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Polyvalent DNA prime and envelope protein boost HIV-1 vaccine elicits humoral and cellular responses and controls plasma viremia in rhesus macaques following rectal challenge with an R5 SHIV isolate

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Abstract

Immunization of macaques with multivalent DNA encoding gp120 genes from HIV-1 subtypes A, B, C and E and a *gag* gene followed by boosting with homologous gp120 proteins elicited strong anti-gp120 antibodies capable of neutralizing homologous and to a lesser degree heterologous HIV-1 isolates. Both Env- and Gag-specific cell mediated immune (CMI) responses were detected in the immunized animals. Following rectal challenge with an SHIV isolate encoding HIV-1_{Ba-L} *env*, plasma viremia in the infected immunized animals was significantly lower than that observed in the naïve animals. Further, one of six immunized animals was completely protected whereas all six naïve animals were infected. These results demonstrate that a vaccine based on priming with a polyvalent DNA vaccine from multiple HIV-1 subtypes followed by boosting with homologous Env proteins elicits anti-HIV-1 immune responses capable of controlling rectal transmission of SHIV_{Ba-L}.

Keywords

AIDS; mucosal; neutralizing antibody; non-human primate; pre-clinical

Introduction

As high-titered neutralizing antibodies and a broad cellular immunity have been demonstrated to be inversely correlated with HIV-1 disease progression in humans [25, 31],

it has been suggested that a prophylactic HIV-1 vaccine should elicit both humoral and cell-mediated immune responses for the containment of viral infection [13]. Although DNA vaccines prime both humoral and cellular immune responses, the level of such responses is often quite low in non-human primates and humans. Hence, efforts have been directed toward boosting immune responses by combining DNA vaccines with different vaccination modalities in the boosting phase. Several pre-clinical studies have clearly demonstrated significant control of plasma viremia against pathogenic SHIV challenge with prime-boost vaccines, which contained a DNA prime component directed primarily at eliciting CMI responses [1, 4, 7, 12, 28, 30].

Passive immunization experiments have demonstrated the protective effects of HIV-1 Env-specific neutralizing antibodies against SHIV transmission [10, 15, 17–19]. Hence, several vaccines have been designed and evaluated for immunogenicity and efficacy against SHIV and SIV challenge with Env-based immunogens [2, 14]. Generally, Env-based vaccines composed of subunit protein have been shown to be safe and effective in eliciting antibody responses in immunized hosts. However, recently completed phase III clinical trials with recombinant Env proteins failed to demonstrate protective efficacy against HIV-1 infection [26]. Previously it was demonstrated that anti-Env antibody responses were broadened significantly following immunization with a cocktail of Env proteins in vaccinia-primed macaques [6]. However this cocktail included Env antigens mainly from clade B viral isolates and did not provide sterilizing protection against SHIV challenge unless immunization included Env protein of the viral challenge strain [6]. Polyvalent Env vaccines representing Env antigens from different subtypes of HIV-1, that are currently circulating globally, have not been well evaluated. Although DNA immunization is an attractive approach to deliver polyvalent Env vaccines, administration of DNA vaccines has not been effective in eliciting high-titer antibody responses either in non-human primates or in human studies. Previous studies in small animal models have suggested that high-titer Env-specific antibody responses could be elicited with limited Env protein boosting when the host was first primed with Env-expressing DNA vaccines [3, 27, S. Wang, unpublished data]. Here we report immunogenicity of multivalent DNA vaccines with protein boost in rhesus macaques. Macaques were first immunized with DNA vaccines encoding *env* genes from multiple clades of HIV-1 of R5 phenotype followed by boosting with homologous gp120 proteins. In addition, a Gag-expressing DNA vaccine was included in the priming phase to elicit CMI responses against Gag antigen. Our results demonstrate that boosting of DNA-primed macaques with gp120 proteins elicits cross-reactive neutralizing antibody responses directed against T-cell line adapted (TCLA) strains and to a lesser extent against primary isolates of HIV-1. Mucosal challenge of these immunized animals with an R5 SHIV isolate led to complete protection in one of the immunized macaques and a significant containment of plasma viremia in the remaining immunized animals compared with naïve controls.

Materials and Methods

Immunogens

The Env and Gag immunogens used in this study are listed in Table 1. Codon optimized HIV-1 gp120 *env* genes coding from HIV-1 isolates of subtype A (92UG037.8), B

(92US715.6), and E (93TH976.17) and the p55 *gag* gene coding for subtype C isolate (96ZM651) were synthesized commercially. Codon-optimized gp120 *env* gene coding for subtype C isolate (96ZM651) was received from Dr Beatrice Hahn (University of Alabama, Birmingham, AL, USA) whereas codon-optimized gene for Ba-L gp120 (subtype B) was received from Dr Marvin Reitz (Institute of Human Virology, Baltimore, MD, USA). The *env* and *gag* gene inserts were individually subcloned in the DNA vaccine vector (pSW3891). The pSW3891 was derived from pJW4303 [16] with the modifications including deletion of the SV40 origin sequence and replacement of ampicillin resistance gene by the kanamycin resistance gene. The pSW3891 contains CMV immediate early promoter and its associated intron A sequence. The *env* gp120 inserts were fused in frame with a human tissue plasminogen activator (tPA) leader sequence. There was no leader sequence in the *gag* DNA vaccine. Both Env and Gag plasmids were prepared commercially (Puresyn Inc., Malvern, PA, USA) and were shown to contain >90% supercoil structure with low level of endotoxin (<20 EU/mg). Each plasmid was shown to be biologically active as transfection of 293T cells with these plasmids resulted in high level of transient expression of gp120 and Gag proteins (data not shown).

Recombinant HIV-1 gp120 proteins were produced in CHO cells stably transfected with gp120 expression plasmids. Each codon-optimized gp120 gene was inserted into the expression plasmids in-frame with the tPA signal peptide to facilitate efficient secretion of the gp120 proteins into the media. These plasmids contained the neomycin phosphotransferase II (*neptII*) and the mouse dihydrofolate reductase (*dhfr*) genes for selection of stable colonies following transfection. CHO/*dhfr*(-) cells were transfected with the gp120 expression plasmids using lipofection. Stable cell lines secreting gp120 were selected in the presence of G418 sulfate. Cultures were further selected in the presence of increasing levels of methotrexate to enhance gp120 production, cloned by limiting dilution and adapted for growth in protein-free media. The gp120 proteins were purified from the conditioned media using *Galanthus nivalis* lectin agarose. Proteins were formulated with QS-21 adjuvant, which was received from Antigenics Inc. (Woburn, MA, USA).

Animal studies

Indian origin rhesus macaques (*Macaca mulatta*) weighing 4–5 kg were immunized with 3 mg of pooled DNA (500 µg per plasmid) vaccines either by intramuscular (i.m.) or intradermal (i.d.) needle injection in saline and were boosted intramuscularly with 375 µg of pooled recombinant gp120 protein (75 µg per protein). Proteins were pooled and formulated with 100 µg of QS-21 before immunization. The immunized and naïve animals were challenged rectally with SHIV_{Ba-L} (2000 TCID₅₀). The SHIV_{Ba-L} challenge stock was prepared by culturing phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMC) from a SHIV_{Ba-L}-infected pigtail macaque [24]. Each animal was inoculated with 1 ml of undiluted virus stock rectally. This dose of virus consistently infected naïve animals in separate titration studies.

Binding antibody assay

Serum samples were tested for Env gp120- and Gag-specific antibodies using an enzyme-linked immunosorbent assay (ELISA) as described before [23]. Serum titers were

determined as the highest dilution of immune serum producing optical density ($A_{450\text{ nm}}$) greater than or equal to two times the optical density detected with a corresponding dilution of pre-immune serum.

Neutralization assay

SHIV_{Ba-L} isolate was isolated from an infected macaque challenged with SHIV_{Ba-L} and expanded in human PBMC [24]. DJ263 is a clade A virus; JRCSF, ADA, SF162, BAL, Bx08 and JRCSF are well-characterized clade B viruses; TV1 is a clade C virus; CM235 is a clade E virus. All of these viruses use CCR5 co-receptor for entry into the host cells. All primary viral stocks were prepared and titrated in PHA and IL-2-stimulated PBMC. Neutralization assays using cell-free virus were conducted in three different laboratories using different assay formats.

The intracellular p24-Ag (HIV-1 Gag p24 antigen) staining neutralization assay was performed by Dr John Mascola and was conducted in 96-well U-bottom culture plates and measured by flow cytometric analysis as previously described [20].

Neutralization assays were conducted by Dr David Montefiori and were described elsewhere [21]. In this assay neutralization was measured as reductions in luciferase reporter gene expression after multiple rounds of virus replication in 5.25.EGFP.Luc.M7 cells. This cell line is a genetically engineered clone of CEMx174 that expresses multiple entry receptors (CD4, CXCR4, GPR15/Bob) and was transduced to express CCR5 [5]. The cells also possess Tat-responsive reporter genes for luciferase (Luc) and green fluorescence protein (GFP). Briefly, 5000 TCID₅₀ of virus were incubated with multiple dilutions of test samples in triplicate for 1 hour at 37°C in a total volume of 150 μl in 96-well flat-bottom culture plates. A 100- μl suspension of cells (5×10^5 cells/ml of growth medium containing 25 μg diethylaminoethyl (DEAE) dextran/ml but lacking puromycin, G418 and hygromycin) was added to each well. One set of control wells received both cells and virus (virus control) while another set received cells only (background control). Plates were incubated until approximately 10% of cells in virus control wells were positive for GFP expression by fluorescence microscopy (approximately 3 days). At this time, 100 μl of cell suspension was transferred to a 96-well white solid plate (Costar, Cambridge, MA, USA) for measurements of luminescence using Bright Glo substrate solution as described by the supplier (Promega, Madison, WI, USA). Neutralization titers are the dilution at which relative luminescence units (RLU) were reduced by 50% compared with virus control wells after subtraction of background RLUs.

The cell-free infection assay conducted at Advanced BioScience Laboratories, Kensington, MD, USA, was performed using U373-MAGI-CCR5 cells, which express a reporter gene β -galactosidase under HIV-1 LTR promoter. Different dilutions of immune serum were incubated with 200 TCID₅₀ of SHIV or HIV-1 isolates for 60 minutes at 37°C. Serum-virus mixture was then added to U373 cells coated onto 96-well plates and incubated for 48 hours. Cells were thoroughly washed and fresh medium was added to each well. Expression of β -galactosidase was measured after 96 hours to monitor viral infection. Neutralization titers are defined as the dilution of immune sera blocking 50% of viral infection compared with untreated controls.

Neutralization of cell-to-cell transmission was measured by coculturing uninfected CEM cells expressing CCR5 (CEM-CCR5) with either chronically HIV-1-infected CEM-CCR5 or PM1 cells in the presence of the immune serum. Syncytia were scored after 72 hours and the dilution of serum inhibiting 50% of syncytia were defined as the syncytium blocking titer.

Enzyme-linked immunosorbent spot-forming cell assay (ELISPOT)

96-well polyvinylidene difluoride-bottomed plates (Millipore, Billerica, MA, USA) were pre-treated with 70% ethanol and air-dried for 15 minutes. Plates were washed four times with phosphate-buffered saline (PBS) and then coated with 5 µg/ml of mouse anti-human interferon (IFN)-γ capture antibody (Pharmingen, San Diego, CA, USA) in PBS and incubated overnight at 4°C. Following incubation, PBS was removed, plates were blocked with complete RPMI media (RPMI1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 µg/ml each of penicillin and streptomycin). Plates were washed five times with complete RPMI medium. Rhesus PBMC (2.0×10^5 cells/well) were then added to the wells in 100 µl of complete RPMI and were stimulated with either HIV-1 Env or Gag peptides (1 µg/ml) for 18 hours in 200 µl media. As Env and Gag peptides (15mer with 11 amino acids overlap) were dissolved in dimethyl sulfoxide (DMSO), care was taken to ensure that final DMSO concentrations did not exceed 0.5%. Following incubation for 18 hours at 37°C in a CO₂ incubator, plates were washed five times in PBS containing 0.05% Tween-20. Biotinylated anti-IFN-γ (Diapharma, Chester, OH, USA), diluted with PBS containing 2% FBS, 0.05% Tween-20 and 1% bovine serum albumin (BSA), was added to wells at a final concentration of 1 µg/ml. Plates were incubated at 37°C for 2 hours and then washed five times with PBS containing 0.5% Tween-20. Avidin-HRP (Vector Laboratories, Burlingame, CA, USA), diluted in PBS containing 2% FBS, 1% BSA and 0.05% Tween-20 was added to wells at a final dilution of 1:2000. Plates were incubated for 1 hour at 37°C. Following incubation, plates were washed five times with wash buffer and 100 µl of Stable DAB substrate (Research Genetics, Huntsville, AL, USA) was added to each well. Spots were visualized within 3–5 minutes and the reaction was terminated by washing plates thoroughly with water. Plates were dried overnight and spots were counted using the VWR Vista Vision Stereo Zoom Microscope (VWR, West Chester, PA, USA).

Virological assays to monitor challenge outcome

The viral RNA load in plasma of challenged animals was assessed using a nucleic-acid sequence-based amplification assay (NASBA) to quantitate SIV RNA [23, 29]. To confirm virus transmission in the challenged animals, PBMC collected 21 days following virus challenge were subjected to qualitative virus isolation by co-culturing with PHA-activated human PBMC [23].

Results

Immunization schedule

Figure 1 shows the immunization schedule. A total of six macaques were used in this study of which three (51M, 978L, 980L) were immunized with a mixture of six plasmids encoding the codon-optimized *env* gp120 genes and a *gag* p55 gene (Table 1) by i.m. route and another three (991L, 997L, 998L) received the same DNA mixture by i.d. route. During the

primary phase of immunization animals were vaccinated with the multivalent DNA immunogens on weeks 0, 6, 12 and 18 followed by boosting with homologous Env protein in QS-21 adjuvant on weeks 24 and 32. After detection of strong antibody and cell-mediated responses elicited from this primary phase of immunization, animals were subsequently rested for 33 weeks and then boosted with a single multivalent DNA immunization on week 65 followed by two protein boosts on weeks 76 and 93 (secondary immunization phase). Animals were challenged with SHIV_{Ba-L} via the rectal route on week 95, 2 weeks after the last protein boost.

Antibody response in macaques following the primary phase of immunization of DNA prime/protein boost vaccine

Antibody titers to all five Env gp120 proteins following each immunization of DNA and protein were assayed and the results are shown in Fig. 2. The first DNA immunization elicited a very low level of antibody response to Env proteins (data not shown). DNA delivered via the i.m. route elicited slightly higher antibody responses to at least three of five Env gp120 proteins and the antibody titers increased progressively following each DNA immunization. However, boosting of DNA-primed animals with gp120 proteins enhanced antibody titers markedly in both groups of animals to a comparable level. Titers of anti-Gag antibodies were present but at low levels in each group during both DNA and protein immunization phase (data not shown).

Sera harvested 2 weeks after the fourth DNA and the single protein boost were assayed for neutralization of SHIV_{Ba-L} and a few HIV-1 isolates homologous to the DNA vaccines. Sera after DNA immunizations did not neutralize any of the viral isolates tested (data not shown). However, sera collected after a single protein boost elicited neutralizing antibodies to the homologous isolates (Table 2). These sera were then tested for neutralization of heterologous isolates including HIV-1_{MN} and a few primary HIV-1 isolates from different clades from which the immunogens were derived. As shown in Table 3, HIV-1_{MN} and primary isolates SF162 and Bx08 were clearly neutralized by the sera of all immunized animals with animal 998L demonstrating the highest neutralizing antibody titers among all the animals. A few sera were able to neutralize clade B isolates ADA and JRCSF. In addition, clade^c isolate TV1 was neutralized by three sera whereas clade A isolate DJ263 was neutralized by sera from four animals. No sera were able to neutralize clade E isolate CM235.

Antibody response during the secondary immunization phase

Ideally an effective prophylactic vaccine should maintain high-titered antibodies in serum. Alternatively, if decay in antibody titer is observed, additional immunization with the vaccine should boost the titers markedly. Hence it was of interest to determine the decay of the anti-gp120 antibody response after primary immunization phase and whether such antibody response could be reboosted with the same polyvalent DNA and protein immunizations. After 33 weeks of rest following the second protein boost (on week 32), animals were reboosted with DNA on week 65 and with proteins on weeks 76 and 93. As shown in Fig. 3, anti-gp120 titers against Ba-L gp120 dropped slowly with time following the second protein boost on week 32, but immunization with a single DNA and two protein

boosts increased anti-gp120 titers slightly higher than that observed during the primary immunization phase. Similar results were observed when anti-gp120 titers were assessed against other gp120 proteins included in the immunogen (Table 1) (data not shown). These results clearly demonstrate that although antibodies elicited during the primary immunization phase decayed, such responses could be easily boosted back to the original level with limited immunizations with DNA and protein vaccines.

HIV-1 Gag cellular immune responses elicited following DNA immunizations

HIV-1 Gag antigen was included in the DNA vaccine phase of this multivalent DNA prime/protein boost vaccine to increase the breadth of cellular immune response elicited by the Env-based DNA and protein immunogens. In order to demonstrate that Gag-specific CMI responses were elicited following DNA immunization, PBMC from immunized macaques were isolated 2 weeks after the fourth DNA immunization (week 20) and the frequencies of Gag-specific lymphocytes were measured by IFN- γ ELISPOT assay, using HIV-1 MN Gag peptide pools (15-mer with 11 amino acid overlap, 12 peptides in each pool). As shown in Fig. 4, all three animals in the i.m. immunization group and two of three animals in i.d. immunization group demonstrated CMI responses against one or more of the 10 Gag peptide pools. The numbers of net IFN- γ spot-forming cells ranged from 12 to 300 per million PBMCs for each peptide pool.

Virological status after rectal challenge with SHIV_{Ba-L}

As the immunogens used in the vaccine originated from R5 SHIV isolates, immunized animals were challenged rectally with an R5 tropic SHIV_{Ba-L} isolate encoding HIV-1_{Ba-L} Env homologous to one of the immunogens. As shown in Table 2, this challenge virus is a neutralization-sensitive isolate. Plasma viral RNA loads in the naïve and immunized animals over time are shown in Fig. 5. As expected, all six naïve animals were infected following rectal challenge with SHIV_{Ba-L} (Fig. 5A). Only one macaque (980L) immunized with DNA by the i.m. route was protected from challenge. The second primate, 51M, had significantly lower plasma viremia detected only on days 28 and 35 compared with the rest of the infected animals (Fig. 5B). Overall, RNA load of the i.m. group was significantly lower than the naïve group ($P < 0.033$), whereas the difference in RNA loads between i.d. and naïve groups was approaching significance ($P < 0.058$). When RNA load of both i.m. and i.d. groups were combined, the reduction in viral load was significant ($P < 0.011$), and when the protected macaque (980L) was removed from the comparison, the difference was still significant ($P < 0.024$) (Fig. 5C). The infectious status of each animal was also confirmed by qualitative detection of SIV proviral DNA from PBMC collected after 120 days following challenge by PCR assay with primers specific to *gag* gene. Macaque 980L with no detectable plasma viremia was also negative for proviral DNA in PBMC (data not shown). Interestingly, macaque 51M with detectable plasma viremia on days 28 and 35 was also shown to be negative for proviral DNA in PBMC (data not shown).

Post challenge immune response

Both humoral and cellular immune responses were evaluated in immunized macaques following viral challenge and the results are shown in Fig. 6. As expected, all the immunized animals had high-titered anti-gp120 antibodies on the day of challenge which stabilized

three weeks post-infection (data not shown). Neutralizing antibodies to the challenge virus (SHIV_{Ba-L}) were detected on the day of challenge in all macaques with the highest titer observed in animal 998L (Fig. 6A). Neutralization titers remained steady up to 18 days post-challenge and increased thereafter (Fig. 6A). In contrast, neutralizing antibody to SHIV_{Ba-L} was detected in naïve animals only after 4 weeks (data not shown). Frequency of Ba-L Env-specific lymphocytes was also evaluated in the challenged animals by IFN- γ ELISPOT assay. All the immunized macaques had a significant level of Env-specific CMI response on the day of challenge (Fig. 6B). Although macaque 998L had significantly higher neutralizing antibody and CMI responses compared with other animals, such responses failed to protect this animal from infection.

Discussion

Immunogenicity studies conducted here clearly demonstrate that priming of rhesus macaques with polyvalent DNA encoding *env* genes from multiple clades of HIV-1 and boosting with gp120 proteins homologous to the DNA vaccine was well tolerated and elicited virus-specific antibody responses. Although administration of DNA by the i.m. route elicited slightly higher antibody response compared with the i.d. route, boosting of DNA-primed animals with gp120 protein markedly enhanced the antibody response to a comparable level regardless of the route of DNA administration. High-titered anti-gp120 antibodies elicited by the DNA prime/protein boost vaccine were able to neutralize a number of homologous HIV-1 isolates and to some extent a few heterologous isolates across the clades tested. However, primary clade E isolate was not neutralized by any of the immune sera tested.

The efficacy of DNA prime/protein boost vaccine was evaluated against rectal challenge with a SHIV_{Ba-L} isolate homologous to the administered vaccine. This SHIV isolate encoding HIV-1 Ba-L *env* gene, was shown to be CCR5 tropic and transmits efficiently via the mucosal route without inducing any CD4 decline in the infected macaque [24]. It is possible that this efficacy outcome was most likely determined by the immune response to the Env component and not by the cellular response to SIV Gag antigen as the macaques were immunized with DNA encoding HIV-1 *gag* gene. Only one of six immunized animals was completely protected whereas a second animal was infected but cleared proviral DNA with time. Partial efficacy of live vector prime and subunit protein boost vaccines against homologous X4 virus challenge was noted with non-pathogenic SHIV isolates challenged by the intravenous route [8, 9]. However, such vaccines failed to protect vaccinated animals from infection with more pathogenic SHIV isolates homologous to the vaccine although they reduced plasma viremia compared with the control group [9].

As the sera from immunized animals were able to neutralize the challenge virus, it was of interest to determine whether such responses are primarily responsible for the efficacy of the vaccine. We observed high-titered anti-Env antibodies with neutralizing activity to SHIV_{Ba-L} in the majority of the animals, but the neutralization titer to SHIV_{Ba-L} was not correlated to the challenge outcome as the protected animals had a low level of neutralization titer. As CMI response to Env antigen was also observed in the immunized macaques on the day of challenge, it is possible that both Env-specific CMI and neutralizing antibodies were

responsible for control of plasma viremia in these animals. Indeed recent studies have demonstrated that Env-specific CMI responses elicited by vaccines play a major role in the containment of SIV and SHIV infection in macaques [2, 14, 22].

The genetic variation of HIV-1 represents a major obstacle in the development of an effective vaccine against AIDS. It is expected that multiple viral proteins from different clades would maximize the breadth and potency of anti-HIV-1 immune response presumably due to the presentation of multiple anti-viral epitopes. Previous studies have demonstrated that immunization of mice with DNA vaccines encoding genes from multiple clades elicited immune response without any interference [11]. Further, a polyvalent Env glycoprotein-based vaccine with clade B Env of both primary and TCLA strains elicited a broader antibody response but failed to protect animals from heterologous SHIV challenge [6]. Our present study indicates that a polyvalent vaccine composed of Env from multiple clades and delivered by DNA prime and protein boost is immunogenic in rhesus macaques and elicits neutralizing antibody responses to HIV-1 across clades. This antibody response was able to control viremia against homologous challenge with an R5 SHIV isolate via the mucosal route in several immunized animals. Hence, these findings suggest that a vaccine approach using multiclade Env should be included in clinical trial. Proper selection of *env* genes representing specific immunotypes as well as modification of Env proteins may improve the anti-HIV-1 immune response elicited by the vaccines tested in this protocol and hence needs to be included for future vaccine efficacy trials.

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Fig. 1.

Immunization schedule. Arrows indicate times of vaccination. Six macaques were divided into two groups. Three animals were immunized with polyvalent DNA by the i.m. route and the other three by the i.d. route followed by protein immunization intramuscularly. Animals in both studies were challenged with SHIV_{Ba-L} by rectal route.

Fig. 2.

Anti-gp120 titers in macaques immunized with polyvalent DNA and gp120 protein during the primary immunization phase. Anti-Env titers in the sera of macaques immunized with DNA and gp120 proteins were assayed by ELISA against A, B715, Ba-L, °C and E gp120 proteins as designated in the figure. The schedule of DNA and protein immunizations during the primary phase is shown in Fig. 1. Sera were collected 2 weeks after each immunization. Antibody titers, expressed as mean titers with standard error (SE), are from vaccinated animals where DNA was administered by either i.m. (closed symbol) or i.d. route (open symbol). Titers are based on the dilution of immune serum producing two times the optical density at 450 nm compared with the corresponding dilution of serum from a naïve animal.

Fig. 3.

Anti-gp120 titers in macaques immunized with polyvalent DNA and gp120 protein during the secondary immunization phase. Anti-Ba-L gp120 titers were measured in the immunized macaques by ELISA during both primary and secondary immunization phases. After the primary immunization phase, animals were rested for 33 weeks and were subsequently reboosted with one DNA and two protein immunizations. The schedule of DNA and protein immunizations during the secondary phase is shown in Fig. 1. Sera were collected 2 weeks after each immunization. Antibody titers, expressed as mean titers with standard error (SE), are from vaccinated animals where DNA was administered by either i.m. (closed symbol) or i.d. route (open symbol) and were calculated as described in the legend of Fig. 2.

Fig. 4.

Gag peptide-specific cellular immune responses after the fourth DNA immunization. The numbers of IFN- γ spot-forming cells per million PBMC were measured by ELISPOT assays as described in the Materials and Methods section. Macaques received HIV-1 Czm Gag DNA vaccine as part of the polyvalent DNA vaccine formulation either by i.m. (animals 51M, 978L and 980L) or by i.d. injection (animals 991L, 997L and 998L). Each bar represents the number of spots against one Gag peptide pool. Gag peptide pools used in the study were from HIV-1_{MN} (15-mer with 11 amino acid overlap) with 12 peptides in each pool.

Fig. 5.

Plasma viral RNA load in macaques following SHIV_{Ba-L} challenge. Plasma viral RNA (copies/100 μ l) was determined by nucleic-acid sequence-based amplification assay (NASBA). RNA loads of individual animals from naïve (A) and immunized (B) groups are plotted at different days post-challenge. Average RNA load of both naïve and five infected macaques of vaccinated groups are plotted with SE (C). Difference in viral RNA load between naïve and infected immunized animals was significant ($P = 0.024$).

Fig. 6.

Post-challenge immune response in challenged macaques. (A) Neutralizing antibody titers in sera collected on the day of challenge and at multiple points post-challenge were determined against SHIV_{Ba-L} in U373 cells as described in the Materials and Methods section; (B) ELISPOT assays were conducted with PBMC collected on the day of challenge and at multiple time points post-challenge in the presence of Ba-L gp120 peptide pools as described in the Materials and Methods section. A single pool of peptides consisting of the entire gp120 protein was used in the assay.

Table 1

Characteristics of Env and Gag sequences used as immunogens in the vaccine formulation

HIV-1 immunogens	HIV-1 strains	HIV-1 subtypes	Countries of origin	Coreceptor usage	GeneBank accession no.
gp120-A	92UG037.8	A	Uganda	CCR5	U09127
gp120-B715	92US715.6	B	USA	CCR5	U08451
gp120-Ba-L	Ba-L	B	USA	CCR5	M68893
gp120-C	96ZM651	C	Zambia	CCR5	AF286224
gp120-E	93TH976.17	EA	Thailand	CCR5	U08458
Gag	96ZM651	C	Zambia	N/A	AF286224

All five gp120 immunogens listed were used as both DNA and recombinant gp120 protein vaccines.

Table 2

Neutralization of homologous HIV-1 and SHIV_{Ba-L} isolates by the sera from macaques immunized with polyvalent DNA prime/protein boost vaccine

Animal	Clade B SHIV _{Ba-L} ¹	Clade C HIV-1 _{Czm} ²	Clade B HIV-1 _{B715} ²
51M	82	83	>1280
978L	37	61	>1280
980L	<10	38	89
991L	>160	44	952
997L	41	46	840
998L	>160	54	>1280

Serum was collected after four DNA and one protein boost during the primary phase of immunization. Anti-Env antibody titers were at peak at this time point.

¹ Dilution of immune serum inhibiting 50% of viral infection compared with untreated control. These neutralization assays were conducted with cell-free SHIV_{Ba-L} virus using U373 as target cells as described in the Materials and Methods section.

² Dilution of immune serum inhibiting 50% of syncytium formation compared with untreated control. These neutralization assays were conducted with chronically infected CEM cell lines expressing CCR5 as described in the Materials and Methods section.

Table 3

Neutralization of heterologous HIV-1 isolates by sera from macaques immunized with polyvalent DNA/protein vaccine

Animal	Clade B									
	MN	SF162 ¹	SF162 ²	Bx08 ²	JRCSF ²	ADA ²	BAL ²	Clade C TVI ²	Clade A DJ263 ²	Clade E CM235 ²
51M	217	717	94	72	46	47	55	74	50	0
978L	248	361	79	64	33	39	6	18	40	0
980L	50	463	62	75	23	32	70	41	56	0
991L	236	407	76	49	32	59	54	0	26	0
997L	94	321	87	58	33	52	36	0	57	0
998L	2455	4632	96	91	82	63	72	67	68	0

Serum was collected after four DNA and one protein boost during the primary phase of immunization. Anti-Env antibody titers were at peak at this time point.

¹ Dilution of immune serum inhibiting 50% of viral infection compared with untreated controls.

² Percentage inhibition of viral infection measured at a fixed dilution of immune serum (1:5 or 1:15).