titration of neutralizing activity in sera after six inoculations of protein. Panel A: sera raised against YU2 gp140 (−/GCN4) envelope glycoprotein was tested for neutralization of different HIV-1 isolates at decreasing serum concentrations. Panel B: neutralizing antibody titers in rabbit sera after six inoculations; IC_{50} values for sera raised against YU2 gp140-GCN4 and YU2 proteoliposomes are shown. Values were derived from dose–response data shown in Fig. 2A and not shown. ND: not determined, if the serum did not exhibit significant neutralization of virus at the highest serum dilution tested.

We next performed a titration of the neutralization activity of the purified IgG from sera of the Group B rabbits against the isolates SF162 and YU2 (see Fig. 2, Supplementary Information). We were able to obtain IC_{50} values of the purified IgG after subtraction of any non-specific neutralization by purified prebleed IgG. The IC_{50} values for rabbits 12, 14, 15, and 16 against SF162 were 505, <80, 727, and 3812 μg/ml and against YU2 the IC_{50} values were 240, 188, 2955, and >10,000 μg/ml. The suggestion that sub-saturating concentrations of immune IgG could induce enhancement of viral entry was evident in the titration of IgG from rabbit #16. In the sera, this rabbit had no detectable neutralization activity against YU2. After IgG purification, weak neutralization was observed and as the IgG was serially diluted, enhancement of entry was observed (Fig. 2S, Supplementary Information).

Although all five of the IgG fractions from the EnvPL-primed and YU2 gp140 (−/GCN4) boosted rabbits (group D) could neutralize SF162 to a reasonable level, these IgG fractions exhibited less heterologous neutralization against the more resistant primary isolates. Some apparent neutralization from rabbit 24 against resistant isolates was rendered suspect due to high levels of enhancement induced by the pre-immune IgG from this animal.

Table 4
Breadth of neutralization of 12 primary isolates by the purified IgG isolated from the sera of the rabbits immunized six times with YU2 gp140(−/GCN4) glycoproteins and the YU2 EnvPLs[a]

Immunogen	Rabbit	YU2	ADA	Bal	SF162	JR-CSF	JR-FL	89.6	BL01	BL07	US1	DM004	CM237
YU2	12	99	12	87	88	83	15	97	-95	68	8	-134	-39
gp140	14	100	50	78	97	86	19	99	-19	97	2	-55	-5
(-/GCN4)	15	76	4	14	92	6	-27	51	-19	-31	-75	-201	-106
	16	22	4	-11	65	16	-44	5	-9	9	-55	-109	-57
YU2	22	-21	29	50	83	46	-14	-14	-157	7	25	-17	17
EnvPL	23	112	-5	17	65	33	-27	29	-119	-5	-15	-29	-5
	24	98	11	50	84	67	34	70	52	-33	81	ND	51
	25	*83	-383	22	96	13	*-240	23	-57	19	11	ND	0
	26	*40	*14	*66	*83	*66	*15	0	-287	7	25	ND	*21

50-80% >80%

[a] Negative numbers indicate enhancement of infection by the IgG. Any non-specific neutralization achieved by pre-immune IgG was first subtracted and all values here represent neutralization achieved by immune IgG beyond the pre-immune levels. In a few instances, high pre-immune IgG enhancement may have affected the values*. Color-coded; light blue indicates 50–80% neutralization; dark blue, >80% neutralization.

V3 loop adsorptions

To determine if the YU2 gp140 (−/GCN4) glycoproteins had elicited V3 loop-directed neutralizing antibodies, we performed V3 peptide adsorption studies on the sera that had exhibited the highest level of neutralizing activity against the SF162 virus. The SF162 virus was chosen as the reporter virus since significant neutralization could be achieved and we assumed that using this virus as the indicator strain would be more amenable to define the specificity of the neutralizing activity. The conformation-sensitive V3 loop antibodies 447-D and 2442 (Gorny et al., 2002) were used as controls to validate the peptide adsorption assay. At 50 μg/ml, the V3 loop antibodies 447-D and 2442 were able to neutralize the SF162 primary isolate to 95% and 99%, respectively (Fig. 3, left and Table 5). However, when the antibodies were pre-incubated with the BaL or YU2 V3 loop peptides, but not the control Ebola peptide, nearly all the neutralizing activity of the two V3 loop antibodies was inhibited (Fig. 3, left and not shown). Next, we utilized pooled IgG from rabbits 12, 14, and 15 to determine the peptide concentration range in which all the neutralizing activity present in the IgG that could be inhibited by the V3 loop peptides was blocked (Fig. 3, right). As shown, in the concentration range from 60 to 1.5 μg/ml, approximately 15% of the pooled immune IgG neutralizing activity against SF162 was inhibited. Once the peptide concentration was decreased to 0.15 μg/ml, full neutralizing capacity was again observed in the immune IgG (Fig. 3, right).

When the peptides were used as potential inhibitors of the SF162 neutralizing activity of sera from animals 12, 13 and 15 (group B), only 25–30% of the neutralizing activity was removed (Table 5). The level of V3 loop peptide inhibition was also assayed over a range of anti-serum dilutions with similar results (Supplementary Information, Fig. 3S). Similar results were observed for the ability of the peptides to inhibit the YU2 homologous neutralizing activity from the sera of the YU2 gp140 (−/GCN4) rabbits (Table 5). We then repeated the V3 loop peptide inhibition

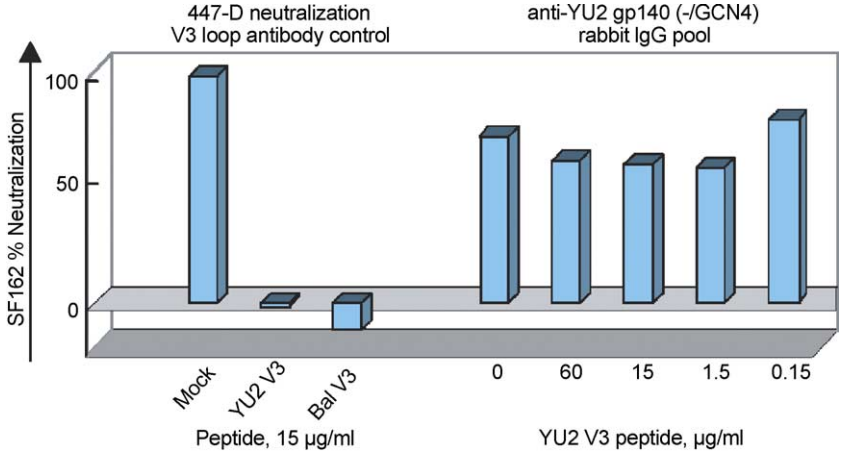


Fig. 3. Depicted in the figure is V3 loop peptide adsorption assay format, optimization and validation. On the left shown is the SF162 neutralization by the conformational V3 loop-directed antibody 447-D and adsorption of neutralization by V3 loop peptides. On the right is neutralization achieved by pooled immune IgG following six inoculations (rabbits 12, 14 and 15) and partial inhibition of neutralization by the serial dilutions of the YU2 V3 peptide.

Table 5
Neutralization of the SF162 virus and V3 loop peptide adsorptions of the sera elicited by six inoculations of YU2 gp140 (−/GCN4) and YU2 EnvPLs[a]

Immunogen	Rabbit	Mock	Scrambled V3		YU2 V3 peptide	
	#	% Neut.	% Neut.	% Inhib.	% Neut.	% Inhib.
YU2 gp140(-/GCN4)	12	87	85	2	71	18
	14	97	97	0	91	6
	15	86	83	4	80	6
	16	47	47	0	31	34
YU2 EnvPL	22	90	84	6	67	26
	23	59	63	-7	41	30
	24	78	69	11	49	38
	25	65	61	5	50	23
	26	60	55	8	15	75

[a] Data are compared to mock (no peptide) and scrambled V3 peptide adsorptions, V3 peptide inhibition (Inhib.) values of SF162 neutralization (Neut.) are shown in red. In the same assay, and as a positive control, the V3 loop antibody 2442 could neutralize SF162 at a level of 100%. When preincubated with 2442, the YU2 V3 loop peptide could inhibit 95% of this neutralization at a peptide concentration of 15 μg/ml.

of SF162 virus neutralization with the gp140 (−/GCN4) antisera and analyzed the YU2 EnvPL anti-sera as well (Table 5). A trend appeared that the EnvPL-elicited serum antibodies seemed to be slightly more sensitive to V3 peptide adsorption than did the sera elicited by the gp140 (−/GCN4) immunogen (Table 5). Interestingly, rabbit 26 anti-sera had approximately 75% of its neutralizing activity inhibited by YU2 V3 loop preadsorption, but very little inhibition by preincubation with the scrambled V3 loop peptide.

When selected individual, purified IgGs were analyzed by this assay, SF162 neutralization by animal 12 was inhibited 27% by the V3 loop peptide and animal 26 by 23%, whereas all other IgG samples were inhibited by 8% or less. Again, no significant neutralization could be inhibited by the Ebola control peptide (not shown).

Discussion

The elicitation of broadly neutralizing antibodies against the HIV-1 envelope glycoproteins poses a major challenge for HIV-1 vaccine development. Monomeric gp120 has proven to be a poor immunogen and to date only a few envelope glycoprotein formulations have been shown to elicit broadly neutralizing antibodies more effectively than monomeric gp120. The complex organization and lability of the functional trimeric envelope glycoprotein spike pose difficulties for the design of faithful antigenic mimics. Here, we tested the ability to elicit neutralizing antibodies in rabbits of two oligomeric envelope glycoprotein formulations: soluble, stable gp140 (−/GCN4) glycoproteins, and solid-phase EnvPLs containing cleavage-defective, HIV-1 gp160ΔCT envelope glycoproteins as compared to monomeric gp120 as an immunogen. By a series of assays designed to analyze the neutralizing specificity of the sera, we show that the trimeric formulations elicit serum antibodies of greater neutralization breadth, that the neutralization is dose-dependent, improves upon repeated inoculation, is IgG-mediated, and importantly, that only a minor fraction of the neutralizing activity is directed against the gp120 V3 loop. We interpret the last observation to indicate that these trimeric YU2 immunogens present more conserved elements of the spike to the immune system.

By deriving the envelope glycoproteins for the generation of EnvPLs from the YU2 molecular clone, the immunogenicity of EnvPLs could be directly compared to that of the previously tested immunogen YU2gp140 (−/GCN4). However, by SDS gel-based estimates, the dose of envelope glycoproteins that could be inoculated into rabbits with soluble YU2gp140 (−/GCN4) constructs was approximately 5-fold greater than the amount of envelope glycoproteins formulated as EnvPLs (data not shown).

To assess the total amount of envelope-directed IgG antibody elicited by the different immunogens, sera were tested by anti-gp120 ELISA. Interestingly, although the dose of envelope glycoproteins administered to animals receiving EnvPLs was approximately 5-fold lower than the soluble protein doses, these animals generated endpoint ELISA titers similar to those of animals inoculated with soluble envelope glycoproteins (Table 1). The particulate presentation of envelope glycoproteins in EnvPLs to the immune system may be more effective for the elicitation of antibodies than the administration of soluble protein by either a depot effect or by more efficient uptake of immunogen by antigen-presenting cells.

After the fourth inoculation, we concluded that the oligomeric envelope glycoprotein formulations elicited greater neutralizing activity than did monomeric gp120. Sera that demonstrated homologous neutralization of the YU2 virus generally neutralized the SF162 isolate and, in most cases, the HXBc2 isolate. However, neutralization of HXBc2 or SF162 did not necessarily imply homologous YU2 neutralization. This observation is consistent with the initial rationale for choosing YU2 for the generation of immunogens. Neutralizing antibodies raised against a neutralization-resistant viral isolate appear to have a greater propensity to neutralize other viral isolates than antibodies directed against a more neutralization-sensitive viral isolate (Yang et al., 2001).

Since we were encouraged by these data, we continued to boost both the soluble gp140 (−/GCN4) rabbits (group B) and the EnvPL-inoculated rabbits (group D). Interestingly, although the ELISA titer of the group B rabbits did not show a large increase, the neutralizing titer for three of the rabbits showed an increase in potency against the 89.6, BaL and JR-CSF isolates. That we boosted neutralizing responses without large increases in binding antibodies suggests that the soluble trimer preferentially presented relevant epitopes and drove relevant responses. This interpretation is supported by an increase in homologous neutralization of the EnvPL rabbits that were inoculated the 5th time with EnvPL and the 6th with the soluble gp140 (−/GCN4) trimers.

We utilized purified immunoglobulins to demonstrate conclusively that the neutralizing activity was IgG-mediated and concentration-dependent. Interestingly, antibodies isolated from the sera of rabbit 16 (gp140 (−/GCN4) group B) displayed no neutralizing activity prior to purification of the IgG. However, following purification, the IgG neutralized SF162 at the 10 mg/ml concentration. This slight gain in potency could simply be due to an effective increase in immunoglobulin concentration following IgG purification or could be due to the elimination of components in the sera that masked a weak, but specific, neutralizing activity. In any case, this example illustrates that purification of the IgG from sera may have a positive influence on the detection of neutralizing activity. The rabbit 16 IgG also weakly neutralized the homologous YU2 strain, but upon serial dilution of the IgG, YU2 entry was enhanced. At the highest concentration tested, the immunoglobulin from this animal also enhanced JR-FL entry. Enhancement of HIV-1 infection by antibodies binding at sub-saturating concentrations has been well described (Homsy et al., 1989; Lund et al., 1995; Robinson et al., 1988; Sullivan et al., 1995, 1998a; Takeda et al., 1988), but the exact mechanisms are unknown. One model suggests that antibodies at sub-saturating concentrations may induce Env conformational changes that may allow for more efficient binding to the cellular receptors. Enhancement could be interpreted as an initial effect of inadequate antibody binding to virions that then proceeds towards neutralization at higher antibody concentrations. Apparently, sera or IgG that display enhancement at the concentrations tested (Table 4) do not possess high enough levels of antibodies that bind to the functional spike to achieve neutralization. In this model, enhancement at least indicates that there are antibodies elicited that can bind to envelope glycoprotein spikes, albeit at levels too low to neutralize. Other models of enhancement include antibody binding to Fc receptors to stabilize the interaction of the virus and the target cell receptors, providing a nonspecific binding component (Tirado and Yoon, 2003). Complement-mediated modes of HIV-1 enhancement have also been proposed (Tirado and Yoon, 2003), but are unlikely in the neutralization format used in this and most studies. As enhancement of HIV-1 infection by Fab fragments has been reported, some mechanisms of enhancement must be independent of Fc-mediated effects.

Analysis of the sera and purified IgG allowed us to detect neutralization of 8 of the 13 isolates analyzed in this study by the gp140 (−/GCN4)-elicited immunoglobulins (7 of 13 were inhibited by two animals or more, including HXBc2). All sera that displayed neutralizing activity prior to purification were able to yield IgG of equal or enhanced neutralization potency, validating the controls and methods used to identify immunoglobulin-mediated viral neutralization in the sera.

When we performed V3 loop adsorptions of anti-Env sera or IgG for either the soluble gp140 molecules or the EnvPLs, most animals displayed a modest reduction in their neutralizing activity. We interpreted these results to indicate that most of the trimer-elicited neutralizing activity is not directed against the V3 loop. However, it is possible that not all V3 loop-directed neutralizing activity was removed by V3 loop peptide adsorption. The data also seemed to indicate that the EnvPLs had slightly less breadth of neutralization and a slightly greater level of V3-loop directed neutralizing antibodies (Table 5). Since the V3 loop is often an immunodominant region of gp120, either the YU2 envelope glycoproteins do not efficiently present the V3 loop or the trimeric constructs obscure or dampen V3-directed antibody responses.

Several factors may account for the better elicitation of neutralizing antibodies by the trimeric envelope glycoprotein formulations, including a diminution in the immunodominance of the V3 region. In addition, many epitopes available for antibody binding on monomeric gp120, such as the gp120–gp41 interface and regions along the trimer axis, elicit non-neutralizing antibodies, because these epitopes are not accessible on the functional spike. The presentation of trimeric spikes to the immune system may also limit the number of irrelevant epitopes that are likely presented to the immune system by the conformationally flexible monomeric gp120 (Kwong et al., 2002; Myszka et al., 2000). This study strongly supports the concept that trimeric envelope glycoprotein formulations, either soluble or on a solid phase, are more efficient in eliciting broadly neutralizing antibodies than gp120 in outbred animals. In contrast to the results obtained with gp120, additional boosting of the rabbits with trimeric HIV-1 envelope glycoproteins resulted in progressive improvement in the breadth and potency of neutralizing activity. The less efficient elicitation of neutralizing antibodies observed with EnvPLs compared to YU2 gp140 (−/GCN4) may be due to the smaller dose of envelope glycoproteins inoculated. The EnvPL format may hold promise in immunogen design if the current dose-related limitations can be overcome. These findings support the continued study of trimeric envelope glycoprotein formulations as platforms for the design of improved HIV-1 vaccine components.

Materials and methods

Envelope glycoprotein constructs used for the generation of EnvPLs

The envelope glycoprotein constructs for the generation of EnvPLs were derived from the primary R5 HIV-1 isolates YU2 and JR-FL, and the X4, TCLA isolate HXBc2, as described previously (Grundner et al., 2002). The coding sequences for the 89.6 envelope glycoproteins were obtained from the pSVIIIenv 89.6 expression plasmid (Sullivan et al., 1998b). A cytoplasmic tail truncation was generated by introduction of a stop codon in place of the codon for amino acid 712 (HXBc2 numbering). The C9 peptide tag was added by appending the sequence encoding the peptide TETSQVAPA to the C-termini of all constructs according to the QuikChange (Stratagene) protocol. To create covalently linked gp120–gp41 glycoproteins, the proteolytic cleavage site between gp120 and gp41 was deleted by replacing the arginines 508 and 511 with serines by QuikChange site-directed mutagenesis. The resulting constructs encoding cleavage-deficient gp160ΔCT envelope glycoproteins were subsequently used to generate the EnvPLs. Amino acid residue numbers are designated according to the prototypic HXBc2 sequence, according to current convention. The introduction of the desired mutations was confirmed by DNA sequencing.

Expression of EnvPL and monomeric HIV-1 envelope glycoproteins

Envelope glycoproteins for the generation of EnvPLs were expressed as previously described (Grundner et al., 2002). Briefly, 293T cells were transiently transfected with plasmids expressing the gp160ΔCT glycoproteins using Effectene reagent (QIAGEN) and, for the YU2 and 89.6 envelope glycoproteins, the HIV-1 Tat expressor plasmid pSVTat. Cells were harvested 48 h after transfection, using phosphate-buffered saline (PBS) containing 5 mM EDTA. Monomeric YU2 gp120 was expressed from codon optimized *env* sequences cloned into the CMV-driven pCDNA3.1 expressor plasmid (Stratagene) and transient transfection of 293T cells by Fugene6 (Roche). Following expression in serum-free medium for 5 days, YU2 gp120 was purified to homogeneity over an F105 affinity column (not shown).

Expression and purification of gp140 (−)GCN4 glycoproteins

To produce soluble YU2 gp140 (−/GCN4) proteins, the serum-free-medium-adapted cell line, 293F, (Invitrogen, Carlsbad, CA) was transiently transfected in DMEM medium supplemented with 10% heat-inactivated fetal calf serum (HIFCS) and 0.1 mM MEM non-essential amino acids solution (Invitrogen), with the pCDNA3.1(−)YU2gp140 (−/GCN4) plasmid. Lipofectamine2000 (Invitrogen) was used to transfect the plasmid DNA as per manufacturer's instructions. One day after transfection, fresh serum-free 293 SFM II medium (Invitrogen) was used to replace serum-containing medium. The cell culture supernatants were collected daily, until the 6th day post-transfection. The YU2 GCN4 envelope glycoproteins were purified from the supernatants with an IgGb12 antibody affinity column, eluted from the column with 3 M $MgCl_2$ containing 20 mM Tris–HCl, pH 7.4 and dialyzed three times against 2 l of PBS containing 0.5 M NaCl. The proteins were concentrated with Amicon Ultra 30,000 MWCO Centrifugal Filter Devices (Millipore, Bedford, MA) and analyzed by SDS PAGE, gel filtration and Blue native gels. As analyzed by reducing and non-reducing SDS gels, the YU2 gp140 (−/GCN4) proteins were highly pure, exceeding 95% homogeneity (not shown). By gel filtration over Superdex 200 or Superose 6 columns and Blue native gels, the proteins were predominantly (approximately 70%) of a molecular weight consistent with trimer as previously reported ((Yang et al., 2000b) and data not shown). The majority of the remainder of the protein oligomers was of a molecular weight consistent with a dimer of trimers, with a small fraction of protein aggregates. The oligomeric mixture from the affinity column was used for inoculations to generate the sera analyzed in this study.

Generation of EnvPLs

EnvPLs containing the envelope glycoproteins from the HIV-1 molecular clones HXBc2, 89.6, JR-FL, and YU2 were generated as described previously (Grundner et al., 2002) with the following modifications. Dynabeads with a diameter of 2.8 μm (M-280) were used for capturing the C9-tagged envelope glycoproteins. The lipid mixture used for the reconstitution of a lipid membrane around the PLs consisted of the lipids 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (POPE) and Dimyristoylphosphatidic acid (DMPA) in a ratio of 6:3:1 and 1% of the head group-modified synthetic lipid 1,2-dioleyl-sn-glycero-3-phosphoethanolamine-*n*-(biotinyl) (Biotinyl-DOPE). All lipids were obtained from Avanti Polar Lipids. Before each inoculation, the PLs were tested for envelope glycoprotein incorporation by FACS analysis. The presence of a lipid membrane around the EnvPLs was confirmed by FACS analysis detecting biotinylated lipids incorporated into the membrane with avidin-FITC (Sigma).

Immunization of rabbits

Groups of five New Zealand White rabbits were inoculated intradermally with 1 ml of monophospholipid A + trehalose dicorynomycolate + cell wall skeleton of tubercule bacillus (MPL+TDM+CWS) Ribi adjuvant emulsion (Sigma) containing the respective antigen. The amounts

of YU2 gp120 and YU2gp140 (−/GCN4) envelope glycoprotein in each inoculum were adjusted to the same molar quantity of the gp120 moiety. Thus, each rabbit received 18.9 μg of YU2 gp120 and 25 μg of YU2 gp140 (−/GCN4). Animals inoculated with EnvPLs received 1 ml MPL+TDM+CWS adjuvant emulsion containing approximately 1.8×10^9 M-280 EnvPLs at 4 to 6 separate dorsal sites. Boosting inoculations were administered 2, 6, 10, 22, and in some cases, 34 weeks after the initial inoculation. Ear bleeding was performed 13 days after the second and 7 days after the fourth and fifth inoculation. The blood was incubated O/N at 4 °C in a Vacutainer SST Gel Clot Activator (Becton Dickinson) and spun for 30 min at 2000 × *g* at 4 °C. The cleared serum was collected and incubated for 30 min at 55 °C to inactivate complement. The sera were then stored at −20 °C.

Purification of rabbit immunoglobulins (IgG)

Rabbit IgG was purified from rabbit sera with the T-Gel Purification Kit (Pierce, Rockford, IL), which purifies the IgG at nearly neutral pH per manufacturer's instructions. Briefly, 87 mg of potassium sulfate was added to 1 ml of rabbit serum until fully dissolved at room temperature (RT). The serum sample was centrifuged at 10,000 × *g* for 20 min, and the supernatant was applied on a T-gel column pre-equilibrated with binding buffer (0.5 M potassium sulfate, 50 mM sodium phosphate, pH 8.0), washed with binding buffer, and eluted with elution buffer (50 mM sodium phosphate, pH 8.0). Upon elution, the buffer was exchanged with PBS, and IgGs were concentrated with Centricon Plus 20 100,000 MWCO concentrators (Millipore). The concentration of each IgG sample was determined by UV absorbance at 280 nm, and adjusted to 50 mg/ml final concentration for the virus neutralization assay.

ELISA for the detection of anti-gp120 reactivity in rabbit serum

To determine the anti-gp120 reactivity in sera from immunized animals, 100 ng of affinity-purified mammalian-expressed YU2 gp120 in PBS was adsorbed onto each well of a high-protein-binding microwell plate (Corning) overnight at 4 °C. After blocking the plates with 100 μl blocking buffer (PBS with 2% dry milk and 5% HIFCS), serial serum dilutions in ELISA blocking buffer were incubated in each well for 1 h at RT. After three washes with PBS/0.2% Tween-20, a secondary Anti-Rabbit-IgG-HRP antibody (Sigma) was added in washing buffer at a 1:5000 dilution for 1 h at RT. Following three washes, the ELISAs were developed with 100 μl TMB Peroxidase substrate (KPL). The reaction was stopped by adding 100 μl 1 M HCl to each well. The optical density at 450 nm was read on a microplate reader (Molecular Devices). Endpoint titers were defined as the last reciprocal serum dilution at which the absorption at 450 nm was greater than two-fold over the signal detected with pre-immune serum.

HIV-1 single-round neutralization assays

The single round intracellular p24-antigen flow cytometric HIV-1 neutralization assay has been described previously (Mascola et al., 2002a). Because animal sera can exhibit a degree of nonspecific effects on HIV-1 neutralization assays ranging from slight enhancement of infection to occasional nonspecific neutralization (data not shown and John Mascola, personal communication), we sought to control for such activities in a rigorous manner in our methods development. To be able to assess the bona fide neutralizing reactivity in individual sera relative to the baseline activity of pre-immune serum in these animals, sera were tested individually and compared to the corresponding individual pre-immune sera.

The assay was performed by incubating 40 μl of virus stock in 96-well culture plates with 10 μl of heat-inactivated rabbit serum or purified IgG in duplicate. This volume of virus represented a multiplicity of infection of approximately 0.1 and roughly 20,000 $TCID_{50}$ (determined by a 14-day titration assay) of HIV in each well. Following incubation for 30 min at 37 °C, 20 μl of peripheral blood mononuclear cells (PBMC; 1.5×10^5 cells) were added to each well. PBMC were maintained in IL-2 culture medium containing 1 μM indinavir, and the cells were fed on day 1 with 150 μl of Il-2 culture medium containing indinavir. One day after infection, permeabilized cells were stained for intracellular p24-Ag using the KC57 anti-p24 antibody, followed by quantitation of HIV-1 infected cells by flow cytometry. The percentage of neutralization was defined as reduction in the number of p24-positive cells compared with the number in well incubated with corresponding pre-immune serum. For example, if there was a 30% reduction of p24-positive cells by the addition of pre-bleed serum, then the post-immune serum neutralization was calculated from this reduced level of viral entry to take into account non-specific serum effects. Besides the pre-bleed negative control, every assay always includes at least two concentrations of the monoclonal antibodies 2G12 and 2F5 and HIVIgG as positive controls to ensure reproducible results.

For the measurement of infection with pseudotyped GFP reporter viruses, infected cells were washed once in FACS buffer and directly detected. Live cells initially gated by forward and side scatter were analyzed for intracellular expression of p24-Ag or GFP. The number of p24-Ag-positive cells or GFP-positive cells was determined using a bivariate plot of fluorescence versus forward scatter; the gate was set on mock-infected cells. To enumerate infected PBMC, cells were washed, fixed and permeabilized, and stained with the KC57 anti-p24 antibody. After forward and

side scatter gating, at least 50,000 events were counted. Final quantitation of p24-Ag-positive cells was done by subtraction of background events in mock-infected PBMC (usually less than 10 positives per 50,000 events). The percent neutralization was defined as reduction in the number of p24-Ag-positive cells and GFP-positive cells, respectively, compared to the number in control wells with individual prebleed serum or no antibody, respectively.

To obtain the IC_{50} and IC_{80} data, serial dilutions of anti-serum or IgG were incubated with virus as described above. Anti-serum dose–response curves were fit with a nonlinear function, and the inhibitory dilution that neutralized 50% and 80% (IC_{50} and IC_{80} respectively) of virus was calculated by a least-squares regression analysis. Statistical analysis of IC_{50} titers was performed using the non-parametric Mann Whitney rank order test (GraphPad Prism software package V3.0, GraphPad Software, Inc. San Diego, CA).

V3 loop adsorption assays

Peptides corresponding to the BaL and YU2 V3 loops were purchased from SynPep (Dublin, CA). Both are 23mers starting with sequence TRPNNN and were provided at >95% purity by HPLC. V3 competition studies were performed in the format of the standard neutralization assay by incubating rabbit sera with the indicated peptide at a concentration of 15–30 μg/ml (6–12 μM) for 30 min at 37 °C prior to the addition of virus. Controls included mock peptide (PBS) and a mixture of Ebola envelope 15mer peptides and a scrambled V3 loop peptide (IGPGRAFTKPNNNFYTTGTRKSIH). Initial studies had indicated that concentrations of the BaL and YU2 peptides equal to or exceeding 15 μg/ml could completely block anti-V3 mAb 447-mediated neutralization of SF162 viral entry (Fig. 3 and data not shown). These peptides could also block approximately 90% of the neutralizing activity of the V3 loop-directed antibody 2442.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2004.09.022.

References

Barnett, S.W., Rajasekar, S., Legg, H., Doe, B., Fuller, D.H., Haynes, J.R., Walker, C.M., Steimer, K.S., 1997. Vaccination with HIV-1 gp120 DNA induces immune responses that are boosted by a recombinant gp120 protein subunit. Vaccine 15 (8), 869–873.

Belshe, R.B., Gorse, G.J., Mulligan, M.J., Evans, T.G., Keefer, M.C., Excler, J.L., Duliege, A.M., Tartaglia, J., Cox, W.I., McNamara, J., Hwang, K.L., Bradney, A., Montefiori, D., Weinhold, K.J., 1998. Induction of immune responses to HIV-1 by canarypox virus (ALVAC) HIV-1 and gp120 SF-2 recombinant vaccines in uninfected volunteers. NIAID AIDS Vaccine Evaluation Group. Aids 12 (18), 2407–2415.

Berman, P.W., Gregory, T.J., Riddle, L., Nakamura, G.R., Champe, M.A., Porter, J.P., Wurm, F.M., Hershberg, R.D., Cobb, E.K., Eichberg, J.W., 1990. Protection of chimpanzees from infection by HIV-1 after vaccination with recombinant glycoprotein gp120 but not gp160. Nature 345 (6276), 622–625.

Binley, J.M., Sanders, R.W., Clas, B., Schuelke, N., Master, A., Guo, Y., Kajumo, F., Anselma, D.J., Maddon, P.J., Olson, W.C., Moore, J.P., 2000. A recombinant human immunodeficiency virus type 1 envelope glycoprotein complex stabilized by an intermolecular disulfide bond between the gp120 and gp41 subunits is an antigenic mimic of the trimeric virion-associated structure. J. Virol. 74 (2), 627–643.

Bruck, C., Thiriart, C., Fabry, L., Francotte, M., Pala, P., Van Opstal, O., Culp, J., Rosenberg, M., De Wilde, M., Heidt, P., et al., 1994. HIV-1 envelope-elicited neutralizing antibody titres correlate with protection and virus load in chimpanzees. Vaccine 12 (12), 1141–1148.

Burton, D.R., Saphire, E.O., Parren, P.W., 2001. A model for neutralization of viruses based on antibody coating of the virion surface. Curr. Top. Microbiol. Immunol. 260, 109–143.

Connor, R.I., Korber, B.T., Graham, B.S., Hahn, B.H., Ho, D.D., Walker, B.D., Neumann, A.U., Vermund, S.H., Mestecky, J., Jackson, S., Fenamore, E., Cao, Y., Gao, F., Kalams, S., Kunstman, K.J., McDonald, D., McWilliams, N., Trkola, A., Moore, J.P., Wolinsky, S.M., 1998. Immunological and virological analyses of persons infected by human immunodeficiency virus type 1 while participating in trials of recombinant gp120 subunit vaccines. J. Virol. 72 (2), 1552–1576.

Daar, E.S., Li, X.L., Moudgil, T., Ho, D.D., 1990. High concentrations of recombinant soluble CD4 are required to neutralize primary human immunodeficiency virus type 1 isolates. Proc. Natl. Acad. Sci. U.S.A. 87 (17), 6574–6578.

Earl, P.L., Broder, C.C., Long, D., Lee, S.A., Peterson, J., Chakrabarti, S., Doms, R.W., Moss, B., 1994. Native oligomeric human immunodeficiency virus type 1 envelope glycoprotein elicits diverse monoclonal antibody reactivities. J. Virol. 68 (5), 3015–3026.

Farzan, M., Choe, H., Desjardins, E., Sun, Y., Kuhn, J., Cao, J., Archambault, D., Kolchinsky, P., Koch, M., Wyatt, R., Sodroski, J., 1998. Stabilization of human immunodeficiency virus type 1 envelope glycoprotein trimers by disulfide bonds introduced into the gp41 glycoprotein ectodomain. J. Virol. 72 (9), 7620–7625.

Fouts, T.R., Binley, J.M., Trkola, A., Robinson, J.E., Moore, J.P., 1997. Neutralization of the human immunodeficiency virus type 1 primary isolate JR-FL by human monoclonal antibodies correlates with antibody binding to the oligomeric form of the envelope glycoprotein complex. J. Virol. 71 (4), 2779–2785.

Gorny, M.K., Williams, C., Volsky, B., Revesz, K., Cohen, S., Polonis, V.R., Honnen, W.J., Kayman, S.C., Krachmarov, C., Pinter, A., Zolla-Pazner, S., 2002. Human monoclonal antibodies specific for

conformation-sensitive epitopes of V3 neutralize human immunodeficiency virus type 1 primary isolates from various clades. J. Virol. 76 (18), 9035–9045.

Grundner, C., Mirzabekov, T., Sodroski, J., Wyatt, R., 2002. Solid-phase proteoliposomes containing human immunodeficiency virus envelope glycoproteins. J. Virol. 76 (7), 3511–3521.

Homsy, J., Meyer, M., Tateno, M., Clarkson, S., Levy, J.A., 1989. The Fc and not CD4 receptor mediates antibody enhancement of HIV infection in human cells. Science 244 (4910), 1357–1360.

Kwong, P.D., Doyle, M.L., Casper, D.J., Cicala, C., Leavitt, S.A., Majeed, S., Steenbeke, T.D., Venturi, M., Chaiken, I., Fung, M., Katinger, H., Parren, P.W., Robinson, J., Van Ryk, D., Wang, L., Burton, D.R., Freire, E., Wyatt, R., Sodroski, J., Hendrickson, W.A., Arthos, J., 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature 420 (6916), 678–682.

Li, Y., Kappes, J.C., Conway, J.A., Price, R.W., Shaw, G.M., Hahn, B.H., 1991. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue: identification of replication-competent and -defective viral genomes. J. Virol. 65 (8), 3973–3985.

Lund, O., Hansen, J., Soorensen, A.M., Mosekilde, E., Nielsen, J.O., Hansen, J.E., 1995. Increased adhesion as a mechanism of antibody-dependent and antibody-independent complement-mediated enhancement of human immunodeficiency virus infection. J. Virol. 69 (4), 2393–2400.

Mascola, J.R., Louwagie, J., McCutchan, F.E., Fischer, C.L., Hegerich, P.A., Wagner, K.F., Fowler, A.K., McNeil, J.G., Burke, D.S., 1994. Two antigenically distinct subtypes of human immunodeficiency virus type 1: viral genotype predicts neutralization serotype. J. Infect. Dis. 169 (1), 48–54.

Mascola, J.R., Snyder, S.W., Weislow, O.S., Belay, S.M., Belshe, R.B., Schwartz, D.H., Clements, M.L., Dolin, R., Graham, B.S., Gorse, G.J., Keefer, M.C., McElrath, M.J., Walker, M.C., Wagner, K.F., McNeil, J.G., McCutchan, F.E., Burke, D.S., 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. The National Institute of Allergy and Infectious Diseases AIDS Vaccine Evaluation Group. J. Infect. Dis. 173 (2), 340–348.

Mascola, J.R., Lewis, M.G., Stiegler, G., Harris, D., VanCott, T.C., Hayes, D., Louder, M.K., Brown, C.R., Sapan, C.V., Frankel, S.S., Lu, Y., Robb, M.L., Katinger, H., Birx, D.L., 1999. Protection of Macaques against pathogenic simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing antibodies. J. Virol. 73 (5), 4009–4018.

Mascola, J.R., Stiegler, G., VanCott, T.C., Katinger, H., Carpenter, C.B., Hanson, C.E., Beary, H., Hayes, D., Frankel, S.S., Birx, D.L., Lewis, M.G., 2000. Protection of macaques against vaginal transmission of a pathogenic HIV-1/SIV chimeric virus by passive infusion of neutralizing antibodies. Nat. Med. 6 (2), 207–210.

Mascola, J.R., Louder, M.K., Winter, C., Prabhakara, R., De Rosa, S.C., Douek, D.C., Hill, B.J., Gabuzda, D., Roederer, M., 2002a. Human immunodeficiency virus type 1 neutralization measured by flow cytometric quantitation of single-round infection of primary human T cells. J. Virol. 76 (10), 4810–4821.

Mascola, J.R., Louder, M.K., Winter, C., Prabhakara, R., De Rosa, S.C., Douek, D.C., Hill, B.J., Gabuzda, D., Roederer, M., 2002b. Human immunodeficiency virus type 1 neutralization measured by flow cytometric quantitation of single-round infection of primary human T cells. J. Virol. 76 (10), 4810–4821.

Moore, J.P., McKeating, J.A., Huang, Y.X., Ashkenazi, A., Ho, D.D., 1992. Virions of primary human immunodeficiency virus type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. J. Virol. 66 (1), 235–243.

Myszka, D.G., Sweet, R.W., Hensley, P., Brigham-Burke, M., Kwong, P.D., Hendrickson, W.A., Wyatt, R., Sodroski, J., Doyle, M.L., 2000. Energetics of the HIV gp120-CD4 binding reaction. Proc. Natl. Acad. Sci. U.S.A. 97 (16), 9026–9031.

Parren, P.W., Burton, D.R., Sattentau, Q.J., 1997. HIV-1 antibody-debris or virion? Nat. Med. 3 (4), 366–367.

Parren, P.W., Marx, P.A., Hessell, A.J., Luckay, A., Harouse, J., Cheng-Mayer, C., Moore, J.P., Burton, D.R., 2001. Antibody protects macaques against vaginal challenge with a pathogenic R5 simian/human immunodeficiency virus at serum levels giving complete neutralization in vitro. J. Virol. 75 (17), 8340–8347.

Pilgrim, A.K., Pantaleo, G., Cohen, O.J., Fink, L.M., Zhou, J.Y., Zhou, J.T., Bolognesi, D.P., Fauci, A.S., Montefiori, D.C., 1997. Neutralizing antibody responses to human immunodeficiency virus type 1 in primary infection and long-term-nonprogressive infection. J. Infect. Dis. 176 (4), 924–932.

Roben, P., Moore, J.P., Thali, M., Sodroski, J., Barbas III, C.F., Burton, D.R., 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 that show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. 68 (8), 4821–4828.

Robinson Jr., W.E., Montefiori, D.C., Mitchell, W.M., 1988. Antibody-dependent enhancement of human immunodeficiency virus type 1 infection. Lancet 1 (8589), 790–794.

Sattentau, Q.J., Moore, J.P., 1995. Human immunodeficiency virus type 1 neutralization is determined by epitope exposure on the gp120 oligomer. J. Exp. Med. 182 (1), 185–196.

Sullivan, N., Sun, Y., Li, J., Hofmann, W., Sodroski, J., 1995. Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. J. Virol. 69 (7), 4413–4422.

Sullivan, N., Sun, Y., Binley, J., Lee, J., Barbas III, C.F., Parren, P.W., Burton, D.R., Sodroski, J., 1998a. Determinants of human immunodeficiency virus type 1 envelope glycoprotein activation by soluble CD4 and monoclonal antibodies. J. Virol. 72 (8), 6332–6338.

Sullivan, N., Sun, Y., Sattentau, Q., Thali, M., Wu, D., Denisova, G., Gershoni, J., Robinson, J., Moore, J., Sodroski, J., 1998b. CD4-Induced conformational changes in the human immunodeficiency virus type 1 gp120 glycoprotein: consequences for virus entry and neutralization. J. Virol. 72 (6), 4694–4703.

Takeda, A., Tuazon, C.U., Ennis, F.A., 1988. Antibody-enhanced infection by HIV-1 via Fc receptor-mediated entry. Science 242 (4878), 580–583.

Tirado, S.M., Yoon, K.J., 2003. Antibody-dependent enhancement of virus infection and disease. Viral. Immunol. 16 (1), 69–86.

Wrin, T., Loh, T.P., Vennari, J.C., Schuitemaker, H., Nunberg, J.H., 1995. Adaptation to persistent growth in the H9 cell line renders a primary isolate of human immunodeficiency virus type 1 sensitive to neutralization by vaccine sera. J. Virol. 69 (1), 39–48.

Wyatt, R., Sodroski, J., 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science 280 (5371), 1884–1888.

Yang, X., Farzan, M., Wyatt, R., Sodroski, J., 2000a. Characterization of stable, soluble trimers containing complete ectodomains of human immunodeficiency virus type 1 envelope glycoproteins. J. Virol. 74 (12), 5716–5725.

Yang, X., Florin, L., Farzan, M., Kolchinsky, P., Kwong, P.D., Sodroski, J., Wyatt, R., 2000b. Modifications that stabilize human immunodeficiency virus envelope glycoprotein trimers in solution. J. Virol. 74 (10), 4746–4754.

Yang, X., Wyatt, R., Sodroski, J., 2001. Improved elicitation of neutralizing antibodies against primary human immunodeficiency viruses by soluble stabilized envelope glycoprotein trimers. J. Virol. 75 (3), 1165–1171.

Yang, X., Lee, J., Mahony, E.M., Kwong, P.D., Wyatt, R., Sodroski, J., 2002. Highly stable trimers formed by human immunodeficiency virus type 1 envelope glycoproteins fused with the trimeric motif of T4 bacteriophage fibritin. J. Virol. 76 (9), 4634–4642.

Zhang, Y.J., Fredriksson, R., McKeating, J.A., Fenyo, E.M., 1997. Passage of HIV-1 molecular clones into different cell lines confers differential sensitivity to neutralization. Virology 238 (2), 254–264.