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Gene gun-based DNA immunization studies in mice using an HIV-1 gp120 expression vector demonstrated that modest effects on the strength and quality of gp120-specific immune responses could be elicited via the codelivery of vectors encoding murine IL-2, IL-7, or IL-12. However, much more dramatic effects on gp120-specific immune responses could be elicited by administering fewer immunizations over a longer time frame.

In the nonhuman primate model, synergistic effects on the induction of HIV or SIV gp120-specific antibody titers were observed when gene gun immunizations were boosted with either recombinant subunit or recombinant vaccinia virus vaccines. In addition, a measurable vaccine effect was observed in rhesus macaques, in that lower virus loads and higher CD4 counts were observed in gene gun-vaccinated animals relative to naive controls following a heterologous challenge with SIVB670. Protection was not correlated with the strength of gp120-specific antibody titers.

In the swine model, very strong humoral responses were observed in a three dose regimen using as little as 0.5 ug of DNA per immunization. These responses were equivalent to those elicited following administration of a commercial adjuvanted recombinant subunit vaccine.

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Oct 26, 1996

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INTRODUCTION

Nucleic acid vaccines have potential to mimic several characteristics of live attenuated viral or bacterial vaccines since they induce the de novo production of microbial antigens, leading to the presentation of correctly folded conformational determinants, and the induction of MHC class I-restricted cytotoxic T lymphocyte (CTL) responses. Because plasmid DNA-based vaccines are noninfectious and incapable of replication, they may be regarded as an attractive alternative to the use of live attenuated or live recombinant viruses that generally carry a finite risk of pathogenicity. Recent activity in the development of candidate DNA vaccines has involved two parallel tracks based on the method of delivery. While the first reported DNA or genetic vaccine involved the intracellular delivery of an antigen-encoding plasmid vector to the skin of mice using a gene gun (1), subsequent DNA vaccine reports demonstrated that intramuscular inoculation of naked plasmid DNA was effective as well (2-14). Both methods elicit humoral, cellular, and protective immune responses and represent an attractive strategy for developing a new generation of safe and effective vaccines for various infectious diseases.

While the majority of reports in the emerging area of DNA vaccination have involved intramuscular inoculation, the mechanism of DNA uptake into skeletal muscle cells is relatively inefficient and poorly understood (15). In addition, recent data suggest that muscle transfection efficiencies in higher animals, such as ferrets and nonhuman primates, are considerably reduced relative to rodents (16, 17). These observations, along with the fact that skeletal muscle is generally not considered a major immunological inductive site, suggest that alternative routes and methods of DNA vaccine delivery could result in considerably stronger responses using smaller quantities of DNA. While concern over the amount of DNA required to elicit specific responses may be largely one of economy, there is a limit to the amount of DNA that can be administered as a single DNA vaccine dose and still remain practical.

The potential of gene gun-based gene transfer methods to effectively deliver DNA vaccines was recognized several years ago since this technology achieves the direct intracellular deposition of small quantities of DNA. While this advantage alone has the potential to dramatically reduce the amount of DNA required per immunization, the ability of gene guns to target the skin provides a simple means of delivering DNA to a major immunological inductive site (18, 19).

Our research efforts during this second year of funding have continued to focus on the development of candidate gene gun-based DNA vaccines for HIV, as well as the optimization of gene gun vaccine technology in general, in both rodent and pig models. This approach is based on the observation that live attenuated vaccine approaches for HIV still appear to offer the best efficacy (SIV / rhesus monkey model) and the fact that DNA-based vaccines can mimic certain characteristics of live vaccines, such as the induction of antibodies recognizing correctly folded antigens, and the capacity to elicit cytotoxic T lymphocyte responses. Progress during this year of funding in the rodent model resulted in data demonstrating that the quality and strength of gp120-specific antibody and cytokine responses can be manipulated via alterations in the immunization regimen (number and timing of vaccine doses). It now appears possible to tailor

individual immunization regimens to specifically enhance certain types of responses, in that longer resting periods between immunizations favor the induction of IgG2a and IFN-γ, so-called Th1-like responses that may be important for protection against HIV infection and/or disease progression.

Progress in the nonhuman primate model has demonstrated that antibody responses elicited via gene gun-based DNA immunization can be dramatically enhanced via boosting with either recombinant subunit or recombinant virus-based vaccines. There appears to be a synergistic effect in the overall strength of antibody responses when more than one vaccination strategy is combined. In addition, a live SIV challenge study in gene gun-immunized rhesus macaques resulted in an approximate 100-fold reduction in virus load (relative to naive controls) following a heterologous challenge.

Finally, progress in the pig model has resulted in the optimization of gene gun vaccine delivery conditions such that the administration of three gene gun doses of a hepatitis B surface antigen-encoding vector results in geometric mean antibody titers that are equivalent to those seen in animals immunized with a commercially available recombinant subunit vaccine. The ability to elicit such potent responses in a large animal model using only 0.5 ug of DNA per vaccine dose demonstrates the potential this vaccine technology may have in future human clinical trials.

RESULTS

Murine Model - Manipulation of gp120-specific immune responses

Background: Recent activity in the characterization and manipulation of immune responses following direct DNA delivery *in vivo* in mouse models has demonstrated that direct intradermal and intramuscular inoculation of plasmid DNA results in the induction of T helper 1-like (Th1) responses characterized by IFN-γ production and predominantly IgG2a antibodies (20-25) More recently, this phenomenon was shown to be dependent upon the adjuvant properties of specific CpG-containing bacterial DNA sequences, termed immunostimulatory sequences (ISS), that result in enhanced immunogenicity, with preferential augmentation of Th1 cytokine production (25).

In contrast to intradermal and intramuscular inoculation, we recently demonstrated that direct intracellular DNA delivery to epidermal keratinocytes using a gene gun elicits antibody responses in mice that consist mainly of the IgG1 subclass, as well as cytokine responses that shift to progressively stronger T helper 2-like (Th2) profiles (IL-4 > IFN-γ) with successive immunizations (21, 26). This phenomenon may be related to the fact that immune responses elicited via epidermal gene gun delivery are not significantly affected by the adjuvant properties of bacterial plasmid DNA that are apparent following parenteral inoculation. This idea is also supported by the observations that strong responses can be elicited by gene gun delivery using very small quantities of DNA (27), the IgG1-to-IgG2a ratio is not affected by titration of the DNA inoculum from 25 pg to 40 μg (T. R. Roberts and J. R. Haynes, unpublished), and immune

responses in rodents and pigs are not diminished when plasmid backbones lacking ISS elements are substituted (manuscript in preparation).

Inasmuch as the route of DNA delivery (gene gun versus parenteral inoculation) influences the quality and types of immune responses elicited, the potential to further manipulate these responses via adjuvantation (28) or cytokine gene codelivery (22, 29-31) continues to be an intriguing possibility. Previously, it was shown that the codelivery of vectors encoding a cytokine such as GM-CSF can augment antigen-specific responses following both intramuscular or epidermal plasmid DNA delivery (22, 32). However, the potential to qualitatively modulate the types of responses elicited (i.e. Th1 vs. Th2) by cytokine vector codelivery has not been described.

In the past funding year, we demonstrated that vectors encoding interleukins -2, -7, and -12 can independently enhance antigen-specific IFN-γ production and suppress both IgG1 and IL-4 responses, when codelivered with an HIV-1 gp120 vector to the epidermis via gene gun treatment. Interestingly, more dramatic effects on immune responses were observed when, in the absence of cytokine gene codelivery, the resting period between gene gun immunizations was lengthened. These data are consistent with the potential to manipulate the types of responses elicited via gene gun-mediated DNA immunization, and suggest that the particular dosing regimen can have marked effects. Details of these findings follow.

Recent data: To determine if cytokine gene codelivery could modulate the quality of responses elicited via gene gun-mediated DNA immunization, a series of mice received either 1, 2, or 3 consecutive epidermal DNA immunizations consisting of an HIV-1 gp120 expression vector, with or without an additional vector encoding murine IL-2, -7, or -12. Successive immunizations were at 1 month intervals and animals in each group were sacrificed two weeks following their final immunization. Gp120-specific IFN-γ and IL-4 production levels were measured in an *in vitro* antigen stimulation assay in the absence of exogenous cytokine addition. Serum samples were also collected for measurement of the relative levels of gp120-specific IgG1 and IgG2a antibodies. Figure 1 shows the IgG isotype data for those animals that received either two or three gp120 DNA immunizations (boost 1 and boost 2, respectively). In the absence of cytokine vector codelivery, gp120-specific IgG1 responses rose markedly between the second and third immunizations (panels A and B, "None") while IgG2a responses remained modest or declined slightly (panels C and D, "None"). These data are consistent with earlier results showing an apparent Th1-to-Th2-like shift in gp120-specific responses with successive immunizations since the relative IgG1-to-IgG2a ratio increased by approximately 25-fold between the second and third doses (26). Interestingly, this selective enhancement of IgG1 responses following the third immunization was significantly suppressed by the codelivery of the IL-7 and IL-12 vectors (panel B). IgG1 responses in mice that received IL-2 vector codeliveries were also suppressed, but the data were of borderline significance.

Additional evidence for the modulation of gp120-specific responses via cytokine gene codelivery was obtained from the IFN-γ and IL-4 production patterns (Figure 2). Using the gp120 vector alone, mean gp120-specific IFN-γ production levels were weak in animals that received either 1, 2, and 3 immunizations (panels A, B, and C, "None"), with no IFN-γ activity

being detected in the splenocyte supernatants derived from 9 of 12 animals. However, cytokine vector codelivery resulted in dramatically enhanced IFN-γ production *in vitro*, particularly in animals that received two immunizations (panel B). Consistent with these results, IL-4 production *in vitro* was suppressed in supernatants derived from animals that received cytokine vector codeliveries (panel D). In the latter case, IL-4 activity was not detected in direct splenocyte supernatants, but was detected in the supernatants of nylon wool-purified T cells from boost 1 animals following antigen stimulation (T cells were not purified from the splenocytes of animals from the primary and boost 2 immunization groups). Interestingly, IL-4 activity was only detected in animals that received the gp120 vector alone or the gp120 + IL-2 vectors. IL-7 and IL-12 vector codelivery resulted in a complete suppression of detectable IL-4 production from purified T cells. These data, when combined with the IFN-γ and IgG isotype data demonstrate that the quality of antigen-specific responses elicited via epidermal DNA immunization can be modulated toward a Th1 pattern via cytokine vector codelivery.

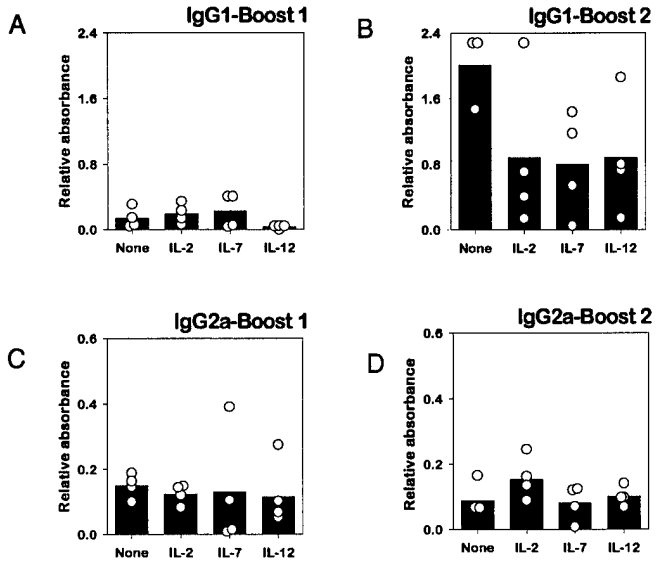

Figure 1. Measurement of HIV-1 gp120 specific IgG1 and IgG2a antibody levels in DNA-immunized mice following cytokine DNA codelivery. All mice received epidermal gene gun immunizations consisting of 1 μg of an HIV-1 gp120 expression vector either alone, or in combination with 1 μg of an IL-2, IL-7 or IL-12 vector as indicated. Four groups of four mice each were immunized twice at 0 and 4 weeks (Panels A and C), while an additional four groups of four mice each received three immunizations at weeks 0, 4 and 8 (Panels B and D). HIV-1 gp120 specific IgG1 (Panels A and B) and IgG2a (Panels C and D) levels were measured two weeks following the last immunization and expressed as relative absorbance. Solid bars indicate the arithmetic means. Open circles show data points for individual animals.

Since earlier reports are consistent with the possibility that multiple and frequent immunizations are responsible for the tendency of epidermal DNA immunization to elicit Th2-like responses, the possibility of modulating these responses by administering fewer immunizations over a longer time period was investigated. To this end, additional groups of mice in the experiments described above received only two epidermal DNA immunizations consisting of an HIV-1 gp120 expression vector, with or without an additional vector encoding

murine IL-2, -7, or -12. However, in this case, the resting period between the two immunizations was extended to three months.

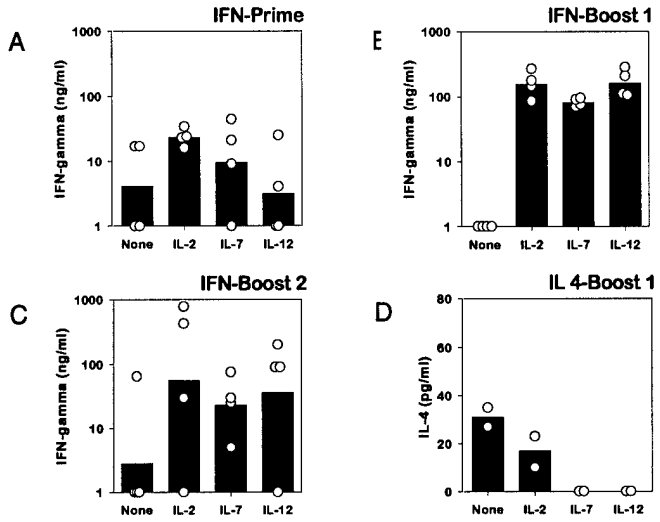

Figure 2. Measurement of antigen specific IFN-γ and IL-4 production in DNA-immunized mice following cytokine DNA codelivery. Immunizations were performed as described in Figure 1, but included an additional set of mice immunized only once. Spleen cells collected at weeks 4, 6, and 10 following one, two or three immunizations, respectively, were used to measure IFN-γ and IL-4 production following *in vitro* stimulation with recombinant HIV-1 gp120. Panel A, IFN-γ production by splenocytes derived from animals immunized once; B, IFN-γ production after two immunizations; C, IFN-γ production following three immunizations; D, IL-4 production from nylon wool-purified T cells after two immunizations. Solid bars indicate the geometric mean in panels A-C, or the arithmetic mean in panel D. Open circles represent data points for individual animals.

Figure 3a ("None") shows that, in the absence of cytokine gene codelivery, gp120-specific IgG1 responses in the two dose, long resting-period animals were reduced approximately 4-fold when compared to animals that received three immunizations over a shorter time period (compare with Figure 1b, same ELISA as Figure 3a). However, gp120-specific IgG2a levels in the two dose, long rest-period animals (Figure 3b, "None") were 4-fold stronger than those seen in animals that received the more aggressive, three dose regimen (compare with Figure 1d). While the IgG1-to-IgG2a ratios in the two dose, long rest-period animals were very similar to those seen in the short rest-period group following their second dose, total IgG levels in the former group were 4-fold stronger (data not shown). These data, along with previous results, are consistent with the idea that the number of doses, rather than time, influences the quality of the responses following epidermal gun immunization, while elongation of the resting period between doses may increase the magnitude of a given response.

Additional support for the idea that the resting period length influences response magnitude can be seen from the IFN-γ and IL-4 data shown in Figures 3c and 3d, respectively. By increasing the resting period to three months, IFN-γ production following the booster immunization increased by over 1000-fold relative to animals that received 1, 2, or 3 immunizations in the more aggressive regimen. In addition, IgG2a and IL-4 production increased as well. In the latter case, IL-4 activity was detected in direct supernatants from antigen-stimulated splenocytes at levels higher than those observed in the short rest-period

experiment using purified T cells. Thus, by simply increasing the resting period from one to three months, the magnitude of the gp120-specific IgG2a, IFN-γ, and IL-4 responses following two doses was significantly enhanced.

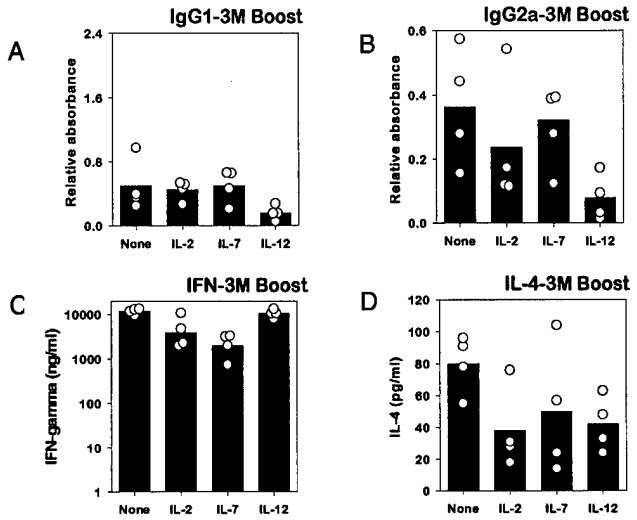

Figure 3. Measurement of HIV-1 gp120 specific antibody and cytokine responses in long rest period animals. Four groups of four mice each received two epidermal gene gun immunizations at weeks 0 and week 12 containing 1 μg of the gp120 expression vector either alone, or in combination with 1 μg of an IL-2, IL-7 or IL-12 vector as indicated. HIV-1 gp120 specific IgG1 (Panel A) and IgG2a (Panel B) levels were assayed two weeks following the second immunization. Panel C, IFN-γ production by total splenocytes following *in vitro* stimulation; panel D, IL-4 production as measured in the same supernatants. Solid bars indicate the arithmetic means, except for panel B, in which the geometric mean is represented. Open circles represent data points for individual animals.

It is interesting to note, that in the context of the longer resting period, cytokine gene codelivery provided no additional stimulus in Th1-associated responses. Rather, IgG2a, IFN-γ, and IL-4 responses were measurably reduced by cytokine gene codelivery in several groups, suggesting that cytokine manipulation of immune response quality may be more difficult under conditions where antigen-specific responses have been augmented in general. Thus, the future role that cytokine gene codelivery may play in DNA immunization strategies will require further investigation.

The dramatic enhancement in IFN-γ production in the context of the longer period is not inconsistent with our earlier reports, demonstrating a Th2 bias in antigen-specific responses elicited via gene gun-mediated DNA immunization, since IL-4 production was markedly enhanced as well. Because of the dominance of IL-4 over Th1 cytokines (33), it can still be argued that, even in the context of longer resting periods, gene gun-mediated DNA immunization leads to responses with significant Th2 character due to IL-4 and IgG1 production. Indeed, the only reports of significant antigen-specific IL-4 production following DNA immunization have been associated with the gene gun route (21, 26), since direct intramuscular or intradermal inoculation of naked DNA results specifically in Th1 responses (20-25). This observation indicates that gene gun-based DNA immunization via the epidermis may be uniquely suited for the elicitation of antigen-specific anti-inflammatory immune responses, as compared to other routes of direct *in vivo* DNA delivery.

Monkey Model

Background: The direct intracellular delivery of plasmid DNAs, encoding a variety of antigens, to the epidermis of laboratory animals using the *Accell* gene gun system results in the induction of significant humoral, cellular, and protective immune responses after one or two immunizations in mice, ferrets, pigs, and nonhuman primates using microgram quantities of DNA (34). However, gene gun-based DNA immunization studies employing vectors encoding antigens from HIV-1 and SIV have typically required several booster immunizations to achieve significant immune responses, presumably due to the low efficiency of these vectors (26, 35, 36). In the past funding year, we demonstrated that immune responses specific for SIV gp120 elicited via gene gun immunization in rhesus monkeys can be markedly improved by increasing the length of the resting period between immunizations. In addition, we demonstrated that gene-gun based DNA immunizations using HIV-1 and SIV expression vectors effectively prime for the induction of very strong antibody responses in rhesus macaques that received recombinant subunit or live recombinant vaccinia virus booster immunizations. Finally, a live heterologous SIV challenge of gene gun-immunized rhesus macaques resulted in an approximate 100-fold reduction in virus load at multiple time points following challenge, with evidence for elevated CD4 counts and some delay in disease progression. These results were achieved despite the presence of very low antibody responses.

Recent data:

Subunit boosting: In the first funding year (94-95) we reported that rhesus monkeys which received a series of gene gun-based HIV-1 DNA immunizations using vectors encoding the entire HIV-1_{LAI} gag-pol-env open reading frame or HIV-1_{LAI} gp120 developed relatively low antibody responses specific for recombinant gp120 and p24 from HIV-1_{IIIB}, but mounted significant anamnestic responses recognizing IIIB antigens following a single booster immunization with a recombinant subunit vaccine containing the gp120 and p24 products from HIV-1_{SF2} (Chiron Corp.). This study was completed in the present funding year in which antibody responses specific for the gp120 and p24 antigens from both the IIIB and SF2 strains were measured following the single recombinant subunit boost. In addition, a second recombinant subunit boost was administered, resulting in even higher titers.

In this study, five rhesus macaques were vaccinated with six consecutive DNA doses spaced four to six weeks apart, in which each dose consisted of 10 μg of vector DNA encoding HIV-1 gag-pol-env (pcHIVpal) or gp120 (pcENV-t). These animals developed only low to modest antibody responses against HIV-1 gp120 (IIIB and SF2) (Table 1). However, after a single recombinant subunit booster immunization consisting of HIV-1_{SF2} gp120 and p24, all five animals showed a greater than log-fold elevation in their antibody responses specific for HIV-1 gp120 (Table 2). A second subunit boost resulted in further amplification of these responses (Table 3). Similar antibody titers were detected against gp120 from HIV-1 strains SF2 and IIIB, demonstrating that DNA immunization with the IIIB sequences effectively primed for the induction of vigorous heterologous responses against SF2, even though the responses specific for IIIB following the DNA immunizations alone were relatively weak.

Antibody responses against HIV-1 p24 (IIIB and SF2) are also shown in Tables 1-3. Similar to the gp120 data, three out of five animals (J342, L620, and M228) previously immunized with DNA expressing HIV-1 p24 showed markedly elevated responses against p24 following a single subunit booster immunization (Table 2). In contrast, the two animals that were immunized with DNA encoding only gp120 (J538 and N340) did not develop p24-specific IgG responses after a single subunit boost. Moreover, after a second subunit boost, the p24 responses detected in these animals were 10 to 40 times lower than the responses detected in the animals that received p24 gag DNA immunizations prior to boosting (Table 3). These data demonstrate the effectiveness of epidermal DNA vaccines in priming for the induction of vigorous antibody responses following routine recombinant subunit immunization, in that considerable synergy between the two routes appears evident. Further evidence in support of synergy between DNA priming and subunit boosting comes from a parallel group of monkeys that received a series of four gp120/p24 subunit vaccinations, in the absence of DNA priming. These animals developed gp120 and p24-specific antibody titers that were approximately 10-fold lower that those observed in the DNA-primed animals that received two subunit booster immunizations (Susan Barnett, personal communication, Chiron Corp.).

TABLE 1: Reciprocal endpoint HIV-1 (IIIB) gp120 and p24 IgG titers following five 10 μg doses of either pcHIVpal or ENV/t DNA.

	Reciprocal Titer				
Monkey:	*J342*	*L620*	*M228*	*J538*	*N340*
DNA:	*pcHIVpal*	*pcHIVpal*	*pcHIVpal*	*ENV/t*	*ENV/t*
Capture antigen					
HIVgp120 (IIIB)	11,200	2,400	4,800	<25	800
HIVp24 (IIIB)	4,800	2,400	3,200	<25	<25

TABLE 2: Reciprocal endpoint HIV-1 (IIIB and SF2) gp120 and p24 IgG titers following five 10 μg doses of either pcHIVpal or pc-Env/t DNA plus one recombinant subunit booster immunization (50 μg gp120 (SF2) + 25 μg p24 adjuvanted with MF59).

	Reciprocal Titer				
Monkey:	*J342*	*L620*	*M228*	*J538*	*N340*
DNA:	*pcHIVpal*	*pcHIVpal*	*pcHIVpal*	*ENV/t*	*ENV/t*
Capture antigen					
HIVgp120 (IIIB)	170,700	136,200	102,400	1,600	10,200
HIVgp120 (SF2)	59,800	51,200	34,100	400	4,400
HIVp24 (IIIB)	76,800	23,500	12,800	<25	<25
HIVp24 (SF2)	153,600	42,700	25,600	<25	<25

TABLE 3: Reciprocal endpoint HIV-1 (IIIB and SF2) gp120 and p24 IgG titers following five 10 μg doses of either pcHIVpal or pc-Env/t DNA plus two recombinant subunit booster immunizations (50 μg gp120 (SF2) + 25 μg p24 adjuvanted with MF59).

	Reciprocal Titer				
Monkey:	*J342*	*L620*	*M228*	*J538*	*N340*
DNA:	*pcHIVpal*	*pcHIVpal*	*pcHIVpal*	*ENV/t*	*ENV/t*
Capture antigen					
HIVgp120 (IIIB)	409,600	182,000	131,700	76,800	36,600
HIVgp120 (SF2)	>409,600	250,300	204,800	51,200	19,900
HIVp24 (IIIB)	102,400	307,200	76,800	6,000	6,000
HIVp24 (SF2)	307,200	409,600	84,100	9,000	10,700

SIV gp120-specific IgG responses in rhesus macaques following gene gun vaccination and recombinant vaccinia virus boosting. In the previous funding period we reported that rhesus monkeys which received a series of consecutive gene gun-based DNA vaccine doses using a vector combination encoding SIV_{mac239} gp160 and gp120 developed modest antibody titers over time that waned considerably following the seventh gene gun DNA immunization. A large number of consecutive immunizations were administered in an effort to drive the responses to higher and higher levels, but following the seventh dose, decreasing rather than increasing titers were evident. Because of modest SIVgp120-specific responses in these animals, three were selected for boosting with a live recombinant vaccinia virus encoding SIV_{mac239} gp120 (Dennis Panicali, Therion Biologics) after a rest period of 36 weeks, to determine whether or not a combination of gene gun and recombinant virus immunizations would result in synergy. As a control, the remaining three animals received an additional gene gun DNA immunization after the same 36 week resting period. Two weeks following receipt of the vaccinia boost, SIV gp120-specific IgG titers in these animals increased dramatically to greater than 1:2,000,000, as shown in Figure 4. The 36 week resting period between the last DNA immunization and the vaccinia boost was likely insignificant in itself, since the three animals that received the additional gene gun DNA immunization after 36 weeks exhibited no further increase in titers.

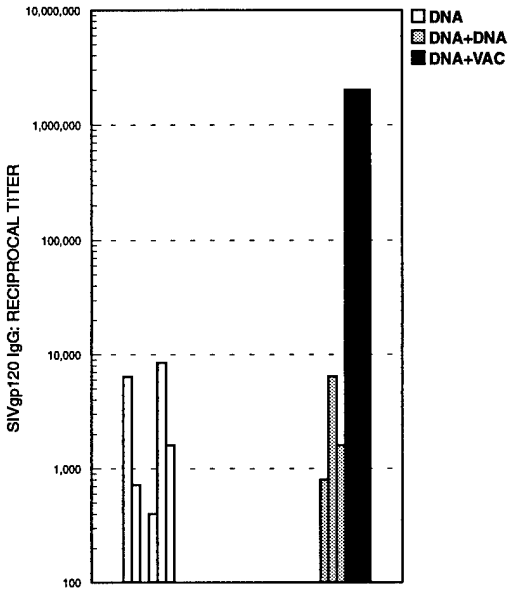

Figure 4. SIV gp120-specific antibody titers in gene gun immunized monkeys following their seventh gene gun immunization (left bars), or following an additional gene gun or recombinant vaccinia virus (gp120) immunization (right bars).

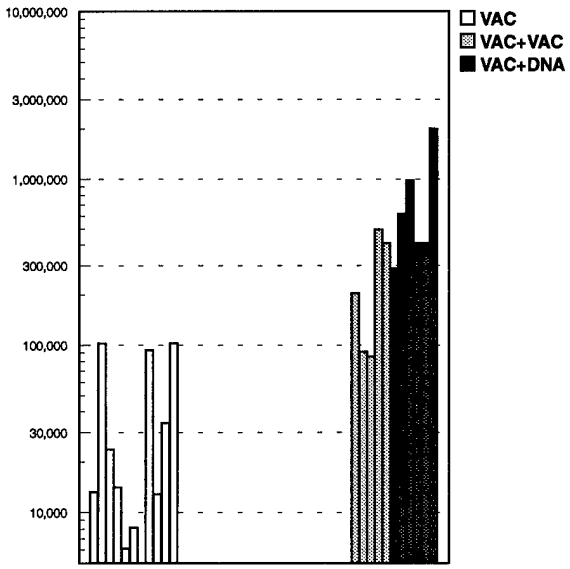

Figure 5. SIV gp120-specific antibody titers in recombinant vaccinia virus-immunized monkeys (left bars) and after boosting with either recombinant vaccinia virus or gene gun immunization (right bars).

As a control to this experiment, 15 monkeys that were previously immunized with a single dose of a recombinant vaccinia virus expressing SIV gp120 from either the mac239 or B670 strains were divided into two groups for either gene gun DNA (9 animals) or further recombinant vaccinia virus (6 animals) boosting, to determine if synergy would be seen using the reverse combination. In the vaccinia boost group, animals previously primed with either the B670 or mac239 viruses were boosted with the corresponding recombinant vaccinia. For the gene gun boost, all vaccinia primed animals received SIV_{mac239} gene gun DNA boosts. gp120-specific antibody titers in these boosted animals are shown in figure 5 in which vaccinia-primed animals

that received a recombinant vaccinia boost exhibited enhanced responses, but those animals that received the gene gun boost mounted an approximately 3-fold stronger antibody titer (note that sera from all 15 animals was not available at the time this assay was performed). These data, in addition to those described above for the HIV DNA immunizations and recombinant subunit boosting, demonstrate that a significant synergy in antibody titers can be realized by combining gene gun-based DNA immunization with either recombinant subunit or recombinant live virus vaccinations.

Live SIV challenge of rhesus monkeys vaccinated via gene gun and recombinant vaccinia virus routes. Because of the robust SIV gp120-specfic antibody titers generated in animals that received a combination of gene gun and recombinant vaccinia virus immunizations, these animals were challenged with 10 monkey infectious doses of SIV_{B670} 19 weeks after their final booster immunization. Preliminary PCR assays conducted 2 weeks post challenge indicated that all animals became infected, demonstrating that sterilizing immunity was not elicited in any group. Measurement of gp120-specific antibody responses by ELISA following challenge showed anamnestic responses developing in all vaccinated animals by 4 weeks following the challenge. These data are shown in Figure 6. Interestingly, gp120-specific titers in the gene gun only group, which were very low at the time of challenge, increased dramatically by 6 weeks. gp120 titers in all four vaccinated groups were essentially equivalent by 6 weeks post-challenge, and were 5 to 10-fold higher than those seen in the naive control group.

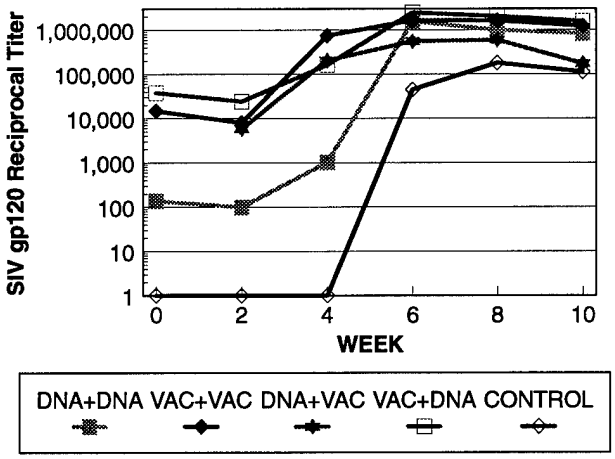

Figure 6. SIV gp120-specific IgG responses following challenge.

Evidence for a partial protective effect in the four vaccinated groups was investigated by examining virus loads in a quantitative-competitive PCR assay for SIV sequences conducted by Dr. Murphey-Corb at the Tulane Primate Center. Blood samples were collected from each experimental and control monkey at three time points following challenge and the SIV viral burden was measured. These data are shown in Figure 7 below.

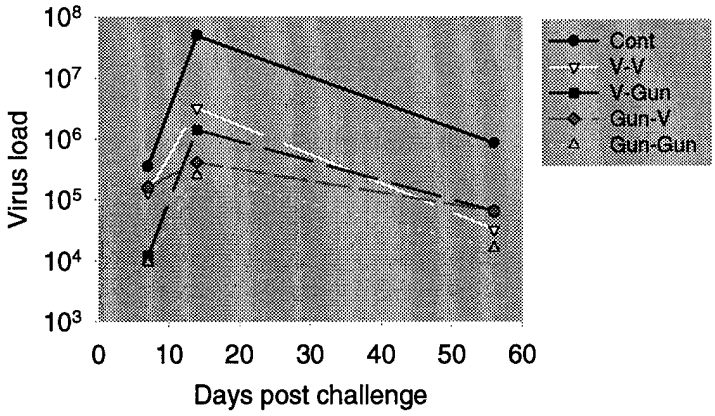

Figure 7. Virus load in vaccinated monkeys post challenge.

Interestingly, all vaccinated groups showed evidence for a measurable reduction in virus load relative to the naive control group at all three time points post-challenge, however, there was no correlation between the extent of virus load reduction and the antibody titer at the time of challenge. Moreover, the gene gun-only group, which showed the lowest viral burden at all time points, had essentially no detectable gp120-specific antibody titers at the time of challenge (Figure 6, above). Thus, the apparent protective effect observed in this case were likely independent of gp120-specific antibody responses, at least those detected by the ELISA methods used here.

Further evidence for a partial vaccine effect was observed when CD4 cell counts in each group were followed relative to their initial baseline at the time of challenge. These data are shown in Figure 8 below. Again, the group which maintained higher CD4 cell counts relative to the initial baseline was the gene gun-only vaccine group, despite that fact that this group exhibited extremely low antibody titers at the time of challenge. Taken together, these data are consistent with the induction of a tangible vaccine effect in these animals, via gene gun-based DNA immunization, that was capable of providing a measurable reduction in virus load following challenge. Moreover, these results were observed in the face of a heterologous challenge (SIV_{mac239} vaccine - SIV_{B670} challenge) with a vigorously pathogenic virus.

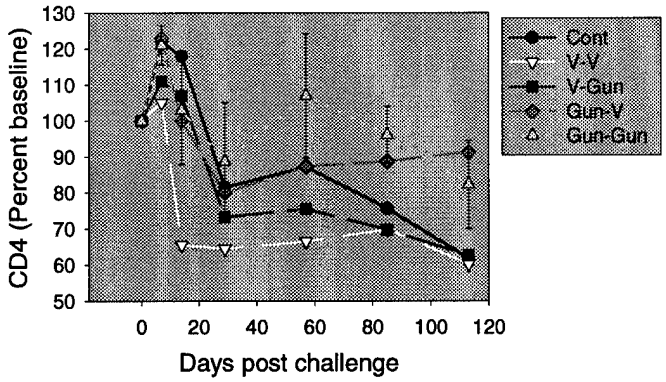

Figure 8. Relative CD4 cell counts in vaccinated monkeys post-challenge.

As of day 180 post-challenge, a number of animals in the vaccinated and control groups developed advanced disease symptoms such as diarrhea and splenomegaly and were euthanized. While there are not enough animals in these groups to show a statistical significance, the numbers of animals that developed advanced symptoms in each group is reflective of the above CD4 cell count and viral burden data in that the gene gun only group showed the greatest delay in disease progression. The number of animals in each group that developed advance disease, and the times of their sacrifice as of day 180 post-challenge are as follows:

- Naive control: 3/5 (60%) terminated at weeks 17, 33, and 34.
- Vac-Vac group: 3/6 (50%) terminated at weeks 27, 30, and 32.
- DNA-Vac group: 2/3 (66%) terminated at weeks 17 and 33.
- Vac-DNA group: 4/9 (44%) terminated at weeks 24, 27, 31, and 32.
- DNA-DNA group: 1/3 (33%) terminated at week 34.

Effect of elongated resting periods on the induction of SIV gp120-specific antibody responses via gene gun immunization in rhesus macaques. In view of the murine model data described above which indicated that augmentation of antigen-specific responses can be achieved by the administration of fewer gene gun immunizations over a longer time frame, a study to investigate this phenomenon was initiated in rhesus macaques. In this trial, 4 naive monkeys received a series of three gene gun DNA immunizations using the SIV gp120/gp160 vector combination described above, in which the resting periods between immunizations were increased to 14-16 weeks. DNA dosages in these animals were either 4 ug (2 animals) or 10 ug (2 animals) per immunization. SIV gp120-specific antibody titers in these animals over a 32 week period are shown in Figure 9 below, along with data from a previous experiment in which another group of 4 animals received a total of 6 similar immunizations (4 ug) over the same time frame. These data are consistent with those shown above in the murine model in which fewer doses over a longer time frame can significantly augment responses. The effects that such modifications in the immunization regimen may have on protection has not yet been investigated.

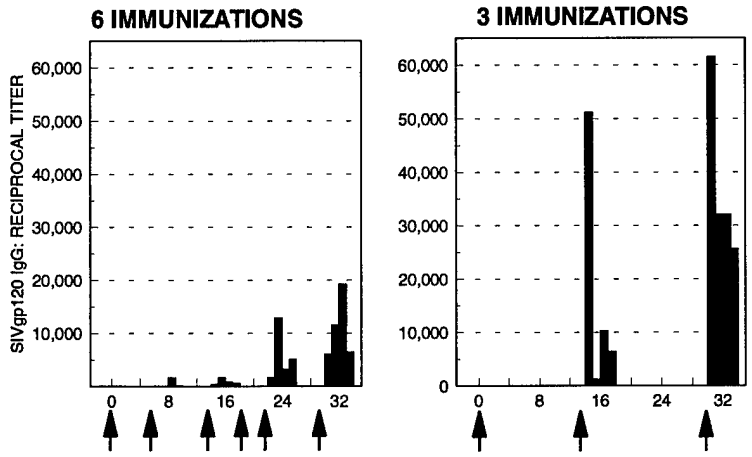

Figure 9. Effect of the length of resting period and number of immunizations on SIVgp120-specific antibody titers in gene gun-immunized rhesus macaques.

Swine Model

Background. Our previous efforts in utilizing the swine model for the development of gene gun-based DNA immunization technology for eventual use in humans centered on use of a swine influenza hemagglutinin expression vector in immunization and challenge studies. In the first funding year, we demonstrated the ability to elicit vaccine protection via gene gun immunization in swine that was equivalent to that elicited via conventional inactivated virus immunization. More recently, we elected to shift our focus in the swine model to the use of a vector encoding the hepatitis B surface antigen for further optimization of gene gun delivery technology in a large animal model. The shift to the hepatitis B vector for development of potential clinical immunization conditions was based on the presence of a surrogate marker for vaccine protection against HBV infection in humans (10 mIU/ml antibody titer) and our company's direct involvement in the development of a human gene gun-based HBV DNA vaccine.

Recent data. Figure 10 below shows the resultant antibody titers in a series of gene gun-immunized pigs in which a vector encoding the hepatitis B surface antigen, driven by the human CMVintronA promoter, was administered to groin skin in recently weaned Landrace pigs. In this study, individual animals received two gene gun immunizations, spaced 2 months apart, in which each immunization consisted of either 2, 6, or 12 gene gun shots per immunization. In this case, each shot contained 0.25 mg of gold and 0.25 ug of HBV vector DNA. Thus, the 2, 6, and 12-shot animals each received 0.5, 1.5, and 3.0 ug of vector DNA per immunization, respectively. Immune responses at week 8 (post prime), week 9 (1 wk post-boost), and week 12 (4 wk post boost) are show in Figure 10 below.

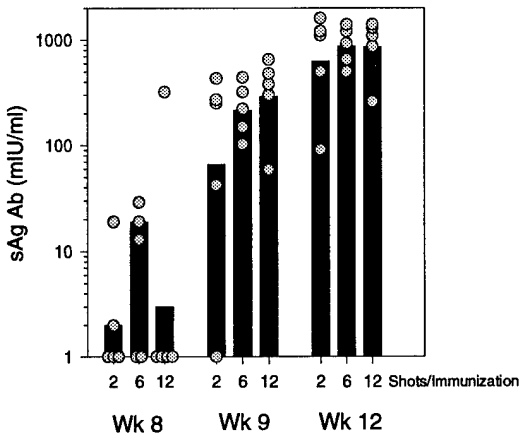

Figure 10. HBsAg-specific antibody titers in pigs using 2, 6, or 12 shots per immunization.

HBsAg-specific titers in gene gun immunized pigs, as measured using a commercial ELISA kit from Abbott, clearly show that strong antibody responses can be elicited with as few as 2 shots (0.5 ug DNA) per immunization. Antibody titers in all animals 4 weeks post-boost were markedly higher than the surrogate protective standard in humans (10 mIU/ml). In addition, there was no significant difference in geometric mean titers between the 2, 6, and 12-shot groups, indicating that 2 tandem gene gun shots per immunization may be all that is required to elicit strong humoral responses in a large animal model.

Because of the significant immunogenicity observed in this experiment, the 12-shot cohort was terminated and the 2 and 6-shot groups received a second and final booster immunization. For the final immunization, the 2-shot group received a standard 0.5 ug - 2 shot immunization at week 16, while the 6-shot group received the Engerix B recombinant subunit hepatitis B vaccine to determine if any synergy could be detected between DNA and protein immunization as described above in the monkey studies. HBsAg-specific antibody responses in these two groups are shown in Figure 11 below.

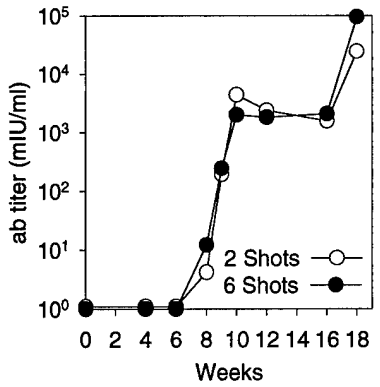

Figure 11. HBsAg-specific antibody titers in the 2 and 6 shot groups through the third and final immunization.

Pigs in the 2-shot group exhibited an additional booster response following their third immunization with a geometric mean titer of 24,000 mIU/ml, which was more than 3 orders of magnitude above the recognized protective standard in humans. In addition, animals in the 6-shot group, which received the commercial protein vaccine for their final dose, developed a geometric mean titer of 100,000 mIU/ml, consistent with the possibility of some synergy between the gene gun and protein vaccine strategies, since this level of antibody titer was higher than that observed in pigs immunized with three doses of the protein vaccine alone (see below).

To determine how the sAg-specific responses in the 2-shot gene gun pigs compared with those that could be achieved in similar animals vaccinated with the Engerix B product, an additional 4 naive pigs were vaccinated with the Engerix B vaccine using the identical regimen (three doses at 0, 8, and 16 weeks). Individual and geometric mean titers in the 2-shot and Engerix B groups are compared in Figure 12 below.

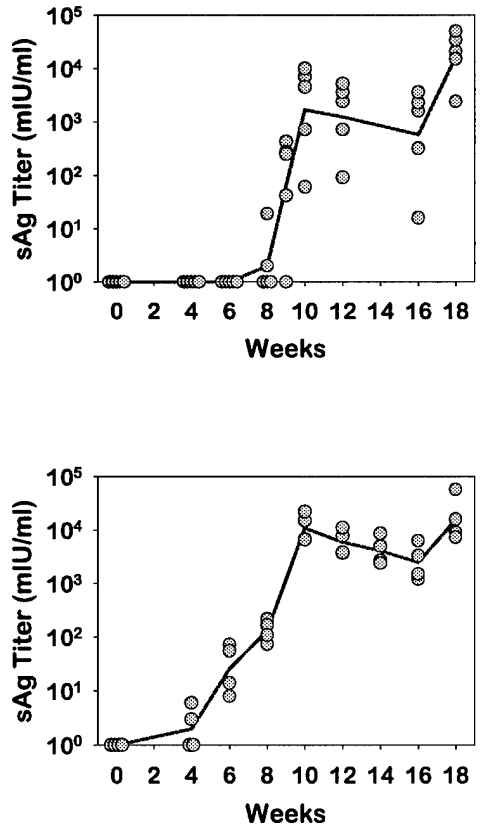

Figure 12. Comparison of geometric and individual titers in 2-shot gene gun- (top panel) and Engerix B- (bottom panel) immunized animals.

The above data demonstrate that very strong humoral responses can be elicited in large animals such as pigs using as little as 0.5 ug of DNA per immunization, in that the final titers after the full three dose regimen were identical between the 2-shot gene gun animals and the Engerix B-immunized controls. Interestingly, immune responses following the primary and first booster immunizations were higher in animals that received the commercial subunit vaccine,

suggesting that responses that develop following gene gun immunization are more dependent upon boosting.

Additional studies in the pig model have indicated that modifications in gene gun design which result in gold particle delivery over a larger surface area result in similar levels of antigen expression, but with a marked reduction in the acute, transient erythema that typically develops in pig skin following gene gun delivery. In a preliminary immunization trial examining the responses that develop after two 2-shot gene gun doses, sAg-specific titers were markedly reduced in animals that received immunizations using the modified gun design. Thus, our initial interpretation is that a mild, transient redness or erythema is an important component of an effective immunization, and that future studies should optimize delivery conditions to achieve such reactions. Additional efforts are underway to examine the feasibility of using the new gun design, coupled with increased gold dosages, to achieve DNA vaccine delivery to larger surface areas, without a reduction in the mild, acute reaction. This strategy may be successful in generating gene gun vaccination conditions in which a single shot using the modified device will be equivalent to two shots using the present device.

CONCLUSIONS

Further development of DNA vaccine strategies in the murine model demonstrated the importance of the timing and number of immunizations in influencing both the quality and strength of resulting immune responses. While cytokine gene co-delivery exhibited a measurable effect on the elicitation of gp120-specific responses following gene gun immunization, the administration of fewer gene gun doses over a longer time frame yielded more dramatic effects on the quality and intensity of such responses. Preliminary results from nonhuman primate studies are consistent with the likelihood that similar augmentative effects on immune responses can be achieved in larger animals by using less aggressive immunization regimens.

Additional studies in the nonhuman primate model demonstrated that strong synergistic antibody responses can be elicited when gene gun immunizations are combined with either recombinant subunit or recombinant vaccinia virus immunizations. However, in an SIV immunization and challenge study, there was no apparent correlation between the level of SIV gp120-specific antibody responses at the time of challenge, and virus burden at multiple time points following challenge. In fact, the animals that exhibited the lowest virus load, highest CD4 levels, and which were the slowest in progressing to advanced disease, were those animals that had essentially no detectable gp120-specific antibody titers at the time of challenge (gene gun-only group). Further attempts to optimize these apparent protective responses, through the use of longer resting periods, and heterologous boosting strategies will be the focus of the third year of funding.

Progress in the swine model using the hepatitis B surface antigen vector demonstrated that very strong antibody responses can be elicited in large animals using as little as 0.5 ug of DNA per dose. In a dose titration study, the use of greater than 2 gene gun shots per dose was not necessary to elicit maximal responses, and these responses were equivalent to those observed following use of a commercial adjuvanted subunit vaccine for HBV. Preliminary data are

consistent with the need to induce a mild, transient erythema in the gene gun target sites in order to achieve optimal responses. Future experiments will continue to focus on the development of alternative gene gun designs that will elicit maximal responses with only a single shot per immunization.

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