

Immunogenicity and Protective Efficacy of Oligomeric Human Immunodeficiency Virus Type 1 gp140

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The biologically active form of the human immunodeficiency virus type 1 (HIV-1) envelope (Env) glycoprotein is oligomeric. We previously described a soluble HIV-1 IIIB Env protein, gp140, with a stable oligomeric structure composed of uncleaved gp120 linked to the ectodomain of gp41 (P. L. Earl, C. C. Broder, D. Long, S. A. Lee, J. Peterson, S. Chakrabarti, R. W. Doms, and B. Moss, *J. Virol.* 68:3015–3026, 1994). Here we compared the antibody responses of rabbits to gp120 and gp140 that had been produced and purified in an identical manner. The gp140 antisera exhibited enhanced cross-reactivity with heterologous Env proteins as well as greater neutralization of HIV-1 compared to the gp120 antisera. To examine both immunogenicity and protective efficacy, we immunized rhesus macaques with oligomeric gp140. Strong neutralizing antibodies against a homologous virus and modest neutralization of heterologous laboratory-adapted isolates were elicited. No neutralization of primary isolates was observed. However, a substantial fraction of the neutralizing activity could not be blocked by a V3 loop peptide. After intravenous challenge with simian-HIV virus SHIV-HXB2, three of the four vaccinated macaques exhibited no evidence of virus replication.

Human immunodeficiency virus type 1 (HIV-1) vaccine development is currently focused on the design of immunogens that will stimulate both the humoral and cellular arms of the immune system (13, 14, 35, 36, 38, 45, 66). Strategies employing DNA and live viral vectors, alone or in combination, have been shown to elicit cellular responses (11, 18, 20, 24, 33, 37, 44, 65, 69). A vigorous humoral response, however, is best achieved by the inclusion of a protein boost (3, 16, 18, 29, 30). While antibodies may not be sufficient to confer protection, they may be critical in reducing viral loads during the initial stages of infection and allowing time for maturation of the cellular response, as has been found with murine virus infections (2, 34, 58).

The envelope (Env) glycoprotein is the major target of neutralizing antibodies to HIV-1 and consequently is the best candidate for stimulation of humoral immunity. The extensive variability of the Env protein, however, presents a major obstacle in designing an appropriate immunogen. Clinical studies with soluble gp120 vaccines have elicited antibodies with a narrow neutralization specificity, an inability to neutralize primary isolates (5, 6, 12, 31, 41, 43), and qualitatively different binding reactivity than those induced by HIV-1 infection (4,

75). Unlike sera from HIV-1-infected people, vaccinee sera react preferentially with epitopes on denatured gp120, bind efficiently to gp120 peptides, do not bind well to heterologous envs, and neutralize only T-cell line-adapted (TCLA) strains of HIV-1. In addition, vaccinee sera contain a preponderance of anti-V3 reactivity, accounting at least in part for the restricted neutralization properties (12, 46).

The monomeric structure of the gp120 vaccines may have been a factor in their poor immunogenicity. Several lines of evidence indicate that epitope exposure on oligomeric and monomeric Env differs in important ways. Many monoclonal antibodies that react well with monomeric gp120 do not react efficiently with oligomeric Env (50, 51, 68, 78), suggesting that dominant epitopes on gp120 are obscured on the oligomer. Some studies have indicated that binding of monoclonal antibodies to oligomeric but not monomeric Env correlates with neutralization (26, 54, 64, 67). In addition, sera from HIV-1-infected individuals contain predominantly antibodies to conformation-dependent epitopes, including those directed at oligomeric Env (49, 73). Another deficiency of the gp120 vaccines is the absence of epitopes in gp41 (25, 28), including one to which a very broadly neutralizing monoclonal antibody has been mapped (52). Thus, oligomeric Env may display epitopes important in eliciting antibodies capable of binding to the Env protein on virions and infected cells.

A second-generation vaccine would retain the epitopes present on oligomeric Env. In an attempt to make an immunogen that maintains native, conserved epitopes, we constructed an oligomeric Env, gp140, from the IIIB strain of HIV that contains all of gp120 and the ectodomain of gp41 (23). To allow efficient secretion, the gene was truncated just upstream of the transmembrane domain. In addition, 12 amino acids

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were deleted from the proteolytic cleavage site between gp120 and gp41 to prevent their dissociation. The Env protein thus generated is soluble, is secreted from infected cells, and efficiently binds soluble CD4. It is almost completely oligomeric, as judged by sucrose density gradient centrifugation, size exclusion chromatography, and chemical cross-linking. Immunization of mice with this soluble oligomeric Env induced primarily conformation-dependent antibodies, while those elicited by immunization with monomeric Env were mostly linear (23). In addition, the monoclonal antibodies generated to oligomeric gp140 bound efficiently to Env on the surface of HIV-1-infected cells, indicating the presence of common structural elements (23). Furthermore, greater binding of antibodies in HIV-positive human sera to soluble oligomeric than to monomeric Env has been shown (63, 77), and human and murine monoclonal antibodies that react preferentially with oligomeric Env have been identified (10, 57, 60). Nevertheless, some quantitative differences in the binding of nonneutralizing monoclonal antibodies to gp140 and membrane-associated Env have been reported (54).

The purpose of the present study was to compare the immunogenicity of similar preparations of gp140 and gp120 in rabbits and then test the better immunogen as a vaccine in rhesus macaques. The rabbit experiments indicated that the gp140-induced antibodies had better cross-reactivity in a binding assay and higher neutralizing titers against laboratory-adapted isolates than did gp120-induced antibodies. Therefore, rhesus macaques were immunized with purified gp140 and challenged with the homologous simian-HIV virus SHIV-HXB2. All animals were significantly protected, and no evidence of virus replication was detected in three of the four. Although our study did not include a direct comparison of the two immunogens in macaques, another group has found similar protection of macaques immunized with gp120 in QS21 (P. Berman et al., unpublished data).

MATERIALS AND METHODS

Viruses. Recombinant vaccinia viruses vPE50 (15) and vPE12B (23), expressing the HIV-1 BH8 gp120 and gp140 Env proteins, respectively, were used for production of soluble, secreted Env proteins used for immunizations. The following recombinant vaccinia viruses expressing gp160 from different HIV-1 isolates (name of isolate in parentheses) were used: vCB29 (JRCSF), vCB34 (SF2), vCB36 (RF), vCB43 (Ba-L) (9), vCB51 (BK132) (22), and vBD3 (89.6) (21). HIV-1 NL4-3 and HIV-1 MN, used for neutralization assays with rabbit sera, were obtained from M. Martin (National Institute of Allergy and Infectious Diseases) and the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, respectively. For neutralization assays with monkey sera, the following viruses were used: HIV-MN (from R. Gallo) and HIV-1 SF2 (from the NIH AIDS Research and Reference Program, donated by J. Levy). SHIV-HXBc2 and SHIV-89.6 were propagated in either H9 cells or human peripheral blood mononuclear cells (PBMC).

Purification of soluble Env proteins. Soluble gp120 and gp140 were produced by infection of BS-C-1 cells (ATCC CCL26) with recombinant vaccinia viruses at a multiplicity of infection of 5 to 10 PFU per cell. Two hours after infection, the medium was replaced with OptiMEM (Gibco-BRL, Grand Island, N.Y.), and infection was allowed to proceed for 24 to 36 h. Medium was harvested, and Env protein was purified by lentil lectin affinity chromatography (Amersham Pharmacia Biotech AB, Uppsala, Sweden) as previously described (23). For immunization of monkeys, the Env protein was concentrated with Centrprep-30 concentrators (Amicon, Beverly, Mass.) and further purified by Superdex-200 (Amersham Pharmacia Biotech AB).

Immunization of rabbits. New Zealand White rabbits were housed at Spring Valley Laboratories, Woodbine, Md. Rabbits (three per group) were immunized three times with 70 μ g of lentil lectin-purified gp120 or gp140 formulated with

MPL-SE adjuvant (Ribi ImmunoChem, Hamilton, Mont.), a 1.0% (vol/vol) squalene oil-in-water emulsion containing 250 μ g of monophosphoryl lipid A per ml, according to the manufacturer's specifications. Immunizations were performed at 4- to 6-week intervals, and the rabbits were bled 12 days after each immunization.

Purification of rabbit IgG. Immunoglobulin Gs (IgGs) were purified from rabbit sera by absorption to HiTrap protein G columns (Amersham Pharmacia Biotech AB). Serum samples were diluted 10-fold with phosphate-buffered saline (PBS) prior to passage over the column. IgG was eluted with 0.1 M glycine-HCl (pH 2.7) into tubes containing the appropriate volume of Tris-HCl (pH 9.0), shown to neutralize the eluate. The buffer was then exchanged with PBS, and the IgG was concentrated using Centrprep-30 microconcentrators (Amicon). The concentrations of individual samples were determined by the A_{280} and adjusted to 2 mg/ml. As a control, IgG was also purified from preimmune sera. No reduction in virus replication was observed with the control sera, and no cell toxicity was observed with any of the samples.

Neutralization of HIV-1 with IgG from immunized rabbits. The endpoint assay described in the 1997 Division of AIDS Virology Manual for HIV Laboratories was employed to determine the neutralizing activity of the IgG samples from rabbits immunized with gp140 or gp120 (71). In this assay, sequential dilutions of virus were mixed with a constant amount of IgG for 2 h, and residual virus infectivity was then determined. The percent neutralization was determined by comparison with virus infectivity observed with IgG from serum samples taken prior to immunization. Details of the protocol are as follows. Threefold serial dilutions of virus stock ranging from 54 to 0.22 tissue culture infectious doses (TCID₅₀)/5 μ l were prepared, and 25 μ l of each dilution was mixed with 25 μ l of a constant concentration of rabbit IgG in microcentrifuge tubes. For neutralization of HIV-1 NL4-3, concentrations of 1,000, 500, 250, 125, 62.5, 31.3, and 15.6 μ g/ml were used. The same concentrations, excluding the lowest concentration, were used for neutralization of HIV-1 MN. Cell suspension (200 μ l, 0.5 \times 10⁶/ml) was plated in the wells of 96-well flat-bottomed plates. After incubation for 2 h at 37°C, 10 μ l of virus-IgG mixture was added to each well. Each virus-IgG mixture was assayed in quadruplicate. Half of the medium was changed on day 7, and reverse transcriptase activity was assayed on day 14.

Reverse transcriptase assay. Supernatants from HIV-1-infected cultures were mixed with reverse transcription reaction mixture [50 mM Tris-HCl (pH 7.8), 63 mM KCl, 4.2 mM MgCl₂, 0.85 mM EDTA, and 0.08% NP-40 plus 4.2 μ g of poly(A) and 0.13 μ g of oligo(dT) per ml] containing [α -³²P]dTTP (Amersham Pharmacia Biotech AB) and incubated for 2 h at 37°C. Then 5 μ l of the reaction mixture was spotted onto DEAE filter paper and washed twice with 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filter paper was exposed to X-ray film overnight. The TCID₅₀ values were calculated using the formula of Reed and Muench (61). Percent neutralization was determined by comparison of pre- and postimmunization IgG samples.

Immunization and challenge of monkeys. Six juvenile rhesus macaques were obtained from the Oregon Regional Primate Research Center (Beaverton, Ore.) and housed at Bioqual, Inc., Rockville, Md., in accordance with guidelines described in the Guide for the Care and Use of Laboratory Animals. Four monkeys were immunized with 300 μ g of purified oligomeric gp140 in 100 μ g of QS-21 adjuvant (Aquila Biopharmaceuticals, Inc., Framingham, Mass.) at 0, 4, 8, and 24 weeks. The two control monkeys received 100 μ g of QS-21 in PBS at the same times. All six animals were challenged 3 weeks after the final immunization with 10 animal infectious doses of SHIV-HXBc2, obtained from Yichen Lu (Virus Research Institute, Cambridge, Mass.) (39). Immediately after administration of the challenge virus, several immunized animals experienced various degrees of anaphylaxis, which was most severe in animal 18001. The likely explanation was the presence of trace amounts of serum proteins contaminating the gp140 and subsequent use of medium containing fetal bovine serum as the diluent for the challenge virus. Sera from the animals were determined, retrospectively, to contain antibodies to albumin (T. VanCott, unpublished).

Neutralization of SHIV-HXBc2, SHIV-89.6, HIV-1 SF2, and HIV-1 MN with sera from immunized monkeys. Neutralization was measured in MT-2 cells as described previously (48). Titers of neutralizing antibodies are reported as the reciprocal of the serum dilution that protected 50% of cells from virus-induced cell killing as measured by neutral red dye uptake. Fifty percent protection corresponds to approximately 90% reduction in Gag antigen synthesis in this assay (12). The same assay was used to measure reductions in neutralization titers in the presence of 20 μ g of V3 peptide per ml (19). Additional neutralization assays with SHIV-HXBc2 were performed in human PBMC as described (47). Neutralization titers in the PBMC assay are reported as the reciprocal serum dilution at which p27 synthesis was reduced 80% relative to the corresponding preimmunization serum sample for each animal tested.

p27 ELISA. For the enzyme-linked immunosorbent assay (ELISA), Immunon-2 (Dynex Technologies, Chantilly, Va.) 96-well U-bottomed plates were coated overnight with 0.5 μg of SIV p27 (Advanced Bioscience Laboratories, Kensington, Md.) per ml. Twofold serum dilutions of monkey sera were incubated overnight at 37°C in block buffer containing 5% goat serum and 0.02% sodium azide. After washing, horseradish peroxidase-conjugated anti-monkey IgG was added for 30 min, followed by BM Blue substrate. After 30 min, absorbance was read at 370 and 492 nm. All assays were performed in duplicate.

SHIV cocultivation from PBMC. The PBMC fraction was isolated from whole blood samples by gradient centrifugation (Ficoll-Hypaque; Pharmacia, Piscataway, N.J.). Cell viability was determined using trypan blue. Cells were resuspended in medium at a concentration of 2×10^6 cells/ml. PBMC and target cells (174 \times CEM) were mixed and added to 96-well tissue culture plates in a total volume of 200 μl . Sample cells were assayed at six 10-fold dilutions starting at 1.5×10^6 /ml. Target cells were used at 3.0×10^5 /ml. Each dilution of sample cells was assayed in replicates of six. The experiment was carried out over a 28-day period. Culture medium was removed and replaced twice weekly. On days 10, 17, and 24, cell density was reduced by removal of approximately 75% of the cells in each well. On days 7, 14, 21, and 28, culture supernatants were collected and virus replication was monitored using the p27 ELISA antigen capture assay (Coulter/Immuntotech). The challenge virus was used to infect 174 \times CEM cells and served as the positive control. Uninfected 174 \times CEM cells served as the negative control. A sample was considered positive for virus replication if the optical absorbance of a given well in the antigen capture ELISA was equal to or greater than three times the background reading.

Quantitative PCR of SHIV RNA. Determination of the concentration of viral RNA in the plasma was performed as described (74).

RESULTS

Comparative immunogenicity of gp120 and gp140 in rabbits. The gp120 and gp140 used for these studies were prepared using previously described recombinant vaccinia viruses vPE50 and vPE12B, respectively (15, 23). The soluble proteins were purified by lentil lectin chromatography from the serum-free medium of cells infected with the recombinant viruses. Virtually all of the gp140 was oligomeric, whereas approximately 60% of the gp120 remained monomeric. To compare the relative immunogenicity of gp120 and gp140, three rabbits were immunized three times with 70 μg of protein formulated in Ribi adjuvant MPL-SE. Reciprocal endpoint ELISA titers were determined using IIIB gp120. Twofold-higher titers were achieved by immunization with gp140 ($341.3 [\pm 118] \times 10^3$) than with gp120 ($170.7 [\pm 59] \times 10^3$). Since gp120 was the captured antigen, differences between the responses were not due to the presence of gp41 in the gp140 immunogen. To explore this difference further, binding to several heterologous gp160s and their respective shed gp120s was analyzed. For this purpose, antigens were prepared from lysates and medium from cells infected with recombinant vaccinia viruses expressing Env proteins from HIV-1 isolates IIIB, JRCSF, SF2, RF, Ba-L, 89.6, and BK132. Results are shown in Table 1 as the serum dilution at which 50% binding was observed. With each heterologous Env tested, the binding of anti-gp140 serum was two- to threefold greater than binding with anti-gp120 serum. This was true for binding to both gp160 and gp120. In addition, serum from a rabbit immunized with reduced, denatured gp140 was analyzed. This serum showed intermediate levels of binding, suggesting that epitopes exposed on the native gp140 are responsible for the enhanced binding.

We assayed the neutralization of two laboratory-adapted isolates, HIV-1 NL4-3 and MN (Table 2). Because of nonspecific effects of whole sera, IgG was purified, and results are given as the IgG concentration resulting in 50, 90, or 95% reduction in infectious titer. With the homologous HIV isolate,

NL4-3, each of the anti-gp140 samples exhibited greater than 50% neutralization at the lowest IgG concentration used (15.6 $\mu\text{g}/\text{ml}$). They also exhibited 90% neutralization, one at the lowest concentration of IgG tested. In contrast, the anti-gp120 IgG samples had 50% neutralization of NL4-3 with 43 to 755 $\mu\text{g}/\text{ml}$, and none of the samples exhibited 90% neutralization. In addition, neutralization of the heterologous isolate, MN, was greater with the anti-gp140 IgGs than with the anti-gp120 IgGs. Together, these results suggested that the neutralizing activity generated by immunization with gp140 was not due entirely to anti-V3 activity. In addition, a low level of neutralization of isolate BZ167 was observed with gp140 IgG but not with gp120 IgG (data not shown).

Immune responses to vaccination of rhesus macaques with gp140. To test the immunogenicity of oligomeric gp140 in a nonhuman primate for which a homologous challenge was available, four rhesus macaques were immunized with purified oligomeric gp140. For this study, the lentil lectin-purified gp140 was further purified by chromatography on Superdex-200. A typical elution profile is shown in Fig. 1A. The fractions designated oligomer in the figure were pooled and used for immunizations. The leftmost peak is due to a buffer change from sample loading and does not contain gp140 or other proteins. Figure 1B demonstrates the purity of the gp140 compared to the starting material. Amino acid analysis of the purified protein verified that the sample was composed primarily of gp140 (data not shown). Intramuscular immunizations were performed at 0, 4, 8, and 24 weeks using 300 μg of gp140 in QS-21 adjuvant. Two control animals received PBS in QS-21.

Humoral immune responses were monitored during the immunization period. Binding titers, as measured by ELISA using IIIB gp120, were boosted incrementally with each successive immunization but were not sustained (data not shown). Furthermore, the animals mounted neutralizing antibodies against the homologous virus, SHIV-HXB2, that were detectable in both the MT-2 and PBMC assays (Table 3). Titers ranged from 187 to 868 after three immunizations and increased to 313 to 1,315 after the fourth immunization, as measured in MT-2 cells. The titers achieved with four immunizations of gp140 are comparable to those found in monkeys infected with SHIV-HXB2 for 21 to 100 weeks (47). In addition, monkeys immunized with IIIB gp120 in QS-21 achieved similar neutralizing antibody titers after four immunizations (D. Montefiori, unpublished). Titers in the PBMC blast assay were on average 6.6 times lower than in the MT-2 assays but followed the same rank order in both assays.

The breadth of neutralizing activity was examined with two heterologous TCLA strains of HIV-1 and SHIV-89.6 (Table 4). Neutralization of HIV-1 SF2 and HIV-1 MN occurred with sera from four and three animals, respectively, although the titers in each case were relatively low. In addition, serum from animal 18102 also neutralized SHIV-89.6. Serum samples obtained 2 weeks post-final boosting were also tested at 1:4 for the ability to neutralize six primary isolates of HIV-1 grown and assayed in human PBMC (56). No significant neutralization was observed (data not shown).

To determine the role of antibodies to V3 determinants, we assayed neutralization of SHIV-HXB2 after incubating serum samples for 2 h in the presence of a saturating concentration of IIIB V3 peptide. The amount of V3 peptide required was

TABLE 1. Binding of rabbit sera to heterologous Envs

Animal no.	Immunogen	Serum dilution giving 50% binding											
		gp160					gp120						
		RF	Ba-L	89.6	BK132	IIIB	JRCSF	RF	89.6	BK132	IIIB	JRCSF	
2219	gp120	3,640	4,920	3,290	870	21,020	2,810	4,780	2,750	2,440			
2220	gp120	2,020	2,870	2,150	1,760	23,160	5,510	4,450	1,990	4,100			
2221	gp120	1,280	2,240	1,590	690	11,590	2,250	2,330	1,250	2,120			
Avg ± SD		2,313 ± 1,207	3,343 ± 1,401	2,343 ± 866	1,107 ± 573	18,590 ± 6,156	3,523 ± 1,743	3,853 ± 1,330	1,997 ± 750	2,887 ± 1,063			
2164	gp140	5,550	10,420	3,720	1,730	31,540	5,000	7,400	3,800	5,550			
2165	gp140	7,220	17,540	6,650	1,200	41,250	12,740	12,420	10,460	7,820			
2166	gp140	5,290	9,690	3,600	2,360	15,180	6,160	7,410	4,550	6,360			
Avg ± SD		6,020 ± 1,047	12,550 ± 4,337	4,657 ± 1,727	1,763 ± 581	29,323 ± 13,176	7,967 ± 4,174	9,077 ± 2,895	6,270 ± 3,648	6,570 ± 1,159			
2144	Denatured gp140	4,920	6,050	3,170	4,830	9,510	2,340	4,180	2,620	7,720			

TABLE 2. Neutralization of laboratory-adapted isolates^a

Animal no.	Immunogen	Concn of rabbit IgG required (µg/ml)			
		NL4-3			MN (50%)
		50%	90%	95%	
2219	gp120	755	>1,000	>1,000	>1,000
2220		43	>1,000	>1,000	>1,000
2221		107	>1,000	>1,000	>1,000
2164	gp140	<15.6	161	579	850
2165		<15.6	<15.6	401	262
2166		<15.6	293	571	<200

^a Concentration of rabbit IgG required to neutralize 50, 90, and 95% of virus.

determined empirically using increasing concentrations of peptide. As shown in Table 5, a significant amount of neutralizing activity in sera from all four animals was not blocked by the linear V3 peptide, consistent with the demonstration of neutralization of several heterologous isolates.

Challenge of rhesus macaques with SHIV-HXB2. To test the protective efficacy of immunization with oligomeric gp140, the four immunized and two control monkeys were challenged with 10 macaque infectious doses of SHIV-HXB2. The challenge was performed intravenously 3 weeks after the fourth protein immunization, when neutralizing antibodies were expected to be at a peak. Infection was monitored by cocultivation of PBMC with CEM×174 cells, levels of viral RNA in plasma, development of anti-p27 antibodies, and maintenance of neutralizing antibodies.

The TCID₅₀ values of virus cultured from the PBMC at various times after challenge are given in Table 6. The control animals exhibited a peak of viremia at 2 weeks after challenge. In contrast, very little, if any, virus was observed in the PBMC of the immunized monkeys. Animals 17951 and 18102 were completely negative at all time points. A very small amount of virus was cultured from animal 18066 at 2 weeks postchallenge

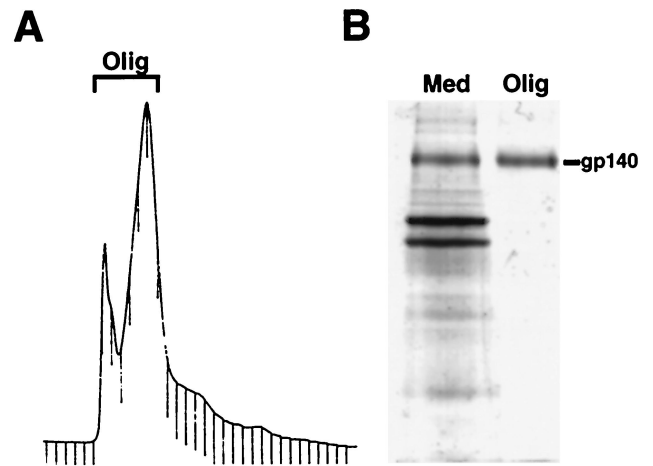


FIG. 1. Purification of oligomeric gp140. (A) Superdex-200 A_{280} elution profile of gp140. The bracketed fractions contain oligomeric gp140, as shown by chemical cross-linking (not shown). These fractions were pooled, concentrated, and used for immunization of macaques. (B) Coomassie blue staining of proteins in the medium of infected cells (Med) and in the purified Env (Olig) from panel A.

TABLE 3. Neutralizing antibody titer to SHIV-HXB2 in rhesus macaque sera^a

Animal no.	Immunogen	Titer at study wk									PBMC assay, wk 26
		MT-2 cell assay									
		10	12	20	26	31	33	37	53	150	
17951	gp140	187	134	<20	424	147	49	23	<20	<20	45
18001		348	159	40	844	563	690	471	469	2,016	250
18066		296	109	<20	313	70	36	13	<20	20	35
18102		868	510	81	1,315	686	408	543	153	88	280
18024	None	<20	<20	<20	<20	4,032	705	510	968	4,380	
18062		<20	<20	<20	<20	<20	25	79	793	4,514	

^a Neutralizing antibody titers are given as the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing. Immunizations were performed on weeks 8 and 24. SHIV-HXB2 challenge was on week 27.

and was not observed at any other time point. Animal 18001 was positive at three isolated time points after challenge; however, the level was more than 100-fold less than that seen in the controls.

Viral RNA in the plasma was determined by a quantitative reverse transcription-PCR assay (Table 7), in which the limit of detection was 300 to 1,200 copies/ml of plasma, depending on the volume of sample available for the assay. No RNA was detected in three of the four immunized monkeys (17951, 18066, and 18102). A low but detectable amount of RNA was found in plasma from animal 18001 at 3 weeks postchallenge.

Development of p27 antibodies was also monitored after challenge (Table 8). Both naïve controls exhibited a rise in anti-p27 antibodies. Immunized animal 18001 also developed antibodies to p27, although the levels were consistently lower than those found in the two controls. In contrast, the other three animals did not develop anti-p27 antibodies, consistent with lack of infection. In addition, serum samples from the two controls and vaccinated animal 18001 reacted with p27 in a commercial HIV-2 Western blot kit (data not shown) and immunoprecipitated metabolically labeled p27 prepared from a recombinant vaccinia virus-infected cell lysate (data not shown).

We monitored the SHIV-HXB2 neutralizing titers for more than 2 years after challenge. Typically, infected animals demonstrated high, sustained neutralizing antibody titers to the challenge strain, while those that were protected exhibited waning titers. Three immunized animals, 17951, 18066, and 18102, lost neutralizing activity against SHIV-HXB2, whereas the two control animals and 18001 have maintained high levels of binding (data not shown) and neutralizing (Table 3) antibodies.

DISCUSSION

Induction of strong humoral immunity is likely to be an important property of a successful HIV vaccine. Passive administration of neutralizing antibodies in both the SCIDhu mouse (27) and macaque (1, 32, 40, 42, 70) models has shown that protection is possible in the absence of cell-mediated immunity. Since the Env protein is the major target for neutralizing antibodies, use of soluble gp120 has been the primary strategy for induction of such antibodies. Initial studies utilizing monomeric gp120 yielded disappointing results in that only low levels of neutralizing antibodies were detected (17, 31, 41, 43). One possible means of eliciting a better response is to use

an oligomeric form of the Env protein that resembles that found on virus particles and infected cells.

The goals of the present study were twofold. First, we wanted to directly compare the immunogenicity of gp120 and gp140 in a small-animal model. To this end, we expressed both proteins from the IIB isolate of HIV-1 in mammalian cells. The proteins were produced, purified, and formulated identically. After formulation with adjuvants, we found no alteration in antibody recognition or oligomeric status (data not shown). The immunogenicity of the two proteins was examined in rabbits. The second goal was to test the immunogenicity and protective efficacy of oligomeric gp140 in the rhesus macaque model, for which a homologous challenge, SHIV-HXB2 (39), was available.

The results obtained from immunization of rabbits demonstrated that gp140 generated antibodies that were higher in titer and more cross-reactive than those generated by gp120. Because immunization with gp140 yielded antibodies with greater binding to both heterologous gp120s and gp160s, the enhancement could not be accounted for entirely by the presence of gp41 epitopes on the gp140 immunogen. Although the enhancement was only two- to threefold, it was consistently observed with six different proteins, including those from CXCR4- and CCR5-utilizing viruses. In addition, antibodies to denatured gp140 were not as cross-reactive as were those to oligomeric gp140. This result suggested that conserved epitopes on oligomeric gp140 were responsible for the enhanced cross-reactivity. In addition, better neutralization of HIV-1 NL4-3 as well as of the heterologous HIV-1 MN and BZ167

TABLE 4. Neutralizing antibodies generated by IIB gp140 immunization^a

Animal no.	Immunogen	Neutralizing antibody titer to:			
		SHIV-HXB2	SHIV-89.6	HIV-1 MN	HIV-1 SF2
17951	gp140	424	<10	22	48
18001		844	<10	23	44
18066		313	<10	<10	19
18102		1,315	16	33	48
18024	None	<20	<10	<10	<10
18062		<20	<10	<10	<10

^a Neutralizing antibody titers are given as the reciprocal serum dilution at which 50% of cells were protected from virus-induced killing. Serum was from week 26.

TABLE 5. Neutralization of SHIV-HXB2—adsorption with V3 peptide^a

Animal no.	Titer		% Non-V3 ^b
	-V3 peptide	+V3 peptide	
17951	323	192	59
18001	422	174	41
18066	204	100	49
18102	1,241	284	23

^a Neutralizing antibody titer is given as the reciprocal serum dilution at which 50% of cells were protected from virus-induced cell killing.

^b Percentage of neutralizing activity found in the presence of V3 peptide.

was found with sera from rabbits immunized with gp140 than with those from rabbits immunized with gp120.

There have been some attempts to predict the immunogenicity of a protein from its antigenicity. Thus, data obtained from phage display library panning of HIV-positive human sera predicted that gp140 would generate a weaker neutralizing antibody response than gp120 (53). In fact, direct comparison of the two immunogens in rabbits demonstrated that gp140 elicited the stronger neutralizing antibody response. In agreement with our results, VanCott et al. (76) demonstrated neutralization of some primary isolates by sera from rabbits immunized with a similar oligomeric Env. Using a recombinant vaccinia virus prime and SIV protein boost, Polacino et al. (59) found that boosting with gp160 gave more effective protection than did gp130. In another study, oligomeric SIV Env was reported to yield protection from challenge, while monomeric Env did not (55).

Our second goal was to test the effectiveness of the oligomeric gp140 immunogen in a nonhuman primate model. We wanted to exploit the potential for studying HIV-1 Env by using the SHIV challenge model. Because of constraints on the use of rhesus macaques for such studies, we did not perform a comparative analysis with gp120, although such a study was performed by another group (Berman et al., unpublished). Results from our study demonstrated that strong binding and neutralizing antibody responses could be achieved by immunization with oligomeric gp140. A compilation of results from several studies showed that neutralizing antibody titers of greater than 200 on the day of challenge were sufficient to afford protection against challenge with SHIV-HXB2 (D. Montefiori, unpublished). After four immunizations with oligomeric gp140, neutralizing antibody titers to SHIV-HXB2

TABLE 6. Isolation of virus from PBMC of rhesus macaques^a

Animal no.	Immunogen	TCID ₅₀ /10 ⁶ cells at wk postchallenge:				
		2	3	4	6	8
17951	gp140	NI	NI	NI	NI	NI
18001		NI	3	NI	NI	1
18066		1	NI	NI	NI	NI
18102		NI	NI	NI	NI	NI
18024	None	10,000	1	1	1	1
18062		10,000	10	1	1	NI

^a Values given are TCID₅₀/10⁶ cells. NI, not infected. Samples from week 10 through week 26 were also assayed. Animals 18001, 18024, and 18062 were positive at one time point during this period; animals 17951, 18066, and 18102 were negative.

TABLE 7. Plasma viral RNA^a

Animal no.	Immunogen	Copies of RNA/ml of plasma at indicated wk postchallenge:				
		0	2	3	8	53
17951	gp140	<1,200	<3,000	<1,200	<1,200	<300
18001		<1,200	<3,000	1,600	<1,200	<300
18066		<1,200	<1,200	<1,200	<1,200	<300
18102		<1,200	<1,200	<1,200	<1,200	<300
18024		<1,200	94,000	11,000	<1,200	<300
18062	None	<1,200	n.t.	8,600	<1,200	<300

^a Values given are copies of RNA per milliliter of plasma. The sensitivity of the assay was dependent on the volume of sample available; more volume was used with week 53 samples than with other weeks. 0 wk, day of challenge; n.t. = not tested.

ranged from 313 to 1,315, values that are similar to those found in monkeys infected with SHIV-HXB2. Comparable values, ranging from <25 to 824, were found in macaques immunized with IIB gp120 in QS-21 (D. Montefiori, unpublished). Significantly, the neutralization elicited by gp140 immunization was not completely blocked by a linear V3 peptide. The proportion of blocking by the V3 peptide was similar to that found in SHIV-HXB2-infected macaques (19), suggesting that other, more relevant epitopes may be accessible on the gp140 oligomer. In contrast to these results, sera from macaques immunized with IIB gp120 elicited almost exclusively V3-dependent neutralization of SHIV-HXB2 (D. Montefiori, unpublished). In addition, several heterologous SHIV and HIV-1 isolates were neutralized, albeit to low levels, with sera from subsets of the gp140-immunized animals. Particularly notable was one case of neutralization of SHIV-89.6, an isolate whose neutralization properties are quite different from those of IIB and which shows some resemblance to primary isolates (19).

Following SHIV-HXB2 challenge, there was no evidence of viral infection in three of the four immunized animals. In comparison to the naïve controls, the fourth animal exhibited a substantial decrease in viral load as measured by detection of both virus in the PBMC and viral RNA in the plasma. Because the challenge virus was nonpathogenic, evaluation of viral replication was limited to the 3- to 4-week time period immediately following challenge. Env and Gag serology, however, was monitored long term to confirm the infection status of the animals. In our study, the three animals that did not exhibit viremia immediately following challenge have shown no evi-

TABLE 8. p27 ELISA endpoint titers^a

Animal no.	Immunogen	Titer at wk postchallenge:				
		0	6	12	34	124
17951	gp140	0.2	0.2	0.1	0.1	0.1
18001		0.2	0.1	25.6	25.6	25.6
18066		0.8	0.1	0.1	0.1	0.1
18102		1.6	6.4	1.6	1.6	0.8
18024		0.1	12.8	102.4	409.6	819.6
18062	None	0.1	25.6	51.2	51.1	204.8

^a Titers are the reciprocal serum dilution × 10³.

dence of anti-p27 antibodies in a 2-year follow-up. In addition, their neutralizing antibody titers declined to background levels, further supporting the conclusion that they resisted infection. In contrast, both controls as well as one immunized animal exhibited properties characteristic of SHIV-HXB2-infected macaques, i.e., development of p27 antibodies and maintenance of SHIV-HXB2 neutralizing antibodies.

In contrast to our findings, Berman et al. (7) demonstrated protection against HIV-1 challenge in chimpanzees immunized with gp120 but not with soluble gp160, a protein produced from a truncated gene similar to ours. Several factors could account for this difference, including different Env purification protocols, the oligomeric status and integrity of the proteins, and a lower neutralizing antibody titer in the gp160-immunized animals on the day of challenge.

In summary, our study in rabbits demonstrated that immunization with oligomeric gp140 resulted in a qualitative improvement over immunization with gp120. In a follow-up study in macaques, we showed that oligomeric gp140 elicited strong homologous neutralizing antibodies and protected against homologous challenge. Importantly, not all of the neutralization was attributable to reactivity against the V3 loop. However, this did not translate into robust neutralization of heterologous viruses, and no neutralization of primary isolates was observed. In addition, we found no improvement in the level of neutralizing antibodies or protection in comparison to macaques similarly immunized with gp120 (Berman et al., unpublished). Clearly, additional modifications are needed to achieve broader and more potent neutralization, particularly with respect to divergent primary isolates.

There may be ways to further improve the gp140 immunogen. Until recently, only uncleaved oligomers could be purified due to the lability of gp120-gp41 interactions. A report from Binley et al. (8) demonstrated that the association between the subunits can be stabilized by the introduction of a disulfide bridge. In addition, Yang et al. (79) described the formation of gp140 oligomers that were stabilized by fusion to GCN4. Further modifications, such as elimination of potential N-linked glycosylation sites (62) or deletion of variable loops (72), may be means of exposing neutralizing epitopes. More importantly, cleaved and uncleaved gp140s from more clinically relevant, CCR5-utilizing primary isolates need to be tested.

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