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Induction of Neutralizing Antibodies against Human Immunodeficiency Virus Type 1 Primary Isolates by Gag-Env Pseudovirion Immunization

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A major challenge for the development of an effective HIV vaccine is to elicit neutralizing antibodies against a broad array of primary isolates. Monomeric gp120-based vaccine approaches have not been successful in inducing this type of response, prompting a number of approaches designed to recreate the native glycoprotein complex that exists on the viral membrane. Gag-Env pseudovirions are noninfectious viruslike particles that recreate the native envelope glycoprotein structure and have the potential to generate neutralizing antibody responses against primary isolates. In this study, an inducible cell line was created in order to generate Gag-Env pseudovirions for examination of neutralizing antibody responses in guinea pigs. Unadjuvanted pseudovirions generated relatively weak anti-gp120 responses, while the use of a block copolymer water-in-oil emulsion or aluminum hydroxide combined with CpG oligodeoxynucleotides resulted in high levels of antibodies that bind to gp120. Sera from immunized animals neutralized a panel of human immunodeficiency virus (HIV) type 1 primary isolate viruses at titers that were significantly higher than that of the corresponding monomeric gp120 protein. Interpretation of these results was complicated by the occurrence of neutralizing antibodies directed against cellular (non-envelope protein) components of the pseudovirion. However, a major component of the pseudovirion-elicited antibody response was directed specifically against the HIV envelope. These results provide support for the role of pseudovirion-based vaccines in generating neutralizing antibodies against primary isolates of HIV and highlight the potential confounding role of antibodies directed at non-envelope cell surface components.

An effective human immunodeficiency virus type 1 (HIV-1) vaccine is the best hope for controlling the AIDS pandemic. In 2004, there were approximately 40 million HIV-infected individuals worldwide, with a reported 5 million newly infected persons and 3 million AIDS-related fatalities (1). Highly active antiretroviral therapy has improved the quality of life and prolonged the survival of infected patients in developed countries. However, access to antiretroviral therapy is limited throughout much of the developing world, and the effectiveness of highly active antiretroviral therapy is frequently limited by the development of resistance and by toxicity. Therefore, there is an urgent need to develop a safe, affordable, and efficacious vaccine.

One of the major obstacles to the development of an effective vaccine has been the inability to design an immunogen that is capable of eliciting broadly cross-reactive neutralizing antibodies against primary HIV-1 isolates. The HIV envelope glycoprotein complex is the logical target for neutralizing antibody responses, and antibodies that bind the virion-associated HIV-1 envelope glycoprotein complex with high affinity can prevent infection of susceptible cell types (29, 43, 44). Passive antibody transfer experiments in animal models have proven that neutralizing antibodies can confer protection against HIV or simian/human immunodeficiency virus infection (3, 11, 14, 26, 28, 41). Although these results established that antibodies of the right type and of sufficient titer can be protective, efforts to develop vaccines based on gp120 subunit constructs have been disappointing so far. Antibodies elicited by monomeric-subunit vaccination strategies react primarily with the V3 loop or with linear epitopes on gp120 that are poor neutralization targets on primary HIV-1 isolates (4, 5, 18, 27, 34, 36, 45). Antibodies elicited by gp120 subunit immunization also appear to have weaker binding affinities to oligomeric Env than to monomeric gp120 (12, 13, 32, 35, 40). The limitations of the monomeric gp120 vaccine approach were demonstrated most dramatically by the failure of the VaxGen bivalent gp120 vaccine to provide protection from HIV infection in humans in phase III trials (47).

The failure of monomeric gp120 vaccines emphasizes the need for new approaches to elicit antibodies against the native, trimeric Env complex. Several strategies to address this, including the use of soluble gp140 trimers (23, 38, 39, 42, 49), solid-phase proteoliposomes incorporating oligomeric Env (16, 17), and HIV-1 pseudovirions (19, 31, 37), are under investigation. Pseudovirions are viruslike particles (VLPs) that are capable of exhibiting the native Env trimer on their membrane surface. Previous studies have established that Gag-Env pseudovirions incorporating primary isolate Env are stable and resist CD4-induced shedding of gp120 (19). When utilized as

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immunogens, HIV-1 and simian immunodeficiency virus (SIV) pseudovirions have been shown to induce both cellular and humoral immune responses in animal immunization protocols (9, 10, 31, 46). Simian/human immunodeficiency virus pseudovirions have been shown to activate human dendritic cells in vivo, upregulating expression of cell surface activation markers and major histocompatibility complex molecules (52). However, the potential of purified HIV-1 pseudovirions bearing primary isolate envelope glycoproteins to elicit broadly cross-reactive neutralizing antibodies requires further investigation.

We report here the immunogenicity of Gag-Env pseudovirions incorporating the R5 HIV-1 BaL Env. Our results demonstrate that envelope glycoproteins presented on immature HIV-1 pseudovirions can generate in guinea pigs antibodies that are capable of neutralizing both homologous and heterologous primary HIV-1 isolates. The magnitude and breadth of neutralization activity against a panel of HIV-1 isolates of primary clade B were greater for sera generated from pseudovirion immunization than for recombinant BaL gp120. A significant amount of reactivity against cell surface components other than Env was also generated and must be considered in ongoing and future studies employing pseudovirion immunogens.

MATERIALS AND METHODS

Cell lines and plasmids. The human kidney cell line 293T was maintained in DMEM (Dulbecco's modified Eagle medium, high glucose) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, and 2 mM penicillin-streptomycin (DMEM-GM). XC-18 cells (see description and construction below) were maintained in DMEM-GM supplemented with 100 μg/ml Geneticin, 100 μg/ml hygromycin, and 0.5 μg/ml puromycin. TZM-bl cells are HeLa cell clones that were engineered to express CD4 and CCR5 and to contain integrated reporter genes for firefly luciferase and *Escherichia coli* β-galactosidase under the control of an HIV-1 long terminal repeat (LTR) (48). TZM-bl cells were maintained in DMEM-GM. pVRC-3900 is an expression plasmid encoding a codon-optimized HIV-1 $Pr55^{Gag}$ polyprotein (22) and was kindly provided by Gary Nabel (VRC, NIH). The envelope expression plasmid pcDNA3.1(Zeo)-BaLgp160opt was produced by subcloning the codon-optimized BaL *env* sequence from pMR1W1-9 into pcDNA3.1(Zeo) (Invitrogen, San Diego, CA). pRevTRE is a retroviral, tetracycline (Tet)-responsive vector that expresses a gene of interest under the control of the Tet response element (BD Biosciences, Clontech, Palo Alto, CA). The Tet-responsive HIV-1 pseudovirion expression plasmid, pRevTREGagEnv, was produced by subcloning the *gag* sequence from pVRC-3900 and the BaL gp160 sequence from pcDNA3.1(Zeo)-BaLgp160opt into pRevTRE. The retroviral packaging plasmid, pCL-Ampho, expresses the amphotropic envelope glycoprotein (4070A) from murine leukemia virus (33) and was used to produce pseudotyped HIV-1 virions by transient cotransfection of 293T cells together with pNL4-3Env$^-$. pNL4-3Env$^-$ is a proviral expression construct deficient in its ability to express Env. This construct expresses the HIV-1 NL4-3 provirus but contains a frameshift mutation at the 5′ end of *env* (nucleotide 5950) that prevents expression of the envelope glycoprotein (6). The vesicular stomatitis virus G protein expression construct pHCMV-G was provided by J. Burns (University of California, San Diego) (51).

Production and purification of immature Gag-Env pseudovirions. To produce immature HIV-1 pseudovirions, the XC-18 cell line was constructed to express *gag* and *env* genes under a tetracycline-inducible expression system. XC-18 was generated using retroviral transduction of HIV-1 codon-optimized *gag* and BaL *env* gene sequences into 293 Tet-On cells. *gag* and *env* were cloned into the retroviral vector pRevTRE (Clontech, Palo Alto, CA) under the control of the modified tetracycline-regulated cytomegalovirus promoter and separated by an internal ribosome entry site sequence. Transduced cell clones were maintained in selection media containing 100 μg/ml Geneticin, 100 μg/ml hygromycin, and 0.5 μg/ml puromycin and were screened for p24 and gp120 release by enzyme-linked immunosorbent assay (ELISA). The XC-18 clone was selected for production of high levels of p24 and gp120, expanded, and used for production of vaccine doses. For production of pseudovirions, supernatants from doxycycline-induced XC-18 cells were harvested on day 5 and day 10 and clarified by centrifugation, filtered through a 0.45-μm filter, and pelleted through a 20% sucrose cushion (100,000 × *g* for 3 h at 4°C). The pellets were resuspended in 1.0 ml of Tris-buffered saline (TBS) and stored at −80°C. The quantity of particle-associated envelope glycoprotein was determined using a gp120 capture ELISA as previously described (19).

Gradient analysis of Gag-Env pseudovirions. HIV-1 Gag-Env pseudovirions were analyzed by centrifugation on linear 20 to 60% sucrose gradients. Pseudovirions were produced by doxycycline induction of the XC-18 cell line. Typically, three 150-cm^2 flasks of XC-18 cells were induced at 20 to 30% confluence and incubated for 48 h at 37°C in 5% CO_2. The cells were then washed, and labeling media were applied for 16 to 18 h. Pseudovirion preparations were metabolically labeled using 75-μCi/ml ^{35}S-labeled cysteine/methionine applied in cysteine- and methionine-deficient DMEM. Labeled pseudovirions were harvested after overnight incubation, clarified by low-speed centrifugation, filtered through a 0.45-μm filter, and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 × *g* for 3 h at 4°C). Particle pellets were then resuspended in 1 ml of phosphate-buffered saline (PBS) and overlaid on linear 20 to 60% sucrose gradients. Ultracentrifugation was performed at 100,000 × *g* overnight at 4°C in a Beckman SW41 rotor. Equal fractions were collected, and the density of each fraction was determined with a refractometer. Samples were subsequently diluted in PBS, disrupted using radioimmunoprecipitation assay buffer (1% NP-40, 0.1% sodium dodecyl sulfate [SDS] in PBS), and immunoprecipitated using HIV positive patients' sera. Analysis was performed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Growth of primary and pseudotyped HIV-1 and SIV isolates. Human peripheral blood mononuclear cells (PBMCs) were isolated from fresh heparinized blood by standard Ficoll-Hypaque gradient centrifugation methods and used to produce primary HIV-1 viral stocks. A panel of genetically diverse R5 clade B primary HIV-1 isolates (4, 20), including BaL, SS1196.1 (SS1196), 6101.10, QH0692.42, BG1168.1, and Pvo.4 (Pvo), have been recently described as part of a panel of subtype B isolates intended for standardized assessment of neutralization (24). PBMCs were resuspended in RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum and 50 μg/ml gentamicin (RPMI 1640-GM), aliquoted at 2.5×10^7 cells/ml, and stored in liquid nitrogen. Primary HIV-1 isolates were propagated in PBMCs stimulated with 5 μg/ml phytohemagglutinin and 5% interleukin 2 (IL-2). The IL-2/phytohemagglutinin-stimulated cells were infected using a high-titer seed stock of virus minimally passaged in PBMCs. One ml of virus was transferred to the flask containing freshly stimulated PBMCs and incubated overnight at 37°C in 5% CO_2. The cells were washed extensively and resuspended in 30 ml of RPMI with IL-2. Virus production was monitored every 2 days by measuring p24 concentrations of supernatants using a p24 capture ELISA. Typically, the virus was harvested three times; the first harvest was on day 5 postinfection, with subsequent harvests every 2 days. The virus-containing supernatants were collected, clarified by centrifugation, and filtered through a 0.45-μm filter. The virus was then aliquoted into 1-ml sterile screw-cap cryovials and stored at −80°C.

The pseudotyped HIV-1 isolate used, NL4-3ΔE/MLV (referred to later in this report as simply MLV), was produced via calcium phosphate cotransfection of 293T cells with pNL4-3Env$^-$ and pCL-Ampho. Supernatants were harvested 48 h posttransfection, clarified by centrifugation, passed through a 0.45-μm filter, and stored in cryovials at −80°C. Titration of pseudotyped virus was determined using the 50% tissue culture infectious dose ($TCID_{50}$) assay in TZM-bl cells described below. The R5-tropic SIV isolate used, $SIV_{mac}251$, was obtained from Ron Desrosiers through the AIDS Research and Reference Reagent Program (7). The chronically infected HUT 78/$SIV_{mac}251$ cell line was maintained in RPMI 1640-GM at 0.5×10^6 to 1.0×10^6 cells/ml. Early-passage virus was collected from cell culture supernatants, clarified by centrifugation, passed through a 0.45-μm filter, and stored in liquid nitrogen. Virus production was monitored and determined by $TCID_{50}$ analysis in TZM-bl cells.

Electron microscopy. Immature Gag-Env pseudovirions were produced and purified as described above. Sucrose-purified pseudovirion pellets were fixed in 2% gluteraldehyde in phosphate buffer, postfixed with 1% osmium tetroxide, stained with 1% uranyl acetate, dehydrated in ethanol, and embedded in Spurr resin. Thin sections were cut with an ultramicrotome and analyzed with a Philips model 3000 electron microscope.

Guinea pig immunizations. Each immunization protocol contained a group of three female Hartley guinea pigs (Elm Hill Breeding Labs, Chelmsford, MA). For induction of anti-Env antibodies, each animal was inoculated intradermally with 300 μl of adjuvanted pseudovirions containing 5 μg of particle-associated BaL gp120 (groups A, B, and C) or 5 μg of recombinant, soluble BaL (sBaL) gp120 (group D). Recombinant gp120 for immunization was obtained from the MicroQuant Facility at the Institute for Human Virology, Baltimore, MD. Intradermal immunizations were delivered dorsally at two to four sites per animal

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on weeks 0, 2, 4, and 8. Group A animals received inoculations of Gag-Env pseudovirions adjuvanted with TiterMax Gold (CytRx, Los Angeles, CA) as recommended by the manufacturer. Group B animals received inoculations of Gag-Env pseudovirions adjuvanted with 100 μl of a 13-mg/ml aluminum hydroxide colloidal suspension (Sigma-Aldrich, St. Louis, MO) and 100 μg of CpG oligodeoxynucleotide (ODN) sequence 2007 (21). A prime-boost immunization regimen, in which the first two prime immunizations were Gag-Env pseudovirions adjuvanted with an alum/CpG ODN 2007 combinatorial adjuvant and two subsequent inoculations were composed of sBaL gp120 adjuvanted with TiterMax Gold (CytRx, Los Angeles, CA) as described above, was designed for group C animals. Group D animals were immunized with sBaL gp120 adjuvanted with TiterMax Gold. Serum samples were collected from test bleeds on weeks 0, 2, 4, 6, 8, and 10 postimmunization. Serum was heat inactivated (56°C, 1 h) and stored at −20°C until use.

ELISA detection of anti-gp120 reactivity in guinea pig serum. The anti-gp120 reactivity of immunized animal sera was determined by coating 20 ng of affinity-purified BaL gp120 in PBS onto each well of a high-protein-binding-capacity microtiter plate (MaxiSorp; Nalge Nunc International, Rochester, NY) overnight at 37°C. The plates were blocked with 150 μl of 7.5% fetal calf serum in PBS for 1 hour at 37°C. The plates were washed four times with wash buffer (0.2% Tween 20 in PBS) prior to the addition of 100 μl of serial serum dilutions in ELISA sample diluent (10% fetal calf serum, 0.2% Tween 20 in PBS) for 2 hours at 37°C. The plates were washed four times as previously described, and a secondary donkey anti-guinea pig immunoglobulin G-horseradish peroxidase (Biomeda, Foster City, CA) antibody was added at a 1:3,000 dilution in sample diluent for 1 hour at 37°C. Following four washes, the ELISA was developed with 100 μl of an Immunopure TMB (3,3′,5,5′ tetramethylbenzidine dihydrochloride) substrate kit (Pierce, Rockford, IL). The reaction was stopped by the addition of 100 μl of 4 N H_2SO_4 to each well, and the optical density was read at 450 nm on a microplate reader. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

Titration of HIV-1 and SIV isolates. The titration of primary HIV-1 isolates and $SIV_{mac}251$ was performed using a $TCID_{50}$ assay in TZM-bl cells. The single-round infection assay was performed in 96-well flat-bottom culture plates seeded with 100 μl of TZM-bl cells at 100,000 cells per ml. The cells were incubated overnight at 37°C in 5% CO_2 prior to the addition of 100 μl of virus serially diluted in quadruplicate in DMEM-GM supplemented with 40 μg/ml of DEAE dextran. The final assay concentration of DEAE dextran was 20 μg/ml. Cells were incubated for 48 h, and 100 μl of supernatant was removed from each well prior to the addition of 100 μl of Bright Glo substrate (Promega, Madison, WI). Measurement of infectivity involved transfer of 150 μl of cell/substrate mixture to black 96-well solid plates (Costar, Corning, NY), and luminescence activity was quantified using a Packard plate luminometer. $TCID_{50}$ endpoint titers were determined using the Reed-Muench formula.

HIV-1 single-round neutralization assay. Neutralization was measured as a function of the reductions in luciferase reporter gene expression after a single round of virus infection in TZM-bl cells as described previously (30). TZM-bl cells were obtained from John Kappes and Xiaoyun Wu through the NIH AIDS Research and Reference Reagent Program. These cells are engineered to express CD4 and CCR5 and contain integrated reporter genes for firefly luciferase and *E. coli* β-galactosidase under control of an HIV-1 LTR. Primary HIV-1 isolates ($TCID_{50}$, 100 to 200) were incubated with serial dilutions of test samples in triplicate in a total volume of 150 μl containing 33.3 μg/ml of DEAE dextran for 1 hour at 37°C in 96-well flat-bottom culture plates. This mixture was then added to the corresponding well of a 96-well flat-bottom culture plate containing adherent TZM-bl cells. The cells were seeded at 10,000 cells per well in 100 μl of growth media and incubated overnight at 37°C in 5% CO_2. The final concentration of DEAE dextran was 20 μg/ml. One set of control wells received cells plus virus (virus control), and another set received cells only (background control). After a 48-h incubation, 100 μl of cells was transferred to a 96-well black solid plates (Costar, Corning, NY) for measurements of luminescence using Bright Glo substrate solution as described by the supplier (Promega, Madison, WI). Neutralization titers are the dilutions at which relative light units (RLU) were reduced by 50% compared to those of virus control wells after subtraction of background RLUs.

HIV-1 single-round Env competition neutralization assay. Affinity-purified, recombinant BaL gp120 was incubated with serially diluted guinea pig sera prior to addition of the primary R5 isolate BaL. Two hundred ng/ml of recombinant BaL gp120 was incubated with serial dilutions of test serum samples in triplicate in a total volume of 100 μl containing 33.3 μg/ml of DEAE dextran for 1 hour at 37°C in 96-well flat-bottom culture plates. BaL virus ($TCID_{50}$, 100) in a 50-μl volume containing 33.3 μg/ml of DEAE dextran was then added, and the plates were incubated for 1 hour at 37°C. This mixture was then added to the corresponding well of a 96-well flat-bottom culture plate containing adherent TZM-bl cells, and neutralization was detected as described above.

HIV-1 single-round V3 peptide competition neutralization assay. BaL V3 peptide purified by high-performance liquid chromatography (KSIHIGPGRAFYTTG) was incubated with serially diluted guinea pig sera prior to addition of the primary R5 isolate BaL. Fifty μg/ml of V3 peptide was incubated with serial dilutions of test serum samples in triplicate in a total volume of 100 μl containing 33.3 μg/ml DEAE dextran for 1 hour at 37°C in 96-well flat bottom culture plates. BaL virus ($TCID_{50}$, 400) in a 50-μl volume containing 33.3 μg/ml of DEAE dextran was then added, and the plates were incubated for 1 hour at 37°C. This mixture was then added to the corresponding well of a 96-well flat-bottom culture plate containing adherent TZM-bl cells, and neutralization was detected as described above.

Adjuvant effect on HIV-1 pseudovirion Env retention. The effect of alhydrogel (alum), an adjuvant used to enhance the immunogenicity of Gag-Env pseudovirions, was determined in regard to particle retention of envelope glycoproteins. Briefly, 5 μg of particle-associated gp120 was concentrated through a 20% sucrose cushion and resuspended in 200 μl of TBS. The purified Gag-Env pseudovirions were incubated overnight with 33.3 μl of a 13.1-mg/ml aluminum hydroxide colloidal suspension (Sigma-Aldrich, St. Louis, MO) and 33.3 μg of CpG ODN sequence 2007 at 4°C. The adjuvanted pseudovirion suspension was then dialyzed overnight with phosphate-buffered saline at 4°C, resulting in the subsequent release of alum-absorbed Gag-Env pseudovirions. The suspension was clarified by low-speed centrifugation, filtered through a 0.45-μm filter, and then purified by ultracentrifugation through a 20% sucrose cushion (100,000 × *g* for 3 h at 4°C). Particle pellets were then resuspended in 200 μl of TBS, and the quantity of particle-associated envelope glycoprotein was determined using a gp120 capture ELISA as previously described (19).

RESULTS

Design and production of pseudovirions. The major purpose of this study was to define the neutralizing antibody response generated by immunization with Gag-Env pseudovirions. We had previously established that Gag-Env pseudovirions bearing the HIV-1 BaL isolate Env were stable and did not lose significant amounts of gp120 when exposed to soluble CD4, incubation at 37°C, or storage and pelleting through sucrose (19). Transient transfection of 293T cells was used as the production method for this prior study. For the present study, we sought to develop a production method that would avoid the need for transfection, would produce pseudovirions of uniform consistency, and could be adapted to large-scale production. To do this, we generated a cell line bearing codon-optimized *gag* and *env* cassettes under the control of a tetracycline-regulated promoter in 293 cells (293 Tet-On; Clontech). This cell line, XC-18, was utilized to generate enough Gag-Env pseudovirions to immunize multiple groups of guinea pigs. The inducible production of Gag and Env by XC-18 is shown in Fig. 1A and B. This figure demonstrates that while Gag production by XC-18 was tightly controlled, Env production was not. Nevertheless, Env production and release from XC-18 was efficient, resulting in secretion of up to 100 ng/ml gp120 in the cellular supernatant. Vaccine doses sufficient to carry out immunization of multiple groups of guinea pigs were obtained from large-scale cultures of XC-18. Gradient analysis of Gag and Env content of pseudovirions produced by XC-18 is shown in Fig. 1C. We noted that the Env protein from this source of pseudovirions was almost entirely in the form of gp120, with minimal uncleaved precursor gp160 present (Fig. 1C). Before the XC-18 production system was optimized, some animals in this study received vaccine lots that were supplemented with pseudovirions that had been produced by transient transfec-

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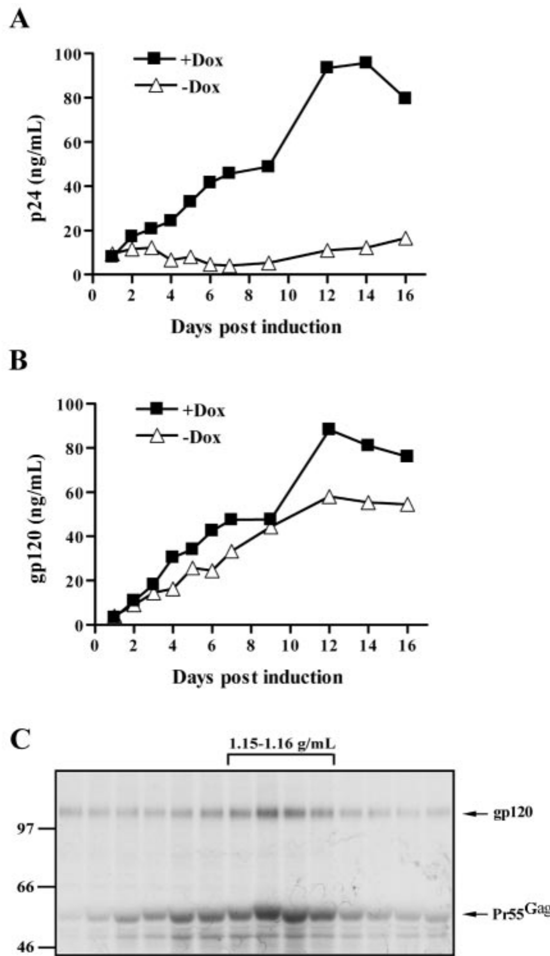

FIG. 1. Production of Gag-Env pseudovirions by XC-18 cell line. Cells were induced by addition of 2 μg/ml doxycycline (Dox). (A) p24 antigen release in cellular supernatants was measured at the times indicated. Closed squares indicate induced cell culture, and open triangles indicate uninduced cells. (B) Envelope glycoprotein secretion into cellular supernatants harvested at time points following induction as measured by gp120 capture ELISA. (C) Determination of sedimentation pattern of pseudovirions harvested from induced XC-18 supernatants by sucrose density-equilibrium gradient analysis. Metabolically labeled, doxycycline-induced XC-18 cell supernatants were concentrated through a 20% sucrose cushion, resuspended, and subjected to centrifugation on 20 to 60% sucrose gradients. Fractions were collected from the top of the gradient and immunoprecipitated using pooled HIV-positive patients' sera prior to analysis by SDS-polyacrylamide gel electrophoresis and autoradiography.

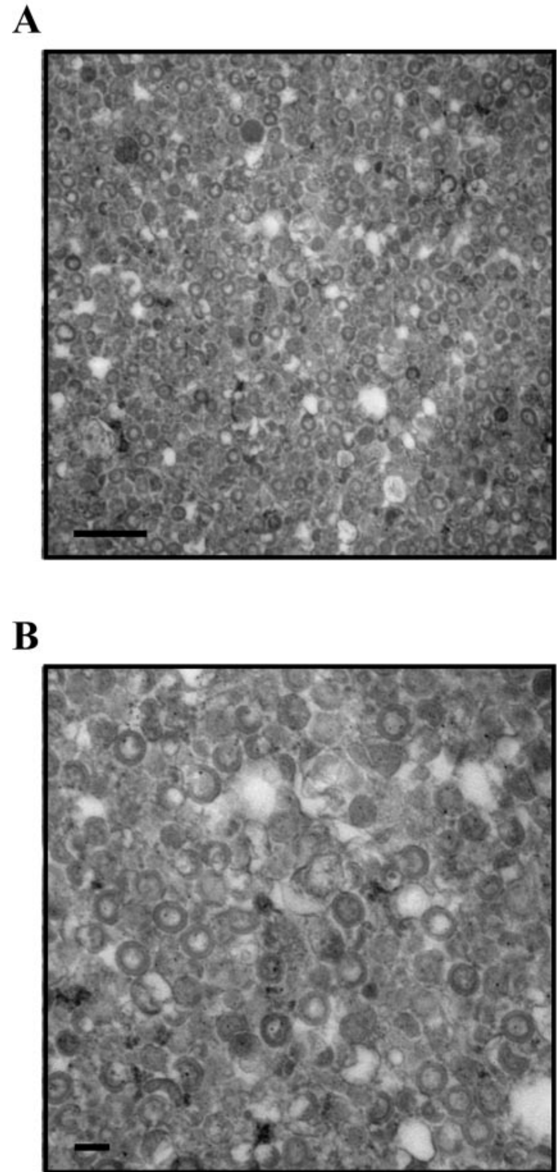

FIG. 2. Electron micrographs of immature HIV-1 VLPs produced from doxycycline-induced XC-18 cellular supernatants. Supernatants were concentrated through 20% sucrose cushion. Results from examination of pelleted VLPs are shown. (A) Magnification, ×49,500; bar = 500 nm. (B) Magnification, ×105,000; bar = 100 nm.

tion of 293T cells. Purified preparations from these lots maintained Gag:Env ratios similar to those of lots from XC-18.

To prepare Gag-Env pseudovirions for immunizations, cellular supernatants were concentrated through a 20% sucrose cushion. We assessed the purity and stability of the pelleted material by electron microscopy. Although some misshapen particles and microvesicles were observed in this preparation, the fields examined revealed primarily intact immature particles (Fig. 2A and B). Based upon Env content (Fig. 1) and morphological assessment (Fig. 2), we judged that the concentrated, sucrose cushion-purified particle preparations were suitable to test the hypothesis that Gag-Env pseudovirions can generate antibodies against the native Env trimer.

Pseudovirion immunization protocol. Guinea pigs were immunized with purified pseudovirions at 0, 2, 4, and 8 weeks according to the groups depicted in Fig. 3. The gp120 content of each production lot was measured, and 5 μg of particle-associated gp120 was administered per dose to each animal via the intradermal route. The first group tested received unadjuvanted pseudovirions (not depicted in Fig. 3). Responses for this group were suboptimal (data presented below), prompting the evaluation of several adjuvant strategies. Group A animals received pseudovirions adjuvanted with TiterMax Gold (CytRx Corp.), a block copolymer, water-in-oil emulsion. Group B

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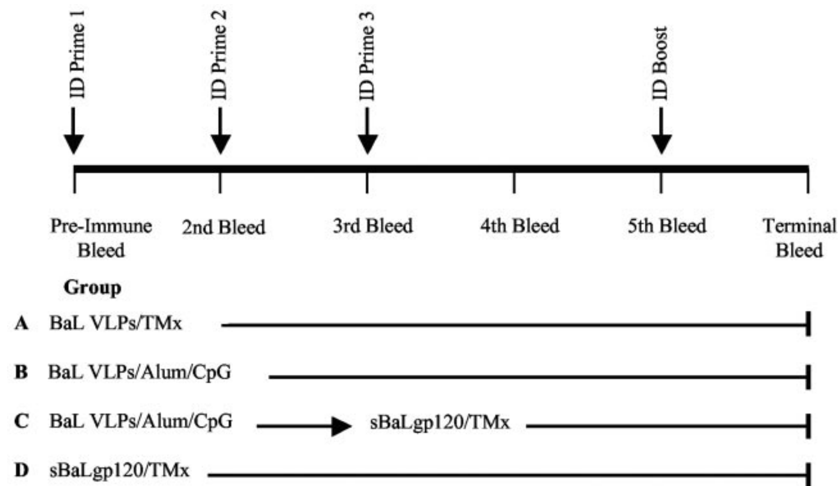

FIG. 3. Schematic diagram of guinea pig immunization protocol. Frequency and distribution of inoculations and bleeds are indicated for each of four groups (A to D). Within individual groups, a horizontal line indicates repeated administration of the same immunogen. A vertical bar denotes termination of the group protocol. TMx, TiterMax Gold; ID, intradermal.

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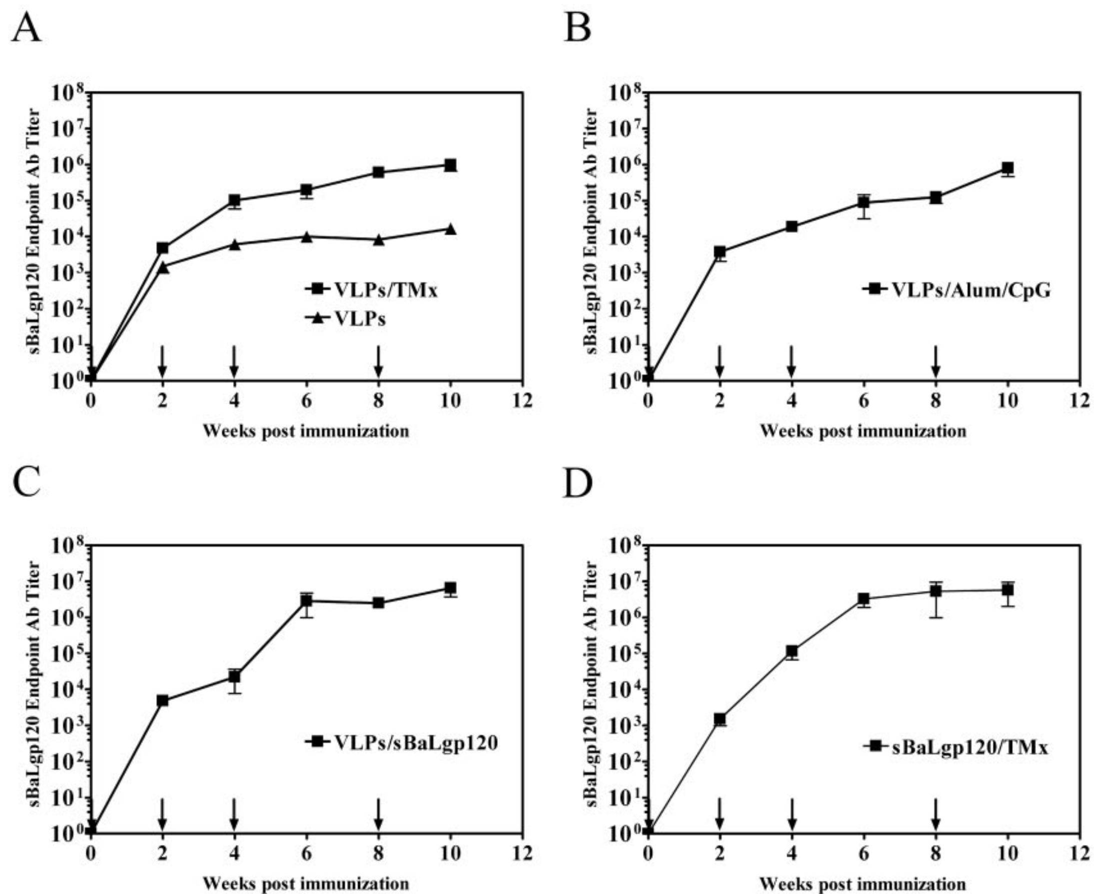

FIG. 4. gp120-specific antibodies raised by pseudovirion and soluble envelope glycoprotein inoculations were quantified by endpoint antibody (Ab) binding titration. (A) Anti-gp120 endpoint antibody titers for guinea pigs immunized with 5 μg of particle-associated BaL gp120 adjuvanted with TiterMax Gold (TMx) and for unadjuvanted pseudovirions. (B) Anti-gp120 endpoint antibody titers for guinea pigs immunized with 5 μg of particle-associated BaL gp120 adjuvanted with alum and CpG ODN sequence 2007. (C) Anti-gp120 endpoint titers for guinea pigs immunized with 5 μg of particle-associated BaL gp120 adjuvanted with alum/CpG ODN 2007 for the first two inoculations at weeks 0 and 2 followed by subsequent inoculations with 5 μg of soluble BaL gp120 adjuvanted with TiterMax Gold. (D) Endpoint antibody titers for guinea pigs immunized with 5 μg of soluble BaL gp120 adjuvanted with TiterMax Gold.

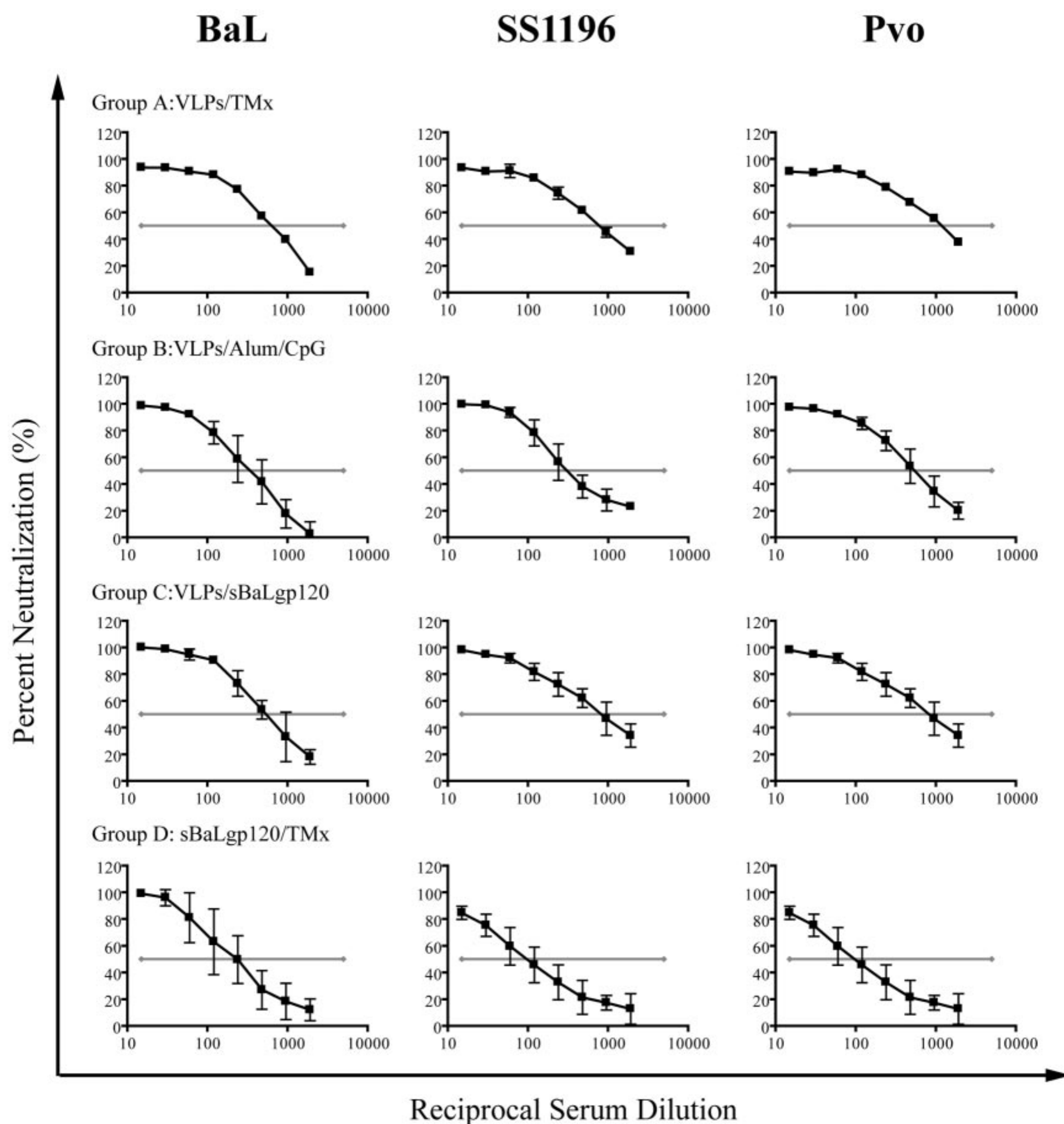

FIG. 5. Neutralization of HIV-1 BaL, SS1196, and Pvo by sera generated from guinea pig immunization protocol. Titrations of neutralizing activity were measured using a single-round infectivity assay in TZM-bl cells and quantitated by a reduction in RLU. Fifty-percent neutralization activity levels are indicated by horizontal gray lines. Neutralizing antibody titers are expressed as the serum dilutions required to reduce RLU by 50%. TMx, TiterMax Gold.

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received particles adjuvanted with aluminum hydroxide and CpG oligodeoxynucleotides (alum/CpG). This approach was chosen in an attempt to preserve the native structure of Env on the particle surface that might be disrupted by an emulsion. Group C animals were primed with pseudovirions adjuvanted with alum/CpG and boosted with soluble BaL gp120 protein adjuvanted with TiterMax Gold. Group D was included as a comparator arm; these animals received soluble gp120 adjuvanted with TiterMax Gold according to the same schedule.

Measurement of binding antibodies. Measurement of anti-gp120 antibodies was performed using an antibody detection ELISA that employed BaL gp120 as the coating antigen. Anti-gp120 antibody responses over time for each group are shown in Fig. 4. Unadjuvanted pseudovirions produced measurable but modest responses, with maximum endpoint titers of 2×10^4 (Fig. 4A). Use of the water-in-oil emulsion enhanced the anti-gp120 titers by more than 100-fold (Fig. 4A). The use of the alum/CpG adjuvant and of TiterMax Gold resulted in similar final titers (Fig. 4B). Group C demonstrated a dramatic increase in titer following the 4-week dose of soluble gp120 (Fig. 4C) and achieved the highest overall titer, which was similar to that seen with four doses of recombinant gp120 adjuvanted with TiterMax (Fig. 4D). Overall, the groups receiving recombinant gp120 developed gp120-binding antibody titers that were 0.5 to 1.0 logs higher than those of groups immunized with adjuvanted pseudovirions alone.

We considered the likelihood that some adjuvants would denature the Gag-Env pseudovirions, thus potentially negating

TABLE 1. Mean neutralization activity

Group: immunogen[a]	% Neutralization	Titer to HIV-1 isolate (± SD):							
		BaL	SS1196	6101	QH0692	BG11698	Pvo	MLV	$SIV_{mac}251$
A: VLPs/TMx	50	659 (42.0)	824 (120)	1,370 (377)	1,510 (61.6)	1,190 (165)	1,220 (117)	151 (8.62)	364 (98.3)
	90	92.3 (53.2)	72.3 (46.2)	152 (5.50)	50.7 (62.1)	101 (27.6)	101 (31.9)	0.00 (0.00)	66.0 (55.1)
B: VLPs/alum/CpG	50	377 (199)	324 (127)	803 (616)	629 (165)	687 (386)	607 (236)	98.7 (45.9)	148 (53.2)
	90	78.0 (26.4)	77.7 (14.0)	109 (57.9)	102 (32.9)	101 (37.0)	93.0 (27.7)	0.00 (0.00)	54.3 (14.1)
C: VLPs/sgp120	50	672 (312)	824 (521)	1,330 (782)	1,100 (595)	1,170 (520)	774 (267)	106 (55.6)	154 (75.9)
	90	126 (34.9)	83.7 (35.9)	171 (113)	69.7 (27.3)	93.0 (49.4)	85.0 (39.7)	0.00 (0.00)	46.7 (16.0)
D: sgp120/TMx	50	229 (165)	121 (69.0)	216 (141)	118 (68.3)	126 (49.0)	90.0 (52.1)	25.0 (2.65)	66.0 (22.1)
	90	63.0 (42.5)	0.00 (0.00)	15.7 (14.0)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	5.0 (7.1)

[a] sgp120, soluble gp120; TMx, TiterMax Gold.

the advantage posed by this immunization strategy in presenting unique epitopes for the development of neutralizing antibodies. Denaturation of the envelope would be expected to result in the release of free gp120 from the particle. To address this, pseudovirions were combined with alum/CpG, and then the alum/CpG was removed by dialysis against PBS. Filtered and dialyzed particle preparations were then pelleted through a 20% sucrose cushion. This resulted in a rise in the Gag:Env molar ratio from 32:1 (starting preparation) to 45:1 (postadjuvantation exposure and release). These data suggest that the alum/CpG adjuvant preparation retains the majority of gp120 on the surface of the pseudovirion particle. However, it was not possible to perform a similar experiment with the TiterMax Gold adjuvant due to the nature of the emulsion.

Neutralization assays employing primary isolates of HIV. We next performed neutralization assays against the homologous BaL viral isolate. Neutralization assays were performed using uncloned virus that was grown in PBMCs. The assay was performed using TZM-bl, a HeLa-based reporter cell line that expresses CD4, CCR5, and CXCR4 and incorporates a luciferase expression cassette under control of the HIV LTR. Neutralization curves for each group of animals against BaL are shown in Fig. 5. Neutralization of BaL was more efficient with sera from animals from each of the pseudovirion-containing regimens (groups A, B, and C) than with sera from animals immunized with soluble gp120 alone (group D). The magnitude of this effect was revealed by comparison of serum titers achieving 50% neutralization (NT_{50}) (Fig. 5 and Table 1). Regimens that included pseudovirions demonstrated mean NT_{50} values that ranged from 1.6-fold (alum/CpG group) to 2.9-fold (pseudovirion prime/gp120 boost) higher than those of the soluble gp120 arm (Table 1). These data indicate that the quality of the antibody response was higher for pseudovirion groups, despite equal or better binding antibody responses, as indicated by the anti-gp120 titers (Fig. 4).

To define the breadth of neutralization observed with pseudovirions versus that observed with soluble gp120, we evaluated a panel of five additional HIV-1 primary isolates. Neutralization curves for two of these isolates, SS1196 and Pvo, are shown in Fig. 5. Surprisingly, the sera were able to neutralize each of these primary isolates (Table 1). The serum dilutions at which 50% neutralization was achieved were again significantly higher for sera generated by pseudovirion-containing regimens than for that generated with soluble BaL gp120, and this was observed for each individual isolate tested. The mean and standard deviations for NT_{50} and NT_{90} values

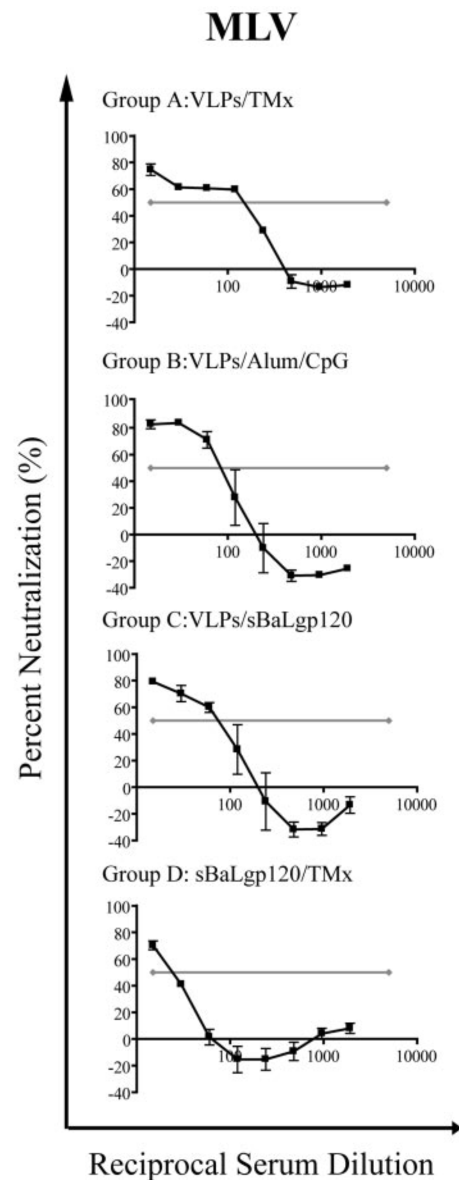

FIG. 6. Neutralization of an amphotropic MLV Env-pseudotyped HIV-1 virus by sera generated from guinea pig immunization regimens. Titrations of neutralizing activity were measured using a single-round infectivity assay in TZM-bl cells and quantitated by a reduction in RLU. Fifty-percent neutralization activity levels are indicated by horizontal gray lines. Neutralizing antibody titers are expressed as the serum dilutions required to reduce RLU by 50%. TMx, TiterMax Gold.

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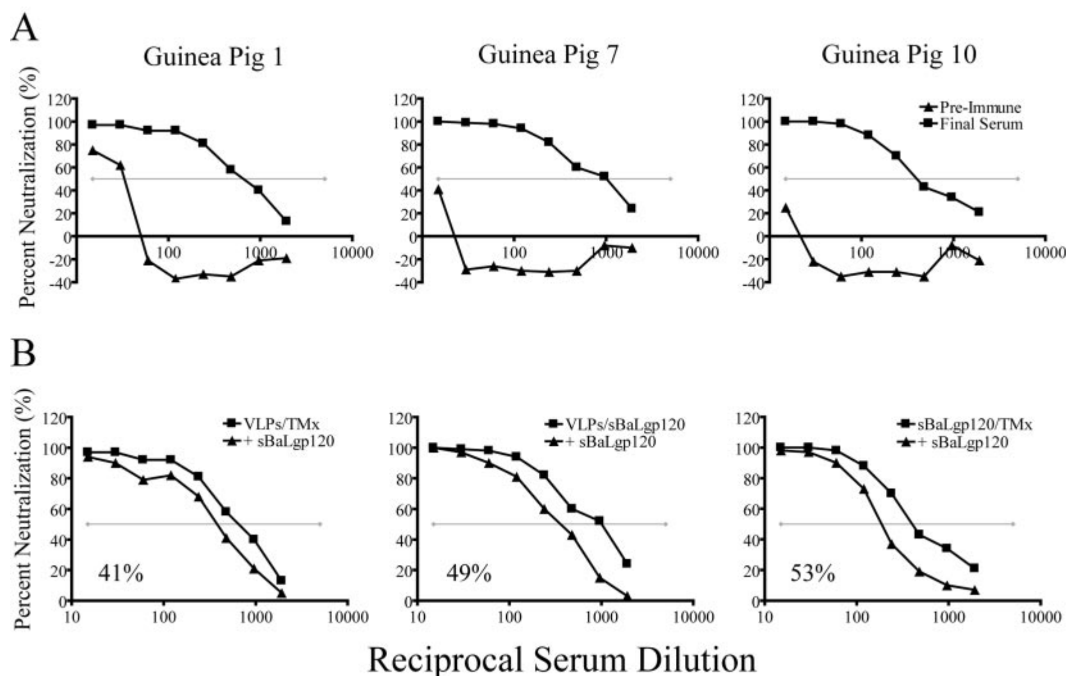

FIG. 7. Specificities of neutralization of HIV-1 BaL by sera generated from guinea pig immunization protocol. Titrations of neutralizing activity were measured using a single-round infectivity assay in TZM-bl cells and quantitated by a reduction in RLU. (A) Neutralization activity of prebleed (triangles) and terminal bleed (squares) serum samples from a representative guinea pig from each pseudovirion immunization group. The representatives from each group had the greatest anti-gp120 reactivity, as determined by endpoint antibody binding titration. (B) Identical guinea pig sera were used in a competition assay with soluble BaL gp120 to determine specificities of sera generated using pseudovirion (VLP) immunizations. Serial dilutions of guinea pig sera were incubated with 200 ng/ml BaL gp120 for 1 h prior to adding HIV-1 BaL virus, which was followed by an additional 1-h incubation period and performance of the neutralization assay described in Materials and Methods. The percent reduction in 50% neutralization titer is indicated in the lower left corner of the respective titration curve set. TMx, TiterMax Gold.

are summarized in Table 1. No significant differences were observed between groups A and C in mean 50% neutralization titers ($P = 0.15$; paired t test), indicating that gp120 boosting of pseudovirion-primed animals was equivalent to the administration of four pseudovirion doses. The titers achieved with alum/CpG were significantly lower than those of these two arms ($P < 0.001$) but were still markedly higher than the NT_{50} values from soluble gp120-immunized animals ($P < 0.01$). Overall, these results indicated an unexpectedly broad neutralizing response, with neutralizing antibody titers that were significantly higher in each case than those seen with soluble gp120 immunization.

We were encouraged by the magnitude and breadth of the neutralizing antibody activity observed in this study. However, we considered the possibility that the magnitudes of these titers may have been due at least partially to responses directed against viral cell surface components other than gp120 or gp41. HIV virions and viruslike particles are known to incorporate a multitude of cell surface proteins, and responses against these components can be neutralizing (2, 15). To examine this possibility, we measured the abilities of the guinea pig sera to neutralize a gp160-deficient virus that was pseudotyped with the amphotropic murine leukemia virus envelope glycoprotein MLV. As expected, there was minimal neutralization of this virus induced by sera from animals that had received soluble BaL gp120 immunization (Fig. 6, group D). Animals that had received pseudovirion immunizations, however, did demonstrate measurable neutralization of MLV (Fig. 6, groups A to C). We compared the titer of this activity to that seen for neutralization of the six HIV isolates tested (Table 1). Although the NT_{50} values for MLV clearly represent significant neutralization above background, they were in each case much lower than the titers seen for neutralizing the HIV isolates. As an additional control, we assessed the ability of sera from each immunization group to neutralize $SIV_{mac}251$, as HIV envelope-specific neutralizing activity should not cross-neutralize SIV. Neutralization of $SIV_{mac}251$ that was well above the background level and that was also of the greatest magnitude for those groups receiving adjuvanted pseudovirions was observed (Table 1, rightmost column). Neutralization titers for SIV remained significantly below the titers for HIV, similar to what had been observed for MLV. We conclude that the titers for the six isolates presented in Table 1 represent a combination of activity directed against the HIV Env complex and neutralization activity against cell surface components. The magnitude of the HIV-specific responses indicates that the majority of the neutralization activity measured was directed against Env rather than against cellular proteins on the viral membrane.

The values obtained by this immunization regimen did not represent intrinsic, nonspecific neutralization present in guinea pig sera. Preimmune sera were tested against each viral isolate, and, where relevant, these values were subtracted from the results for the final bleed sera. Figure 7A demonstrates the

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differences seen with preimmune and final bleed serum from the animals in each of the three pseudovirion-immunized groups that demonstrated the highest neutralizing antibody titers. In each case, at the dilutions where 50% neutralization was achieved, there was no neutralizing activity present in the preimmune serum.

To further demonstrate that the neutralization activity we observed in this study represented HIV-specific neutralization, we repeated the neutralization assays for selected animals in the presence of 200 ng/ml of recombinant BaL gp120. A significant shift to the left of the neutralization curve was observed in each case, representing partial adsorption of anti-gp120 activity (Fig. 7B). The magnitudes of the reductions in NT_{50} ranged from 41 to 53% (Fig. 7B). The same experiment was performed with MLV, and no shift was observed (data not shown). These data indicate that a significant portion of the neutralizing activity in the sera from immunized guinea pigs was due to anti-gp120 antibodies. We were not surprised to note that the adsorption effect was only partial, as this procedure would not adsorb anti-gp41 activity and may not remove some conformation-dependent neutralizing activity against epitopes not present on the monomer. In addition, the amount of protein may not have been sufficient to saturate the anti-gp120 activity, and as demonstrated above there was also a component of activity directed against cell surface proteins.

V3 loop peptide neutralizing antibody competition was performed with sera from the same three animals used in the gp120 competition experiments shown in Fig. 7. A 15-amino-acid peptide representing the Bal V3 loop was placed in wells at a concentration of 50 μg/ml together with serial dilutions of guinea pig sera and assessed for the ability to inhibit neutralization of BaL virus. Neutralization of BaL by the monoclonal antibody 447-52D (specific for the V3 loop) was completely inhibited by V3 peptide under the conditions of this assay. Neutralization by serum from an animal receiving soluble gp120 immunization (Group D) was inhibited by 53%, while serum from the animal receiving pseudovirion priming followed by soluble gp120 boosting was inhibited by 21%. The neutralization conferred by serum from a representative animal from Group A (pseudovirion prime/pseudovirion boost) demonstrated no detectable inhibition by the V3 loop peptide. These results suggest that a significant portion of the neutralization activity elicited by soluble Bal gp120 was directed against the V3 loop, while pseudovirion immunization raised neutralizing antibodies directed primarily at non-V3 determinants on gp120.

DISCUSSION

The inability of current HIV vaccine regimens to generate a broad neutralizing antibody response against primary isolates leaves a major gap in our vaccine armamentarium. There is broad consensus that generation of such antibodies at high titer in vaccinated individuals in combination with vectors that elicit strong cellular immune responses is desirable. Immunogens that recreate the native, trimeric envelope glycoprotein structure on a lipid membrane have the potential to avoid generating undesirable antibodies directed against epitopes on gp120 that are not exposed on native virions. Whether antibodies raised by such membrane-bound primary isolate Env complexes can elicit strong neutralizing antibody responses remains to be determined.

The primary goal of this study was to define the ability of Gag-Env pseudovirions to generate antibodies capable of neutralizing primary HIV-1 isolates. Pseudovirions represent one means of presenting the Env glycoprotein complex in its native form. One of the limitations of the pseudovirion HIV vaccine approach has been the inefficient production of pseudovirion particles when production is based on transient transfection of mammalian cells. The feasibility of performing studies using pseudovirion immunogens was enhanced in our study by the development of an efficient mammalian cell-based production system. The use of this mammalian cell line facilitated standardization of particle production for our guinea pig study and should facilitate scale-up to levels that are feasible for vaccine development in the future. This production method resulted in pseudovirions bearing gp120-gp41 heterodimers that had been efficiently cleaved from the gp160 precursor.

In this study, anti-Env endpoint antibody titers of high magnitude were elicited using several pseudovirion immunization regimens. HIV-1 pseudovirions have been reported to stimulate innate and adaptive immune responses in the absence of adjuvants, and it has therefore been suggested that adjuvants may be unnecessary with pseudovirion approaches (10). However, we found that with the pseudovirions produced from the XC-18 cell line, anti-gp120-binding antibody titers were greatly enhanced either through the use of a block copolymer adjuvant or with alum/CpG. Notably, the group of animals that received unadjuvanted pseudovirions did not develop measurable neutralizing antibody responses against any of the isolates examined. We conclude that although anti-gp120 antibodies can be elicited by naked pseudovirions, adjuvants will be needed for optimal humoral responses.

The neutralizing antibody responses against a panel of HIV-1 primary isolates that were elicited by pseudovirion immunization in this study were remarkably broad, and the antibodies were of significantly higher titer than those elicited by soluble BaL gp120 alone. These responses were at least partially directed against epitopes on gp120, as indicated by adsorption with recombinant gp120. A limitation of our study, however, was the development of a significant neutralizing antibody response that was directed against cellular proteins incorporated on the pseudovirion membrane. Anticellular antibodies may be generated from xenogeneic immunization of small animals with pseudovirion immunogens that incorporate human cell surface antigens (30). These antibodies can neutralize virus by inhibiting envelope-CD4 binding, by interfering with viral attachment via adhesion molecule-ligand interaction, and by providing overall steric hindrance. The contribution of anticellular activity in this study was shown by the neutralization of MLV-pseudotyped, HIV Env-deficient virus and by the neutralization of SIV_{mac}251. The presence of antibodies that bound to cells was confirmed by fluorescence-activated cell sorter analysis of producer 293 cells using immunized guinea pig sera (data not shown). We note that the level of neutralization of MLV-pseudotyped virus was relatively low compared with the neutralization of intact HIV isolates. Nevertheless, the presence of a measurable level of cross-reacting antibody prevents us from providing a definitive neutralization titer that is directed entirely against HIV Env. The potentially con-

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founding effect of this activity will need to be taken into consideration in future evaluation of pseudovirion immunogens. It will be important to determine if the use of production methods based on primate cells or other cells from other species will diminish this effect. Adsorption of anti-cell surface activity by incubation of sera with the producer cell type is another option for dealing with this problem. However, in our study we were unable to completely remove this activity, despite several rounds of adsorption with 293 cells.

Differences in the magnitudes of neutralizing antibody activities elicited against primary isolate viruses in this study did not correlate well with studies examining the relative ease of neutralization of this panel of viruses (24). Why would pseudovirion-induced responses generate broad responses that do not correlate with the differential sensitivities of these isolates to neutralizing monoclonal antibodies or to sera pooled from HIV-infected patients? We can speculate that this activity is directed against a conserved epitope or epitopes present on the membrane-bound trimer and that these epitopes may differ from those recognized by the currently available neutralizing monoclonal antibodies or those commonly recognized by HIV-infected patients' sera. Further evaluation of this optimistic hypothesis, however, requires the development of methods that will remove the confounding issue of anti-cell antibody responses.

The neutralization of primary HIV isolates using sera from rabbits immunized with proteoliposomes bearing YU-2 Env trimers has recently been reported (16). Proteoliposome immunization resulted in enhanced neutralization of primary isolates compared with that induced by soluble gp120 alone, similar to findings in our study. The YU-2 proteoliposomes were also able to induce a breadth of neutralization of HIV primary isolates that was enhanced compared to that induced by soluble gp120. This approach is not likely to be complicated by the presence of cellular proteins on the membrane surface that can elicit neutralization of viral particles.

The titers of neutralizing antibodies we found in this study raised by soluble gp120 against primary isolate viruses were relatively high compared to results from a number of studies of monomeric HIV glycoprotein immunization in the literature (8, 27, 45, 50). While it is possible that this reflects the high level of anti-gp120 antibodies raised by the immunization protocol employed, it is also important to point out that the methods may not be directly comparable. The fact that our studies were performed with guinea pigs and utilized a luciferase reporter cell line (TZM-bl) may account for some of the differences. However, another recent study from guinea pigs immunized with the same Bal gp120 protein reported NT_{50} values for Bal and SS1196 that were comparable with and even slightly higher than ours (25), supporting the validity of these results. Efforts at standardizing neutralizing antibody reporter cell assays and test virus panels are under way (24) and should help in placing these results within the context of those from other laboratories. A more important aspect of the present study was the ability of pseudovirion-associated glycoproteins to increase the magnitude of the neutralizing antibody response in the same animal model and using the same assay format.

HIV-1 pseudovirions remain an attractive platform for the development of immunogens capable of eliciting broadly cross-reactive neutralizing antibodies. Results presented here suggest that neutralizing antibody responses of greater magnitude than those of soluble gp120 can be generated by pseudovirion immunization. This study also highlights the potential confounding influence of anticellular antibodies on the interpretation of neutralizing antibody titers resulting from pseudovirion immunization. It will be important to address this in the future through development either of new production systems or of efficient means of adsorption of this activity.

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