JOURNAL OF VIROLOGY, Aug. 2005, p. 10210–10217 Vol. 79, No. 16
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.16.10210–10217.2005

Formaldehyde-Treated, Heat-Inactivated Virions with Increased Human Immunodeficiency Virus Type 1 Env Can Be Used To Induce High-Titer Neutralizing Antibody Responses

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Received 3 January 2005/Accepted 4 May 2005

The lack of success of subunit human immunodeficiency virus (HIV) type 1 vaccines to date suggests that multiple components or a complex virion structure may be required. We hypothesized that the failure of current vaccine strategies to induce protective antibodies is linked to the inability of native envelope structures to readily elicit these types of antibodies. We have previously reported on the ability of a formaldehyde-treated, heat-inactivated vaccine to induce modest antibody responses in animal vaccine models. We investigated here whether immunization for HIV with an envelope-modified, formaldehyde-stabilized, heat-inactivated virion vaccine could produce higher-titer and/or broader neutralizing antibody responses. Thus, a clade B vaccine which contains a single point mutation in gp41 (Y706C) that results in increased incorporation of oligomeric Env into virions was constructed. This vaccine was capable of inducing high-titer antibodies that could neutralize heterologous viruses, including those of clades A and C. These results further support the development of HIV vaccines with modifications in native Env structures for the induction of neutralizing antibody responses.

Although it has been 20 years since human immunodeficiency virus type 1 (HIV-1) was first isolated, the virus remains an emerging pathogen worldwide, with 14,000 to 16,000 new infections occurring daily. The field has developed potent chemotherapeutic strategies to treat HIV infection, which have dramatically reduced the number of AIDS cases and progression to disease in the United States and Europe. Nonetheless, these regimens have not been uniformly successful, and they remain economically impractical for treatment of the epidemic in the developing world. Therefore, it is clear that studies must be targeted at the identification and development of protective HIV vaccine immunogens. Cogent arguments exist for a variety of HIV-1 vaccine strategies, including one based on inactivated virions. This technology has worked successfully for a variety of viral, including retroviral, vaccines (29, 41, 48). Moreover, dendritic cells pulsed with inactivated autologous virions have been successfully used in a phase 1 trial as a therapeutic HIV vaccine (33).

Recombinant protein subunit vaccines based on X4-tropic viral isolates represented the first generation of HIV candidate vaccine strategies. We now recognize that antibody responses to monomeric envelope proteins generally elicit weak responses to homologous viruses and are generally unable to neutralize heterologous primary viral isolates (9, 24, 34, 35). In light of these data, attention has been focused on generating oligomeric envelope proteins which can be used as vaccine immunogens, such as soluble gp140 oligomers. Unfortunately, it has been exceedingly difficult to generate stable secreted forms of trimeric envelope (5, 54). Similarly, DNA vaccine strategies to date have generally resulted in the induction of low-titer antibody responses. Whereas plasmid DNA vaccination and a number of recombinant-vector-based strategies have been shown to induce cellular immune responses against internal proteins (1–4, 10, 18, 19, 22, 23), the ability of current vaccine candidates to induce protective neutralizing antibody responses has been limited. While it is increasingly clear that cell-mediated immune responses will be a critical component of vaccine-induced protection, it is also apparent that these responses are not likely to be sufficient. Therefore, it is crucial to test vaccine strategies aimed at inducing protective antibody responses.

Studies using vaccination with immunogens containing V3 sequences have generally elicited antibodies that recognize linear clade-specific epitopes (15, 28, 34, 49). Attempts have also been made to modify gp120, for instance, by deleting variable loops or glycan residues. These too have failed to generate high-titer heterologous antibody responses. For instance, vaccines based on HIV DH12- or 89.6-derived Env containing deletions in variable loops failed to induce heterologous neutralizing antibodies at all (31, 42), and similar constructs based on HXB2 generated low-level heterologous neutralizing antibodies in mice and rats (30, 46). Other approaches, such as gp120-CD4 cross-linked immunogens, have elicited neutralizing antibodies against a panel of primary viruses in macaques, but clear determinations as to whether these responses were against gp120 or CD4 (20, 50) have not been made. Finally, studies attempting to use chimeric gp120 molecules with C3d elicited higher antibody titers than gp120 alone, but the antibodies were not able to neutralize heterologous viruses (25).

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The use of whole killed virions provides a complex antigen source and is another approach that could be taken to develop an effective HIV-1 vaccine. The technology for a whole killed virion vaccine has been available for many years and has proven to be effective in the development of a number of human and veterinary vaccines, including those for retroviruses which infect cats (feline immunodeficiency virus) (52, 53) and horses (equine infectious anemia virus) (29). In general, three concerns are cited as reasons for not exploring virion-based vaccines for HIV: (i) a belief in the inability to retain gp120 on virions, (ii) the xenoreactivity observed with early inactivated simian immunodeficiency virus (SIV) vaccine preparations, and (iii) safety concerns surrounding whole virion preparations as vaccines. We previously addressed a number of these concerns in vitro (27). In those studies we demonstrated that virus could be inactivated by at least 7 logs and not only maintain, but also enhance, capacity for binding to broadly reactive, conformation-dependent neutralizing antibodies. In addition, we have shown that treatment of these preparations with sublethal doses of formaldehyde can result in retention of gp120 on the virion. Studies with mice and nonhuman primates indicated that these preparations were immunogenic and were HIV specific, but the titers that were induced after vaccination were modest (40). Therefore, we modified the immunogen in an attempt to increase its immunogenicity.

We describe here a vaccine containing a point mutation in the gp41 cytoplasmic tail that has been reported to reduce the rate of endocytosis of HIV Env (17) and SIV Env (32, 45), resulting in increased expression of these proteins on the surface of infected cells and, in the case of SIV, resulting in increased incorporation of Env into virions (55). This conserved tyrosine (32) at position 706 (of HIV_{SX}) in gp41 serves to direct the removal of cell surface Env that is not associated with Gag and thus would not be incorporated into virions during the budding process. In this manner, HIV-infected cells may gain a level of protection from immune surveillance. We report here on the ability of a vaccine incorporating this mutation in gp41 to induce broad neutralizing antibody responses in mice and rabbits.

MATERIALS AND METHODS

HIV immunogens. Vaccine immunogens were based on the full-length infectious molecular clone HIV_{SX} (37) and were prepared by transfection of 293T cells grown in Nunculon triple flasks as previously described (26). Prior to initiation of the experiments described in this paper, we restored a well-described HLA A2-restricted cytotoxic-T-lymphocyte epitope in *gag* (7, 8) that is missing in the wild-type HIV_{SX}. This change was made because we felt it might be important to restore this epitope (SLYNTVATYL) for future studies of cell-mediated immunity. This resulted in the generation of the infectious molecular clone that is called HIV_{SXSL9}. The wild-type envelope proteins of these two clones are identical. For mutation of the tyrosine at position 706 to a cysteine, a 3.2-kb fragment of HIV_{SXSL9}, spanning the EcoRI and XhoI sites (nucleotides 5744 to 8900), was subcloned into pBluescript SK(+) (Stratagene, Calif.) (pBlue-env). PCR site-directed mutagenesis was performed using primers 5′ GTTAGGCAG GGCTGCAGCCCATTATCGTTTC 3′ (forward) and 5′ GAAACGATAATG GGCTGCAGCCCTGCCTAA C 3′ (reverse) and confirmed by DNA sequencing. The StuI/BlpI fragment, containing the Y706C mutation, was inserted back into HIV_{SXSL9}, resulting in HIV_{SXSL9}(Y706C).

Cell-free supernatants were treated with Benzonase (Merck) for 2 h at 37C to digest contaminating nucleic acids. Benzonase-treated virus stocks were subjected to ultraflitration through a 300-kDa-molecular-mass-cutoff device using a Pall Mini-ultrasette tangential flow ultrafiltration device. These samples were subjected to ultracentrifugation in a fixed-angle rotor for 2 hours at an average relative centrifugal force of 40,000 × *g*. Virus pellets were resuspended in serum-free medium (AIM V), and formaldehyde (10% ultrapure; Polysciences) was freshly diluted in phosphate-buffered saline (PBS) and added to the virus for a final concentration of 0.02%. After incubation, an equal volume of 0.2% bovine serum albumin in PBS was added to quench residual aldehyde. The buffer was removed by diafiltration in a 100-kDa ultrafiltration device (Millipore) by centrifugation at 10,000 rpm. The filtrate (approximately 95% of the volume) was removed by aspiration, and PBS was added to the upper cell to reconstitute the sample to the original volume. After mixing by inversion, the device was centrifuged at 10,000 rpm. This process was repeated a total of four times, resulting in a 160,000-fold dilution of the low-molecular-weight molecules, including residual aldehydes. Thermal treatment of HIV-1 was performed as described previously (27). In brief, samples were loaded into thin-wall 0.5-ml microtubes and subjected to three successive 10-min incubations at 62°C in a heat block filled with water. Inactivation was confirmed by infection of allogeneic pools of peripheral blood mononuclear cells (PBMC) with undiluted supernatants as previously described (27).

Reagents used in capture enzyme-linked immunosorbent assays (ELISAs). Monoclonal antibody (MAb) 2G12 was a gift of H. Katinger, MAb IgG1b12 was a gift of D. Burton, MAb 17b was a gift of J. Robinson, MAb 447-52D was a gift of S. Zolla-Pazner, and soluble cD4 (sCD4) was obtained from the AIDS Reagent Repository (21). Plasmid CDM7-CD4Eγ1, coding for CD4-IgG (12), was a gift of D. Camerini and was transfected (25 μg) into 293 T cells (5×10^6) by standard methods (44). Supernatant was collected at 48 h, titrated, and used at a 1:10 dilution for all assays.

gp120 capture ELISA. Capture of gp120 was performed as described previously (27, 36). In brief, 80 μl of clarified culture supernatant was incubated with 20 μl of human anti-gp120 MAb or with CD4-IgG (2 to 10 μg/ml) in a U-bottom microtiter plate. Where appropriate, the sample was preincubated with 2 ng/well of sCD4 in PBS with 0.2% bovine serum albumin for 30 min at 37°C. Samples and antibodies were allowed to react in the liquid phase for 45 min at 37°C. Triton X-100 was added to a final concentration of 1% for 15 min at 37°C. This concentration of detergent will not disrupt the immune complex (47). At the end of the incubation period, the contents were transferred to an ELISA plate precoated with sheep anti-gp120 (International Enzyme). The gp120-Ab complex was captured onto the plate at 37°C for 60 min. After washing, the plate was incubated with goat anti-human immunoglobulin G (IgG) (horseradish peroxidase conjugated; Accurate Chemical) for 45 min at 37°C. Following a final wash, 200 μl of tetramethyl benzidine substrate was added to each well and left for 20 min. The reaction was terminated by addition of 4 N H_2SO_4 (final concentration of 0.8 N) and read at 450 nm (Molecular Dynamics). A standard serial dilution of concentrated HIV_{SX} was used as a standard to normalize gp120 binding in all assays.

Western blotting. Viral supernatants were collected at 48 h posttransfection and filtered through a 0.22-μm filter. Equivalent amounts of virus (10 ng of p24) were lysed in 2× Laemmli buffer (nonreducing) or in 2× Laemmli buffer containing 5% β-mercaptoethanol (reducing) and separated on a 4 to 12% bis-Tris gel (Invitrogen). HIV envelope was detected using a cocktail of human MAbs against envelope (2G12, 1B12, 48D, 694/98-D, 670-30-D, 447-52-D, C2FS, 2-1C, and 23E), and total HIV-1 was visualized using the enhanced chemiluminescence assay

Mice. Six- to 8-week-old female BALB/c mice were purchased from Charles River Laboratories. All mice were allowed a 1-week period of acclimatization prior to initiation of experiments. Mice were vaccinated subcutaneously with vaccines containing 1 μg of gp120 as determined by ELISA in the presence of 10 μg QS 21 (kindly provided by C. Kensil, Antigenics, Inc.) as an adjuvant. Blood was obtained 2 weeks after the final vaccination by cardiac puncture. All protocols were approved by the Animal Research Committee at UCLA.

New Zealand rabbits. New Zealand rabbits were purchased from Charles River Laboratories. All rabbits were allowed a 2-week period of acclimatization prior to initiation of experiments. Rabbits were vaccinated intramuscularly with vaccines containing 1 μg of gp120 as determined by ELISA in the presence of 15 μg QS 21 as an adjuvant. The rabbits received a total of six vaccinations. Blood was drawn under anesthesia prior to each vaccination and 2 weeks after each vaccination. All protocols were approved by Animal Research Committees at UCLA.

Virus stocks for MAGI neutralization assays. Virus stocks from primary isolates from clades A, C, and E were propagated on allogeneic pools of PBMC. Infectious viral titers were determined on allogeneic pools of PBMC. Half-log dilutions of viral stocks were applied to the cells for 16 h. Supernatants were changed at day 7 and 14 and harvested at day 21 to determine the 50% tissue culture infective dose ($TCID_{50}$) for each virus. $TCID_{50}$s were calculated by the method of Reed and Muench (43).

Virus neutralization assays. Virus neutralization was measured on MAGI CCR5 cells (National Institutes of Health [NIH] AIDS Reagent Repository) (13). In these assays, viral inocula (100 to 200 $TCID_{50}$) were incubated for 30 min at 37°C with an equal volume of serum at various dilutions as indicated. Neutralization was calculated as a percent reduction in β-galactosidase production relative to the virus control at 48 h after infection. Spots were quantitated using an automated system (Immunospot; Cellular Technologies). Samples were run in triplicate. Control human sera were obtained from the AIDS Reagent Repository (51). Control animal sera were obtained from either unvaccinated animals (rabbits) prior to vaccination or age-matched mice that were not vaccinated with HIV immunogens. All serum samples were heat treated at 55°C for 30 min prior to assay. All animal sera were subjected to preabsorption against the cell substrate (293T cells) in which the immunogens were prepared to remove any nonspecific anti-cell substrate antibodies that might be present. For this purpose, serum samples were subjected to three rounds of adsorption against an equal volume of packed cells at 4°C. The first incubation lasted 2 h (to remove high-affinity anti-cell substrate antibodies with low on/off rates), the second incubation lasted 1 h (to remove intermediate-affinity antibodies), and the third incubation lasted 30 min (to remove low-affinity antibodies). Analysis of the serum by indirect immunofluorescence assay demonstrated removal of antibodies which bound to uninfected cells by the end of the third incubation in murine sera (data not shown). (Data demonstrating removal of nonspecific antibodies in rabbit sera can be found in Fig. 4.) The 50% inhibitory concentration was determined and neutralization titers expressed as the reciprocal of the plasma dilution conferring 50% inhibition.

FACS analysis of 293T or MAGI cells for xenoreactive antibodies. The removal of xenoreactive antibodies in the sera of immunized animals by the end of the third round of adsorption was confirmed by fluorescence-activated cell sorter (FACS) analysis. For this purpose, 293T or MAGI cells (5×10^5 cells) were incubated with serum, either unadsorbed or after each adsorption, at a 1:20 dilution in FACS buffer (PBS containing 2% fetal bovine serum) for 20 min at 25°C. The cells were washed with FACS buffer and then incubated with Cy5 goat anti-rabbit IgG at a 1:300 dilution (Zymed Laboratories). As controls, cells were incubated with sera alone, secondary antibody alone, or unadsorbed preimmune serum followed by secondary antibody. After being washed with FACS buffer, the cells were analyzed using a FACSCalibur flow cytometer (Becton Dickinson).

RESULTS

Env content on virions is enhanced by gp41 mutation. It is now believed that of the estimated 72 envelope spikes/virion, there may be only 7 to 14 trimers/particle (14). This might, however, be one contributing factor to the poor immunogenicity of HIV envelope. We examined the hypothesis that increased expression of oligomeric Env on virions might lead to improved neutralizing antibody responses. For this purpose, we constructed a mutation in the endocytosis and sorting signal in gp41 of HIV_{SXSL9} (Y706C) which has been reported to result in better expression of Env at the surface of SIV-infected cells (45).

We measured Env content and function by a number of assays. First, we asked whether Env in virions bearing the Y706C mutation was functional, and thus properly folded, by testing its ability to mediate infection of primary peripheral blood lymphocytes. For this purpose, we transfected 293T cells with DNA encoding replication-competent virions bearing wild-type or mutant Env. As can be seen in Table 1, we observed a >2-log increase in infectivity of virions bearing the Y706C mutation, indicating that Env was properly folded on virions bearing the Y706C mutation. Next, we measured binding of Env by two different reagents (CD4-IgG and the monoclonal antibody 2G12) by ELISA. As can also be seen in Table 1, we observed an approximately 10-fold increase in binding to both of these reagents in virions containing the Y706C mutation versus wild-type Env. Supernatant p24 levels after the transfection were similar (2.8 to 3.5 μg/ml), supporting the conclusion that virions contained more envelope on a per-particle basis. Because this ELISA measures both gp160 and gp120, we examined these preparations by Western blotting to discern whether Env bearing the Y706C mutation was properly processed. As can be seen in Fig. 1a, the ratio of gp160 to gp120 is similar to that in the wild type. Finally, we asked whether Env on the surface of Y706C virions remained in an oligomeric form by examining virions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing and nonreducing conditions to identify the presence of oligomers and monomers. Under these conditions, disulfide-linked oligomers can be detected in the absence of reducing agents but will migrate as monomeric proteins in their presence (5). As can be seen in Fig. 1b, Y706C-containing virions contain oligomeric Env (visible in lane 4) that is reduced to its monomeric molecular weight (lane 2) upon addition of a reducing agent.

TABLE 1. Assay of virions containing a mutation of the endocytic signal Y706C in gp41[a]

Virus	$TCID_{50}$/ml	CD4-IgG (ng/ml)	2G12 (ng/ml)
HIV_{SXSL9}	$10^{5.42}$	21	26
$HIV_{SXSL9,Y706C}$	$10^{7.9}$	243	220

[a] Infectivity titers were determined in PBMC by limiting dilution as previously described (26). Env concentration was determined by ELISA. CD4-IgG or 2G12 indicate which capture reagent was used. Sample input was normalized by p24 content. Of note, because we wanted to determine whether these Env modifications affected protein folding, the data regarding concentration of gp120 were obtained with samples that were not heat treated. Each of these samples demonstrated at least a 180% increase in binding to CD4-IgG after heat treatment compared to its isogenic unheated control (data not shown). This increase in binding indicates that the Env-modified virions show enhanced binding to conformation-dependent reagents after heat treatment in the same manner as we have observed for wild-type envelopes.

Envelope modifications which result in increased Env incorporation affect induction of neutralizing antibodies after vaccination of mice. In order to address whether the changes we observed in Env also translated into differences in the ability of virions bearing this mutation to serve as vaccine immunogens, we vaccinated BALB/c mice ($n = 5$/group) with formaldehyde-stabilized, heat-inactivated virions containing either wild-type or mutant Env a total of four times at 5-week intervals in the

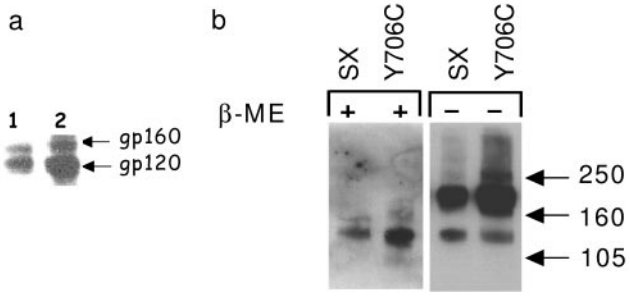

FIG. 1. Virions containing a mutation of the endocytic signal Y706C in gp41 have increased Env as measured by Western blotting. DNA was transfected into 293T cells with calcium phosphate as previously described (26), and samples were analyzed on a 4 to 12% bis-Tris gel and probed with a cocktail of anti-Env monoclonal antibodies (39). Sample input was normalized by p24 content. (a) Lanes: 1, SX_{SL9}; 2, $SX_{SL9Y706C}$. (b) Samples were lysed in 2× Laemmli buffer plus 5% β-mercaptoethanol (β-ME) (reducing, lanes 1 and 2) or in 2× Laemmli buffer (nonreducing, lanes 3 and 4).

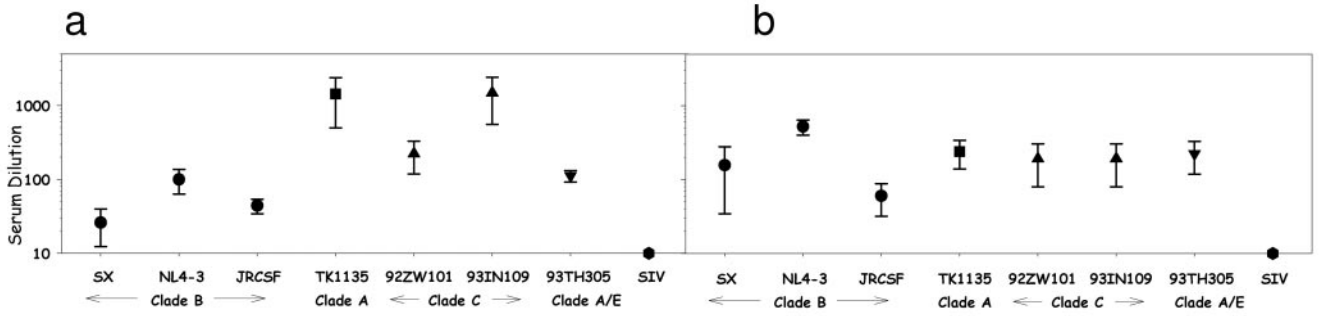

FIG. 2. Potent neutralizing antibody titers are induced in mice after vaccination with formaldehyde-stabilized, heat-inactivated virions bearing the Y706C mutation. Mice were vaccinated with formaldehyde-stabilized, heat-inactivated virions in the presence of 10 μg of QS 21. Sera obtained 2 weeks after the fourth and final vaccination were heat inactivated and subjected to three successive rounds of adsorption against an equal volume of packed 293T cells, which resulted in the removal of >90% of xenoreactive antibodies as measured by immunofluorescence assay. The viral inoculum remained constant (150 $TCID_{50}$) and was incubated for 30 min at 37°C with serial dilutions of serum (final serum concentration of 1:20 to 1:10,240) to determine approximate 50% end point titers. Virus without serum served as the marker for 100% infection. Virus-neutralizing and nonneutralizing polyclonal antibodies diluted in normal mouse serum were included as controls. Neutralization was calculated as percent reduction in β-galactosidase production in MAGI cells relative to the virus control at 48 h after infection. Spots were quantitated using an automated system (Immunospot; Cellular Technologies). (a) Vaccination of mice with formaldehyde-stabilized, heat-inactivated $SX_{SL9Y706C}$ virions. (b) Vaccination of mice with formaldehyde-stabilized, heat-inactivated SX_{SL9} virions. Error bars indicate standard deviations.

presence of 10 μg of the adjuvant QS-21. We measured the ability of serum drawn 2 weeks after the final vaccination to neutralize a panel of viral isolates, including those from clades A (TK1135), B (SX, JRCSF, and NL4-3), C (92ZW101 and 93IN109), and A/E (93TH305). Serum samples were adsorbed against 293T cells prior to assay as described in Materials and Methods. End point titers against virus bearing Env from HIV_{JRFL} (HIV_{SX}) following immunization with either vaccine were similar to those previously reported (40) and are consistent with the relative resistance to neutralization reported for HIV_{JRFL} (6). Remarkably, high-titer antibody responses (>1:1,000) were observed against non-clade B viruses after vaccination with formaldehyde-treated, heat-inactivated virions bearing the Y706C mutation. As can be seen in Fig. 2a, 50% end point titers of up to 5,120 against primary viruses from clades A and C were observed in some mice after vaccination with Y706C-bearing formaldehyde-treated, heat-inactivated virions. Geometric mean titers (GMTs) (n = 5 mice) after vaccination with mutant Env against HIV TK1135 and 93IN109 were >1:1,400, whereas the GMTs against these primary isolates after vaccination with formaldehyde-treated, heat-inactivated virions bearing wild-type Env were six- to eightfold lower. Of importance, neither vaccine resulted in serum antibodies capable of neutralizing virus bearing an SIV Env.

Envelope modifications which result in increased Env incorporation affect induction of neutralizing antibodies after vaccination of rabbits. It can be reasonably argued that mice do not represent the best model for antibody induction, by virtue of the ease with which antibodies can be generated in mice and a relatively high degree of nonspecific neutralization that has historically been observed with these animals. Therefore, we next vaccinated New Zealand White rabbits. Rabbits represent a genetically outbred population with which antibody studies can be reliably conducted. Moreover, they appear to be a reasonable model for predicting antibody responses in nonhuman primates (11). Rabbits (n = 3/group) were vaccinated with formaldehyde-stabilized, heat-inactivated virions bearing the Y706C mutation that was demonstrated to result in increased Env on virions. For these experiments, rabbits were vaccinated via the intramuscular route in the presence of 15 μg of QS 21 according to the schedule depicted in Fig. 3.

Virus-neutralizing antibody titers were assayed at a 1:20 dilution before vaccination and at 2 weeks following the third and sixth vaccinations at dilutions from of 1:20 to 1:1280. Serum samples were adsorbed against 293T cells prior to assay as described in Materials and Methods. Sera were reacted with uninfected 293T cells and uninfected MAGI cells prior to and following adsorption. As can be seen in Fig. 4, unadsorbed sera from vaccinated animals contained xenoreactive antibodies at a 1:20 dilution that were successfully removed by our adsorption protocol. These xenoreactive antibodies were not present in the sera from unvaccinated animals. A similar reduction in reactivity was seen when sera were incubated with MAGI cells, where unadsorbed sera had a mean fluorescence intensity of 1,779, which decreased to 85 after one incubation, to 28 after

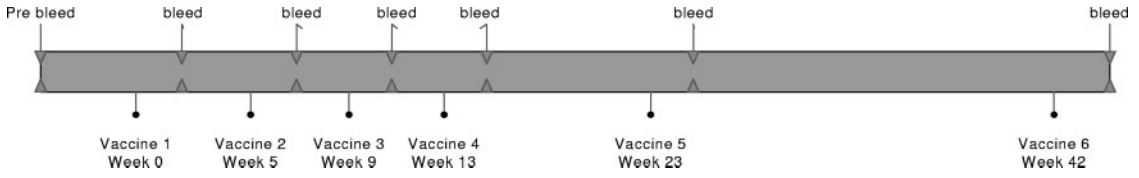

FIG. 3. Scheme for vaccination of rabbits with Env-modified, heat-treated virions. Animals were vaccinated as indicated. Blood samples were drawn 2 weeks after each vaccination. Vaccination was intramuscular in the presence of 15 μg of QS-21.

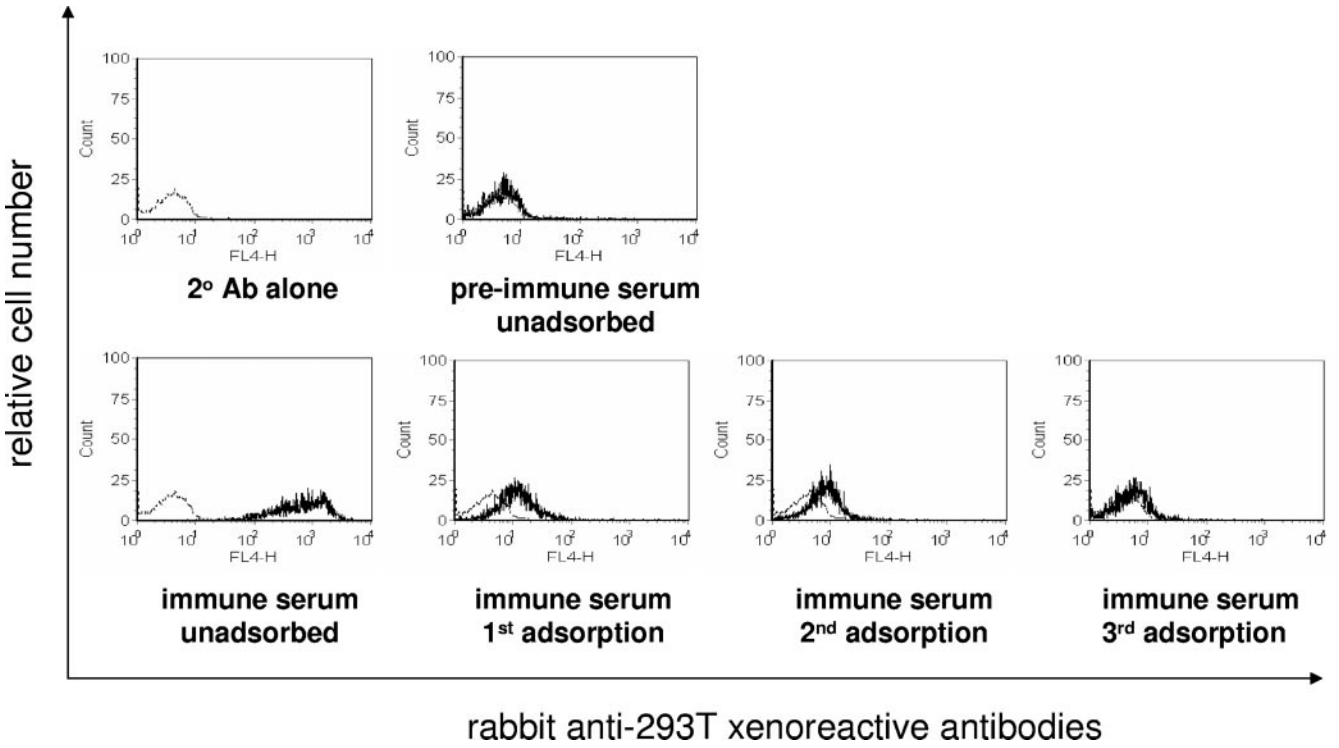

FIG. 4. Preabsorption of immune sera with 293T cells results in removal of xenoreactive anticellular antibodies. Representative sera from rabbit 3058 were analyzed prior and after each adsorption for binding to 293T cells by flow cytometric analysis as described in Materials and Methods. The histograms indicate relative cell number (*y* axis) as a function of relative amount of anti-293T xenoreactive binding (*x* axis). 293T cells stained with Cy5 goat anti-rabbit IgG alone are represented by the dashed histogram area, and cells stained with either preimmune sera or immune sera unadsorbed or adsorbed are represented by the black histogram area.

two incubations, and to 16 after the final incubation (data not shown).

When these sera were tested for neutralization activity, no samples had detectable neutralizing activity at the starting serum dilution of 1:20 prior to vaccination. Neutralizing antibodies (50%) were observed at a 1:20 dilution after three vaccinations against all HIV isolates tested and were detected after six vaccinations at titers ranging from 20 to 640 (Table 2). For comparison, human polyclonal sera from persons infected with clade B viruses were able to neutralize these same viral isolates at concentrations of 1:20 to 1:80 in this assay. Finally, these sera failed to neutralize virions bearing SIV Env at a 1:20 dilution in this assay. Taken together, these data suggest that broad HIV-specific neutralizing antibodies can be induced following vaccination with formaldehyde-stabilized, heat-inactivated virions bearing increased Env.

DISCUSSION

We demonstrate here that vaccination with formaldehyde-stabilized, heat-inactivated virions bearing increased Env can induce neutralizing antibodies in mice and rabbits. We previously showed retention of several major neutralization epitopes on viral envelope following treatment with formaldehyde and heat. Moreover, we found that binding of gp120 to some of these epitopes was enhanced greater than 1.8-fold following thermal treatment. These induced sites include epitopes recognized by potent neutralizing antibodies, including that recognized by the MAb 17b, which has been postulated to be partially occluded or cryptic in native virions (27). These preparations were also shown to be immunogenic in mice and nonhuman primates (40). In the current proof-of-

TABLE 2. Serum end point neutralizing antibody titers measured in MAGI cells[a]

Rabbit no.	Titer with:				
	HIV_{SXSL9} (clade B)	TK1135 (clade A)	93IN109 (clade C)	93TH305 (clade A/E)	SIV
3065	40	80	20	640	<20
3249	160	80	20	320	<20
3058	80	160	20	40	<20
GMT	93	106	20	333	<20
Human polyclonal sera	80	20	40	40	

[a] Rabbits were vaccinated at weeks 0, 5, 9, 13, 23, and 42. Sera drawn 2 weeks after vaccination were heat inactivated and subjected to three successive rounds of adsorption against an equal volume of packed 293T cells. The viral inoculum remained constant (150 $TCID_{50}$) and was incubated for 30 min at 37°C with serial dilutions of serum (final serum concentration of 1:20 to 1:1,280) to determine approximate 50% end point titers. Virus incubated in serum from prevaccination bleeds served as the marker for 100% infection. Polyclonal sera from persons infected with clade B viruses were used as a positive control for neutralization. Neutralization was calculated as percent reduction in β-galactosidase production in MAGI cells relative to the virus control incubated in the presence of normal rabbit serum (at a concentration of 1:20) at 48 h after infection. Spots were quantitated using an automated system (Immunospot; Cellular Technologies).

principle study, we demonstrate that formaldehyde-stabilized, heat-inactivated, envelope-modified virions can induce higher-titer antibodies in small-animal HIV vaccine models.

Inactivated vaccines are theoretically advantageous, since they represent a complex mixture of viral antigens closely resembling native virions. Vaccines of this nature have successfully been used as preventive vaccines for a number of viral, including retroviral, diseases (29, 41, 48). Recently, dendritic cells pulsed with autologous inactivated virions were successfully tested in a phase 1 trial as a therapeutic vaccine (33). Ideally, inactivation would result in conservation of linear and conformational epitopes required for both humoral and cellular immune responses. We have previously demonstrated inactivation protocols that result in exposure of normally cryptic neutralization epitopes (27). We reasoned that it might be possible to enhance the immunogenicity of HIV vaccines beyond that achieved by native virions by this means. In the current study, we have incorporated a genetic mutation which results in increased Env incorporation into virions and which is also capable of inducing potent antibody responses when used as a vaccine in small animal models.

The combination of a low number of trimers on the virion surface and the recently described balance of functional/nonfunctional viral envelope structures (38) may aid in shielding virus from immune recognition. For instance, of the estimated 72 spikes on the virion, current data indicate that only 7 to 14 of these represent functional trimers (14). In part, HIV might have evolved to incorporate the minimum number of spikes necessary for infection without drawing the attention of the humoral immune system. As such, it seems that nonfunctional molecules outnumber functional structures. These nonfunctional molecules appear to induce nonneutralizing antibodies and may include unprocessed gp160 as well as molecules that more approximate monomeric, as opposed to oligomeric, gp120 in structure. Conversely, functional trimers are believed to be capable of inducing neutralizing antibodies (38). Tipping the balance in favor of increased functional structures may increase the likelihood of vaccines inducing protective antibodies. In order to evaluate whether increased numbers of oligomers on the virion surface may affect the immunogenicity of the vaccine, we constructed a mutation in the endocytosis and sorting signal in gp41 of HIV_{SXSL9} (Y706C) which has been reported to result in better expression of Env at the surface of SIV-infected cells (45) and has been shown to increase the incorporation of SIV Env into virions (55). This tyrosine appears to direct the endocytosis of free Env at the surface of cells, which may aid in preventing immune-mediated destruction of HIV-infected cells. Data presented here (Fig. 1) indicate that this mutation results in a 10-fold increase in Env on the surface of virions and that this Env is oligomeric. Vaccination of animals with immunogens containing this mutation resulted in broad and high-titer neutralizing antibody responses. These data suggest that modifications in viral envelope that alter native structures can influence neutralizing antibody titers against heterologous viral isolates.

The finding that neutralization was more potent against heterologous viral isolates compared with the vaccine strain was unexpected but suggests that modifications in viral envelope that alter native structures can dramatically influence neutralizing antibody titers against heterologous viral isolates. It is possible that the epitopes recognized by antibodies generated after vaccination with formaldehyde-stabilized, heat-treated Y706C virions are more exposed on the non-clade B isolates. Previous studies have used HIV Env expression plasmids containing a truncation in the gp41 ectodomain generated by frameshift mutation in this region of gp41 to demonstrate enhanced binding to antibodies recognizing the CD4 binding site, the CD4 induced site, and, to a lesser extent, the immunodominant epitope of the gp41 ectodomain itself (16), suggesting that the antigenic structures of both gp120 and gp41 can be altered by changes in the ectodomain of gp41. If increased exposure of neutralizing epitopes is coupled with an increase in the total number of molecules of Env present with that epitope available for the immune system to see, it is possible that antibody responses might be higher against viruses that are more susceptible to neutralization through those epitopes. Alternatively, it is possible that the antibodies generated here have higher affinity for structures on these non-clade B isolates. Future work will be required to map the epitope(s) against which these antibodies are reactive to discern the mechanism by which these antibodies mediate more potent neutralization of the non-clade B immunogens. Ultimately, these findings suggest that a clade B immunogen can be modified and used to generate antibodies that might protect against infection with non-clade B viruses.

ACKNOWLEDGMENTS

We thank J. P. Moore, S. Shapiro, B. Lee, O. Yang, J. Zack, S. Kung, and D. S. An for helpful discussions. We thank G. Miller and B. Gordon for technical assistance; J. Mitchell for administrative assistance; the UCLA Center for AIDS Research HIV Virology Laboratory for performing the p24 assays; and the UCLA Jonsson Comprehensive Cancer Center and Center for AIDS Research Immunology and FACS Cores, D. Burton, H. Katinger, D. Ho, D. Camerini, and C. Kensil for providing reagents. The following reagents were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: sCD4-183 from Pharmacia, MAGI-CCR-5 from Julie Overbaugh, and HIV-1-neutralizing sera (1 and 2) from Luba Vujcic, FDA, Center for Biologics Evaluation and Research. We thank IAVI for its scientific support.

This work was supported by NIH grants R21AI42687, 1R01AI052012, and CA016042; the VA Merit Review Entry Program; the James B. Pendelton Charitable Trust; the Burch Trust; the Center for AIDS Research of the University of California at Los Angeles (National Institutes of Health grant AI028697).

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