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Work proceeded well during the past year. Our experiment to optimize DNA vaccination in primates has been completed and almost all samples are analyzed. We found that optimal DNA vaccination conditions in Macaques are quite different from those that most workers are using and this could account for the weak and transient responses and lack of protection observed to date. We have also completed the design of the challenge experiment and have been vaccinating animals with SIVmac *env* containing plasmids. We collected samples at monthly intervals and now are in the 4th month post-vaccination. Animals will be reinjected in December and challenged with SIVmac 251 February, 1998.

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Title Page

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"Genetic Immunization for Lentiviral Immunodeficiency Virus Infection and Disease"

ARMY GRANT PROGRESS REPORT 1996 to 1997

November 1997

I. Introduction

We had two goals for the third year of this contract. The first goal was to complete our experiment to optimize DNA vaccination in Macaques using reporter antigens from influenza virus and *E. coli*. The results, our interpretations of these experiments, and their implications for the challenge assay are discussed in section II of this report. Our second goal was to design and begin the SIV challenge experiment. The experimental protocol and our progress in the challenge experiment is discussed in section III along with the schedule for completion of the extended contract.

The motivation for our optimization experiments was the poor quality of the immune responses induced by DNA vaccination in primates (1). We were especially troubled by the results published by Lu et al. (1) which showed weak and transient immunity induced only after many DNA injections. These results were exactly the opposite of studies in mice which showed that a single injection of plasmid DNA gave lifetime cellular and humoral immunity to the encoded antigen (2, 3). *One interpretation* of these experiments is that nucleic acid vaccination in primates is fundamentally different from vaccination in rodents and would require adjuvants or co-stimulation for an effective immune response. *An alternative interpretation* is that the vaccination conditions used were not optimal for primates. This idea is reasonable because we still have no idea how DNA enters muscle or dermal cells. In the absence of any mechanism, it is not possible to estimate how to scale doses and volumes from rodents to primates, a size difference of 500 fold. These considerations lead to what we optimistically call our optimization experiment in which we investigated the effect of DNA dose and injection volumes for intradermal and intramuscular DNA vaccination in Macaques.

The details and results of the optimization experiments are discussed in section 2. A brief summary of the results:

1. Relatively small amounts of DNA (40 to 200 μg) are required for optimal vaccination

2. Injection volume plays a role for the intramuscular route.

3. Two (perhaps one) injections are sufficient to produce both humoral and cellular immune responses in all injected animals. The resulting titers are similar to those seen in rodents.

4. The kinetics of immune responses are delayed compared to rodents with humoral and cellular responses observed 2 to 3 months after the initial vaccination.

In the progress report for last year we discussed a number of alternative approaches to these vaccine experiments including a possible mucosal immunization routes, the use of cytokines or co-stimulatory genes as adjuvants and the use of a

replication defective provirus for immunization. All of these proposals were designed to compensate for the poor immune responses observed by others. We no longer feel that these measures are necessary because we can induce immunity in Macaques which is similar in titer to those which produce protective immunity in mice. Thus our challenge experiment will be relatively simple relying on nucleic vaccination alone to produce sufficient systemic humoral and cellular immunity to, hopefully, provide significant protection from challenge.

II. Optimization Experiment

This is a preliminary report of the data obtained in the optimization experiment [GIM01]. Some samples remain to be assayed and others have been assayed but the data analysis has not been finished.

Introduction

The immune responses induced by nucleic acid vaccination in primates have generally been disappointing with large numbers of injections and large amounts of DNA needed to produce a small and transient immune response (1, 2). These results contrast strongly to nucleic acid vaccination in rodents where a single injection produces long lasting immunity (3, 4, 5). This encouraged us to investigate injection conditions in order to optimize vaccination in primates.

We utilized three groups of three animals in these experiments. The variables in our experiments were the route of injection (intramuscular [im] and intradermal [id]), the amount of plasmid DNA injected (for id and im), and the volume of injection (for im) [Table 1]. Three parameters were measured in each animal using the antigen genes ß-galactosidase, influenza virus nucleoprotein (NP) and hemagglutinin (HA). A summary of the protocol is shown in Table 1 and 2.

Table 1.
Summary of Injection Parameters

Exp.	Antigen	Route	Vary	Range
1	NP	im	DNA	50, 200, 800 μg
2	β-gal	im	Volume	100, 500, 2500 μl
3	HA	id	DNA	20, 80, 320 μg

Table 2.
Experimental design for inoculating 9 rhesus monkeys with DNA expressing 3 foreign genes

Group	NP DNA intramuscular (vary DNA)	β-gal DNA intramuscular (vary volume)	HA DNA intradermal (vary DNA)
A	50μg DNA 500 μl volume	200 μg DNA 100 μl volume	20 μg DNA 200 μl volume
B	200 μg DNA 500 μl volume	200 μg DNA 500 μl volume	80 μg DNA 200 μl volume
C	800 μg DNA 500 μl volume	200 μg DNA 2500 μl volume	320 μg DNA 200 μl volume

We used three animals per group. They were injected with plasmid DNA at week 0 and 7. Blood samples were obtained at week 0, 3, 7, 10 and 14 weeks after the initial immunization. Samples were assayed for CTL, antigen specific cytokine release, antigen specific proliferation and antibody.

CTL. The PBMC isolated from each bleed were restimulated for 5 days in culture with killed autologous feeder cells which were infected with recombinant vaccinia expressing the antigen (6). Effector cells were then assayed by a standard ^{51}Cr release assay.

Proliferation and cytokine release. PBMC were also restimulated in the presence of protein antigen for 7 days. At this time the culture supernatant was frozen and stored and RNA was extracted from the cells. The RNA samples were analyzed by RT-PCR for the expression of IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ and cell supernantants were analyzed by ELISA for some of these cytokines. Proliferation assays were done after restimulation for 5 days. Cells were pulsed with ^{3}H-thymidine, harvested and counted.

Antibody. The plasma obtained at each time was assayed for IgG antibody titer using purified antigen protein as the solid phase antigen in ELISA. Serial dilutions from 1/20 to 1/2560 were assayed for each sample.

A. Intramuscular Injection – Vary Amount of DNA

The first optimization experiment investigated the effect of varying the amount of plasmid DNA injected. The antigen used was influenza nucleoprotein (NP). Nine macaques were divided into three groups with each group receiving either 50, 200 or 500 μg of DNA. The injection volume was 500 μl in all cases. The CTL data on all 9 animals is summarized in Table 3, and the antibody data is summarized in Table 4.

Table 3
CTL Response After Intramuscular NP DNA Immunization
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	50	500	-	-	-	-
A	26024	50	500	-	-	-	-
A	26787	50	500	-	-	-	-
B	26728	200	500	-	-	+	+
B	26214	200	500	-	-	-	+
B	26267	200	500	-	-	+	+
C	26159	800	500	-	-	+	-
C	25456	800	500	-	-	+	+
C	21049	800	500	-	-	-	-

Table 4
IgG Antibodies After Intramuscular NP DNA Immunization
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	50	500	-	-	-	nd
A	26024	50	500	-	+ +	+ + +	nd
A	26787	50	500	-	+ +	+ +	nd
B	26728	200	500	+	+ +	+ + +	nd
B	26214	200	500	-	+	+ + +	nd
B	26267	200	500	-	-	-	nd
C	26159	800	500	-	-	-	nd
C	25456	800	500	-	-	+ +	nd
C	21049	800	500	-	-	-	nd

nd -- not done
+ indicates OD 0.5-1.0
+ + indicates OD 1.0-2.0
+ + + indicates OD >2.0

These results produced our first surprise. We detected CTL activity at the mid and high DNA doses but antibody was optimally induced at the low and mid doses. The CTL results may partly be a kinetic effect and it remains possible that we could have observed CTL at the lowest DNA dose if we could have afforded to keep the animals for longer times.

We obtained another blood sample for animal 26728 which was taken almost a year after the first injection. The animal was still strongly positive for anti-NP CTL. This indicates that nucleic acid vaccination in primates can produce the same

long-lasting immune responses we have observed in rodents.

Antibody production is clearly optimal at lower doses. Antibodies appear earliest and give the highest titers in group B. It will be interesting to analyze the 14 week time points to see if the levels in group C increase and if the levels in the other groups continue to increase. These experiments await the production of more antigen.

These results differ from those seen in mice where CTL are induced at DNA doses where no antibody is observed. Similarly, the kinetics of immunity appear to be different in rodents and primates. In mice, CTL appear within a week of vaccination whereas IgG antibodies first appear at 2 to 3 weeks. Part of the differences may be explained by postulating that the CTL assay is more sensitive in rodents but much remains unexplained at this time.

One firm conclusion of these experiments is that the optimal DNA doses required for intramuscular nucleic acid vaccination are approximately the same for rodents and primates. Higher doses may be inhibitory.

B. Intramuscular Injection – Vary Injection Volume

This experiment tested the effect of injection volume into muscle. Animals received a constant amount of DNA (200 μg) in volumes of 100 μl, 500 μl and 2500 μl. The CTL data is shown in Table 5 and the antibody data is summarized in Table 6.

Table 5
CTL Response After Intramuscular β-gal DNA Injection
Vary Injection Volume

				Time (weeks)			
Group	Animal	Amount Injected (μg)	Injection Volume (μl)	3	7	10	14
A	27877	200	100	-	-	-	-
A	26024	200	100	-	-	-	-
A	26787	200	100	-	-	-	-
B	26728	200	500	-	-	-	+
B	26214	200	500	-	-	-	-
B	26267	200	500	-	-	+	+
C	26159	200	2500	-	-	+	-
C	25456	200	2500	-	-	+	+
C	21049	200	2500	-	-	+	-

Table 6
IgG Antibodies After Intramuscular β-gal DNA Immunization
Vary Injection Volume

Group	Animal	Amount Injected (μg)	Injection Volume (μl)	Time (weeks) 3	7	10	14
A	27877	200	100	-	-	-	nd
A	26024	200	100	-	-	-	nd
A	26787	200	100	-	-	-	nd
B	26728	200	500	-	-	+ +	nd
B	26214	200	500	-	+	+ +	nd
B	26267	200	500	-	-	-	nd
C	26159	200	2500	-	-	-	nd
C	25456	200	2500	-	-	+	nd
C	21049	200	2500	-	-	-	nd

nd -- not done
+ indicates OD 0.5-1.0
+ + indicates OD 1.0-2.0
+ + + indicates OD >2.0

No immune response, either cellular or humoral, was found at the lowest injection volume (group A). Optimal antibody induction was seen in group B whereas optimal CTL occurs at the highest injection volumes. This may also be a kinetic effect as described above. Our conclusion is that for intramuscular nucleic acid vaccination, primate vaccination appears to require substantially increased injection volumes but about the same amounts of DNA as compared to rodents.

C. Intradermal Injection – Vary amount of DNA

We have also investigated the amount of DNA requires for intradermal immunization. The CTL data are summarized in Table 7. The antibody levels remain to be determined as shown in Table 8.

Table 7
CTL Response After Intradermal HA DNA Injection
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (ml)	Time (weeks) 3	7	10	14
A	27877	20	100	-	-	+	+
A	26024	20	100	-	-	+	-
A	26787	20	100	-	+	-	+
B	26728	80	100	-	-	+	+
B	26214	80	100	-	-	-	+
B	26267	80	100	-	-	+	+
C	26159	320	100	-	-	+	-
C	25456	320	100	-	-	+	+
C	21049	320	100	-	-	+	-

Table 8
IgG Antibody Response After Intradermal HA DNA Injection
Vary amount of DNA

Group	Animal	Amount Injected (μg)	Injection Volume (ml)	Time (weeks) 3	7	10	14
A	27877	20	100	nd	nd	nd	nd
A	26024	20	100	nd	nd	nd	nd
A	26787	20	100	nd	nd	nd	nd
B	26728	80	100	nd	nd	nd	nd
B	26214	80	100	nd	nd	nd	nd
B	26267	80	100	nd	nd	nd	nd
C	26159	320	100	nd	nd	nd	nd
C	25456	320	100	nd	nd	nd	nd
C	21049	320	100	nd	nd	nd	nd

nd – not done

All of the animals develop a CTL response at some time after vaccination regardless of the DNA dose. Most animals were positive by 10 weeks. For this reason, we have decided to use intradermal immunization in the challenge experiment. Remarkably, we find that CTLs are induced by as little as 10 μg of DNA and do not depend much on the DNA dose. We have previously demonstrated that intradermal injection in rodents required 5 to 10 fold lower amounts of DNA than does intramuscular injection (3). The antibody data for this experiment has not yet been analyzed.

Cytokine Secretion

We have measured antigen specific cytokine secretion for all antigens as a function of time after immunization. The cytokine levels for IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ have been measured for each time point and for each antigen by RT-PCR and some will be confirmed by ELISA. We are still in the process of analyzing these data but the following can be said.

1. We appear to see an increase in antigen specific IL-6 expression at increasing times after injection.
2. We may also see increases in IL-10, TNF-α and IFN-γ although these are more equivocal.
3. We see no induction of IL-2 or IL-4 at any times. However, we cultured the cells for longer than most experiments thus may have missed some of the earlier cytokines such as IL-2.

It is clear from these data that we see neither a pure Th_1 or a Th_2 response. We do not know at this time if this result is because of our restimulation conditions or if it is the usual response seen in primates. These data are similar to that reported in the recent papers by Letvin (7, 8) on T cell responses after nucleic acid vaccination in Macaques.

Work Remaining

The remaining data to collect is antibody titers after HA immunization. This has been delayed by lack of adequate antigen but we have just completed making a recombinant baculovirus expressing HA and we expect to complete the antibody assays in a month.

Conclusions

Several conclusions can be drawn from these data about plasmid DNA inoculation conditions and induction of immune responses in rhesus macaques. First, antibody and CTL responses can be induced with a maximum of only two DNA vaccinations. Secondly, IgG antibodies are not seen until 7 to 10 weeks after the first injection which is substantially slower than in rodents. Antibody titers continue to increased at each time point and it remains to be determined how long this increase will continue and what the final titers will be. However, it seems likely that the final titers will approach those obtained by rodent vaccination. Finally, increasing the amount of DNA injected may to suppress immune responses, especially humoral responses. Optimal amounts of plasmid appear to be in the range of 100 to 200 μg for intramuscular injection. Finally, there is no trace of the transient antibody response reported by several authors after injection of envelope genes (1, 2). Although we can not rule out that the observed differences are due to the different antigens used, we feel it is much more likely that the reported transient responses are due to non-optimal injection conditions. In support of this interpretation, we find that injection of more than 200 μg of DNA, decreases the IgG antibody levels observed at 10 weeks. Most of the published experiments have used substantially more than 1 mg of DNA for each injection in each animal. The suppression of the immune response at high DNA levels may be due to inhibition of antigen expression. Studies with reporter genes in mice have demonstrated that expression levels decrease when large

amounts of DNA are injected (G. Rhodes, unpublished).

CTL induction by intramuscular injection of plasmid DNA may require higher levels of plasmid than induction of antibodies (compare Tables 3 and 4). This is a puzzling result and is the opposite of that obtained in mice. More experiments are needed to determine the generality of this observation.

The antigen specific cytokine secretion is similar to that reported by Letvin (7,8) with secretion of IFN-γ and TNF-α but no apparent IL-2 or IL-4 production. IL-6 levels were not reported. However, our restimulation conditions involved much longer culture times than most people use and it is not clear if this effects our results. We are currently examining several restimulation conditions in our experiments with the SIV envelope vaccination.

These data demonstrate that a sustained immune response can be generated in non-human primates with one or two injections of relatively small amounts of plasmid DNA. The responses are qualitatively similar to that seen in rodents in terms of levels of immunity induced and perhaps also in the duration of the induced immunity but responses are delayed from the 2 to 3 weeks seen in rodents to 2-3 months observed in these experiments. These results have some bearing on the design of our challenge experiments in this grant. Because the immune responses take 2 to 3 months to develop, the vaccination protocols tend to be lengthy and experimental protocols will have to be long.

Overall, this experiment defined nucleic acid vaccination conditions in Macaques which give immune responses comparable to those seen in rodents. The immunization conditions that we find are quite different than those published by other groups using Macaques. These data now set the stage for our challenge experiment [GIM02].

III. Challenge Experiment

A. Experimental Design

We have constructed four vectors which express various forms of the *env* antigen. The names, sizes and physical forms of the expressed antigens are summarized in Table 9. The plasmids range in size from pND14-G1 which expresses the gp130 protein to pND14-G4 which expresses gp160, the full length envelope. Our original intention was to separately inject each group of animals with one of these plasmids. This protocol would test whether antigen dimerization is required for the production of neutralizing antibodies, whether the LLP region of gp160 inhibits an immune response, whether soluble antigen is as effective as membrane bound antigen in producing neutralizating antibodies, and whether truncation of the cytoplasmic tail of *env* produces conformational changes in the extra-cellular domain which effects the production of neutralizing antibodies. Although these experiments are important in order to determine the mechanism of immune mediated protection, we feel that a first challenge should be designed to maximize the chance of protection and to yield data which give some indication of which components of immunity contribute to the protection.

Table 9
Expression Vectors and Antigen

Plasmid	Protein	Physical Form	Comments
pND14-G1	gp130	Secreted monomer	
pND14-G2	gp140	Secreted dimer (multimer)	
pND14-G3	gp160t	Membrane bound multimer	LLP1 deleted
pND14-G4	gp160	Membrane bound multimer	

The actual design of the challenge experiment is shown in Table 10. We have 5 experimental groups A to F. The first four groups have 4 animals and are vaccinated in the manner shown. The last group has two animals and will serve as the unvaccinated control.

Group A animals are vaccinated with both soluble forms of the antigen. Group B animals are vaccinated identically but will be boosted with gp130 protein a month before challenge. Protein boosting after nucleic acid vaccination has been shown to increase antibody titers by several hundred fold (9) but is not expected to effect the cytotoxic T cell response. Thus, comparison of Groups A and B will directly test the effect of increasing the antibody titer at constant CTL level.

Group C will be immunized with the membrane bound forms of the antigen. Comparison of groups A and C with thus test for the effects of antigen conformation, LLP and antigen localization on the production of both neutralizing antibodies and protection as discussed above.

Group D animals will be vaccinated with the plasmids which express membrane bound antigen. Later, they will be injected with DNA from a defective provirus which deletes the *int* and *vif* regions. Multiple DNA injections do not increase either antibody titers or cellular immunity under optimal conditions of nucleic acid vaccination (G. Rhodes, unpublished). Thus, this vaccination protocol should produce cellular immune responses to the *gag*, *tat*, *rev*, *nef*, and the N-terminal portion of *pol* gene products but should not effect either the cellular or humoral responses to envelope generated by vaccination with the plasmids pND14-G3 and pND14-G4. Therefore, any differences in protection between groups C and D can be attributed to the broader immune response which recognized multiple antigens. The experimental questions to be tested in the challenge experiments are summarized in Table 11.

We plan to use an oral challenge for the animals. We chose this route because 100% infectivity can be obtained (10). If warranted, animals can be rechallenged with an intravenous dose at a later time.

Table 10
Challenge Groups

Group	First DNA	Second DNA	Protein Boost	Comments
A	G1 + G2	G1 + G2	-	gp130 & gp140 as antigen
B	G1 + G2	G1 + G2	+	Antibody titers boosted 500 fold
C	G3 + G4	G3 + G4	-	gp160 and gp160t as antigen
D	G3 + G4	G3 + G4 Delta *int*	-	Immunity against *env, gag, nef*
E	none	none	-	Unvaccinated Control

The size of the groups are 4 animals each for A to D and 2 control animals in group E.

Table 11
Experimental Questions

Compare Groups	Test
A & B	Vary antibody titer at constant CTL
A & C	Effect of antigen structure and form
C & D	CTL to multiple antigen at constant antibody

C. Schedule

Our experimental schedule is shown in Table 12. The experiment started in August with the initial DNA vaccination. Animals were injected intradermally with the plasmids shown in Table 10. We chose intradermal vaccination because all injected animals responded in the optimization experiments. The two plasmids were injected at separate intradermal sites in order to avoid any possible interaction of the antigen forms and also to avoid any toxic effects produced by any individual antigen. We plan to give the second intradermal plasmid immunization next month. Animals in group D will also be vaccinated intramuscularly at the same time with the proviral DNA. Group D will receive a second proviral DNA injection in February. Group B animals will be boosted with protein antigen at the same time. Challenge will occur in March, 1998 and virological studies will are planned monthly for three months.

Table 12
Injection Schedule

Month	Date	Blood Sample	Injections
0	8/21/97	+	*env* DNA id
1	9/21/97	+	
2	10/21/97	+	
3	11/21/97	+	
4	12/21/97	+	1. *env* DNA id 2. provirus DNA im
5	1/21/98	+	
6	2/21/98	+	1. Protein Boost im 2. Provirus DNA im
7	3/21/98	+	Challenge
8	4/21/98	+	
9	5/21/98	+	
10	6/21/98	+	

Abbreviations: id, intradermal; im, intramuscular

C. Assays

We are obtaining blood samples at monthly intervals. Pre-challenge samples are being assayed for antibody, neutralizing antibody and antibody avidity. Cellular immunoassays include antigen specific proliferation and cytokine secretion and cytotoxic T cell assays. All of these assays are currently in progress by the different

labs involved in this experiment. In addition, talks are underway with Dr. D. Montefiori of Duke University to measure neutralizing antibodies in some of our serum samples. This should allow us to compare the titers we see with those of the many other labs whose samples he has assayed.

Post-challenge virological assays will include virus isolation by co-cultivation, analysis of viral DNA by PCR and by bDNA, and measurement of CD4/CD8 ratio at monthly intervals after challenge. Additionally, we will monitor antibody titers after challenge to determine if an increase occurs which may indicate infection.

IV. Conclusion and Schedule

The challenge experiment was begun in August 1997 and will be concluded in June 1998. Data analysis will require another 2 months so the entire contract will be completed within the 1 year no cost extension which was granted last year.

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