Sequential Immunization with a Subtype B HIV-1 Envelope Quasispecies Partially Mimics the In Vivo Development of Neutralizing Antibodies

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Received 19 November 2010/Accepted 4 March 2011

A major goal of human immunodeficiency virus type 1 (HIV-1) vaccine efforts is the design of Envelope (Env)-based immunogens effective at eliciting heterologous or broad neutralizing antibodies (NAbs). We hypothesized that programming the B-cell response could be achieved by sequentially exposing the host to a collection of Env variants representing the viral quasispecies members isolated from an individual that developed broad NAbs over time. This ordered vaccine approach (sequential) was compared to exposure to a cocktail of Env clones (mixture) and to a single Env variant (clonal). The three strategies induced comparable levels of the autologous and heterologous neutralization of tier 1 pseudoviruses. Sequential and mixture exposure to quasispecies led to epitope targeting similar to that observed in the simian-human immunodeficiency virus (SHIV)-infected animal from which the Env variants were cloned, while clonal and sequential exposure led to greater antibody maturation than the mixture. Therefore, the sequential vaccine approach best replicated the features of the NAb response observed in that animal. This study is the first to explore the use of a collection of HIV-1 env quasispecies variants as immunogens and to present evidence that it is possible to educate the B-cell response by sequential exposure to native HIV-1 quasispecies env variants derived from an individual with a broadened NAB response.
involved in CD4 and chemokine receptor binding. Mutations associated with changes in susceptibility to autologous NAbs are located in regions of Env that are exposed and may be accessible to antibodies (33). NAbs target these relatively exposed regions of Env, as shown by the isolation of human MABS that target these regions from HIV-infected subjects (46). Escape from autologous NAbs (41, 58) is due to alterations in Env characterized by entropic masking (27), flexibility in size and the positioning of the variable loops (10, 16), amino acid sequence variation (25), and glycosylation changes (8, 58). Indeed, during the course of infection, the location of potential N-glycosylation sites (PNG) is altered (3) and the number of PNGs is increased (44). Recent studies (36, 43) demonstrated that multiple pathways are involved in escape from autologous NAbs in clade C-infected patients and the pathways are context dependent, as they vary from patient to patient and during the course of infection. These pathways include the evolution of the V3 to V5 region of env, the cooperation of V1/V2 with the ectodomain of gp41 (43), or the evolution of the V1/V2 and C3 regions (36). Other proposed escape mechanisms include the expression of a nonfunctional decay Env (13, 35, 39). We recently showed that in viremic controllers there is continuous viral escape and selection by autologous NAbs (33). Therefore, the resulting NAbs appear to exert selective pressure on the viral quasispecies, resulting in the fixation of env mutations that alter Env charge, shape, or epitope exposure, in turn resulting in a dynamically changing B-cell response.

A number of approaches have been attempted to design Env immunogens capable of eliciting broad, heterologous NAbs (reviewed in reference 22). These designs include inactivated viruses, monomeric secreted Env, stabilized Env trimers, the stabilization of Env intermediate fusion states, structural analogs of conserved Env epitopes grafted onto scaffolds, and polyvalent or consensus/ancestral Env sequences. To date, only low levels of NAbs have been detected in vaccine studies using these immunogens, with antibodies typically neutralizing only a subset of easier-to-neutralize tier 1 viruses. Previous studies showed that trimeric gp140 is more efficient at inducing an immune response than monomeric gp120 (2, 5, 54, 61), but only marginally so. Because NAbs target native Env trimers on the surface of virions, it may be necessary to recapitulate native Env conformation in vaccines. One such strategy is the use of DNA vaccines based on expression plasmids injected intramuscularly or intradermally. The antigen of interest then is made in vivo with the concomitant development of both humoral and cellular immunity directed to the full-length native trimer. Several studies have shown that weak autologous and heterologous NAbs can be elicited by a combination of DNA prime/protein boost immunization (9, 29, 34, 51, 56), thereby suggesting that structures in the native Env are important for eliciting NAbs (50). Our prior studies exploring the use of ancestral DNA vaccines delivered intradermally by a Gene Gun showed that binding antibodies (BAbs) were elicited in a DNA dose-dependent manner (19) and that DNA vaccination followed by a boost with monomeric gp120 protein elicited weak NAb responses (17) that were poorly cross-reactive. We sought to improve upon these results by exploring a novel idea for env-based vaccines.

Understanding how the virus can elicit a broad NAb response may provide clues as to how to generate these responses during vaccination. We hypothesized that immunization with a collection of env quasispecies variants would result in the development of broader NAbs than immunization with any single env gene alone. To test this hypothesis, we utilized a collection of env quasispecies variants that evolved sequentially in a macaque that developed a moderate cross-NAb response following infection with SHIVSF162P3. This unvaccinated animal (A141) developed autologous NAbs quickly and showed evidence of gradual broadening, with its serum neutralizing eight heterologous variants, including four standard subtype B viruses, by the end of the study at day 670 (26). The major goal of the present study was to test the quasispecies vaccine concept and to investigate whether exposing rabbits to the same quasispecies in sequential order as it developed over time in A141 could elicit the same moderate breadth of response by immunization as that seen in A141. We compared antibody responses of rabbits exposed to the sequential evolution of env clones (sequential group) to those of rabbits receiving a cocktail of env clones present in the quasispecies (mixture group) or repeated exposure to the same env sequence (clonal group). Clonal and sequential vaccination resulted in greater antibody maturation than the mixture approach. We found that the three vaccine strategies led to the development of comparable levels of binding and neutralizing antibodies. A slight enhancement of heterologous neutralization against HIV-1 SS1196.1 was observed in the sequential group. In addition, NAbs elicited by the mixture and sequential strategies targeted the same epitope on Env as A141. Taken together, our data indicate that the sequential vaccine strategy best replicated the features of the NAb response observed in macaque A141.

MATERIALS AND METHODS

Animals. Female New Zealand White rabbits were housed at R&R Research, Marysville, WA. Immunizations were performed on rabbits of 6 pounds of weight or greater. All procedures followed IACUC-approved protocols by the Seattle Biomedical Research Institute (Seattle, WA) and R&R Research (Marysville, WA). A rhesus macaque of Indian origin (A141) was part of previously described studies (9, 26), and only serum samples from this animal were used in the current study.

Site-directed mutagenesis. The mutations of interest in A141 were introduced in the codon-optimized gp160 plasmid expression vector (Novartis, Emeryville, CA) by site-directed mutagenesis with the QuickChange multi-site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two hundred twenty mutations (labeled according to HxB2 Env numbering) were introduced to generate the 15 plasmid clones present in the quasispecies (mixture group) or env gene alone. To test this hypothesis, we utilized a quasispecies HIV-1 vaccination elicits NAbs 5263...
immunization. New Zealand White rabbits were immunized intradermally with a Gene Gun (Bio-Rad, Hercules, CA) at a pressure of 400 lb/in². A total of 36 g DNA each, given in clusters of three nonoverlapping positions at six shaven sites (lower back, inside back legs, and abdomen). Animals were vaccinated with DNA at weeks 0, 4, 10, 20, 24, and 32. Protein boosts, consisting of 50 µg recombinant gp140 protein mixed with an equal volume of MF-59 adjuvant (Novartis, Emservile, CA), were delivered intramuscularly by needle injection at weeks 41 and 47. Blood was collected 2 weeks after each immunization, and serum was separated and stored at −20°C until IgG purification.

IgG purification. IgG from individual rabbit sera was purified by affinity chromatography at weeks 12, 22, 26, 34, 43, and 49. Briefly, individual samples (9 ml) of rabbit sera were mixed with 1 ml 10 g/ml DEAE-dextran (Fisher Biotech, Fair Lawn, NJ) and layered on top of a 4 ml column of DEAE-Sepharose FF (Sigma, St. Louis, MO). The plates were read at 650 nm in a SpectraMax190 plate reader.

Neutralization assay. IgG purified from the following time points from each animal were tested: 12, 22, 26, 34, 43, and 49 weeks. Serum samples from week 49 also were assessed for heterologous neutralization. Pooled purified rabbit IgG from a previous immunization study (17) was included as a control. The TZM-bl neutralization assay was performed in flat-bottom 96-well plates as previously described (57). Briefly, 200 TCID₅₀/ml of virus was added to serum samples or serial dilutions of rabbit IgG and incubated in a total volume of 150 µl medium (DMEM, 10% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin) for 1 h at 37°C. Each well then received 100 µl of TZM-bl cells resuspended in medium at 1 × 10⁵ cells/ml in the presence of 7.5 µg/ml DEAE-dextran. Forty-eight hours later, 150 µl of medium was removed from each well, and cells were lysed for 2 min directly in the neutralization plate, using 100 µl of Bright-Glo luciferase assay substrate (Promega, Madison, WI), and immediately analyzed for luciferase activity with a luminometer (Victor; PerkinElmer, Waltham, MA). The IC₅₀ of each sample was calculated by nonlinear regression analysis using GraphPad Prism 5.0 software to achieve 50% neutralization of each virus at the 50% inhibitory concentration (IC₅₀), and an increase in neutralization was measured by a decrease in the IC₅₀. As a negative control, a pool of prebleed IgG was used, which never neutralized the tested pseudoviruses (data not shown). All values were calculated with respect to virus-only wells with the following formula: [value for virus only minus value for cells only] minus [value for serum minus value for cells only], divided by (value for virus minus value for virus only) for all values.

Peptide competition neutralization assay. Serum from macaque A141 at day 670 postinfection was tested for the neutralization of SF162 in the presence of linear peptides derived from each of the variable regions of Env or in the presence of a control peptide (V3 scrambled) as previously described (15). IgG samples purified from the week 49 time point from each rabbit were tested in the presence of the V1 peptide (TNLKNATNKSNWKEMDRIEIK) or the V3 peptide (PNPNTRKSTIPGRAYATGD) from SF162 (Invitrogen, Carlsbad, CA) or the V3 scrambled peptide (PNPNTRKSIERYRGAPGATGD) (Gen- script, Piscataway, NJ). Briefly, serial dilutions of rabbit IgG (30 µl) were incubated with 10 µg/ml of the peptide of interest (30 µl) for 1 h at 37°C before the addition of 200 TCID₅₀/ml of virus in a total volume of 90 µl medium (DMEM, 10% fetal calf serum, 1% L-glutamine, 1% penicillin-streptomycin) for an additional 1 h at 37°C. Each well then received 100 µl of TZM-bl cells resuspended in medium at 1 × 10⁵ cells/ml in the presence of 7.5 µg/ml DEAE-dextran. Forty-eight hours later, 90 µl of medium was removed from each well and cells were lysed for 2 min directly in the neutralization plate, using 100 µl of Bright-Glo luciferase assay substrate (Promega, Madison, WI), and immediately analyzed for luciferase activity with a luminometer (Victor, PerkinElmer, Waltham, MA). The IgG concentration necessary to achieve 50% neutralization was determined, and data were reported as the fold increase in the IC₅₀ above the neutralization measured without peptide divided by the IC₅₀ at 30 µg/ml of peptide. The IC₅₀ of each sample was calculated with respect to virus only: [value for virus only minus value for cells only] minus [value for serum minus value for cells only] divided by (value for virus minus value for cells only).

SPR assay. All surface plasmon resonance (SPR) experiments were performed at 25°C on a Biacore T200 (GE Healthcare, Piscataway, NJ) using a CM4 sensor chip and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.05% F20). Protein A (Pierce, Rockford, IL) was covalently immobilized on all flow cells at a concentration of 50 µg/ml in acetate buffer, pH 4.5, using standard amine coupling to a level of 950 resonance units (RU). IgG samples from four of the highest responders in each group were tested at weeks 12 and 43. The antibodies were captured onto the protein A surface on flow cells 2 to 4, leaving flow cell 1 as a reference. The antibodies were noncovalently immobilized to a level of 1,500 (week 12 IgG), 1,000 (week 43 IgG), and 80 RU (positive-control Mab VRC01) by being flowed for 20 to 30 s at a concentration of 15 µg/ml. This resulted in an R₅₀₀ of 7 to 10 for the week 12 samples and 30 to 50 for the week 43 samples, because only a fraction of the total IgG was antigen specific. Monomeric SF162 gp120 was injected over the sensor surface at the following concentrations: 0, 0.41, 1.23, 3.7, 11.11, 33.33, and 100 nM. All concentrations had a 6-min association period and a 2-min dissociation period, except for the 33.33 and 100 nM concentrations, which had a 1-h dissociation (24). To ensure the accurate quantitation of drift and assay reproducibility, blank replicate injections for 1 h were used and gp120 at 11.11 nM was tested twice, respectively. In all experiments, the binding of gp120 to the reference surface was negligible, and the binding to the reference surface did not change with concentration.
Samples were maintained at 15°C before injection. The regeneration of the capture complex was achieved using a 30-s pulse of 10 mM glycine, pH 1.75, 0.05% P20. The data were analyzed using T200 evaluation software. All data were double reference subtracted using the averages from two blank injections. Data were fit to a 1:1 model without any bulk refractive index term despite the inherent multiphasic shape of some sensorgrams due to the immobilization of a polyvalent ligand. Therefore, the measured off-rate is an apparent $k_d$ (off-rate), $t^{-1}$ because the ligand is polyonal IgG.

**Statistical analyses.** Statistical analysis was done using SAS statistical software, version 9.2. Analyses of BAB levels, SF162 and d56 B3 neutralization from samples drawn in weeks 12 to 49, and $k_d$ rates were determined using mixed-effect models to account for the serial correlation of the repeated measures obtained from each animal. These analyses used log-transformed BAB levels, log-transformed SF162 and d56 B3 IC50 levels, and log-transformed $k_d$ rates as their outcome variables. Due to the presence of floor effects (no observed neutralization, which resulted in the substitution of the maximum tested concentration for a measured IC50), the log-transformed IC50 for d417 D15 and SS1196.1 were not normally distributed. These outcomes were modeled as ordinal variables using a generalized estimating equation with a multinomial link function, with the correlation between repeated measures taken into account in the analysis. These ordinal regression analyses modeled the association of immunization group with the probability of having a lower IC50 (higher neutralization). The targeting of peptides V1 and V3 was analyzed through a mixed-effect model of the log-transformed SF162 IC50 that treated the assessments of the four different preincubations as repeated measures for each animal, specifying an unstructured correlation matrix for the repeated measures to allow for nonuniform variances. The association of the immunization strategy with the neutralization of the heterogeneous variants at week 49 was analyzed using Welch’s analysis of variance (ANOVA) of the log-transformed IC50. Neutralization breadth scores were computed as described by Piantadosi et al. (38) and are the sum of the number of viruses (SF162, d56 B3, d417 D15, d487 A6-1, and d670 A8-9) for which the neutralization IC50 was below the median for that antigen. A single median was computed for each antigen using all measurements from all animals and time points. The association-of-breadth scores with immunization strategy and time point was analyzed using ordinal regression as described above.

**Nucleotide sequence accession numbers.** All A141 sequences included in this study have been deposited in GenBank (accession numbers JF419573 to JF419587).

## RESULTS

**Selection of clones and generation of env variants.** Fifteen variants among 41 env clones that represented the major Envelope variants present during the development of the heterologous NAB response in macaque A141 (26) were selected using the following inclusion criteria: (i) dominant sequence at each time point; (ii) multiple unique sequences per time-point; (iii) more variants at time points with more viral sequence diversity; (iv) all mutations that became fixed over time; (v) key changes in PNG sites; and (vi) the sequence at the last time point that was the most divergent from the inoculum. These clones (Fig. 1) include the inoculum at day 0 (d0; SF162), two variants at day 56 (d56 A4-5 and d56 B3), three variants at day 215 (d215 A5-1, d215 A5-6, and d215 A5-C7), three variants at day 417 (d417 D8, d417 D15, and d417 D16), three variants at day 487 (d487 A6-1, d487 A6-2, and d487 A6-6), and four variants at day 670 (d670 A8-2, d670 A8-9, d670 A8-11, and d670 A8-15). Each clone has between 4 and 27 amino acid (aa) changes compared to the sequence of the inoculum and contains between 20 and 25 PNGs (Table 1). As seen in Fig. 1, nonsilent mutations accumulated through the quasispecies and accrued in the variable regions as well as in the constant regions of envelope, such as V1/V2, C3, and gp41. Site-directed mutagenesis was used to reconstruct each mutation that was found in the natural variants arising over time in macaque A141 at the five different time points. The selected Env were expressed in codon-optimized gp160 DNA plasmid vectors.

The expression of Env was assessed by the transfection of 293T cells, and all 15 Env were expressed at the cell surface as measured by IgG b12 binding in an immunofluorescence assay (data not shown), and most of them were able to form syncytia and entry-competent pseudovirions (data not shown).

**Envelope-specific binding antibody responses are elicited by the three vaccine strategies.** Female New Zealand White rabbits were immunized intradermally by a Gene Gun with 36 µg of plasmid DNA/rabbit/time point during a period of 32 weeks to approximate, on an accelerated scale, proportional exposure to the quasispecies in A141 (Fig. 2). Because all sequences were derived from an individual animal infected with SHIVSF162P3, all rabbits were immunized with env from the viral inoculum at the first vaccination time point. For the following five time points the rabbits received the clonal, sequential, or mixture vaccine strategy. In the clonal group, rabbits received the SF162 env used in the original viral inoculum. The mixture group was immunized with a cocktail of all 15 quasispecies env sequences given at each of the five remaining time points. Rabbits in the sequential group received an ordered immunization with five cocktails of env sequences derived from each of the five different time points to recapitulate the changes in the viral quasispecies over time. The clonal group was boosted with the trimeric clonal protein SF162 gp140, and both the sequential and the mixture group were boosted with the late variant trimeric protein d670 A8-9 gp140. This late variant was chosen as a representative Env for the d670 clones because it has 25 PNGs (mean, 24.5 PNGs for d670 variants) and 24 amino acid changes compared to the sequence of SF162 (mean, 26.75 aa changes for d670 variants) (Table 1). This boosting strategy was used to maximize potential neutralizing antibody responses in each group. The two gp140 protein boosts (50 µg/animal) were delivered at weeks 41 and 47 in the presence of MF-59 adjuvant.

The binding antibody (BAB) responses in rabbits were assessed by measuring SF162 gp120-specific binding antibodies using a kinetic ELISA (Fig. 3). IgG samples purified by affinity chromatography were tested after the third DNA prime, subsequent DNA primes, and protein boosts. There was similar variability among the BAB titers in each vaccine group, with some animals generating higher titers than others. Overall, BAB titers increased during the DNA primes in the sequential group ($P = 0.0015$) and in the clonal group ($P < 0.001$) but not in the mixture group ($P = 0.57$). BAB titers were significantly increased in all groups by the protein boosts ($P < 0.001$) regardless of the boosting agent used. The second protein boost did not further increase these titers, which decreased slightly (clonal, $P = 0.002$; mixture, $P = 0.004$; sequential, $P = 0.035$). Overall, BAB titers were not statistically different between the three vaccine strategies ($P > 0.05$).

**Efficient neutralization of clonal HIV SF162 is achieved by all three vaccine approaches.** Purified IgG samples were tested for the neutralization of HIV SF162, which was neutralized by A141 serum samples at day 670 postinfection (26). Similarly to the BAB data, neutralization was variable among individual rabbits, with weak responders and strong responders in each vaccine group (Fig. 3). All IgG samples except 7207 in the mixture group neutralized SF162 efficiently after the DNA primes. In the clonal group, SF162 neutralization improved moderately during the DNA primes ($P = 0.003$), and it was
significantly higher after the last DNA prime than at the earlier time points ($P < 0.004$), as measured by a decrease in $IC_{50}$. Neutralization was enhanced by protein boosting in all three groups (clonal, $P = 0.0005$; mixture, $P = 0.0027$; sequential; $P = 0.0002$), with an $IC_{50}$ of less than $10 \mu g/ml$ achieved by five or more animals in each group regardless of the protein used for boosting. The second protein boost significantly reduced SF162 neutralization in the clonal group ($P < 0.004$) but not in the other two groups. Overall, there was no statistically significant difference in the neutralization of SF162 between the three vaccine regimens ($P > 0.05$). However, there was a strong correlation between binding antibody and neutralizing antibody levels against SF162 ($P < 0.0001$), and this correlation does not differ between groups ($P = 0.3$).

**Variable levels of autologous neutralization by all three vaccine groups.** To investigate the autologous antibody response generated by the three vaccine strategies, we measured the neutralization of an early Env variant, d56 B3, two middle variants, d417 D15 and d487 A6-1, and a late variant, d670 A8-9 (Fig. 4), by constructing pseudoviruses with each of these env genes. The neutralization pattern of d56 B3 was very similar to that obtained with SF162. For some rabbits, such as 7222, 7180, and 7203, the neutralization of d56 B3 was more effective than that of SF162, and we suggest that these results are due to the sequence similarities between the two viruses (only a 5-aa difference). As seen with the neutralization of SF162, we observed both weak and strong responders in each vaccine group. In addition, statistical analysis demonstrated that there was a strong positive correlation ($P < 0.0008$) at every time point between d56 B3 and SF162 neutralizations. For all groups the neutralization of d56 B3 was effective during the DNA primes and was significantly enhanced by the protein boosts (clonal, $P < 0.0001$; mixture, $P < 0.0001$; sequential, $P = 0.0007$). Four to eight animals in each group reached an $IC_{50}$ of $10 \mu g/ml$ or less after the protein boosts, but the second protein boost decreased the neutralization of d56 B3 in all groups ($P < 0.0001$). There was no statistically significant difference between vaccine strategies ($P > 0.05$). The neutralization of d417 D15 was not as efficient as the neutralization of the clonal SF162 or the early variant d56 B3, as the $IC_{50}$
were 1 to 2 log_{10} higher at all time points tested (Fig. 4). However, some similar patterns were observed, including the presence of weak and strong responders in each group, and efficient boosting by the proteins (clonal, \( \text{P} = 0.011 \); mixture, \( \text{P} = 0.018 \); sequential, \( \text{P} = 0.013 \)). The reduction of NAbs directed to d417 D15 after the fourth DNA prime were not statistically significant when the groups were analyzed individually. The boosting effect was pronounced for d417 D15, with an at least 1 log_{10} improvement for most samples. Six to seven rabbits in each group had an IC_{50} of less than 500 \text{ng/ml} after the protein boosts. The neutralization of d417 D15 was not significantly different among the vaccine strategies (\( \text{P} = 0.05 \)). The neutralization of d487 A6-1 and d670 A8-9 was weak in all vaccine groups (Fig. 4). The sequential group appeared to neutralize d487 A6-1 better than the clonal or the mixture group; however, there was no statistical difference between groups regarding the neutralization of these last two variants. For the autologous viruses d56 B3, d417 D15, d487 A6-1, and d670 A8-9, neutralization breadth scores were generated (38). Protein boosts significantly increased the breadth scores in all vaccine groups (clonal, \( \text{P} = 0.012 \); mixture, \( \text{P} = 0.033 \); sequential, \( \text{P} = 0.019 \)), and this increase was significantly larger in the clonal group than in the mixture group (\( \text{P} = 0.034 \)). There was no significant difference in autologous breadth scores between vaccine groups, but autologous neutralization breadth was associated with SF162 BAbs (\( \text{P} = 0.03 \)) and SF162 NAbs (\( \text{P} = 0.005 \)).

Weak heterologous neutralization elicited by all three vaccine strategies. We assessed the broadening of the NAb response of the three vaccine strategies by measuring the neutralization of heterologous viruses among those neutralized by animal A141 (26), such as SS1196.1, 89.6, TRO.11, REJO4541.67, QH0692.42, 7165.18, 6535.3, and 6101.1. The neutralization of SS1196.1 (Fig. 5) was weak but detectable after the DNA primes for several samples in each group. Protein sequences were aligned to HXB2 (accession no. AF033819) and were analyzed with the N-GlycoSite program using the HIV Sequence Database from the Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). Green boxes indicate the addition of a PNG compared to the number of PNGs found in HIV\text{SF162}, and purple boxes indicate the removal of a PNG. 1, Presence of PNG at a given amino acid position; 0, absence of PNG at a given amino acid position.

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![FIG. 2. Immunization strategies. Rabbits were vaccinated with six DNA primes at weeks 0, 4, 10, 20, 24, and 32, followed by two protein boosts in the presence of MF-59 at weeks 41 and 47. All three groups were immunized with SF162 \text{env} at week 0. The clonal group then was given SF162 \text{env} five additional times and was boosted twice with SF162 gp140 protein. The mixture group was immunized with a cocktail of all 15 quasispecies \text{env} sequences given five times. The sequential strategy was an ordered immunization with five cocktails of \text{env} sequences derived from five different time points to recapitulate the changes in the viral quasispecies over time. Both the sequential and the mixture groups were boosted with d670 A8-9 gp140 protein.](http://jvi.asm.org/Downloaded from May 1, 2019 by guest)
tein boosts were efficient at improving the neutralization of SS1196.1 in all groups \((P < 0.03)\). Indeed, an IC_{50} of 2,000 \(\mu g/ml\) or less after the first protein boost was achieved by 6/8 animals in the sequential group but by only 3/8 animals in the clonal and mixture groups. Out of these responders in the sequential and clonal groups, 67% reached an IC_{50} of <500 \(\mu g/ml\), whereas only 33% reached this level in the mixture group. Furthermore, some samples achieved an IC_{50} of 80 \(\mu g/ml\) or less after the first protein boost (7168 in the sequential group and 7213 in the mixture group). However, the protein boosts did not induce neutralization in rabbits that did not respond to the DNA primes, such as 7186 in the clonal group, 7109 in the sequential group, and 7207 in the mixture group. Statistical analysis demonstrated that the effect of the first protein boost on the neutralization of SS1196.1 was stronger in the sequential group than in the clonal and mixture groups \((P = 0.0064)\). In addition, the neutralization of SS1196.1 was associated with the neutralization of SF162 \((P = 0.004)\), d56B3 \((P = 0.002)\), and d417 D15 \((P = 0.0008)\) and the autologous breadth score \((P = 0.0009)\). When modeled together, both the strength and the breadth of the neutralization of autologous viruses predicted the neutralization of SS1196.1 \((P = 0.02)\).

To further our investigation of the broadening of the NAb response, we measured the neutralization of heterologous isolates 89.6, TRO.11, REJO4541.67, QH0692.42, 7165.18, 6535.3, and 6101.1 by serum samples after the last protein boost (data not shown). Serum from macaque A141 neutralized these viruses, but it did so less efficiently than SS1196.1 \((P = 0.02)\). Little to no neutralization of these specific heterologous viruses was observed in any vaccine group.

**Preferential targeting of the V3 loop by macaque A141 and rabbits immunized with sequential and mixture strategies.** Peptide competition neutralization assays were performed to determine which variable regions of Env are targeted by the antibodies generated in animal A141. We performed these experiments with linear peptides derived from the different
variable loops of SF162 Env, which is the original inoculum virus for macaque A141. Serum from A141 at day 670 postinfection was tested for the neutralization of the pseudovirus of interest (d56 B3, d417 D15, d487 A6-1, and d670 A8-9) in a TZM-bl cell assay. The IgG concentration (µg/ml) required to reach 50% neutralization at each time point is reported as the IC<sub>50</sub> (µg/ml).

FIG. 4. Elicitation of autologous neutralizing antibodies. Serially diluted IgG samples from the three vaccine groups were tested for the neutralization of the pseudovirus of interest (d56 B3, d417 D15, d487 A6-1, and d670 A8-9) in a TZM-bl cell assay. The IgG concentration (µg/ml) required to reach 50% neutralization at each time point is reported as the IC<sub>50</sub> (µg/ml).

There are up to four amino acid changes (out of 23 aa) in the sequence of V1 and up to two amino acid changes (out of 22 aa) in the sequence of the V3 region of the 15 variants compared to the SF162 amino acid sequences used for the linear peptides. These differences may affect antibody affinity toward the peptide of interest. In addition, competition experiments performed with linear peptides may not identify neutralizing antibodies that target conformational epitopes. Despite these limitations, we observed a differential epitope targeting among vaccine regimens when IgG samples purified after the last protein boost were tested against SF162. Antibodies targeting the V3 loop were found in the mixture group (V3 peptide versus V3 scrambled peptide, \(P = 0.0011\)) and in the sequential group (V3 peptide versus V3 scrambled peptide, \(P = 0.0001\)), whereas antibodies targeting V1 were found in the clonal and mixture groups (V1 peptide versus no peptide, \(P < 0.0001\)). In the clonal group, V1 and V3 were equally targeted by NAbs.
In contrast, both the mixture and the sequential strategies preferentially targeted the V3 loop over the V1 loop (mixture, $P = 0.0002$; sequential, $P = 0.0039$) (Fig. 6B). Furthermore, the targeting of V3 over V1 is statistically different between the groups that were immunized with the quasispecies and with SF162 (mixture versus clonal, $P = 0.0002$; sequential versus clonal, $P = 0.0027$); however, due to the experimental design, this cannot be separated from the effect of the immu-

(P > 0.05).
The inhibition of neutralization by the V3 peptide was not complete (data not shown), implying that NAbs target the V3 loop region as well as other conformational and/or linear epitopes. Taken together, these results suggest that NAbs elicited by the three vaccine strategies differentially targeted exposed epitopes on Env and that the sequential strategy elicits epitope targeting similar to that obtained with A141.

Greater antibody maturation to monomer gp120 induced by the clonal and sequential strategies. Antibody maturation is likely to be important for effective vaccination, as mature antibodies generally have higher affinity and avidity for their antigen. To investigate whether the three different vaccine strategies induced antibody maturation, surface plasmon resonance (SPR) experiments were performed. Since the evolution of the off-rates ($k_d$) is indicative of the antibody maturation as measured by a decrease in $k_d$ over time (49), we assessed the binding of SF162 gp120 protein to purified IgG of four of the highest responders in each group at an early time point (week 12) and a late time point (week 43). All groups showed a statistically significant decrease in $k_d$ between week 12 and week 43 (clonal, $P < 0.0001$; mixture, $P = 0.0015$; sequential, $P = 0.0417$; #, $P = 0.0249$) (Fig. 7), thereby indicating that all vaccine strategies induced antibody maturation. The mean $k_d$ values at week 12 were not different between groups, but they were significantly higher at week 43 in the mixture group than in the clonal and sequential groups (clonal, $P < 0.0001$; mixture, $P = 0.0015$; sequential, $P < 0.0001$) (Fig. 7), thereby indicating that all vaccine strategies induced antibody maturation. The mean $k_d$ values at week 12 were not different between groups, but they were significantly higher at week 43 in the mixture group than in the clonal and sequential groups (clonal, $P = 0.0249$; sequential, $P = 0.0417$), thus demonstrating that the clonal and sequential approaches induced greater antibody maturation than the mixture group. In addition, the shape of the sensograms was indicative of a homogenous/clonal response in the clonal and in the sequential groups, whereas it was more indicative of a heterogeneous/polyclonal response in the mixture group (see Fig. S4 in the supplemental material). Binding responses ($R_{max}$) increased between week 12 and week 43 in all groups, showing that a greater fraction of the purified IgG was antigen specific (see Fig. S4), a finding that is in agreement with the increase in antibody titers as measured by ELISA (Fig. 3A). VRC01 was used as a positive control, and the values ($k_a$ [on-rate], 3.54E+04 M$^{-1}$ s$^{-1}$; $k_d$, 4.28E-05 s$^{-1}$; and $K_D$ [equilibrium dissociation constant], 1.21E-09 M) are in line with the data published by Wu et al. (60). Taken together, our data show that the clonal and sequential strategies induced greater antibody maturation than the mixture approach.

DISCUSSION

This study is the first, to our knowledge, to utilize clones of native, sequential HIV-1 env variants arising in vivo during infection to elicit NAbs. We explored a new vaccine strategy in which rabbits were sequentially exposed to Env-encoding DNA expression plasmids in an ordered manner designed to recapitulate the changes in the viral quasispecies experienced in vivo over time. Our goal was to determine how closely, if at all, the resulting NAb responses resembled those observed in the SHIV-infected macaque from which the sequences were derived. Fifteen variants representing the env variants from macaque A141 showing a broadened NAb response were used to determine if these sequences could program the B-cell response in a more effective manner than an individual env (clonal strategy) or a cocktail of these same env variants (mixture strategy). Our data demonstrate that this sequential vaccine approach induced the production of binding antibodies, autologous and weak heterologous NAbs. Furthermore, NAbs targeted the V3 loop of Env among other epitopes. This sequential strategy was comparable to or better than vaccination with a single env clone.
or with a cocktail of env variants. In addition, this strategy partially reelicited a similar NAb response profile and the same epitope targeting as those obtained from macaque A141, from which the env sequences were cloned.

The three immunization strategies induced gp120-specific BAbs in all rabbits, and as previously demonstrated (18, 19), several DNA primes were required to mount a measurable immune response. In addition, BAb titers were improved by the protein boosts, which is consistent with the published literature (51). As predicted, the BAb levels were not statistically different between the three vaccine groups. The Env-specific BAb response also gradually increased in macaque A141 (data not shown) and reached very high levels as a consequence of the high viremia during both the acute and chronic phases of infection (6). We assessed the longitudinal development of the neutralization of several autologous viruses included in the vaccine. We hypothesized that if the sequential immunization strategy were effective at educating B cells, statistical differences in the NAb responses, but not in the BAb responses, would be observed among the three vaccines groups, which is consistent with the results described above. Our data show that both SF162 and the earliest variant, d56 B3, were efficiently neutralized by sera from all rabbits. The neutralization of the middle variant, d417 D15, was far less efficient than the neutralization of SF162 and d56 B3. The immunization strategies did not induce NAb against the late clones d487 A6-1 and d670 A8-9, which appear to be more difficult to neutralize. Some heterologous NAb and were not able to reproduce the breadth measured in the individuals from whom the env variants were obtained (4). In addition, Derby et al. demonstrated that a concentration of 10 mg/ml of purified IgG collected after the immunization of macaques with SF162 DNA prime/protein boost was necessary to neutralize SF162 potently and 89.6, 6535, and SS1196.1 moderately, and it showed some hint of the neutralization of REJO4541.67 (15). Therefore, our results are consistent with other immunization studies.

Differences in neutralization sensitivity in some HIV Env proteins have been correlated to changes in variable loop lengths (21, 44) or variation in PNG numbers (44). The tested heterologous viruses had the same V3 length as the A141 Env proteins. Neutralization resistance has been shown to be associated with V1/V2 length (21, 44), and NAb have correlations with shorter V1-V4 lengths in clades other than B; thus, these effects do not explain our observations. Previous studies demonstrated that neutralization resistance correlated with an increase in the number of PNGs (44). We found that both the late autologous variants (d417 D15, d487 A6-1, and d670 A8-9) and the heterologous variants had increased numbers of PNGs in gp120 compared to that of the parental SF162 (Table 2). Indeed, the autologous variants had more PNGs in V1/V2 and V4 regions, and this increase in PNG sites could explain why they are more difficult to neutralize. Some heterologous variants had more PNGs in C1, V1/V2, C2, C3, V4, and V5 regions. Interestingly, SS1196.1 and REJO4541.67 had fewer PNGs than TRO.11 and 7165.18, but only SS1196.1 is neutralization sensitive; thus, variation in PNG number alone cannot explain the measured variation in neutralization resistance as tested in our study. The influence of specific PNG sites and amino acid residues on neutralization sensitivity in the A141 quasispecies is being investigated further.

Neutralization competition assays were performed with linear V1 and V3 SF162 peptides to determine which variable regions of Env are involved in the neutralizing activity of the polyclonal antibodies generated by our vaccine strategies. The targeting of V1 and V3 loops was expected, since both loops

| Envelope | C1 | V1/V2 | C3 | C4 | V5 | C5 | PNGs/gp120 |
|----------|----|------|----|----|----|----|-----------|
| SF162    | 1  | 3    | 1  | 3  | 5  | 1  | 21        |
| d56 B3   | 1  | 3    | 6  | 1  | 3  | 5  | 21        |
| d447 D15 | 1  | 3    | 6  | 1  | 3  | 5  | 21        |
| d447 A6-1| 1  | 5    | 6  | 1  | 3  | 5  | 21        |
| d670 A8-9| 1  | 5    | 9  | 1  | 3  | 5  | 21        |
| REJO4541.67 | 1 | 6 | 9 | 2 | 3 | 1 | 24 |
| TRO.11  | 1  | 4    | 5  | 1  | 3  | 5  | 29        |
| Q/0992.24 | 1 | 4 | 7 | 2 | 3 | 1 | 28 |
| SS1196.1 | 1  | 3    | 5  | 1  | 3  | 5  | 28        |

*Each row represents a viral Env variant. All protein sequences were aligned to HXB2 (accession no. AF033819) and were analyzed with the N-GlycoSite program using the HIV Sequence Database from the Los Alamos National Laboratory (http://www.hiv.lanl.gov/content/sequence/GLYCOSITE/glycosite.html). An increase in the number of PNGs compared to that of SF162 is indicated by a green box, and a decrease is indicated by a purple box.*
sequences were derived from an SHIV-infected macaque after due to the vaccine delivery system used and/or to the fact that broadened NAb response. This strategy did not result in the native HIV-1 quasispecies derived from an individual with anofocusing. Therefore, our data suggest that it is possible to of two of the same HIV envelope variants and V3 loop immu-
tinuously rather than in a cocktail.

In conclusion, immunization with a collection of env quasi-
species variants resulted in the development of autologous and weakly heterologous NABs that were comparable to the ones elicited by the clonal strategy. In addition, our quasispecies vaccine strategy replicated some of the NAB response features obtained from an SHIV-infected animal (from which the quai-
species env variants were cloned), such as the neutralization of two of the same HIV envelope variants and V3 loop immuno-
focusing. Therefore, our data suggest that it is possible to program humoral responses in part by exposure to a subset of native HIV-1 quasispecies derived from an individual with a broadened NAB response. This strategy did not result in the induction of broad heterologous NABs, but this may have been due to the vaccine delivery system used and/or to the fact that sequences were derived from an SHIV-infected macaque after less than 2 years of infection. Naked DNA plasmids have long been considered a weak delivery system to induce protective

immune responses (30, 31), but recent studies showed that DNA prime-protein boost approaches can induce neutralizing antibodies in rabbits and in humans (52, 53). Additional ex-
periments are in progress using env variants cloned from the plasma of HIV-infected human subjects who developed more potent NABs than did macaque A141 to determine the broader utility of this approach, as well as to further explore the use of key individual variants that arise during infection as immuno-
gens. Finally, an ideal HIV Env-based vaccine would generate antibody maturation, specific epitope targeting, and broad neutralization. Our study emphasizes the importance of assessing all of these different aspects of the immune response in immunized animals to determine which immunization ap-
proaches are beneficial to the vaccinee.

ACKNOWLEDGMENTS

We thank William Sutton for assistance with the immunizations and Than-Phuong Chu and Garrett Waagmeester for assistance with the neutralization assays. We are grateful to Dina Kovarik for helpful discussions regarding the experiments and manuscript preparation. We thank Franco Pissani, Shelly Krebs, and Ihlem Messaoudi for their suggestions regarding manuscript preparation. TZM-bl and 293T cell lines were obtained from the NIH AIDS Research and Reference Reagent Program.

This work was supported by National Institutes of Health grants P01 AI087064, P01 AI054564, and RR-000163 and a Global Health Grand Challenges Explorations Grant from the Bill and Melinda Gates Foundation (N.L.H.). Statistical assistance was supported by grant number ULI RR024140 01 from the National Center for Research Resources (NCRR).

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