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Antibody Responses Elicited through Homologous or Heterologous Prime-boost DNA and Protein Vaccinations Differ in Functional Activity and Avidity

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Abstract

Using a gp120 envelope glycoprotein from the JR-FL strain of human immunodeficiency virus-1 (HIV-1) as a model antigen, the goal of the current study was to evaluate the level and quality of antibody responses elicited by different prime-boost vaccination regimens (protein only, DNA only, DNA plus protein) in rabbits. Our data demonstrated that incorporating DNA immunization as a prime in a heterologous prime boost regimen was able to elicit a more diverse and conformational epitope profile, higher antibody avidity, and improved neutralizing activity than immunization with only protein. Additionally, this improved neutralizing activity was observed in spite of similar antibody specificities and avidities seen when only DNA vaccination was used, providing additional evidence that the use of a combination immunization regimen increases the protective antibody response. Insights gained from the current study confirmed that the heterologous DNA prime-protein boost approach is effective in eliciting not only high level but also improved quality of antigen-specific antibody responses, and thus may offer a new technology platform to develop better and safer subunit vaccines.

Keywords

DNA vaccine; Prime-boost; HIV-1

1. Introduction

Despite the obvious need for a better understanding of how to raise functional antibodies through immunization, we still only have limited knowledge regarding to the relationship between the type of immunizations administered and the resulting antibody responses. Given safety concerns associated with live attenuated vaccines and the overall poor immunogenicity of inactivated vaccines, subunit vaccines, using only selected antigens from a complex pathogen, have been considered a more ideal choice. However, after more than 30 years of

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vaccine development, there are only a few subunit vaccines licensed for wide clinical use, including the surface protein-based hepatitis B virus vaccine and the L1 protein-based human papillomavirus (HPV) vaccine [1,2]. Two conditions have been identified as critical for a successful recombinant protein-based subunit vaccine. First, an adjuvant is absolutely necessary as part of a recombinant protein-based vaccine formulation to achieve sufficient immunogenicity in humans [3-7]. Second, the conformation of protein-based vaccine plays a key role in determining the functionality of antibody responses elicited by protein-based subunit vaccines. It was demonstrated in early HPV vaccine studies in animals that intact papilloma virions provided protection against subsequent challenge [8], but vaccination with disrupted or denatured papilloma virion particles failed to provide protection [9-11]. Such dependency on adjuvant and natural antigen conformation may have contributed to the slow development of more recombinant protein-based subunit vaccines.

At the same time, novel vaccine modalities, such as DNA vaccines and viral vector-based vaccines, have become attractive alternative approaches to deliver subunit antigens [12,13]. More interestingly, heterologous prime-boost (i.e., the sequential use of two types of vaccines to deliver the same subunit antigen), can be more immunogenic than repeated administrations of either type of vaccine alone (homologous prime-boost) [14]. Studies previously conducted in our laboratory have demonstrated that the DNA prime-protein boost approach is more effective than using DNA or protein alone in eliciting higher antibody responses in both HIV-1 and influenza vaccine studies [15-18]. While these studies began to identify differences in antibody responses resulting from different vaccination approaches, they lacked a rigorous comparison of the effects of number of immunizations as well as a detailed analysis on the quality of antibody responses. In the current study, we attempt to elucidate how the immunization regimen influences the quality of antibody response. By using HIV-1 gp120 envelope protein as a model antigen, differences in the resulting antibody responses elicited by either homologous or heterologous prime-boost immunizations are analyzed including the ability of the sera to neutralize a diverse panel of sensitive and primary HIV isolates, the specificity of antibodies being generated in a polyclonal sera, as well as the resulting avidity of gp120-specific antibodies.

2. Materials and Methods

2.1 Vaccines

2.1.1 HIV-1 gp120 DNA vaccine—A codon optimized JR-FL gp120 construct in the pJW4303 vector was used for all DNA-based immunizations, as previously reported [16]. DNA was produced in HB101 bacterial cells then isolated and purified using the Qiagen Plasmid Mega Kit (Cat # 12183).

2.1.2 HIV-1 gp120 protein vaccines—Recombinant HIV-1 gp120 proteins were produced from Chinese Hamster Ovary (CHO) cells. The JR-FL gp120 protein produced by Progenics was provided by Dr. John Warren at Division of AIDS, NIAID, NIH. Other gp120 envelope glycoproteins from subtypes A (UG21-9), B (92US715), C (MW959), and E (TH14.12) were all produced in our laboratories. Secreted proteins from stably transfected CHO cell lines were harvested and purified over a lectin affinity column.

2.1.3 Antibodies—The CD4 binding site directed monoclonal antibody, b12, was obtained as a gift from Dr. Dennis Burton. The V3-directed mAb, 447-52D, was provided as a gift from Dr. Susan Zolla-Pazner. The co-receptor binding site antibody 17b was provided by James Robinson. The monoclonal antibodies 2G12, and F105 were obtained through the NIH AIDS Research & Reference Reagent Program.

2.2 Rabbit Immunizations

New Zealand White (NZW) rabbits at 6-8 weeks of age were purchased from Millbrook Farm (Amherst, MA) and housed in the animal facility managed by the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS) in accordance of the protocol approved by UMMS' Institutional Animal Care and Use Committee (IACUC).

When DNA immunizations were given, DNA encoding either JR-FL gp120 or pJW4303 vector control was coated onto 1 micron gold beads at a ratio of 2 µg of DNA per milligram of gold beads and delivered to animals via a Bio-Rad Helios gene gun onto shaved abdominal skin. Each animal received 36 µg of DNA per immunization. Where appropriate, protein immunizations was done consisting of either 50 µg of JR-FL gp120 or 10 µg each of a 5-valent formulation with gp120s from subtypes A (UG21-9), B (JR-FL & 92US715), C (MW959), and E (TH14.12). The amino acid sequences of these five gp120 proteins are included in Supplemental Fig. 1. Prior to injection, 50 µg of gp120 proteins was diluted in 500 µL PBS and mixed with 500 µL Incomplete Freund's Adjuvant (IFA). The 1 mL adjuvanted protein solution was then injected intramuscularly into the back of rabbits. Serum was collected for antibody studies two weeks prior to the first immunization and two weeks after each animal immunization.

2.3 Enzyme Linked Immunosorbent Assay (ELISA)

Recombinant JR-FL gp120 protein was coated onto 96 well microtiter plates (Costar #3369) at 1 µg/mL in 100 µL of PBS for 1 hr at room temperature. Plates were then washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4° in PBS containing 4% whey by weight (whey dilution buffer) and 5% powdered milk. The following morning, plates were washed 5 times in EWB and serially diluted rabbit sera, collected at 2 weeks following the final protein immunization, was added to the wells in a volume of 100 µL. Plates were washed 5 times in EWB and 100 µL of biotinylated anti-rabbit secondary antibody (Vector Labs BA-1000) at 1.5 µg/mL was incubated on the plate for 1 hr at room temperature. Plates were washed 5 times with EWB and incubated with 100 µL of a streptavidin horseradish peroxidase construct (Vector Labs SA-5004) at 500 ng/mL. Plates were washed 5 times with EWB and developed for 3 min in 100 µL of a 3,3',5,5'-tetramethylbenzidine substrate solution (Sigma T3405). The reaction was stopped with addition of 25 µL of 2N H₂SO₄. Endpoint titers as reported are defined as the last dilution of a serially diluted serum sample with greater than double the background optical density of a preimmune serum sample.

2.4 HIV-1 Neutralization Assay

Neutralization assays were done as previously described [19]. HIV-1 pseudovirions were produced through cotransfection of the pSG3^{Δenv} backbone (NIH AIDS Research Reference and Reagent Program) and an Envelope gp160 bearing plasmid in HEK 293T cells. Pseudovirus-containing supernatants were cleared of cell debris by low speed centrifugation. Pseudoviruses were then titered out on the TZM-bl cell line before use. For a typical neutralization assay, 200 TCID₅₀ of pseudovirus was incubated with rabbit sera for 1 hr at 37° C. The virus/sera mix was then added to 10⁵ TZM-bl cells in a final concentration of 20 µg/mL DEAE Dextran. Plates were incubated at 37°C for 48 hours and developed with luciferase assay reagent according to the manufacturer's instruction (Promega). Neutralization was calculated as the percent change in luciferase activity in the presence of preimmune sera versus that of luciferase activity in the presence of immune sera [(Preimmune RLUs – Immune RLUs)/(Preimmune RLUs)]*100.

In peptide adsorption experiments the same neutralization protocol was applied as described above except, prior to the exposure of sera to the pseudovirus, the sera were incubated with 25 µg/mL of a consensus HIV-1 subtype B V3 epitope peptide

(CTRPNNNTRKSIHIGPGRAFYTTEIIGDIRQAHC) for 30 minutes at 37°C. Additionally, in some assays, the JR-FL virus was preincubated with 5 µg/mL soluble CD4 (NIH AIDS Research and Reference Reagent Program) prior to the addition of serum. In these assays, percent neutralization was calculated with the light signal of JR-FL in the presence of sCD4 serving as our baseline light signal.

2.5 Competitive Binding Assays

Competitive binding assays were performed as previously described [20,21] with minor modifications. Pseudovirions bearing the JR-FL Envelope and Vesicular Stomatitis Virus (VSV) glycoprotein were produced with the pSG3^{ΔEnv} backbone in 293T cells. Microtiter plates were coated with 50 µL of monoclonal antibody (mAb) at 5 µg/mL for 1 hr at room temperature. Plates were then blocked in PBS with 3% BSA overnight at 4°C. Rabbit sera heat inactivated at 56°C for 30 min, serially diluted, and incubated with pseudovirus correlating to 2.5 ng of p24/well for 1 hr prior to the addition to the virus/sera mixture to the ELISA wells. Virus/sera mixture was then incubated on the ELISA wells for 3 hrs at room temperature. Plates were washed 5 times with sterile PBS and overlaid with 10,000 TZM-bl cells per well. Plates were incubated for 48 hrs at 37°C. Luciferase activity was determined per the manufacturer's instruction (Promega). Data is reported as the serum dilution at which the luciferase signal is reduced by 50% compared to a serum negative control.

2.6 NaSCN Displacement

JR-FL gp120 was coated onto 96 well microtiter plates (Costar #3369) at 1 µg/mL in 100 µL of PBS for 1 hr at room temperature. Plates were then washed 5 times in PBS containing 0.1% Triton-X (EWB) and blocked overnight at 4°C in PBS containing 4% by weight whey (whey dilution buffer) and 5% powdered milk. Rabbit sera were then added to the plate at either a 1:30,000 or 1:100,000 dilution and incubated at room temperature for 1 hr. Plates were then washed 5 times in EWB. NaSCN was then added at various (0, 1, 2, 3, 4, 5 M) concentrations in PBS for 15 min followed by 5 washes in EWB. Bound IgG was detected as described above. Determination of IgG quantity remaining on the plates was done using linear regression analysis of a standard IgG curve (Southern Biotech 0111-01). Data is reported as the NaSCN concentration required to displace 50% of IgG initially bound on the plate.

2.7 Statistical Analysis

All statistical analyses were performed in the Graphpad Prism software program. Reported p values were derived from data subjected to a two tailed Mann-Whitney test.

3. Results

3.1 Study Design and Immunization Schedule

In this study rabbits were immunized in one of five schedules in order to provide a direct comparison as to the relative immunogenicities of homologous vs. heterologous prime-boost vaccinations. These approaches (summarized in Fig 1) utilize HIV-1 JR-FL gp120 as a model antigen delivered as either a DNA vaccine or a recombinant protein vaccine.

Groups of NZW rabbits were immunized with one of the following regimens: 1) five JR-FL gp120 DNA immunizations; 2) five JR-FL gp120 protein immunizations; 3) three JR-FL gp120 DNA immunizations followed by two JR-FL gp120 protein immunizations. The first two schedules are homologous prime-boost using the same type of vaccine. The third schedule is a heterologous prime-boost which administered DNA and protein immunizations to the same animals at different time points. Consistent with previous studies, three DNA-based immunizations were given in the priming phase, followed by two protein-based immunizations

in the boosting phase [15,22]. Two additional immunization schedules were included as controls. In one schedule, rabbits received three empty DNA vaccine vector immunizations followed by two JR-FL gp120 protein boosts. The purpose of this control is to exclude the non-specific effect by a DNA plasmid as the prime. Another control group received a polyvalent (five) gp120 protein vaccine, which included the JR-FL gp120 and another four gp120 proteins from other HIV-1 isolates (Supplemental Fig 1), as the boost in order to compare the difference between the polyvalent and monovalent boosts. In all of the above studies, the “priming” immunizations were given at Weeks 0, 2, and 4 with “boosting” immunizations given at Weeks 8 and 12 (Fig 1).

3.2 Generation of gp120 specific antibodies as measured by ELISA

The overall immunogenicity for each animal group was first determined by the binding titers of serum IgG for each individual rabbit against the JR-FL gp120 antigen by ELISA (Fig 2). All rabbits, regardless of immunization regimen, generated a significant gp120-specific antibody response. Despite the observation that rabbits receiving five protein immunizations tended to generate a slightly lower binding antibody response, endpoint serum dilution titers among these groups were statistically indistinguishable from each other (data not shown). Additionally, variation of individual animals within a single group was also minimal, with no animals deviating more than a single dilution step from the group geometric mean titers.

3.3 Analysis of antibody specificity elicited by each immunization regimen

We chose to use a pseudoviral-based competitive binding assay to examine antibodies of a particular specificity capable of binding to an HIV-1 viral envelope spike as previously reported [15,21,23]. Knowing that the V3 loop is an immunodominant epitope of gp120, whose recognition is sometimes responsible for the neutralization of select viruses, we began by assaying sera for the presence of V3-directed antibodies using a known V3-directed monoclonal antibody, 447-52D, in a competitive binding assay (Fig 3A). Consistent with the immunodominant nature of the V3 loop, all immunization regimens elicited high titer antibody responses capable of outcompeting binding of 447 to this domain. In many cases, titers approached or exceeded 1:1000. However, animals that received protein alone immunizations or immunizations with only the empty DNA vaccine vector prime had the lowest V3 loop-targeted antibody responses. The group with the highest antibody responses to this region was the group that received the heterologous DNA prime-protein boost regimen. Rabbits in this group elicited a significantly higher V3-directed antibody response than rabbits that received five protein-based injections ($p=0.02$). DNA alone immunization was also less effective than the heterologous prime-boost approach but the difference was not statistically significant. Interestingly, the group that received a polyvalent gp120 boost had a lower 447-52D-like antibody response than the monovalent (JR-FL gp120) boost, suggesting the polyvalent boost, which included gp120 from different subtypes, may dilute the focus of boost against a subtype B V3 epitope.

Next, we expanded our analysis to antibodies against other important gp120 epitopes. This was conducted by determining any differences in the fine specificity of the antibodies elicited by each prime-boost immunization regimen. First, we looked for the presence of antibodies targeted to a cluster of carbohydrates recognized by the human mAb, 2G12 (Fig 3B). Of the 15 rabbits tested in this study, only one that received the homologous DNA prime-protein boost was capable of outcompeting binding to the mAb 2G12, indicating that antibodies of this specificity are rare with any of the immunization schemes used in the current study. This observation is also consistent with data in HIV-infected humans, indicating that antibodies of this specificity are rarely elicited [24].

Next, we evaluated the rabbit immune sera for the presence of antibodies targeted to the co-receptor binding site by testing competition with the human mAb, 17b (Fig 3C). Again, with only a single rabbit capable of outcompeting binding, and at a titer barely reaching our cutoff of 50% reduction in binding at a 1:40 serum dilution, we determined that antibodies targeted to this domain were also largely absent. This result is also consistent with a previous report showing that the failure to elicit co-receptor-targeted antibodies in rabbits was not unusual [25].

The presence of CD4 binding domain-specific antibodies in sera from immunized animals was also evaluated using either the narrowly neutralizing monoclonal antibody, F105 (Fig 3D), or the broadly neutralizing antibody, b12 (Fig 3E). When the mAb F105 was used as a competitive binding target, five of the six animals that received protein alone immunizations demonstrated no capability of outcompeting binding to this monoclonal antibody. In contrast, all animals that received five DNA immunizations generated antibodies capable of outcompeting binding to F105, and did so with the highest competition titer among all groups with an average reciprocal dilution of approximately 150. Similarly, all three animals in the matched JR-FL DNA prime-protein boost group generated an antibody response capable of outcompeting binding to F105. Interestingly, when the protein boost formulation was changed from a single JR-FL gp120 to five recombinant gp120 proteins from different HIV-1 subtypes, antibodies capable of competing binding to F105 were largely absent, with only one of the three animals capable of doing so at lower serum dilution.

This general trend continued when we tested the ability of the rabbit sera to outcompete binding to a second CD4 binding site mAb, b12, which can neutralize a wide range of primary HIV-1 isolates (Fig 6E). Again, the animals that received only two JR-FL gp120 protein-based immunizations with empty DNA vector prime could not outcompete binding to b12 in any instance. Animals that received immunizations with protein alone, again, only sporadically elicited antibodies targeted to the CD4 binding site; however, they did so at very low titer, barely making our cutoff of a 1:40 dilution. Again, in the animals that received five DNA immunizations, we detected CD4 binding site-directed antibodies at titers very similar to those observed against F105, at approximately a 1:150 dilution. Two out of the three rabbits that received a monovalent DNA prime-protein boost were capable of outcompeting binding to b12. This is one rabbit less than was capable of outcompeting binding to F105 within the same group. However, the two animals that did generate antibodies capable of outcompeting binding to b12 did so with high 50% competition values approaching serum dilution of 1:600. In rabbits that received the polyvalent protein boost, we again noticed that only one out of the three animals were capable of outcompeting binding to b12. This change in the boost formulation might result in the generation of antibodies that have a more diverse recognition for the CD4 binding site, such as those that may target CD4 binding sites from envelopes of other HIV-1 subtypes that are no longer capable of outcompeting binding to the largely subtype B-specific antibodies, F105 and b12.

3.4 Neutralization of Tier 1 HIV-1 viruses

Because the ability to outcompete binding to V3-directed antibodies only confirms the presence of this type of antibody in the immune sera but does not reveal any information about their functionality, we evaluated how well these V3-directed antibodies are capable of neutralizing sensitive isolates of HIV-1. In order to do this, we utilized two viruses with a known sensitivity to V3-mediated neutralization. The first virus was SF162 (Fig 4A). Consistent with the presence of high titer V3-directed antibodies in rabbit sera, this virus was neutralized by serum from every animal in the study. However, the potency at which this was achieved differed between immunization groups. Geometric mean neutralizing antibody (NAb) titers in groups whose immunizations consisted of only a single vaccine modality were all below 1:100.

Specifically, geometric mean NAb titers for animals that received two protein, five protein, or five DNA immunizations were 1:60, 1:38, and 1:88, respectively. In contrast, animals that received three DNA prime immunizations and were boosted with two protein immunization of either matched JR-FL protein or a polyvalent gp120 protein mix, achieved geometric mean NAb titers of 1:754 and 1:334, respectively. The increases in potency seen against SF162 when the heterologous DNA prime-protein boost regimen was used were statistically higher than those seen with the use of two protein immunizations ($p=.024$), five protein immunizations ($p=.028$), and the use of five DNA immunizations ($p=.024$).

Rabbit sera were next tested against NL4-3, an HIV-1 isolate slightly more resistant to neutralization (Fig 4B). Again, consistent with the SF162 neutralization data, the antibody response generated through a heterologous immunization regimen was significantly more potent than that generated through immunization with any single vaccination modality. Animals that received vector primes followed by two protein immunizations were found to be completely incapable of neutralizing this virus. This trend only improved slightly in animals that received five protein immunizations. Within this group, only serum from a single animal was capable of neutralizing the virus, and only at a NAb titer of 1:10. Rabbits that received five DNA immunizations neutralized this isolate with slightly more frequency and potency. Two of the three animals in the group neutralized the virus with a geometric mean NAb titer of 1:19. In contrast to the sporadic neutralization seen with the single modality immunizations, all six rabbits that received heterologous JR-FL DNA prime and either monovalent or polyvalent protein boost were capable of neutralizing NL4-3. Geometric mean NAb titers of 1:143 and 1:132 were achieved for the two DNA prime-protein boost groups, respectively. This demonstrates another 7-fold increase in potency over the next best immune sera from animals that received five DNA immunizations. Again, the potency of neutralization observed when a combination DNA prime-protein boost regimen was administered was significantly higher than the potency of neutralization seen when two protein ($p=0.024$) or five protein ($p=0.028$) immunization were given.

Further investigation of this V3-mediated neutralization led us to investigate its role in neutralizing the autologous HIV-1 JR-FL isolate. Initial screening of all sera against a JR-FL pseudovirus showed that greater than 50% neutralization was achieved only in a single animal in the JR-FL DNA prime-JR-FL protein boost group (Fig 5A). Previous studies have demonstrated that exposure to soluble CD4 (sCD4) sensitizes envelopes to V3-mediated neutralization [26]. We utilized this phenomenon to further study the functionality of the V3-directed antibodies being generated by each immunization regimen. JR-FL pseudovirus was exposed to sCD4 at 5 $\mu\text{g/mL}$ and sera was then added to the virus to determine if any increases in neutralization was observed (Fig 5B). This produced even more striking differences between immunization groups than those observed with SF162 or NL4-3. Against the sCD4 exposed homologous JR-FL virus, rabbits who received either two or five JR-FL protein immunizations were completely incapable of neutralizing the virus. Rabbits that received five DNA-based immunizations fared better with two of three animals capable of neutralizing the virus. When animals that received the JR-FL DNA prime and monovalent protein boost regimen were evaluated, sera from all three animals were capable of neutralizing the sCD4-treated virus. In rabbits that received the JR-FL DNA prime and polyvalent protein boost, two out of three could neutralize the virus. Therefore, the inclusion of the DNA prime in the immunization regimen increased the quality of the V3-directed antibody response, making it more capable of neutralizing not only the V3 sensitive viruses, such as NL4-3, but also the more resistant JR-FL virus upon sCD4 treatment.

Despite evidence that wild type rabbits are not capable of generating antibodies to CD4 inducible sites, such as the co-receptor binding site [25], we wanted to confirm that the observed neutralization against JR-FL was in fact mediated by recognition of the V3 loop. Sera were

first incubated with a JR-FL V3-matched peptide (CTRPNNTRKSIHIGPGRAFYTTEIIGDIRQAHC) at 25 µg/mL prior to the addition to sCD4 exposed JR-FL. As expected, this step eliminated all observed neutralization of the JR-FL virus (Fig 5C).

3.5 Neutralization of Tier 2 primary HIV-1 isolates

While we have already demonstrated some differences in the capacity of sera from each immunization regimen to neutralize sensitive or Tier 1 isolates, we also wanted to determine if there were any differences in the ability of these sera to neutralize other, more resistant primary isolates. To do this we chose to evaluate sera from each immunization group against viruses from the standard NIH Tier 2 clade B primary isolate panel [27]. While, overall, these viruses are much more resistant to neutralization, we observed the superiority of the DNA prime-protein boost approach in neutralizing these viruses (Table 1). When only two protein immunizations were given, none of the animals elicited a NAb response against any of the Tier 2 viruses. When five protein immunizations were given, neutralization of these Tier 2 viruses was also absent with the exception of one animal (Rabbit #620) that was capable of neutralizing the 6535.1 isolate. Furthermore, administration five DNA alone immunizations did not improve neutralization of these viruses; as Rabbits #626 and #627 were not capable of neutralizing any of the Tier 2 viruses while Rabbit #628 neutralized two of the 12 (QH0692.42 and RHPA4259.7) isolates at a 1:10 dilution. The neutralizing activity of sera from animals immunized with a DNA prime-protein boost immunization regimen improved slightly, but not dramatically. Within this immunization group, one of the animals (Rabbit #625) was capable of neutralizing four of the 12 isolates. However, this was not typical as Rabbit #624 could only neutralize one of the 12 isolates and Rabbit #626 could not neutralize any. When animals were given the JR-FL DNA prime followed by a polyvalent protein boost, the consistency of neutralization as well as the breadth of neutralization was further increased. The best neutralizer we encountered, Rabbit #631, neutralized six isolates (6535.3, AC10.0.29, CAAN5342.A2, QH0692.42, SC422661.8, and THRO4156) at a 1:10 dilution. Rabbit #630 neutralized four isolates in the same panel as Rabbit #631. Rabbit #629 however, could not achieve 50% neutralization against any of the viruses tested. The observed neutralization was HIV-1-specific as none of the sera neutralized a control Murine Leukemia Virus (MuLV) pseudotyped virus (Table 1).

3.6 Evaluating the avidity of elicited antibody responses

Previous studies have suggested that a heterologous DNA prime-protein boost approach was able to elicit antibody responses with higher avidity than the homologous DNA or protein alone approaches [16,28]. Importantly, recent evidence has indicated that antibody avidity may correlate to better protection against SHIV challenge in a non-human primate SHIV challenge study [29]. The avidity of rabbit immune sera being elicited by each immunization group in the current study was also evaluated by measuring how tightly the serum elicited by each immunization regimen is capable of binding the gp120 antigen in an ELISA assay. We found that serum avidity differed greatly between immunization regimens (Fig 6). By running bound IgG from our test animals against an IgG standard we were able to calculate the amount of IgG displaced by increasing concentrations of sodium thiocyanate (NaSCN). Sera from animals who received only two protein injections were most easily displaced by NaSCN. Half of all bound IgG was displaced with an average of a 1.9 M solution of NaSCN. When five protein immunizations were given instead of two, 50% of bound sera remained bound to the plate in a 2.8 M solution of NaSCN. Serum avidity increased further with the use of five DNA immunizations with 50% of sera remaining bound at a concentration of 3.4 M NaSCN. The use of a DNA prime-protein boost approach improved this further to an average 50% displacement at 3.6 and 3.9 M NaSCN for the monovalent and polyvalent gp120 boosted groups, respectively. These observed increases in binding avidity with the heterologous DNA

prime protein boost were found to be statistically higher than those seen when only protein-based immunizations were used ($p = 0.024$). Because only relatively small gains in serum avidity were seen with the inclusion of a protein boost, it is likely that the use of a DNA immunization induces an initial antibody response with a higher avidity than is observed with an initial protein immunization. The increase in binding avidity may be one of the reasons as to why the DNA prime-protein boost regimen appears to be superior to either DNA or protein alone in eliciting a functional antibody response.

4. Discussion

Recent studies have suggested that a heterologous prime-boost vaccination approach in which the same antigen is delivered sequentially by different types of vaccines was more effective in eliciting higher immune responses than the homologous prime-boost using same type of vaccines [14]. In the current study we have built upon previous work [15,16] in evaluating humoral responses generated using HIV-1 gp120 antigen as a model antigen delivered by different prime-boost regimens. A more rigorous comparison of immunizations was conducted with two or five protein vaccinations, five DNA vaccinations, or a combination of DNA and protein vaccinations in a rabbit model. We demonstrated that all regimens studied were capable of eliciting an equivalent binding antibody response. However, sera generated by each of these immunization regimens proved to differ greatly in more important characteristics including specificity, functionality, and avidity. This finding will have significant impact on the future development of vaccines.

Different vaccine delivery approaches have been developed based on the available technology at any given time in history. Efficacy and safety have been the main final parameters driving the development of different vaccine delivery approaches. However, little work has been done to compare the detailed parameters among different vaccine delivery approaches.

Unfortunately, immune correlates of protection are not well understood, even for licensed human vaccines. While antibodies are well recognized for playing a major role in protection for many successful vaccines, it is frequently not clear what specific mechanism contributes to such protection. This situation became even more complicated with the discovery of a newer generation of vaccination approaches, such as DNA- or viral vector-based vaccines, and the use of prime-boost strategies employing different types of vaccines; there have been very few studies examining how these newer immunization regimens affect the quality of the final antibody response.

Due to the challenge of developing an HIV vaccine, many novel approaches have been developed and tested with the goal of raising an optimal antibody response to the HIV-1 envelope glycoprotein. The current study utilized some of these novel approaches in order to conduct a detailed analysis on the quality of antibody responses elicited by different prime-boost vaccination strategies.

We hypothesized that the measurement of binding antibodies against a protein antigen by a polyclonal animal serum may not reflect the difference in detailed antibody profiles of such immune sera. It is well-known that the gp120 form of HIV-1 envelope protein-based immunizations typically leads to antibodies targeted to the immunodominant V3 loop. We used this region as the first model antigen determinant to identify a number of differences among different prime-boost immunization regimens. As expected, all immunization regimens used in our study generated antibodies to this domain, all of which were shown capable of outcompeting binding to the V3-directed mAb, 447-52D. However, it became apparent that immunization regimens that included a DNA-based immunization elicited antibodies that were more capable of outcompeting binding to 447-52D. This manifested itself at a functional level and in DNA-immunized animals as it was observed that these animals were more capable of

neutralizing the V3 sensitive isolate, NL4-3. Similarly, only sera from animals that received a DNA prime demonstrated neutralizing activities against the CD4-sensitized primary isolate, JR-FL. Through the use of V3 peptide adsorptions, we further demonstrated that this neutralizing activity was mediated by antibodies recognizing the exposed V3 loop after CD4 treatment.

Every animal in our study generated neutralizing antibodies against SF162, an isolate which is extremely sensitive to neutralization by V3 antibodies. These results indicate that even for the V3 antigen, there are different fine specificities, which can mediate different degrees of neutralization breadth. Additional evidence for this has also been recently reported when different V3-specific human monoclonal antibodies with different levels of breadth on neutralizing activities were identified [30-32]. Results from our study indicated that the less effective protein-based immunizations generated mainly V3-directed neutralizing antibodies only against the highly sensitive SF162 isolate. Other types of V3-directed neutralizing antibodies, such as those capable of neutralizing NL4-3 or other heterologous primary isolates, may require a DNA priming step. Therefore, the DNA priming step either provides a more relevant antigen conformation or generates antibodies with some biophysical quality that is superior than those generated through the protein alone vaccination approach.

Further investigation into epitopes outside of the V3 loop also yielded interesting results. While little to no antibodies targeted to the CD4-induced (17b-like) or glycan (2G12-like) epitopes were seen through immunization with any regimen, a significant number of antibodies capable of outcompeting the CD4 binding site monoclonal antibodies, F105 and b12, were observed. However, these CD4 binding site-directed antibodies were only seen in animals that had received some form of DNA-based immunization. Also interesting to note is data suggesting that the fine specificities of these antibodies can be shifted with different types of protein vaccine boost. Vaccination regimens that included the JR-FL monovalent gp120 formulation as either DNA alone or in a DNA prime-protein boost format, elicited detectable levels of F105 or b12 competition in almost every instance. However, when the JR-FL DNA prime was followed by a polyvalent gp120 protein boost, only a single rabbit was capable of outcompeting binding to F105 and b12. This may reveal a shift in specificity to CD4 binding sites of subtypes other than clade B, or potentially, but less likely, loss of CD4 binding site-directed antibodies altogether. The fact that antibodies to this domain are being generated at all is potentially important based upon evidence that broadly neutralizing activity in some individuals is mediated by antibody recognition of this domain [33]. This fact alone makes the use of DNA vaccines an attractive platform for HIV vaccine development.

While using competition assays to dissect antibody specificity revealed interesting trends, the best evaluation for these comparisons is the neutralizing activity elicited by each regimen. Consistent with previous reports [15,16], animals that received a heterologous DNA prime-protein boost were better capable of neutralizing relevant primary isolates. Neutralization of primary isolates was almost completely lacking in rabbits that received antigen by only a single vaccine modality. In addition to the issue of the method of immunization, and in concurrence with previous data [18], a polyvalent formulation of envelope antigens appears to play an important role in eliciting a broader neutralizing antibody response than immunization with only a single envelope antigen. This may be the result of too much focus on a single envelope leading to a more potent, but less broadly neutralizing antibody response.

Additional data demonstrating that immunization with some form of DNA-based vaccine increases serum avidity to the vaccine antigen is also enlightening. It may be possible that the smaller amount of antigen being produced from the initial DNA immunizations results in a higher avidity antibody response. This may prove to be an important facet of a potential vaccine in light of recent data indicating that serum avidity inversely correlates with viral load after

challenge [29]. This result points to the need for future studies to understand how DNA immunization may affect the generation of B cells that produce high avidity antibodies.

In summary, results from this study have confirmed prior work [15,16] showing that a heterologous DNA prime-protein boost approach elicits a higher quality antibody response as indicated by the fine specificity, the avidity, and the ability to neutralize heterologous HIV isolates. However, when the individual antibody specificities that are being elicited are examined, very little difference can be detected between animals who receive a heterologous DNA prime-protein boost and those that receive only DNA immunizations. This is in spite of significant differences in the ability to neutralize primary HIV-1 isolates. This indicates that much work remains to understanding changes in the fine specificity of antibodies being elicited between immunization regimens and in the identification of potentially new neutralizing epitopes present on the viral glycoprotein. This finding will have an important impact on the future study of antibody profiles related to the development of highly effective vaccines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Study design and immunization schedule. Rabbits were immunized with one of five prime-boost regimens: 1) three times with a DNA vector prime plus two times with JR-FL gp120 protein boosts, 2) five JR-FL gp120 protein immunizations, 3) five JR-FL gp120 DNA immunizations, 4) three times with JR-FL gp120 DNA primes plus two JR-FL gp120 proteins boosts, and 5) three time JR-FL gp120 DNA immunizations plus two 5-valent gp120 protein boosts. The 5-valent protein mixture consisted of gp120s from clades A (UG21-9), clade B (JR-FL + US715), clade C (MW959), and clade E (TH14.12). “Priming” immunizations were administered at Weeks 0, 2, and 4. “Boosting” immunizations were given at Weeks 8 and 12.

Figure 2.

Vaccine elicited antibody titers. Endpoint binding titers from samples collected two weeks after the final boosting immunization were determined against the autologous JR-FL gp120 protein by ELISA. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP). Data are shown as geometric mean within a group.

Figure 3.

Specificity of vaccine-induced antibody responses as determined through monoclonal Ab competition. The ability of serially diluted polyclonal serum to outcompete binding of mAbs to a JR-FL & VSV-G pseudotyped virus was measured. Competition titers indicate the serum dilution preventing 50% of pseudoviral binding to the ELISA plate. A. Competition against the V3 directed mAb 447-52D. B. Competition against the glycan directed mAb 2G12. C. Competition against the co-receptor binding site directed mAb 17b. D. Competition against the narrowly neutralizing CD4 binding site mAb F105. E. Competition against the broadly neutralizing CD4 binding site mAb b12. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120

protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

Figure 4.

Neutralizing activity of serum elicited by different vaccine regimens against sensitive HIV isolates. Rabbit sera collected two weeks after the final boost immunization were tested for their ability to neutralize Tier 1 sensitive HIV isolates in the TZM-bl assay system A. Serum NAb titers against SF162. B. Serum NAb titers against NL4-3. NAb titer is defined as the serum dilution capable of inhibiting 50% of viral infection. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

Figure 5.

Ability of serum to neutralize sCD4 exposed JR-FL. The functionality of V3-directed antibodies elicited by each immunization group was evaluated by their ability to neutralize a sCD4 exposed JR-FL pseudovirus. A. Neutralization of JR-FL prior to exposure to sCD4. B. Neutralization of JR-FL after exposure to sCD4. C. Neutralization of JR-FL after exposure to sCD4 when sera are preincubated with a matched V3 peptide. NAb titer is defined as the serum dilution capable of inhibiting 50% of virus infection. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

Figure 6.

Measurement of serum avidity elicited by different immunization regimens. Sera was evaluated for its ability to be displaced from autologous JR-FL gp120 by increasing molar concentrations of sodium thiocyanate (NaSCN). Dots indicate concentration of NaSCN required to displace 50% of bound IgG. Bars indicate group geometric mean. Immunization groups are abbreviated as follows: three DNA vector immunizations plus two JR-FL gp120 protein boosts (3V + 2P), five JR-FL gp120 protein immunizations (5P), five JR-FL gp120 DNA immunizations (5D), three JR-FL gp120 DNA immunizations plus two JR-FL gp120 protein immunizations (3D + 2P), and three JR-FL gp120 DNA immunizations plus two polyvalent gp120 protein immunizations (3D + 2pP).

Table I

Neutralization of heterologous primary isolates

	3 Vector 2 JR-FL Protein			5 JR-FL Protein			5 JR-FL DNA			3 JR-FL DNA 2 JR-FL Protein			3 JR-FL DNA 2 5-Valent		
	617	618	619	620	621	622	626	627	628	623	624	625	629	630	631
6535.3	-	-	-	53	-	-	-	-	-	-	60	67	-	57	59
AC10.0.29	-	-	-	-	-	-	-	-	-	-	-	-	-	55	65
CAAN5342.A2	-	-	-	-	-	-	-	-	-	-	-	59	-	-	74
PVO.4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
QH0692.42	-	-	-	-	-	-	-	-	61	-	-	61	-	50	50
REJO4541.67	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
RHPA4259.7	-	-	-	-	-	-	-	-	51	-	-	-	-	-	-
SC422661.8	-	-	-	-	-	-	-	-	-	-	-	55	-	52	66
THRO4156.18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TRJO4551.58	-	-	-	-	-	-	-	-	-	-	-	-	-	-	51
TRO.11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
WITO4160.33	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MLV	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Numbers indicate % neutralization at a 1:10 serum dilution

“-” indicates less than 50% neutralization at a 1:10 dilution