

UNCLASSIFIED

AD NUMBER
ADB252231
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Info.; Dec 97. Other requests shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott St, Fort Detrick, MD 21702-5012.
AUTHORITY
USAMRMC ltr, 1 Jun 2001.

THIS PAGE IS UNCLASSIFIED

AD _____

Award Number: DAMD17-94-J-4426

TITLE: A Novel DNA-Based Vaccine Methodology for AIDS

PRINCIPAL INVESTIGATOR: Dexiang Chen, Ph.D.

CONTRACTING ORGANIZATION: Powderject Vaccines, Incorporated
Madison, Wisconsin 53711

REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Distribution authorized to U.S. Government agencies only (proprietary information, Oct 99). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

2000 0324060

NOTICE

USING GOVERNMENT DRAWINGS, SPECIFICATIONS, OR OTHER DATA INCLUDED IN THIS DOCUMENT FOR ANY PURPOSE OTHER THAN GOVERNMENT PROCUREMENT DOES NOT IN ANY WAY OBLIGATE THE U.S. GOVERNMENT. THE FACT THAT THE GOVERNMENT FORMULATED OR SUPPLIED THE DRAWINGS, SPECIFICATIONS, OR OTHER DATA DOES NOT LICENSE THE HOLDER OR ANY OTHER PERSON OR CORPORATION; OR CONVEY ANY RIGHTS OR PERMISSION TO MANUFACTURE, USE, OR SELL ANY PATENTED INVENTION THAT MAY RELATE TO THEM.

LIMITED RIGHTS LEGEND

Award Number: DAMD17-94-J-4426

Organization: Powderject Vaccines, Incorporated

Location of Limited Rights Data (Pages):

Those portions of the technical data contained in this report marked as limited rights data shall not, without the written permission of the above contractor, be (a) released or disclosed outside the government, (b) used by the Government for manufacture or, in the case of computer software documentation, for preparing the same or similar computer software, or (c) used by a party other than the Government, except that the Government may release or disclose technical data to persons outside the Government, or permit the use of technical data by such persons, if (i) such release, disclosure, or use is necessary for emergency repair or overhaul or (ii) is a release or disclosure of technical data (other than detailed manufacturing or process data) to, or use of such data by, a foreign government that is in the interest of the Government and is required for evaluational or informational purposes, provided in either case that such release, disclosure or use is made subject to a prohibition that the person to whom the data is released or disclosed may not further use, release or disclose such data, and the contractor or subcontractor or subcontractor asserting the restriction is notified of such release, disclosure or use. This legend, together with the indications of the portions of this data which are subject to such limitations, shall be included on any reproduction hereof which includes any part of the portions subject to such limitations.

THIS TECHNICAL REPORT HAS BEEN REVIEWED AND IS APPROVED FOR PUBLICATION.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)**2. REPORT DATE**

October 1999

3. REPORT TYPE AND DATES COVERED

Annual (30 Sep 98 - 29 Sep 99)

4. TITLE AND SUBTITLE

A Novel DNA-Based Vaccine Methodology for AIDS

5. FUNDING NUMBERS

DAMD17-94-J-4426

6. AUTHOR(S)

Dexiang Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)Powderject Vaccines, Incorporated
Madison, Wisconsin 53711**8. PERFORMING ORGANIZATION
REPORT NUMBER****E-MAIL:**

dexiang_chen@powderject.com

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Distribution authorized to U.S. Government agencies only (proprietary information, Dec 97). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

A novel DNA-based vaccine strategy, employing a particle delivery device to administer gold beads directly into the cells of the epidermis was tested for the ability to induce humoral and cellular immune responses to the AIDS virus and to generate preclinical data for future human clinical trials. SIV DNA vaccine induction of CTL correlated with significant virus load reduction of up to 10,000-fold when compared to control monkeys following challenge with a pathogenic, heterologous SIV. DNA administration to either the skin or mucosal tissue induced low levels of mucosal antigen-specific CTL and antibody responses. Following intrarectal challenge with SIV, virus loads were below the limit of detection in 4 of 7 DNA vaccinated monkeys and in 1 of 7 control animals. Studies to evaluate a CTL epitope-based DNA vaccine demonstrated the induction of high frequency CD8+ T cell responses in rhesus macaques. Evaluation of strategies to augment or modulate immune responses induced by particle-mediated DNA immunization demonstrated that co-administration of DNA with adjuvants resulted in substantial augmentation of both antibody and CTL.

14. SUBJECT TERMS

HIV, AIDS

15. NUMBER OF PAGES**16. PRICE CODE****17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

DC Where copyrighted material is quoted, permission has been obtained to use such material.

DC Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

DC Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

X For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

X In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

X In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Dexiang Chen
PI - Signature

1/29/00
Date

TABLE OF CONTENTS

Front Cover	..
SF298 Report Documentation Page	2
Foreword	3
Table of Contents	4
Introduction	5
Results	6
Specific Aim 1	6
Specific Aim 2	7
Specific Aim 3	7
Specific Aim 4	15
Key Research Accomplishments	21
Reportable Outcomes	22
Conclusions	25
References	27

INTRODUCTION

A safe and effective vaccine remains the best hope for controlling the human immunodeficiency virus type 1 (HIV-1) pandemic. The rational design of HIV-1 vaccines would be facilitated by knowledge of the immune correlates of protective immunity. Evidence supports a role for both HIV-1-specific antibody and T-cell responses, including cytotoxic T lymphocytes (CTL) in preventing or limiting HIV infection and progression to AIDS (1-5). DNA immunization is a relatively new approach that has proven to be a simple and effective means to generate humoral, cell-mediated, and protective immune responses against a variety of pathogens (6). Direct *in vivo* transfer of plasmid DNA results in expression of foreign antigens within the host's cells. In this aspect, DNA immunization mimics the expression and presentation of antigens after viral infection, providing the advantages of live attenuated or live recombinant viral vaccines without the safety concerns associated with infectious agents.

The purpose of our research was to test a novel DNA-based vaccine strategy for the ability to induce humoral and cellular immune responses against the AIDS virus in animal models, and to generate preclinical data necessary for future human clinical trials. Our strategy employed a particle delivery device ("gene gun") to deliver DNA directly into the cells of the epidermis. This needle-free strategy provides potentially valuable characteristics for military applications, including the potential for large-scale, low cost vaccination and an increased safety profile by avoiding risks associated with blood contamination from needles. To test the feasibility of this strategy for human vaccination against AIDS, our research efforts under this grant focused on 4 specific aims: Optimization of particle-mediated DNA delivery parameters to the skin (specific aim 1); induction of protective immunity against influenza in pigs as a model for vaccination of humans (specific aim 2); evaluation of candidate DNA vaccines for AIDS in the Rhesus monkey SIV model (specific aim 3); and identification of novel strategies to manipulate or augment immune responses induced by DNA immunization (specific aim 4). In this final report, we summarize data generated during the first four years of the funding period from September 30, 1994 to September 30, 1998 and document the details of our most recent findings obtained during the last year (October 1, 1998 – December 30, 1999).

RESULTS

Central to the evaluation of this new vaccine strategy was the use of animal models. Our work focused on using mice, monkeys, and pigs to achieve the specific aims of our proposal.

Specific Aim 1: Optimization of particle-mediate delivery parameters to the skin

To optimize particle-mediated DNA delivery parameters to the skin, we have employed the pig animals model as the most relevant and predictive model to humans. Swine epidermis is morphologically similar to human epidermis (24). In addition swine are similar in size to humans and represent, like humans, an outbred population. Therefore, the pig was chosen as the most relevant animal model for optimizing the gene gun technology for human DNA vaccination. DNA vaccine delivery conditions that function well in swine epidermis may prove to be acceptable for clinical investigations of this technology.

Induction of maximum immune responses using the minimum number of treatment sites in pigs

Particle-mediated DNA immunization results in effective induction of immune responses in mice following delivery of less than 16 nanograms of DNA into a single target site (9, 25). During the first two funding periods, our efforts focused on inducing maximum immune responses in pigs using a one- or two-site treatment to demonstrate practicality for use of the device in the clinical setting. Using a DNA expression vector encoding hepatitis B surface antigen, we demonstrated that very strong antibody responses could be elicited in pigs using as little as 0.5 ug of DNA per dose. In a dose titration study, a two-site treatment was equally effective in the induction of maximal responses as six- or twelve-site treatments (Annual Progress Report, 1994-95).

Role of local inflammation in the induction of immune responses by particle-mediated DNA immunization

Particle-mediated DNA delivery to the skin results in a mild, localized inflammatory response characterized by transient erythema at the target site. Preliminary data in pigs during the first funding year indicated a need to induce erythema (redness) at the target site to induce optimal immune responses. The relationship between the localized inflammatory response that accompanies particle-mediated delivery to the skin and the subsequent immune response was investigated in detail during the second and third funding years in both the pig and monkey models. Studies in the pig demonstrated that optimum immune responses were induced in pigs when higher doses of gold were used, corresponding to an increase in the inflammatory response but decreased antigen production (Annual Progress Report 1996-97). In monkeys, we deliberately manipulated parameters of DNA/gold delivery to induce a broad range of erythema. The results in the monkey model confirm that in the pig and demonstrate that increasing the local inflammatory responses at the skin target site has a beneficial impact on the induction of immune responses following particle-mediated DNA delivery (Annual Progress Report 1997-98). We propose that the transient erythema contributes to recruitment of antigen presenting cells to the affected site, resulting in enhanced antigen processing and presentation.

Specific Aim 2: Induction of protective immunity against influenza in pigs as a model for vaccination of humans

Induction of protective immunity to influenza A by particle-mediated DNA immunization

Influenza A was chosen as a model to further develop particle-mediated DNA immunization because infection with influenza A is similar in swine and humans such that vaccination procedures that provide protection in swine against influenza infection are likely to be applicable to humans as well. The similarity of swine in both skin morphology and size also supports the use of the swine flu challenge as a model for vaccine protection in humans. Research in this mode during the first funding year demonstrated that administration of DNA encoding the hemagglutinin (HA) of an H1N1 influenza virus to the skin or tongue of pigs induced protective responses in pigs following only two administrations of microgram quantities of DNA. Significantly, the protection levels seen in pigs for influenza were similar to those elicited using a commercially available inactivated swine flu vaccine (26).

Induction of protective levels of antibody to hepatitis B surface antigen (HBsAg) in pigs by particle-mediated DNA immunization.

The presence of a surrogate marker for vaccine protection against HBV infection in humans (10mIU/ml) led to experiments to evaluate the potential for particle-mediated DNA immunization to induce protective immunity in pigs to another disease model. Strong antibody responses exceeding 10,000 mIU/ml were induced in pigs following only two immunizations with as little as 500 ng of DNA. These responses were comparable to levels of antibody induced in a parallel set of pigs immunized with a commercial hepatitis B protein vaccine (Annual Progress Reports 1995-96 and 1996-97, 27).

The induction of protective immune responses in the swine model is consistent with the feasibility of using particle-mediated DNA vaccine technology to elicit vigorous and protective responses in animals that are similar to humans in both size and skin morphology.

Specific Aim 3: Evaluation of candidate DNA vaccines for AIDS in the Rhesus monkey SIV model

Induction of HIV-1-specific immune responses in rhesus macaques by particle-mediated DNA immunization and challenge with SHIV

Our efforts have focused on using the rhesus macaque model to evaluate candidate DNA vaccines for AIDS because it allows investigation of vaccine protection against both infection and disease. During the first funding year, we investigated the feasibility of eliciting HIV-1-specific immune responses in rhesus macaques by particle-mediated DNA immunization. HIV antibody responses induced by the DNA vaccines encoding HIV gp120 or *gag-pol-env* were generally weak and required several immunizations to appear (Annual Progress Report 1994-95). However, the DNA immunizations were highly effective in priming for vigorous antibody responses when followed by boosting with recombinant subunit protein vaccines (Annual Progress Report 1995-96, 14, 20). The DNA prime-protein boost regimen did not protect the monkeys from challenge with pathogenic, heterologous SHIV_{SF33} (Annual Progress Report 1996-

97). However, other groups subsequently demonstrated that a similar regimen could protect from infection when monkeys were challenged with nonpathogenic, homologous SHIV_{HXB2}. ().

Induction of SIV-specific immune responses in rhesus macaques by particle-mediated DNA immunization

During the first funding year, we also initiated studies to investigate the potential for particle-mediated DNA immunization to elicit SIV-specific immune responses in rhesus monkeys. The results in this study were similar to the HIV study in that SIV-specific antibody responses were weak and required a number of immunizations to develop. To improve the induction of antibody responses by SIV DNA immunization, we tested a new set of expression vectors in a second study. These vectors encoded SIV gp120 or gp160, the CMV promoter, and associated intron A sequences that were previously shown to augment expression (21). The SIV expression vectors used in the first study did not contain intervening sequences. Monkeys in the second study were immunized with a co-delivery of the two *env* vectors. SIV *env*-specific IgG antibodies were induced more quickly and with fewer immunizations than was observed in the previous study. Significantly, antibody responses induced in these animals were capable of neutralizing homologous SIV after 3 to 4 immunizations. In addition, neutralizing activities against a heterologous SIV were also detected after the 5th or 6th DNA immunization, demonstrating a broadening of the immune response with additional doses (Annual Progress Report 1994-95, 20). These results demonstrate the importance of using an appropriate vector design for inducing immune responses by DNA immunization and demonstrate that DNA immunization can induce neutralizing antibody responses to the AIDS virus in monkeys.

Partial Protection to heterologous, pathogenic SIV challenge in rhesus macaques immunized with SIV DNA

In the second funding year, we demonstrated that weak SIV-specific antibody responses induced in monkeys by DNA immunization could be substantially augmented by combining DNA vaccines with live recombinant vaccinia vaccines in a prime-boost regimen. In contrast, numerous booster immunizations (>6) with DNA resulted in suppression of antibody responses (Annual Progress Report 1995-96, 14, 15). A pathogenic, heterologous SIV challenge of monkeys immunized with multiple consecutive administrations of DNA alone or a combination of DNA and recombinant vaccinia vaccines resulted in significant suppression of viral burden (100-500-fold). Interestingly, the most significant virus load reduction and the only evidence for attenuation of CD4⁺ T cell decline and progression to disease was observed in the animals that were immunized with multiple administrations of DNA alone and had the lowest prechallenge antibody responses (Annual Progress Report 1995-96, 15). In the third funding year, we evaluated the possible mechanisms for this partial protection. Studies with live attenuated SIV vaccines have demonstrated that the induction of protective responses correlates with a maturation of the humoral immune responses (2). Analysis of antibody avidity and conformation, however, indicated that at the time of challenge, the humoral immune responses in the DNA-vaccinated were not "mature" (15, Annual Progress Report 1996-97). In addition, neutralizing antibody responses at the time of challenge had declined to low or undetectable levels, consistent with the dramatic decline in ELISA titers. These results indicate that the antibody responses in these animals at the time of challenge may not have been protective.

DNA vaccine induction of antibody responses does not protect rhesus macaques against a heterologous challenge with SIV

Encouraged by the partial protection in these monkeys despite low pre-challenge antibody titers, we sought to determine if protection might be improved if the animals were challenged before the antibody titers declined. In addition, we previously observed that increasing the interval between consecutive DNA immunizations enhanced the antibody responses. In this trial, 4 monkeys were immunized 3 times, spaced 12-16 weeks apart and following the 3rd immunization developed antibody titers approximately 10-fold higher than peak titers that were measured in the monkeys described above following 6 doses of DNA. The vaccinated monkeys were challenged along with 4 control monkeys 4 weeks following the final immunization. However, there was no significant difference in virus loads between the vaccinated and control animals (Annual Progress Report 1996-97). Taken together, these results suggested that antibody responses did not play a significant role in the SIV virus load reduction observed following multiple DNA immunizations in rhesus macaques.

Kinetics of antibody and CTL induction in rhesus macaques following multiple SIV DNA immunizations

Since DNA immunization induces CTL responses, we speculated that CTL were, at least in part, responsible for the partial protection in our first SIV challenge study. We therefore evaluated the possibility the number of DNA doses influenced the induction of CTL in rhesus macaques. Blood samples were collected for analysis of SIV-specific serum antibody and CTL. Following the 3rd or 4th immunization, the monkeys developed antigen-specific IgG titers of up to 1:100,000, but CTL responses were undetectable. However, following the 5th or 6th dose a dramatic decline in antibody responses occurred in all animals, coincident with the first emergence of CTL. Additional immunizations boosted CTL but antibody responses remained suppressed, demonstrating a reciprocal induction of antibody versus CTL (Annual Progress Report 1996-97). We previously observed similar kinetics of CTL and antibody induction to HIVgp120 following DNA immunization of mice. The reciprocity between HIVgp120-specific CTL and antibody responses in mice is reverse from what we observed in monkeys. That is, CTL responses appear after only one or two immunizations but then decline with the emergence of antibody responses after 3 or 4 DNA immunizations (7). In mice, Th1 responses induced early in the immunization regimen were later replaced by Th2 responses following additional DNA doses, suggesting that the reciprocity may be mediated by cytokine cross-regulation. To determine if a similar mechanism occurred in monkeys, we examined the Th cytokine production profile in monkeys that were immunized with 7 doses of DNA expressing SIV gag-pol-env and demonstrated increased CTL responses and declining antibody responses between the 5th and 7th DNA dose (Annual Progress Report 1997-98). Fresh PBMC were stimulated *in vitro* for 72 hours with recombinant SIVp27 and the supernatants were assayed for the production of IL-4 or IFN- γ using commercial human cytokine detection kits known to cross-react with rhesus cytokines. The results in Figure 1 show that there are high levels of IFN- γ production, but no detectable IL-4 production at the time of CTL induction and antibody decline in these animals. These results suggest that the reciprocity between CTL and antibody responses in monkeys may be due to CD4⁺ T cell-mediated cytokine cross-regulation. Moreover, these results demonstrate

that the type of immune response induced in rhesus monkeys by particle-based DNA immunization can be modulated by the number of doses delivered.

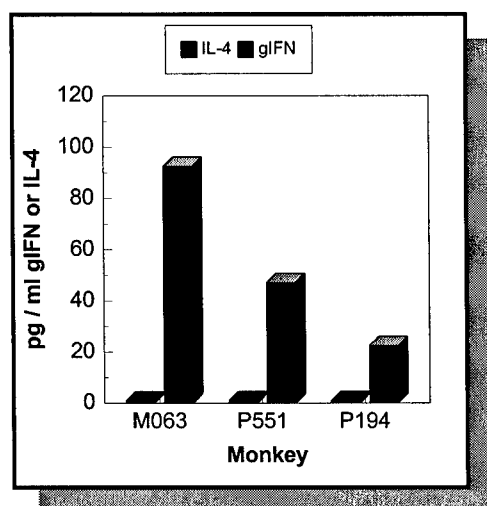


Figure 1: Th cell cytokine production profiles in DNA-immunized rhesus macaques. 1×10^6 PBMC were stimulated *in vitro* with recombinant SIVp27 for 72 hours. Supernatants were collected and assayed for the presence of IL-4 or IFN- γ using commercial human cytokine detection kits previously shown to cross-react with cytokines from nonhuman primates. (Endogen).

Improved protection against SIV infection by increasing the number of DNA immunizations and interval between doses

The reciprocity between CTL and antibody responses observed following multiple DNA immunizations provides a possible explanation for the different protective effects observed in our two SIV challenge studies. The suppression of virus burden following 7 doses of DNA compared to the lack of protection following 3 doses of DNA is likely related to the emergence of CTL following 5 or more DNA doses. The appearance of CTL and protective effects following multiple DNA immunizations suggests that increasing the number of DNA doses to further drive the immune response toward a CTL response may improve DNA vaccine protection against SIV infection. To test this hypothesis, 6 monkeys received 9 consecutive immunizations with DNA encoding SIV_{mac251FR} *gag-pol-env*. A longer resting period of 8-12 weeks was employed because previous studies have shown that increasing the intervals between immunizations enhances both antibody and CTL responses (8, 14). SIV-specific CTL responses were tested after 1, 3, 5, 7, and 9 immunizations. Consistent with previous findings, CTL were not detected until after the 5th or 7th DNA dose (data not shown). Following the 9th DNA dose, significant CTL responses against one or more SIV antigens were detected in 4 of the 5 animals tested (Figure 2).

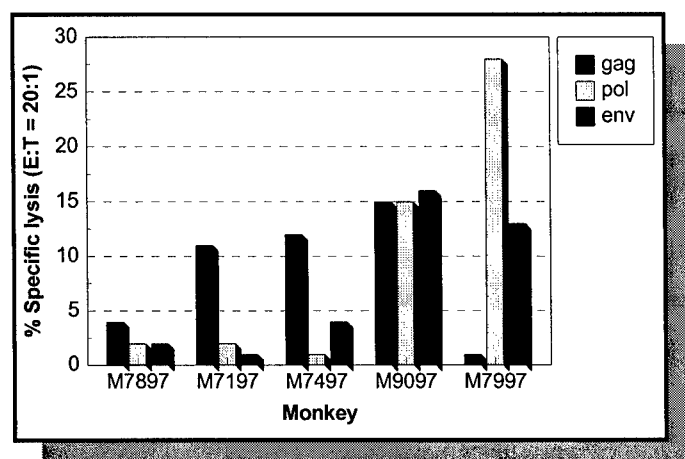


Figure 2: CTL responses in rhesus macaques following 9 consecutive immunizations with DNA encoding SIV_{gag-pol-env}. Three weeks following the 9th DNA immunization, PBMC were purified, stimulated for two weeks *in vitro* with autologous PBMC infected with recombinant vaccinia expressing SIV_{gag-pol-env}, and analysed for cytolytic activity against chromium-labeled EBV-transformed autologous B cells infected with the recombinant vaccinia.

Serum antibody responses were also tested following each immunization. The results in Figure 3 demonstrate a pattern similar to previous studies demonstrating a decline in antibody titer coincident with the first appearance of CTL between the 5th and 7th DNA immunization.

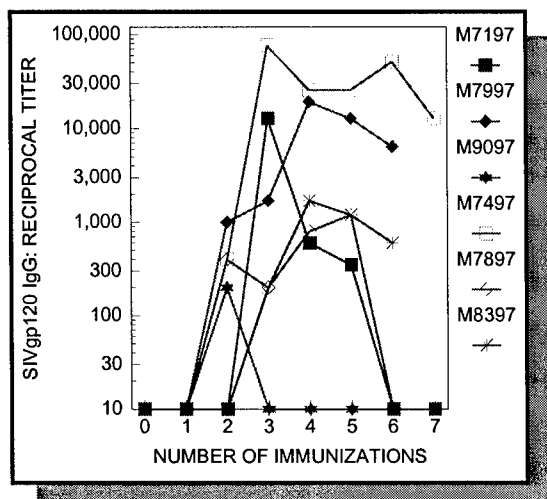
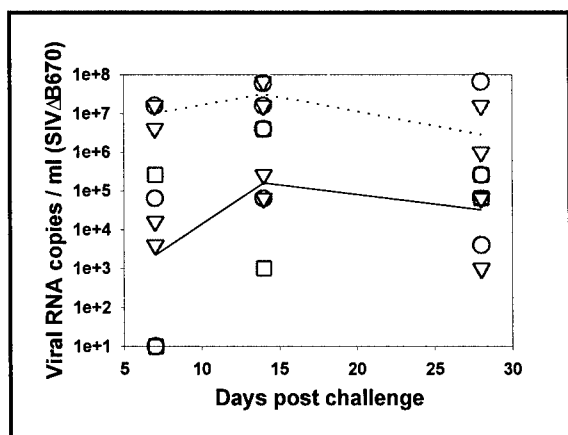


Figure 3: Kinetics of serum antibody responses to SIVgp120 following multiple DNA immunizations in rhesus macaques. Endpoint antibody titers were measured against rSIV_{mac251}gp120 by ELISA.

Three weeks following the 9th DNA immunization, the vaccinated monkeys and 3 naïve control monkeys were challenged by IV injection of 10 AID of the heterologous SIV_{Delta/B670}. Protection was assessed by QC-PCR measurement of plasma viral RNA loads at various timepoints following challenge. Figure 4 shows virus loads for each monkey and compares geometric mean virus loads between the vaccinated and control groups.



Naïve Controls (n=4)
 ▽ ○
 DNA vaccinated (n=6)
 ▽ ○ □

Figure 4: Geometric mean virus loads in rhesus macaques following IV challenge with 10 AID of SIVDelta/B670. Virus loads are expressed as RNA copy number, were determined by QC-PCR at weeks 1, 2, and 4 weeks post-challenge.

The results show that the DNA vaccine reduced virus burden when compared to controls, demonstrating significant but limited protection against the challenge. Significantly, 4 of the 6 vaccinated monkeys showed substantial virus load reductions of 100 to 10,000-fold when compared to the controls. These results demonstrate a significant improvement over previous challenge study where a virus load reduction following challenge with the same virus ranged from 10- to 500-fold when compared to controls.

Induction of mucosal immune responses in rhesus macaques by particle-mediated DNA immunization and mucosal challenge with pathogenic SIV

The mucosal surface is a primary route for HIV infection and recent studies have shown that the gastrointestinal tract is a major target site for infection by immunodeficiency viruses and early CD4+ T cell loss (22). Vaccine induction of mucosal immune responses may therefore be an effective strategy for the development of an efficacious vaccine against HIV. During the last two funding periods, we evaluated the potential for particle-mediated DNA immunization, either in the skin or mucosal surfaces to induce SIV-specific mucosal immune responses and protection from a SIV challenge. We previously reported (Annual Progress Report 1996-97) that 2 administrations of DNA encoding SIV_{mac251FR} *gag-pol-env* to possible mucosal inductive sites (tongue, buccal cavity, and rectal tissues) in monkeys resulted in the induction of low-levels of antigen-specific CTL in the lamina propria. In addition, we observed that targeting DNA to the skin (Annual Progress Report 1997-98) could also induce these mucosal responses.

Administration of DNA to either mucosal or skin tissues resulted in comparable levels of peripheral immune responses. During the final funding year, we tested skin-immunized animals for the induction of mucosal CTL and antibody responses following additional SIV DNA immunizations either to the skin or mucosal tissues. Three weeks following the 5th immunization with SIV DNA, a section of the jejunum and accompanying lymph node were surgically removed for the animal. Mononuclear cells were purified from the tissue and assayed for SIV-specific CTL activity in a chromium release assay. The results in Table 1 demonstrate that DNA administration to the skin or mucosa induced comparable but low levels of CTL in the lamina propria. Saliva and rectal washes were also tested the induction of SIV-specific IgG or IgA responses by ELISA. Positive responses were sporadic, but both IgG and IgA responses were detected. Skin-immunized monkeys showed a higher rate of positive IgG/IgA responses in the mucosal fluids than mucosally-immunized monkeys.

Table 1: Induction of mucosal immune responses following DNA delivery to the skin or mucosa

Monkey	Immunization site	Gut-specific CTL (SIVenv), lytic units/10 ⁶ PBMC	Rectal IgG/IgA (SIVgp120)	Saliva IgG/IgA (SIVgp120)
M7097	Mucosal	12.4*	- / -	- / +
M7297	Mucosal	14.6*	- / -	- / -
M8497	Mucosal	10.0*	- / -	- / -
M7797	Skin	nd	- / -	- / -
M8097	Skin	nd	- / +	+ / +
M12297	Skin	nd	- / -	- / -
M11797	Skin	10.0**	- / -	- / +
M7697	Skin	nd	+ / -	- / -
M11897	Skin	3.5**	+ / +	- / -
M12097	Skin	18.0**	- / -	- / -

Murphey-Corb et. al recently demonstrated that SIV_{env}-specific CTL correlates with protection from a mucosal infection with SIV (23). To determine if vaccine induction of mucosal immune responses could protect from a mucosal challenge, these animals were challenged along with 7 naïve controls intrarectally with 100 AID of heterologous SIV_{Delta/B670}. Protection was assessed by QC-PCR measurement of virus load and is the data are shown in Figures 5 and 6.

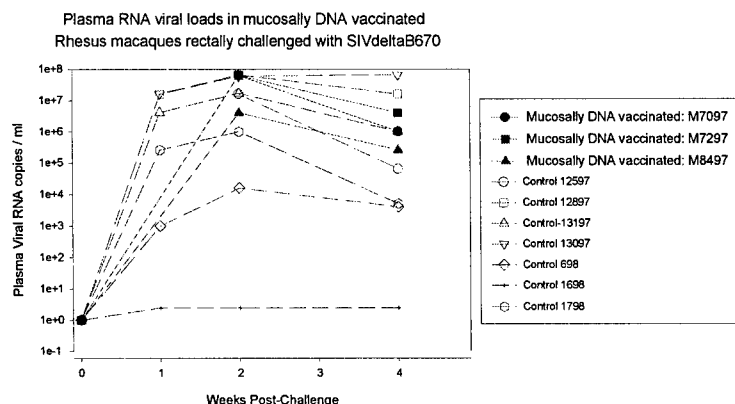
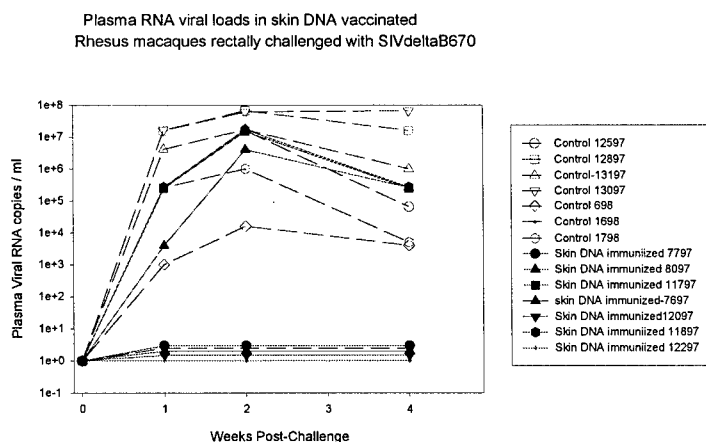


Figure 5: Plasma viral RNA loads following intrarectal challenge with 100 AID of the heterologous SIV_{Delta/B670} in monkeys immunized by particle-mediated delivery of SIV DNA to mucosal tissues (oral and rectal)



Six of the seven control animals became infected and developed acute viremia (Figures 5 and 6). All three mucosally-immunized monkeys (Figure 5) and three of seven skin-immunized monkeys (Figure 6) also became infected and showed no reduction in virus load when compared to the controls. Four of the seven skin-immunized showed evidence of infection following co-culture of PBMC in vitro, but virus loads remained below the limits of detection of the assay at all times. Although mucosal immunization induced CTL in the lamina propria, no protection was

observed. The levels of CTL induced by this regimen were 3-4-fold weaker than levels previously shown to correlate with mucosal protection from SIV infection (23), suggesting that a minimum threshold level of CTL may be required to achieve protection from SIV at the mucosal barrier. In addition, little or no mucosal antibodies were detected in these animals, which may play a role in blocking SIV infection. A single control monkey did not become infected in this study and a broad range of virus loads were seen among all the controls, indicating that consistent intrarectal infections may have been impeded by natural host defenses at the mucosal barrier. Based on this result, protection could not be clearly associated with the vaccination. Nevertheless, 4 of the 7 skin-vaccinated monkeys as compared to only 1 of 7 monkeys in the control group showed no detectable viremia following challenge. This result is consistent with the possibility that DNA immunization to the skin may enhance the ability of the mucosal tissue to resist infection.

Development of a method that induces high frequency CTL responses in nonhuman primates

The requirement for multiple DNA immunizations to induce CTL responses in rhesus monkeys may be due to possibility that antibody / Th2 responses that arise early in the immunization regimen may suppress CTL / Th1 responses. To overcome this issue and induce CTL responses earlier in the immunization regimen, we initiated studies in collaboration with David Watkins at the University of Wisconsin-Madison in our fourth year of funding to evaluate the potential for immunizing with expression vectors encoding specific CTL epitopes. Unlike whole antigens, this approach allows focus of the cytotoxic cellular immune responses toward highly conserved and important epitopes and excludes peptide domains that could induce suppressive antibody or Th2 responses. During the last funding year, we demonstrated that inserting a single SIV gag-specific CTL epitope into the immunodominant region of the hepatitis core antigen resulted in the induction of CTL responses in monkeys after only one or two particle-mediated DNA immunizations with the HBcAg/ SIV CTL epitope hybrid vector. Moreover, levels of CD8+ T cell responses equivalent or greater than that detected in an SIV-infected monkey were also achieved (Annual Progress Report 1997-98). Analysis of HBcAg responses demonstrated a correlation between the frequency of SIV-specific CTL and HBcAg-specific immune responses, supporting the hypothesis that the HBcAg carrier contributes to enhancement of immune responses toward the carried epitope. Significantly, these responses were substantially boosted (MVA) vaccine encoding the same epitope. Very high frequencies (10-18%) of SIV-specific CD8+ T cell responses were detected by tetramer staining of unrestimulated PBMC. These levels are 10-20-fold greater than typically seen in monkeys naturally infected with SIV (1-2%).

During this funding period, we initiated a second study to confirm these results. Three rhesus macaques were primed with 5 doses of DNA encoding the HBcAg/SIV CTL epitope (C>M) hybrid sequences and boosted with MVA as previously described (Annual Progress Report 1997-98). PBMC were isolated before and after the MVA boost. Fresh unrestimulated PBMC were directly assayed for frequency of CD8+ T cell responses by tetramer staining and also stimulated *in vitro* for 2 weeks for cytolytic assays. The results in Table 2 show the induction of high frequency CTL by this method is effective and reproducible in this second set of monkeys.

Table 2: % Tetramer-specific CD8+ T cells in fresh, unrestimulated PBMC

Monkey	Post DNA	Post DNA+MVA
96118	0.58	2.4
96123	0.54	7.5
94004	0.65	20.0

* Background = 0.04%. A positive result is > 0.1%

These monkeys will be challenged with homologous SIVmac239 to evaluate the potential for immune responses to a single CTL epitope to reduce virus load. We plan to continue to explore this method in the context of the potential for developing a polyepitope-based DNA vaccine for HIV.

Specific Aim 4: Modification or augmentation of immune responses induced by particle-mediated DNA immunization.

Our research toward specific aim 4 extensively employed the murine model because of the ease with which new concepts in particle-based DNA immunization can be tested in smaller animals. Certain strategies that were successful in mice were subsequently tested in monkeys and pigs.

Manipulation of immune responses induced by particle-mediated DNA immunization:

Modulation of immune responses by varying the number of immunizations:

In the first funding year, we demonstrated that particle-mediated DNA administration of plasmid expressing HIV-1 glycoprotein 120 (gp120) induced antigen-specific CTL and antibody responses in mice (Annual Progress Report 1994-95, 7). A single immunization of DNA resulted in the induction of MHC class I-restricted CD8+ CTL. CTL responses peaked following a booster immunization but then declined with the first appearance of gp120-specific IgG following additional booster immunizations. Examination of antigen-specific T helper cell responses and antibody isotypes indicated a shift from predominantly Th1-like to Th2-like responses with successive gp120 DNA immunizations. These results indicated that the reciprocity between CTL and antibody responses in this study was mediated through changes in the Th1 and Th2 subsets of CD4+ T cells.

Modulation of immune responses by cytokine gene codelivery

The shift in the type of responses induced with increasing immunizations observed in this study suggested the possibility of purposely manipulating the types of responses elicited, either by varying the number of immunizations, or by codelivering certain immunomodulatory compounds or cytokine expression vectors. In the second funding year (1995-96), we evaluated the feasibility of manipulating immune responses induced by DNA immunization via cytokine gene codelivery. Mice were immunized with DNA encoding HIVgp120 with or without DNA encoding IL-2, IL-7 or IL-12. Following 2 or 3 immunizations, gp120-specific IFN- γ and IL-4 production levels from splenocytes were measured in an *in vitro* antigen stimulation assay. Cytokine codelivery resulted in dramatically enhanced IFN- γ production and a corresponding

suppression of IL-4 production. Enhancement of IFN- γ production was similar for all 3 cytokine genes tested. IL-7 and IL-12, but not IL-2 DNA codelivery resulted in complete suppression of detectable IL-4 production (Annual Progress Report 1995-96, 8). These results demonstrate that the quality of antigen-specific responses elicited via epidermal DNA immunization can be modulated toward a Th1 pattern via cytokine DNA codelivery.

Modulation of immune responses by engineering an immunogenic carrier moiety into the DNA vaccine

The apparent reciprocity between HIVgp120-specific antibody and CTL responses is unique when compared to similar responses induced by particle-mediated DNA immunization to other antigens such as influenza NP (9) and hepatitis B surface antigen (10). In addition, the HIVgp120-specific antibody responses were significantly lower when compared to antibody responses induced following DNA immunization with influenza or hepatitis DNA (7, 8, 9, 10). The reasons for these unique responses to HIVgp120 are not known but could be related to the low level of gp120 antigen production following DNA immunization (unpublished observations) or the possibility that gp120 is a relatively poor immunogen in mice. We therefore investigated the potential of modulating the immune response to HIVgp120 to more closely mimic immune responses against hepatitis antigens by inserting a DNA sequence encoding the major immunodominant epitope of gp120 into the coding sequences for hepatitis B surface (HBsAg) or core antigens (HBcAg). The rationale behind this strategy was to overcome the problems of HIV-1 gp120 production by expressing the major epitope at higher levels, and to enhance immunogenicity of the gp120 sequences by fusion to highly immunogenic hepatitis particulate antigens. The concept of using HBsAg or HBcAg to serve as immunogenic carriers for HIV T cell epitopes has been previously shown for protein-based immunizations (11, 12). We inserted a 15 amino acid gp120 B and T cell epitope into the C-terminus of HBsAg or immunodominant region of HBcAg. In contrast to previous results demonstrating a reciprocal induction of gp120-specific antibody and CTL, immunization with the HBsAg/HIVgp120 hybrid vector resulted in simultaneous induction of both CTL and antibody responses. In addition, the types of Th cell responses were balanced, exhibiting no clear bias towards a Th1 or Th2 phenotype (Annual Progress Report 1994-95). Significantly, both HBsAg/HIVgp120 and HBcAg/HIVgp120 hybrid vectors induced 25-100-fold higher gp120-specific IgG titers than seen previously with the dedicated gp120 vector. These results demonstrate that the antigen influences the type of response induced by DNA immunization. Moreover, HBsAg can be effectively used as a carrier moiety for heterologous epitopes in DNA vaccines and influence the type and potency of the responses elicited to the carried epitopes.

Augmentation of immune responses induced by particle-mediated DNA immunization:

Enhancement of immune responses by increasing the interval between consecutive immunizations.

The relatively weak antibody responses induced by HIV-1gp120 DNA immunization in mice led to efforts to identify strategies to augment these responses. The insertion of HIV-specific epitopes into HBcAg or HBsAg described above was highly effective in enhancing antibody responses induced by DNA immunization, but this strategy was limited to only known T or B cell epitopes. Extensive studies of protein-based vaccines for hepatitis have shown that increasing the interval between immunizations enhances potency of the vaccine (13). Therefore,

during the second funding year (1995-96), we investigated the possibility of augmenting immune responses induced by DNA immunization by increasing the interval between immunizations. In one study, increasing the resting period between HIVgp120 DNA immunizations in mice from one to three months substantially enhanced the magnitude of gp120-specific IgG2a, IFN- γ , and IL-4 responses (Annual Progress Report 1995-96, 8). Similarly, a group of 4 rhesus monkeys that received three immunizations of SIVgp120 DNA spaced 12-14 weeks apart developed 5 to 15-fold higher antibody titers than a second group of 4 monkeys that received 6 SIV DNA immunizations spaced 4 to 6 weeks apart (Annual Progress Report 1995-96, 14). These results demonstrate that immune responses induced by DNA immunization can be enhanced by administering fewer immunizations over a longer period of time.

Enhancement of immune responses by combination vaccine regimens

As described previously, studies in both mice and monkeys have shown that particle-mediated DNA immunization with HIV-1 or SIV expression vectors requires several booster immunizations to achieve significant immune responses. During the second funding year (1995-96), we investigated the possibility of enhancing immune responses in DNA-immunized monkeys by employing consecutive immunization strategies involving priming with DNA and boosting with either recombinant protein vaccines or recombinant vaccinia virus vaccines.

In one study, five rhesus macaques that receive 6 consecutive immunizations of DNA encoding either HIV-1 *gag-pol-env* or HIV-1 gp120 were boosted with rgp120 and rp24 adjuvanted in MF59 (Chiron, Inc.). Antigen-specific antibody responses increased over 100-fold following a single booster immunization with recombinant protein. Significantly, post-boost p24 antibody responses in two animals primed with only gp120 DNA remained low, demonstrating the importance of the primary DNA immunizations in achieving the vigorous antibody titers observed (Annual Progress Report 1995-96, 14, 20).

In a second study, 6 rhesus macaques had developed low to moderate SIVgp120-specific antibody responses following 6 consecutive immunizations with DNA expressing SIVgp120. Three of these animals were boosted with live recombinant vaccinia viruses encoding SIVgp160 and gp120. The remaining 3 monkeys were boosted with another dose of DNA. A group of monkeys previously primed with the vaccinia virus vaccine were boosted with DNA and another group of monkeys were primed and boosted with the vaccinia virus vaccine. In animals primed and boosted with DNA, antibody titers declined. In contrast, monkeys primed with DNA and boosted with vaccinia, developed 100-500-fold higher antibody responses with antigen-specific IgG titers exceeding 1:2,000,000. Monkeys primed with vaccinia and boosted with DNA also developed elevated antibody responses but statistically higher titers were observed in the group that was primed with DNA first. Both combination vaccine groups developed higher titers than animals primed and boosted with vaccinia, demonstrating a synergistic relationship between DNA-based vaccines and recombinant virus-based vaccines or protein-based vaccines (15). The results from these two studies demonstrate the utility of combining DNA immunization with other vaccine modalities to enhance immune responses.

Enhancement of immune responses by co-administration of DNA encoding IL-6

IL-6 is important for end-stage differentiation of B cells and studies have demonstrated that IL-6 is critical for induction of mucosal IgA responses (16). IL-6 also stimulates proliferation of T cells (17) and may enhance immunity by increasing IgG production and cellular immunity for viral clearance. In the fourth funding year (1997-98), we co-delivered DNA encoding hepatitis B surface antigen with DNA encoding IL-6 to determine if IL-6 could enhance peripheral or mucosal immune responses induced by DNA immunization. IL-6 had no effect on the T helper or antibody responses, but had a potent positive effect on CTL responses (Annual Progress Report 1997-98). A previous report demonstrated enhanced mucosal and protective immunity in mice immunized with a co-delivery of genes for influenza and IL-6 and challenged mucosally with influenza (18). However, in our study, IL-6 had no significant effect on HBsAg-specific mucosal immune responses, suggesting enhancement effects by IL-6 codelivery may be antigen-specific.

Enhancement of immune responses by co-administration of DNA and adjuvant

A standard approach for enhancing immunogenicity of protein-based vaccines is to formulate them with adjuvants, such as alum. During the final funding year (1998-99), we investigated the possibility of enhancing immune responses by co-administration of adjuvants with DNA. A number of adjuvants have been shown to augment both antibody and CTL responses. We evaluated the potential for one of these adjuvants, Quil-A (Accurate, Westbury, NY, to augment both CTL and antibody responses induced by particle-mediated DNA immunization.

To identify an optimum method to co-administer DNA and adjuvant, we compared various strategies to deliver the adjuvant and DNA to the same skin target site. In one application, 10 μ g QA was formulated with 1.0 μ g DNA on gold beads (Gold). This results in direct intracellular delivery of gold beads and the associated DNA/adjuvant. In other applications, 50 μ g of QA was dissolved in saline and injected intradermally (ID) or subcutaneously (SQ) at the target site or dissolved in DMSO and applied topically (Topical) to the target site just before particle-mediated delivery of 1.0 μ g DNA. Influenza NP DNA was used in this study because particle-mediated immunization of mice with this DNA results in induction of both antibody and CTL after only as single immunization (9). Mice received a single immunization and splenocytes were collected and pooled for each group 4 weeks post-immunization for analysis of CTL and T helper cell responses. An ELISPOT assay that directly detects individual peptide-specific, IFN- γ -secreting CD8+ T cells without *in vitro* restimulation (19) was used to compare frequencies of CD8+ T cell responses. Figure 1A shows that intracellular co-delivery of Quil-A with DNA on gold beads (NP + QA gold) resulted in a substantial elevation of CD8+ T cell responses when compared to DNA alone (NP only). Significantly, this method of adjuvant co-delivery was superior to the extracellular delivery methods (NP+QA ID, SQ, or Topical) despite the use of 5-fold less adjuvant. Analysis of cytolytic T cell responses in the chromium release assay following stimulation with peptide for 6 days *in vitro* is consistent with these findings (Figure 1B).

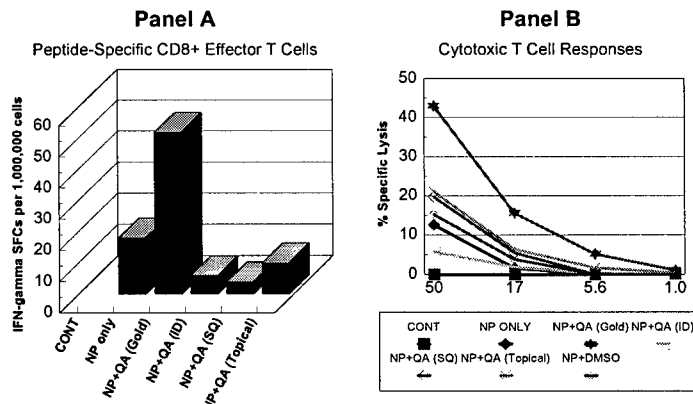


Figure 1: Enhancement of CD8+ T cell responses following co-administration of DNA and Quil-A. Panel A: NP peptide-specific CD8+ effector T cell responses. Frequencies of peptide-specific CD8+ effector T cells were determined by IFN- γ ELISPOT weeks after a single immunization. Data are expressed in spot forming cells (SFC) per 1×10^6 PBMC (N=4) and represent corrected values after background subtraction (PBMC cultured with non-specific peptide). Panel B: influenza NP peptide-specific CTL cytolytic activity.

Based on these findings, subsequent experiments to determine if T helper cell responses and antibody induced by DNA immunization could also be augmented by adjuvantation employed co-administration of DNA and Quil-A on gold beads. Mice received a single immunization with influenza NP DNA and Quil-A on gold beads as described above and serum and splenocytes were collected to analyze for NP-specific antibody and Th cell responses. Serum antibody titers were determined by ELISA are shown in Table 1. The results demonstrate that Quil-A codelivery also promotes significant enhancement of antigen-specific IgG responses.

Table 1: Influenza NP-specific reciprocal endpoint IgG Titers following co-administration of NP DNA and the adjuvant Quil-A

DNA only	DNA + Quil-A
19200	38400
25600	102400
12800	51200
12800	76800
16846	62706

Frequencies of NP-specific Th1 cells secreting IFN- γ or Th2 cells secreting IL-5 were determined by ELISPOT 4 weeks after immunization. Figure 3 demonstrates that intracellular co-administration of DNA and Quil-A on gold beads also results in enhancement of both Th1 and Th2 T helper cell responses, suggesting that Quil-A enhancement of CTL and antibody responses may be mediated by augmentation of T helper cell responses.

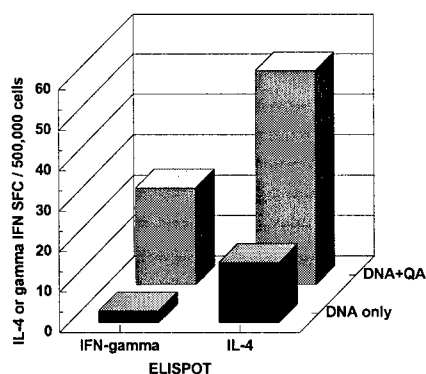


Figure 3: Influenza NP-specific T helper cell responses. Frequencies of NP-specific Th1 cells secreting IFN- γ or Th2 cells secreting IL-5 were determined by ELISPOT 4 weeks after a single immunization following *in vitro* restimulation with viral lysate. Data are expressed in average spot forming cells (SFC) per 1×10^6 PBMC (N=4) and represent corrected values after background subtraction (PBMC cultured in media or with non-specific recombinant protein)

To determine if Quil-A can similarly enhance HIV-1-specific immune responses, mice were primed and boosted with DNA expressing HIVgp120 codelivered with Quil-A on gold beads. Splenocytes were collected and assayed for CTL in a chromium release assay following *in vitro* stimulation with an HIVgp120-specific CTL peptide for 6 days. The results in Table 2 demonstrate that Quil-A enhances HIV-specific CTL induced by DNA immunization.

Table 2: Induction of CTL following co-delivery of Quil-A with DNA expressing HIVgp120 (Ave % specific lysis, 50:1, N=4)

Group	No adjuvant	+ Quil-A
Control	3.2	3.5
HIVgp120 (prime+boost)	14.7	35.4

The mechanism for enhancement of immune responses following intracellular co-administration of DNA and adjuvant on gold beads is not clear. However, it is possible that co-delivery of small amounts of adjuvant within the microenvironment where the DNA is expressed may be an advantage for recruitment of antigen presenting cells or T helper cells or antigen depot effects. Studies are in progress to determine if the adjuvant remains in the cell or is exported.

Taken together, our work toward specific aim 4 demonstrates that immune responses induced by particle-mediated DNA immunization can be modulated or enhanced by a variety of strategies. These results support the possibility of tailoring the immune response induced by DNA vaccination to favor and/or augment the types of responses that may be important for protection against HIV and/or disease progression.

KEY RESEARCH ACCOMPLISHMENTS

- Using pigs as a predictive model for particle-mediated DNA immunization in human skin, we established well-tolerated DNA delivery parameters that resulted in the induction of maximal and consistent immune responses using only 500 ng of DNA per dose.
- We induced protective levels of antibody to hepatitis B surface antigen and protective immune responses to swine influenza in pigs, demonstrating the feasibility of using particle-mediated vaccine technology to elicit vigorous and protective immune responses in animals similar to humans in both size and skin morphology.
- We demonstrated that particle-mediated DNA immunization induces HIV or SIV-specific neutralizing antibody, CTL, type 1 T helper cells, and mucosal immune responses in rhesus monkeys.
- We tested several candidate particle-mediated DNA vaccines for AIDS in the rhesus monkey model and achieved up to a 10,000-fold reduction in virus load in vaccinated animals challenged with a pathogenic, heterologous SIV.
- We observed that SIV-specific CTL responses, but not antibody, correlated with the reduction of virus load following pathogenic, heterologous SIV challenge in DNA-vaccinated monkeys.
- We developed a DNA vaccine-based strategy to induce very high frequency, SIV-specific CD8+ T cell responses in monkeys, exceeding the levels of virus-specific CD8+ T cell frequencies typically found in SIV infected monkeys by 10-20-fold.
- We identified several strategies to tailor the immune response induced by DNA vaccination to favor and/or augment the types of responses that may be important for protection against HIV and/or disease progression. Some of the most promising strategies include co-administration of cytokine genes and adjuvants with vaccine DNA, modulation of the immunization regimen (doses and dosing interval), development of novel DNA vaccine vectors, and combining particle-mediated DNA vaccinations with other vaccine modalities, such as recombinant subunit or viral vector vaccines in a prime-boost regimen..

REPORTABLE OUTCOMES

Manuscripts

1. Fuller, DH and Haynes, JR. 1994. A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. *AIDS Res. Hum. Retroviruses* 10: 1433-1441.
2. Haynes JR, DH Fuller, MD Eisenbraun, MJ Ford, and TM Pertmer. 1994. Accell particle-mediated DNA immunization elicits humoral, cytotoxic, and protective immune responses. *AIDS Res. Hum. Retroviruses* 10(suppl2):S43.
3. Pertmer T M, M D Eisenbraun, D McCabe, S K Prayaga, D H Fuller, and J R Haynes. 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13:1427.
4. Fuller JT, DH Fuller, D McCabe, JR Haynes, and G Widera. Immune responses to hepatitis B virus surface and core antigens in mice, monkeys, and pigs after Accell particle-mediated DNA immunization. 1995. *Annals of the New York Academy of Sciences* 772:282.
5. Fuller DH, M Murphey-Corb, J Clements, S Barnett, and JR Haynes. 1996. Induction of immunodeficiency virus-specific immune responses in rhesus monkeys following gene gun-mediated DNA vaccination. *J Med. Primatol.* 25:236.
6. Fuller JT, DH Fuller, D McCabe, JR Haynes, and G Widera. Gene-gun-mediated DNA immunization with HBV antigens: Immune responses in mice, monkeys, and pigs. In *Vaccines 96: Molecular approaches to the control of infectious disease*. Brown, F., D. Burton, P. Doherty, J. Mekalanos, E. Norrby, eds. Cold Spring Harbor Laboratory Press, New York, NY, p.88.
7. Prayaga SK, M J Ford, and J R Haynes. 1997. Manipulation of HIV-1 gp120 immune responses elicited via gene gun-based DNA immunization. *Vaccine* 15:1349.
8. Fuller DH, Murphey-Corb M, Barnett S, Steimer K, Haynes JR. 1997. Enhancement of immunodeficiency virus-specific immune responses in DNA-immunized rhesus macaques. *Vaccine* 15:924.
9. Fuller, J. T., M. Macklin, B. Drape, D. McCabe, W. F. Swain, J. R. Haynes, and G. Widera. 1997. Gene gun-mediated DNA immunization with HBsAg: efficacy in small and large animals. In *Vaccines 97: Molecular approaches to the control of infectious disease*. Brown, F., D. Burton, P. Doherty, J. Mekalanos, E. Norrby, eds. Cold Spring Harbor Laboratory Press, New York, NY, p.157.
10. Fuller DH, L Simpson, KC Stefano, JE Clements, DL Panicali, RC Montelaro, M Murphey-Corb, and JR Haynes. 1997. Gene gun-based nucleic acid immunization alone or in combination with recombinant vaccinia vectors suppresses virus burden in rhesus macaques challenged with a heterologous SIV. *Immunology and Cell Biology* 75:389.
11. Macklin, M.D, D McCabe, MW McGregor, V Neumann, T Meyer, R Callan, V S Hinshaw, and W F Swain. 1998. Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. *J. Virol.* 72:1491.
12. Allen TM, DH Fuller, TU Vogel, T Shipley, J Fuller, S Steffen, JE Boyson, T Hanke, A Sette, JD Altman, AJ McMichael, and DI Watkins. 1999. CTL epitope-based DNA vaccinations at both dermal and mucosal sites induce SIVgag-specific CD8 responses in rhesus macaques (submitted).

Abstracts and Presentations

1. Fuller, D. H., L. Wilson, M. Wu, T. Shipley, M. Murphey-Corb. Reciprocal induction of antibody and CTL responses in rhesus macaques following multiple immunizations with SIV DNA: Implications for vaccine development. *Abstracts of the 17th Annual Nonhuman Primate Models for AIDS*, New Orleans, LA, Oct. 6-9, 1999.
2. Shipley, T., M. Wu, D. Fuller. Particle-based intracellular co-delivery of DNA with a saponin type adjuvant enhances specific cellular immune responses in mice. *Abstracts of the Second Annual Conference on Vaccine Research*. Bethesda, MD, April 30-May 2, 1999.
3. Fuller D., T. Shipley, J. Fuller, T. Allen, T. Vogel, D. Watkins. Improved induction of specific cytotoxic T lymphocytes in mice and monkeys immunized with chimeric DNA vectors encoding HIV or SIV epitopes fused with hepatitis B core or surface antigen. *Abstracts of the 1999 Keystone symposia: HIV Vaccine Development*, Keystone, CO, Jan. 7-13, 1999.
4. Fuller, D. H., SIV DNA vaccine trial. *Abstracts of the IBC conference on Novel Vaccine Strategies for HIV*. November 17, 1997.
5. Fuller D. H., Genes as vaccines: Gene gun-mediated DNA Immunization of small and large animals. *Abstracts of the IBC Gene Therapy Conference*, May 8-9, 1997.
6. Fuller D. H., L. Simpson, K. S. Cole, R. Montelero, J. Haynes, M. Murphey-Corb. Immune responses induced by Accell gene gun SIV DNA immunization reduce viremia and delay CD4 decline in rhesus macaques challenged with a heterologous SIV isolate. *Abstracts of the Ninth Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS*. May 4-7, 1997.
7. Fuller, D. H., M. Murphey-Corb, L. Simpson, R. Montelaro, K. Stefano-Cole, J. Clements, D. Panicali, J. Haynes, (1996). SIV challenge of rhesus macaques following a prime-boost vaccination regimen with gene gun-based DNA immunization alone or in combination with recombinant vaccinia vectors. *Abstracts of the 14th Annual Symposium on Nonhuman Primate Models for AIDS*.
8. Fuller, D. H. J. Haynes, M. Murphey-Corb, S. Barnett, (1996). Gene gun-based DNA immunization primes for the induction of vigorous immune responses in rhesus macaques when followed by a recombinant subunit or vaccinia boost. *Abstracts of the WHO International Meeting on Nucleic Acid Vaccines*.
9. Fuller, D. H., J. Fuller, M. Murphey-Corb, J. Clements, J. R. Haynes (1995). DNA vaccines: Analysis of HIV-1 and SIV-specific immune responses in rhesus macaques. *Abstracts of the 13th Annual Symposium on Nonhuman Primate Models for AIDS*.
10. Fuller, D. H., T. Pertmer, M. Murphey-Corb, J. E. Clements, J. R. Haynes (1995). Elicitation of immunodeficiency virus-specific immune responses in mice and rhesus monkeys via particle-based DN immunization. *Abstracts of the IBC conference on Gene Therapy and Nucleic Acid Vaccine Strategies*.
11. Prayaga, S. K., D. H. Fuller, M. Murphey-Corb, J. R. Haynes (1994). Microparticle-mediated nucleic acid immunization. *Abstracts of the Cold Spring Harbor Meeting on Molecular Approaches to Control Infectious Diseases*.
12. Fuller, D. H., M. D. Eisenbraun, M. Murphey-Corb, J. R. Haynes, (1994). Anti-SIV and Anti-HIV immune responses induced in rhesus monkeys through *in vivo* particle-mediated DNA immunization. *Abstracts of the 7th Annual Meeting of the National Cooperative Vaccine Development Groups for AIDS*

Patent Applications

1. Nucleic Acid Constructs
2. Genetic Vaccines for Infectious Disease
3. Vaccination method for efficient induction of cytotoxic T lymphocyte response.

Personnel

Joshua Arrington
Dexiang Chen
Katherine Larrivee
Tim Shipley
Mary Wu
Deborah Fuller
Jim Fuller

CONCLUSIONS

DNA immunization represents an attractive alternative to classic vaccination, providing the advantages of live attenuated vaccines without the safety and stability concerns associated with infectious agents. DNA can be administered into the muscle or skin by a needle and syringe. Administration of vaccine DNA by needle or particle-mediated DNA immunization has been shown to be highly effective in the induction of antibody, cell-mediated, and protective immunity to a variety of antigens (6). These results suggested DNA vaccination might be clinically useful for vaccination in humans. However, recent clinical trials employing intramuscular inoculation of DNA pointed towards a need for a considerable improvement or in DNA administration or scale up in the amount of DNA needed to achieve the level of success initially predicted by animal models (28-30). Particle-mediated DNA immunization permits the use of much smaller quantities of DNA than required for administration with a needle and syringe because the DNA is delivered directly into the host's skin cells rather than extracellular spaces. In addition particle-mediated DNA immunization targets the skin, an immunologically active tissue populated by antigen presenting cells (APC) that routinely traffic from skin to lymph nodes (31). Resident APC can be directly transfected by particle-mediated delivery of DNA (32) or take up antigen expressed and secreted by other cells of the epidermis (33, 34). Thus, particle-mediated DNA administration provides the combined advantages of an effective *in vivo* DNA delivery system and the immune competence of the skin as a delivery site. Comparisons of methods for DNA delivery into both mice and monkeys have demonstrated that particle-mediated DNA transfer is significantly more efficient than intramuscular or intradermal DNA inoculations in the induction of antibody responses, requiring 100-500-fold less DNA to induce higher titers of antibody (35-38).

Our work efforts under this grant focused on testing particle-mediated DNA delivery as a novel vaccine strategy for AIDS and readying the technology for future human clinical trials. In the latter aspect, we extensively employed the swine model to achieve the goals of specific aim 1 because of its similarity in both size and skin morphology to humans. We investigated the relationship between physical parameters of DNA/gold delivery, the mild and localized erythema after delivery, and the resulting immune response. Data generated in the pig further refined optimal DNA immunization parameters as a sound basis for future clinical studies. In addition, studies in pigs led to successful fulfillment of our second specific aim, the induction of protective immune responses in pigs as a model for human vaccination. Protective responses to swine influenza and hepatitis B were induced in the pig following only 2 immunizations with as little as 500 ng of DNA. Significantly, the protection levels seen in pigs for were similar to those elicited using commercially available swine flu or hepatitis B vaccines. These and other data encouraged PowderJect Vaccines, Inc. to bring a hepatitis B DNA vaccine into clinical trials. These trials, outside the scope of the research covered under this grant, demonstrated that particle-mediated administration of DNA vaccines could induce protective humoral immunity in humans, the first demonstration of its kind for a DNA vaccine. Moreover, particle-mediated DNA immunization was highly effective in the in the induction of T cell responses, including cytotoxic T lymphocytes, and effectively induced both cell-mediated and humoral immune responses in 100% of the vaccinees (39). Based on these results, PowderJect's gene delivery technology can

be considered as a safe and effective tool for use in future human clinical studies of candidate DNA vaccines for HIV and other diseases.

Our work in testing a particle-based DNA vaccine strategy for AIDS supports the potential for development of such a vaccine. We demonstrated that particle-mediated vaccination of rhesus macaques with SIV DNA resulted in the induction of antigen-specific neutralizing antibody responses, CTL, mucosal immunity and reduced virus load up to 10,000-fold following a intravenous challenge with pathogenic, heterologous SIV. The relationship between prognosis in HIV-1 infection and plasma viremia has been well-established (40). A vaccine capable of achieving significant virus load reduction and improving disease prognosis and reducing viral shedding and transmission may be realistic goal.

Partial protection from the vigorous, heterologous challenges used in our studies was correlated with the induction of CTL, but not antibody. Although no single measure of immunity has been found to be predictive of *in vivo* control of HIV replication, evidence is mounting that cytotoxic T lymphocytes play a significant role. HIV infected patients with strong CTL responses demonstrate reduced acute viremia (40), and high levels of virus-specific CTL are associated with long term survival (41). In contrast, patients with low or undetectable CTL poorly control acute viremia and progress more rapidly to AIDS (42). These results strongly suggest that an effective vaccine against HIV will need to induce cytotoxic T lymphocyte responses. Another promising application of an HIV vaccine that induces CTL is in the treatment of chronic HIV infection. An immunization strategy that induces or boosts HIV-specific CTL may be effective during highly active anti-retroviral therapy (HAART). In particular, vaccine induction of CTL could effectively target residual infected cells and reduce or eliminate virus load.

The best protection in our challenge studies was observed only after numerous DNA vaccinations indicating a potential limitation for DNA vaccination against HIV. However, our work in the evaluation of strategies to augment or manipulate immune responses induced by DNA vaccines indicates it should be possible to achieve the desired protective response with fewer immunizations. It should be noted that the relatively poor immunogenicity of HIV or SIV proteins is likely an antigen-specific issue since particle-mediated DNA immunization has been shown to be highly effective in the induction of potent and protective immune responses against other antigens in mice, monkeys and pigs (9, 18, 25-27, 35).

Taken together, our results in each of the specific aims of this grant and the excellent safety and efficacy outcome of our first clinical trial support particle-mediated DNA immunization for further evaluation of this technology for future human clinical trials as a preventative and/or therapeutic vaccine approach for AIDS.

REFERENCES

1. Emini EA, WA Schleif, JH Nunberg, AJ Conley, Y Eda, S Tokiyoshi, SD Putney, S Matsushita, KE Cobb, CM Jett, JW Eichberg, and KK Murthy. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. *Nature* 355:726-730.
2. Cole KS, Rowles JL, Jagerski BA, Murphey-Corb M, Unangst T, Clements JE, Robinson J, Wyand MS, Desrosiers RC, Montelaro RC. 1997. Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with simian immunodeficiency virus and its association with the development of protective immunity. *J Virol.* 71:5069-79.
3. Borrow P, H Lewicki, BH Hahn, GM Shaw, and MBA Oldstone. 1994. Virus-specific CD8+ cytotoxic T lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. *J. Virol.* 68:6103-6110.
4. Borrow P, H Lewicki, X Wei, MS Horwitz, N Pfeffer, H Meyers, JA Nelson, JE Gairin, BH Hahn, MBA Oldstone, and GM Shaw. 1997. Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nat. Med.* 3:205-211.
5. Ogg GS, X Jin, S Bonhoeffer, PR Dunbar, MA Nowak, S Monard, JP Segal, Y Can, SL Rowland-Jones, V Cerundolo, A Hurley, M Markowitz, DD Ho, DF Nixon, and A. McMichael. 1998. Quantitation of HIV-1-specific cytotoxic T lymphocytes and plasma load of viral RNA. *Science* 279:2102-2106.
6. Donnelly JJ, JB Ulmer, and MA Liu. 1997. DNA vaccines. *Annu. Rev. Immunol.* 15:617.
7. Fuller, DH and Haynes, JR. 1994. A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. *AIDS Res. Hum. Retroviruses* 10: 1433-1441.
8. Prayaga SK, M J Ford, and J R Haynes. 1997. Manipulation of HIV-1 gp120 immune responses elicited via gene gun-based DNA immunization. *Vaccine* 15:1349.
9. Pertmer T M, M D Eisenbraun, D McCabe, S K Prayaga, D H Fuller, and J R Haynes. 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13:1427.
10. Haynes JR, Fuller DH, McCabe DE, Swain WF, and Widera, G. 1996. Induction and characterization of humoral and cellular responses elicited via gene gun-mediated nucleic acid immunization. *Advanced Drug Delivery Reviews* 21: 3.
11. Schlienger K, Mancini M, Riviere Y, Dormont D, Tiollais P, and ML Michel. 1992. Human immunodeficiency virus type 1 major neutralizing determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates. *J. Virol.* 66:2570.
12. Schödel, F., Peterson, D., Hughes, J., Milich, D. 1994. Hepatitis B virus core particles as a vaccine carrier moiety. *Int. Rev. Immunol.* 11:153.
13. Jilg W, F Schmidt, and F Deinhardt. 1989. Vaccination against hepatitis B: comparison of three different vaccination schedules. *J. Infect. Dis.* 160:766.
14. Fuller DH, Murphey-Corb M, Barnett S, Steimer K, Haynes JR. 1997. Enhancement of immunodeficiency virus-specific immune responses in DNA-immunized rhesus macaques. *Vaccine* 15:924.
15. Fuller DH, L Simpson, KC Stefano, JE Clements, DL Panicali, RC Montelaro, M Murphey-Corb, and JR Haynes. 1997. Gene gun-based nucleic acid immunization alone or in

- combination with recombinant vaccinia vectors suppresses virus burden in rhesus macaques challenged with a heterologous SIV. *Immunology and Cell Biology* 75:389.
16. Holmgren J, C Czerkinsky, N Lycke, and AM Svennerholm. 1992. Mucosal immunity: Implications for vaccine development. *Immunobiology* 184: 157-179.
 17. Van Snick, J. 1990. Interleukin-6: an overview. *Annu. Rev. Immunol.* 8: 253-278.
 18. Larsen DL, N Dybdahl-Sissoko, MW McGregor, R Drape, V Neumann, WF Swain, DP Lunn, CW Olsen. 1998. Coadministration of DNA encoding interleukin-6 and hemagglutinin confers protection from influenza virus challenge in mice. *J. Virol* 72:1704.
 19. Lalvani A, R Brookes, S Hambleton, W J Britton, A V Hill, and A J McMichael. 1997. Rapid effector function in CD8+ memory T cells. *J. Exp. Med.* 186:859.
 20. Fuller DH, M Murphey-Corb, J Clements, S Barnett, and JR Haynes. Induction of immunodeficiency virus-specific immune responses in rhesus monkeys following gene gun-mediated DNA vaccination. *J Med. Primatol.* 25:236.
 21. Chapman BS, RM Thayer, KA Vincent, and NL Haigwood. 1991. Effect of intron A from human cytomegalovirus (Towne) immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids Res.* 19:3979.
 22. Veazey R, M DeMaria, LV Chalifoux, DE Shvetz, DR Pauley, HL Knight, M Rosenzweig, RP Johnson, RC Desrosiers, AA Lackner. 1998. Gastrointestinal Tract as a Major Site of CD4⁺ T Cell Depletion and Viral Replication in SIV Infection. *Science* 280:427.
 23. Murphey-Corb M, Wilson LA, Trichel AM, Roberts DE, Xu K, Ohkawa S, Woodson B, Bohm R, Blanchard J. 1999. Selective induction of protective MHC class I-restricted CTL in the intestinal lamina propria of rhesus monkeys by transient SIV infection of the colonic mucosa. *J. Immunol.* 162:540.
 24. Meyer, W., R. Schwarz, and K. Neurand. 1978. The skin of domestic mammals as a model for the human skin, with special reference to the domestic pig. *Curr. Probl. Dermatol.* 7:39.
 25. Eisenbraun, M.D., Fuller, D.H., and Haynes, J.R. 1993. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol.* 12: 791.
 26. Macklin, M. D., D. McCabe, M. W. McGregor, V. Neumann, T. Meyer, R. Callan, V. S. Hinshaw, and W. F. Swain. 1998. Immunization of pigs with a particle-mediated DNA vaccine to influenza A virus protects against challenge with homologous virus. *J. Virol.* 72:1491.
 27. Fuller, J. T., M. Macklin, B. Drape, D. McCabe, W. F. Swain, J. R. Haynes, and G. Widera. 1997. Gene gun-mediated DNA immunization with HBsAg: efficacy in small and large animals. In *Vaccines 97: Molecular approaches to the control of infectious disease*. Brown, F., D. Burton, P. Doherty, J. Mekalanos, E. Norrby, eds. Cold Spring Harbor Laboratory Press, New York, NY, p.157.
 28. MacGregor, R. R., J. D. Boyer, K. E. Ugen, K. E. Lacy, S. J. Gluckman, M. L. Bagarazzi, M. A. Chattergoon, Y. Baine, T. J. Higgins, R. B. Ciccarelli, L. R. Coney, R. S. Ginsberg, and D. B. Weiner. 1998. First human trial of a DNA-based vaccine for treatment of human immunodeficiency virus type 1 infection: safety and host response. *J. Infect. Dis.* 178:92.
 29. Wang, R., D. L. Doolan, T. P. Le, R. C. Hedstrom, K. M. Coonan, Y. Charoenvit, T. R. Jones, P. Hobart, M. Margalith, J. Ng, W. R. Weiss, M. Sedegah, C. de Taisne, J. A. Norman, and S. L. Hoffman. 1998. Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 282:476.

30. Calarota, S., G. Bratt, S. Nordlund, J. Hinkula, A. C. Leandersson, E. Sandstrom, and B. Wahren. 1998. Cellular cytotoxic response induced by DNA vaccination in HIV-1-infected patients. *Lancet* 351:1320.
31. Stingle, G., E. Tschachler, V. Groh, K. Wolff, and C. Hauser. 1989. The immune functions of epidermal cells. *Immunol. Ser.* 46:3.
32. Condon C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2:1122.
33. Klinman, D. M., J. M. Sechler, J. Conover, M. Gu, A. S. Rosenberg. 1998. Contribution of cells at the site of DNA vaccination to the generation of antigen-specific immunity and memory. *J. Immunol.* 160:2388.
34. Iwasaki, A., C. A. Torres, P. S. Ohashi, H. L. Robinson, and B. H. Barber. 1997. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* 159:11.
35. McCluskie, M. J., C. L. Brazolot Millan, R. A. Gramzinski, H. L. Robinson, J. C. Santoro, J. T. Fuller, G. Widera, J. R. Haynes, R. H. Purcell, H. L. Davis. 1999. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol. Med.* 5:287.
36. Leitner, W. W., M. C. Seguin, W. R. Ballou, J. P. Seitz, A. M. Schultz, M. J. Sheehy, and J. A. Lyon. 1997. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from *Plasmodium berghei* malaria parasites. *J. Immunol.* 159:6112.
37. Fynan, E. F., R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. 1993. DNA vaccines: protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci. U.S.A.* 90:11478.
38. Pertmer, T. M., M. D. Eisenbraun, D. McCabe, S. K. Prayaga, D. H. Fuller, and J. R. Haynes. 1995. Gene gun-based nucleic acid immunization: elicitation of humoral and cytotoxic T lymphocyte responses following epidermal delivery of nanogram quantities of DNA. *Vaccine* 13:1427.
39. Roy, MS, MS Wu, LJ Barr, JT Fuller, LG Tussey, S Speller, J Culp, J Burkholder, WF Swain, RM Dixon, G Widera, R Vessey, A King, G Ogg, A Gallimore, DH Fuller. 2000. Clinical evaluation of a nucleic acid vaccine for hepatitis B: Induction of T cell responses and protective antibodies in humans by particle-mediated administration of plasmid DNA. (submitted).
40. Mellors, J.W., Rinaldo, C.R., Gupta, P., White, R.M., Todd, J.A., Kingsly, L.A. 1996. Prognosis of HIV-1 infection predicted by the quantity of virus in plasma. *Science* 272:1167-1170.
41. Rinaldo, C., Huang, X., Fan, Z., et. al. 1995. High levels of anti-HIV-1 memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1 infected long-term nonprogressors. *J Virol* 69:5838-5842.
42. Pantaleo, C., Menzo, S., Vaccarezza, M., et. al. 1995. Studies in subjects with long-term nonprogressive human immunodeficiency virus infection. *N Engl J Med* 332:209-216.
43. Klein, M.R., van Baalen, C.A., Holwerda, A.M., Kerkhof Garde, S.R., Bende, R.J., Keet, I.P., Eeftinck-Schattenkerk, J.K., Osterhaus, A.D., Schuitemaker, H., Miedema, F. 1995. Kinetics of Gag-specific cytotoxic T lymphocyte responses during the clinical course of HIV-1 infection: a longitudinal analysis of rapid progressors and long-term asymptomatics. *J Exp Med* 181:4 pp. 1365-1372.

Brown, Loretta

From: Miller, Virginia M Ms USAMRMC [Virginia.Miller@DET.AMEDD.ARMY.MIL]
Sent: Friday, May 12, 2000 2:57 PM
To: 'lbrown@dtic.mil'
Cc: Pawlus, Judy K Ms USAMRMC
Subject: Correction to ADB252231

Loretta,

Could you please change the "Descriptive Note" for ADB252231 on the citation in DTIC. It should be "Annual Report" and the period of time should be 30 Sep 98 - 29 Sep 99. This change should be made to the cover and 298 in the report as well.

Thank you for your assistance.

Virginia

Virginia Miller
Technical Information Specialist
USAMRMC, MCMR-RMI-S
301-619-7327, FAX: 301-619-2745
virginia.miller@det.amedd.army.mil

*Replace
pages
#52231*

*FAX
DSN 427 - 9244*



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

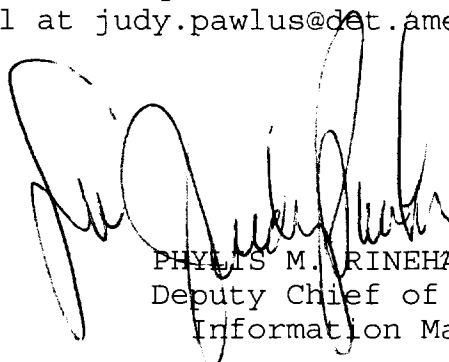
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

DAMD17-94-J-4391
DAMD17-94-J-4426
DAMD17-94-J-4426

ADB247843
ADB240092
ADB252231