

UNCLASSIFIED

AD NUMBER
ADB261755
NEW LIMITATION CHANGE
TO Approved for public release, distribution unlimited
FROM Distribution authorized to U.S. Gov't. agencies only; Proprietary Information; Oct 1999. Other requests shall be referred to U.S, Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, MD 21702-5012
AUTHORITY
USAMRMC ltr, 21 Feb 2003

THIS PAGE IS UNCLASSIFIED

AD _____

GRANT NUMBER:

DAMD17-98-1-8173

TITLE: Gene Painting as a Simple Method of Vaccinating Animals
Against Breast Cancer Micro-Metastases

PRINCIPAL INVESTIGATOR:

De-chu Tang, Ph.D.

RECIPIENT ORGANIZATION:

University of Alabama at Birmingham
Birmingham, Alabama 35294-0111

REPORT DATE: October 1999

TYPE OF REPORT:

Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
504 Scott Street
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.

DTIC QUALITY INSPECTED 3

20010109 059

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

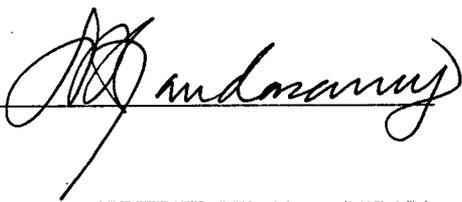
Award Number: DAMD17-98-1-8173

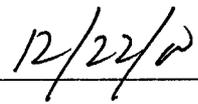
Organization: University of Alabama at Birmingham

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 PAGE			Form Approved OMB No. 0704-0188	
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 the 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 (1 Oct 98 - 30 Sep 99)		
4. TITLE AND SUBTITLE Gene Painting as a Simple Method of Vaccinating Animals Against Breast Cancer Micro-Metastases		5. FUNDING NUMBERS DAMD17-98-1-8173		
6. AUTHOR(S) De-chu Tang, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Alabama at Birmingham Birmingham, Alabama 35294-0111		8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSORING / MONITORING AGENCY REPORT NUMBER		
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY 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.			12b. DISTRIBUTION CODE	
13. ABSTRACT (Maximum 200 words) We are developing a simple and effective method for the delivery of cancer vaccines by noninvasive vaccination onto the skin (NIVS) using a patch. The hypothesis is that a noninvasive vaccine patch can elicit specific immune responses to tumor-associated antigens with resultant eradication of limited numbers of tumor cells in animals with low tumor burden breast cancer. In these studies, we have elicited anti-CEA antibodies by topical application of an adenovirus vector encoding CEA. Furthermore, animals immunized by this novel vaccination modality were well protected against a mammary tumor cell line expressing CEA. When compared to other means of immunization including intramuscular injection of DNA and intranasal inoculation of adenovirus vectors, the skin-targeted vaccine patch appeared to be more protective in a disease setting, probably due to the immunocompetence of the outer layer of skin where antigens were expressed. We envision that patch-based vaccination may emerge as an important technique for the administration of vaccines because the procedure is simple, effective, economical, painless, and safe. It may also boost vaccine coverages due to patient comfort.				
14. SUBJECT TERMS Breast Cancer		15. NUMBER OF PAGES 19		
		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 Limited	

FOREWORD

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

DT

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

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

DT

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

DT

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 Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

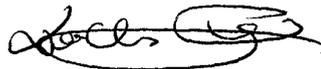
For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45 CFR 46.

DT

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

DT

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



10-29-99

TABLE OF CONTENTS

	<u>Page numbers</u>
SF298	2
Foreword	3
Table of Contents	4
Introduction	5
Body	6-10
Conclusions	11
References	12
Appendices	13-18

INTRODUCTION

The induction or augmentation of tumor-specific immune responses providing protection against neoplastic disease is a promising approach for treating metastatic breast cancers. Genetic immunization potentially may present functional antigenic proteins to the host for recognition by all arms of the immune system, yet is able to delete pieces of tumor antigens that may have deleterious effects. We have demonstrated that the surface of the skin is a convenient site for the inoculation of genetic vaccines. Since the outer layer of skin interfaces directly with the external environment and is in constant contact with innumerable pathogens, immunologic components for the elicitation of both humoral and cytotoxic cellular immune responses must be present along the skin border for counteracting undesirable infections. Evidence supporting the immunologic competence of the outer layer of skin includes: 1) Antigens expressed in the epidermis are more immunogenic than those expressed in the dermis (1), and 2) genetic vaccines inoculated into the epidermis using a gene gun are more potent than those injected intramuscularly (2). Injection of vaccines underneath the epidermis as commonly practiced is likely to bypass an epidermis-associated immune surveillance zone along the border, resulting in insufficient or inappropriate immune responses. The large accessible area of the skin and its durability are other advantages for applying genetic vaccines to this tissue. The immunologic competence of the skin, the ease with which genes can be targeted to defined sites within the skin, the rapid turn-over of skin cells, and our finding that animals can be protected against tumor challenges by noninvasive vaccination onto the skin (NIVS), may allow for the development of a unique method for the administration of vaccines. In these studies, we have demonstrated that anti-CEA (carcinoembryonic antigen) antibodies could be elicited by adenovirus-based NIVS. Furthermore, animals immunized by a skin patch containing AdCMV-hcea (an adenovirus vector encoding human CEA) (3) were protected against challenges by a mammary tumor cell line expressing CEA. Results suggested that vaccination against metastatic breast cancers may be achieved by a noninvasive skin patch. This approach not only may boost vaccine coverages because the procedure requires no specially trained personnel and equipment, but also may be able to elicit potent antitumor immune responses because antigens are expressed in the outer layer of skin which is a very immunocompetent tissue.

BODY

1. Elicitation of anti-CEA antibodies in mice. We have experience in immunizing animals with a noninvasive vaccine patch (3, 4). **Figure 1** shows that antisera against CEA could be induced in mice by a vaccine patch containing AdCMV-hcea (an adenovirus vector encoding CEA) (3). Serum samples were collected from BALB/c mice that had been immunized by intramuscular injection of pGT37 DNA (a plasmid expression vector encoding human CEA) (5), intranasal inoculation of AdCMV-hcea, or noninvasive application of AdCMV-hcea onto abdominal skin using a skin patch. Evidence suggested that only a small fraction of topically applied vectors may be absorbed by the skin (4). **Figure 1** shows that absorption of vectors by unbroken skin could elicit anti-CEA antibodies at a higher titer than that achieved by intramuscular injection of a large dose of DNA, although intranasal inoculation of adenovirus vectors appeared to be more potent than NIVS in eliciting a humoral immune response probably due to more efficient gene transfer in the respiratory tract. Control animals including naïve mice and mice immunized by topical application of an irrelevant vector all failed to elicit anti-CEA.

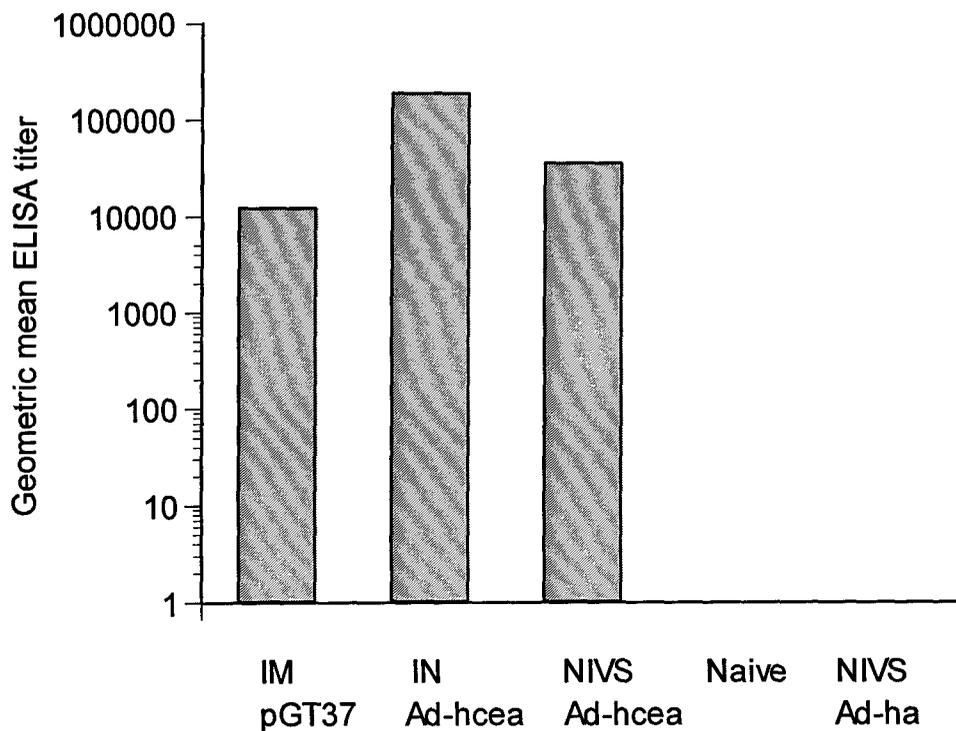


Figure 1. ELISA antibodies generated by the AdCMV-hcea vector in mice. BALB/c mice (3 months old) were immunized by intramuscular (IM) injection of 100 μ g of pGT37 DNA, intranasal inoculation (IN) with a dose of 2.5×10^7 pfu (plaque-forming units) of AdCMV-hcea, or topical application using a patch by incubating 10^8 pfu of AdCMV-hcea with pre-shaved abdominal skin in a noninvasive mode. For patch-based immunization, the vector was spread as a thin film over naked skin with a piece of the Tegaderm patch (3M). Unabsorbed vectors were washed away in an hour. Each animal was immunized

by the specified vector and route for 3 times every 3 weeks. Serum samples were assayed for anti-CEA antibodies 1 week after the last boost. Titers of anti-CEA IgG were determined by ELISA as described (4) using purified CEA (CalBiochem) as the capture antigen. Serum samples and peroxidase-conjugated goat anti-mouse IgG (Promega) were incubated sequentially on the plates for 1 hour at room temperature with extensive washing between each incubation. The end-point was calculated as the dilution of serum producing the same OD₄₉₀ as a 1/100 dilution of preimmune serum. Sera negative at the lowest dilution tested were assigned endpoint titers of 100. IM/pGT37, mice immunized by intramuscular injection of pGT37 DNA; IN/Ad-hcea, mice immunized by intranasal inoculation of AdCMV-hcea; NIVS/Ad-hcea, mice immunized by topical application of AdCMV-hcea; Naïve, non-immunized mice as a control group; NIVS/Ad-ha, mice immunized by topical application of an irrelevant vector AdCMV-PR8.ha (an adenovirus vector encoding an influenza hemagglutinin) as a control group. The data was plotted as geometric mean endpoint ELISA titers, where $n=9$ for IM/pGT37, $n=19$ for IN/Ad-hcea, $n=9$ for NIVS/Ad-hcea, $n=10$ for naïve, and $n=10$ for NIVS/Ad-ha.

2. Construction of a mammary tumor cell line expressing CEA. The murine mammary tumor cell line JC derived from a female Balb/c mouse was obtained from ATCC. A CEA-expressing mammary tumor cell line JC-hcea was constructed by co-transfecting pGT37 (5) with pH β APr-1-neo (6) at a molar ratio of 10:1, followed by selecting transfectants in medium containing G418. G418-resistant clones containing the human CEA sequences were validated by PCR analysis.

3. Elicitation of a protective antitumor immune response by skin-targeted noninvasive vaccine patches. To test the efficacy of a noninvasive vaccine in a disease setting, mice were challenged by subcutaneous injection of 3×10^5 JC-hcea cells, then monitored daily for mortality. **Figure 2** depicts mice immunized by AdCMV-hcea-based noninvasive vaccine patches compared to groups which were immunized by intramuscular injection of pGT37 DNA, intranasal inoculation of AdCMV-hcea, topical application of an irrelevant vector AdCMV-PR8.ha, or received no vaccines. Mice immunized by topical application of AdCMV-hcea were afforded 100% protection from the challenge. Animals immunized by intranasal inoculation of AdCMV-hcea or intramuscular injection of pGT37 were also protected. In contrast, those that were immunized by an irrelevant vector or received no vaccines had the highest mortality rate and sustained significant weight loss before they either died, or slowly recovered. It is interesting to note that protection did not correlate with the titer of anti-CEA (**Figure 1**). It is conceivable that a protective antitumor immune response may also involve cytotoxic T lymphocyte (CTL) responses that have not been measured in these studies.

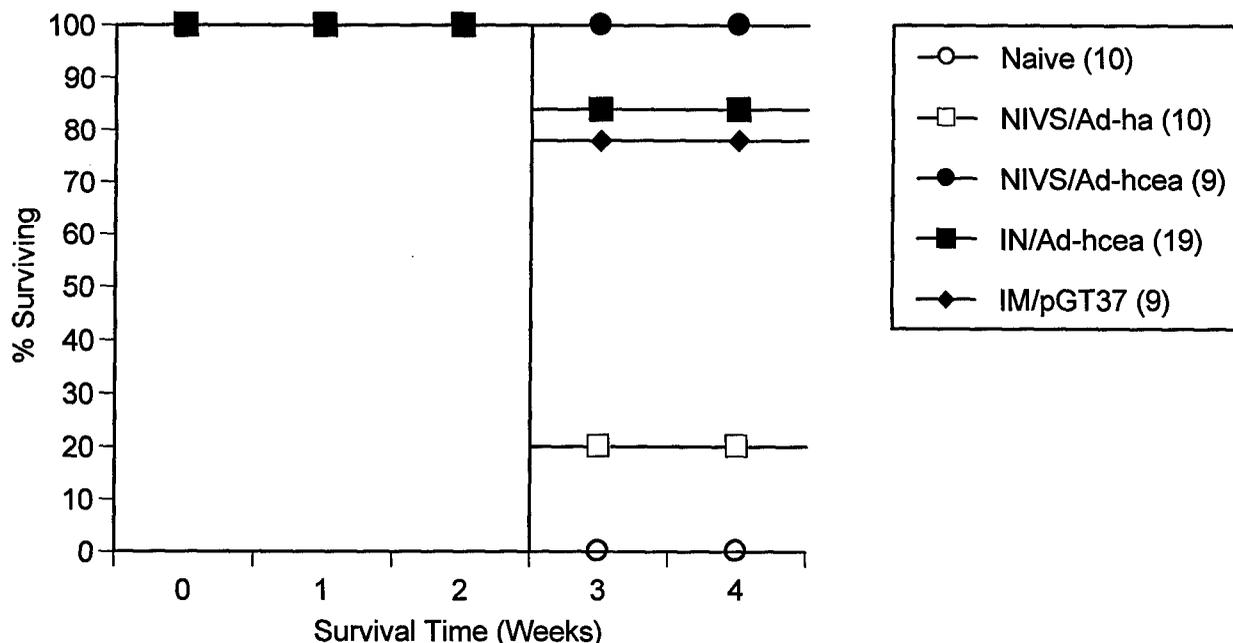
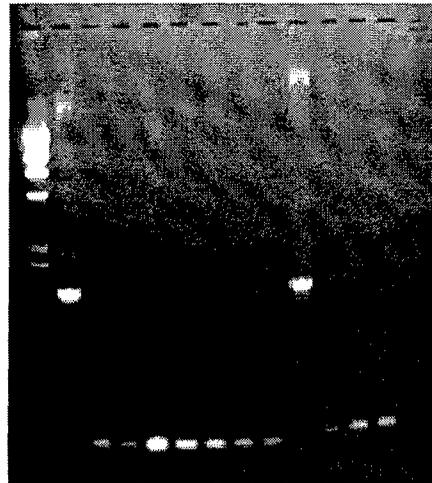


Figure 2. Protection from death after tumor challenge. BALB/c mice (3 months old) were immunized by a variety of vaccination modalities as described in **Figure 1** legend. One week after the last boost, mice were challenged subcutaneously with a lethal dose (3×10^5) of JC-hcea cells and monitored daily for survival. The data was plotted as % survival versus weeks after challenge. Naïve, mice received no vaccines; NIVS/Ad-ha, mice immunized by topical application of AdCMV-PR8.ha; NIVS/Ad-hcea, mice immunized by topical application of AdCMV-hcea; IN/Ad-hcea, mice immunized by intranasal inoculation of AdCMV-hcea; IM/pGT37, mice immunized by intramuscular injection of pGT37 DNA. Numbers in parentheses represented the number of animals for each treatment.

4. Relocation and degradation of foreign DNA after localized gene delivery in a noninvasive mode. In an attempt to determine whether topical application of an adenovirus vector could deliver foreign DNA beyond the inoculation area, we extracted DNA from various tissues, followed by amplification of the transgene as well as the adenovirus type 5 fiber gene by PCR after noninvasive delivery of AdCMV-luc (7) into neck skin. As shown in **Figure 3**, the full-length luciferase and fiber genes could be amplified from neck skin 3 hours post-inoculation. The full-length gene was usually undetectable in neck skin DNA after 1 day or in DNA extracted from other tissues. However, subfragments of both luciferase and fiber genes could be amplified from liver, whole blood, ear, abdominal skin, or lymph nodes using different sets of primers. No foreign DNA was detectable in any of the tissues 4 weeks post-inoculation. Results suggested that topical application of an adenovirus vector could deliver foreign DNA into a localized area in skin, although foreign DNA may be rapidly acquired by other cell types, degraded, and relocated into deep tissues. The elimination of foreign DNA in 4 weeks highlighted the safety of NIVS.



M 1 2 3 4 5 6 7 8 9 10 11 12

Figure 3. Amplification of foreign DNA in various tissues after localized gene delivery in a noninvasive mode. AdCMV-luc was inoculated onto neck skin in a noninvasive mode as described (4). DNA was extracted by DNAZOL (GIBCOBRL), and amplified by the following sets of primers:

Luc5.1: GCGCCATTCTATCCTCTAGA
 Luc3.1: ACAATTTGGACTTTCCGCCC

Luc5.2: GTACCAGAGTCCTTTGATCG
 Luc3.2: CCCTCGGGTGTAATCAGAAT

Fb5.1: CCGTCTGAAGATACCTTCAA
 Fb3.1: ACCAGTCCCATGAAAATGAC

Fb5.2: GGCTCCTTTGCATGTAACAG
 Fb3.2: CCTACTGTAATGGCACCTGT

Luc5.1 and Luc3.1 amplifies the 1.7 Kb full-length luciferase gene; Luc5.2 and Luc3.2 amplifies an 0.52 kb subfragment encompassing the central portion of the luciferase gene; Fb5.1 and Fb3.1 amplifies the 1.7 kb full-length adenovirus type 5 fiber gene; Fb5.2 and Fb3.2 amplifies an 0.55 kb subfragment encompassing the central portion of the fiber gene. Lane M, Molecular weight marker (Lambda DNA cleaved with HindIII); lane 1, full-length luciferase gene amplified by Luc5.1 and Luc3.1 from neck skin DNA 3 hours after NIVS; lane 2, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from neck skin DNA 3 hours after NIVS; lane 3, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from neck skin DNA 20 hours after NIVS; lane 4, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from mouse ear DNA 20 hours after NIVS; lane 5, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from abdominal skin DNA 20 hours after NIVS; lane 6, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from liver DNA 20 hours after NIVS;

lane 7, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from DNA extracted from whole blood 20 hours after NIVS; lane 8, a subfragment of luciferase DNA amplified by Luc5.2 and Luc3.2 from lymph node DNA 7 days after NIVS; lane 9, full-length fiber gene amplified by Fb5.1 and Fb3.1 from neck skin DNA 3 hours after NIVS; lane 10, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from neck skin DNA 3 hours after NIVS; lane 11, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from neck skin DNA 20 hours after NIVS; lane 12, a subfragment of fiber DNA amplified by Fb5.2 and Fb3.2 from mouse ear DNA 20 hours after NIVS. DNA from lymph nodes was extracted by pooling superficial cervical lymph nodes and axillary lymph nodes in DNAZOL solution. DNA was amplified for 35 cycles at optimized annealing temperatures in a Stratagene Robocycler gradient 40 thermal cycler. Amplified DNA fragments were fractionated in 1% agarose gel and stained with ethidium bromide.

CONCLUSIONS

We have demonstrated that noninvasive application of an adenovirus vector encoding a tumor-associated antigen onto the skin could elicit an immune response against the antigen, and protect vaccinees against tumor challenges. Evidence suggested that the efficacy of a "vaccine patch" may be even greater than that achievable by the commonly used intramuscular route, possibly due to the immunocompetence of the outer layer of skin. We envision that a noninvasive vaccine patch may emerge as a novel vaccination modality in a few years because the procedure is simple, effective, economical, painless, and safe. It may also boost vaccine coverages due to patient comfort.

REFERENCES

1. Eisenbraun, M. D., Fuller, D. H., and Haynes, J. R. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization, *DNA Cell Biol.* 12: 791-797, 1993.
2. Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations, *Proc. Natl. Acad. Sci. USA.* 90: 11478-11482, 1993.
3. Tang, D.-c., Shi, Z., and Curiel, D. T. Vaccination onto bare skin, *Nature.* 388: 729-730, 1997.
4. Shi, Z., Curiel, D. T., and Tang, D.-c. DNA-based non-invasive vaccination onto the skin, *Vaccine.* 17: 2136-2141, 1999.
5. Conry, R. M., LoBuglio, A. F., Kantor, J., Schlom, J., Loechel, F., Moore, S. E., Sumerel, L. A., Barlow, D. L., Abrams, S., and Curiel, D. T. Immune response to a carcinoembryonic antigen polynucleotide vaccine, *Cancer Res.* 54: 1164-1168, 1994.
6. Gunning, P., Leavitt, J., Muscat, G., Ng, S.-Y., and Kedes, L. A human beta-actin expression vector system directs high-level accumulation of antisense transcripts, *Proc. Natl. Acad. Sci. USA.* 84: 4831-4835, 1987.
7. Tang, D.-c., Jennelle, R. S., Shi, Z., Garver, R. I., Jr., Carbone, D. P., Loya, F., Chang, C.-H., and Curiel, D. T. Overexpression of adenovirus-encoded transgenes from the cytomegalovirus immediate early promoter in irradiated tumor cells, *Hum. Gene Ther.* 8: 2117-2124, 1997.

DNA-based non-invasive vaccination onto the skin

Zhongkai Shi^{a, b, c, d}, David T. Curiel^{a, b, c, d}, De-chu Tang^{a, b, c, d, e, *}

^a*Vaxin Pharmaceuticals, Inc., Birmingham, AL 35209, USA*

^b*Gene Therapy Center, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA*

^c*Department of Medicine, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA*

^d*Division of Pulmonary and Critical Care Medicine, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA*

^e*Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294-0006, USA*

Received 21 May 1998; received in revised form 3 December 1998; accepted 4 December 1998

Abstract

Non-invasive vaccination onto the skin (NIVS) could improve vaccination programs because the procedure requires no specially trained personnel and may eliminate many problems associated with needle injections. There is also evidence that the efficacy of a skin-targeted vaccine may be optimal when the antigen is expressed within the outer layer that is in constant contact with potential pathogens. We report here that non-invasive gene delivery by pipetting adenovirus- or liposome-complexed plasmid DNA onto the outer layer of skin could achieve localized transgene expression within a restricted subset of skin in mice and the elicitation of an immune response against the protein encoded by the DNA. These results provide a proof of principle that NIVS may appear as a novel method for the administration of DNA-based vaccines. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: DNA-based vaccine; Non-invasive vaccine; Skin-targeted vaccine

1. Introduction

Vaccination usually requires needle injections by medical personnel. Non-invasive vaccination onto the skin (NIVS) by expressing antigens in the outer layer of skin [1] not only may allow the administration of vaccines by individuals without medical training or equipment, but may also elicit more potent immune responses than conventional needle injections given equivalent doses due to the immunocompetence of epidermis along the skin border [2]. We report here that NIVS using DNA-based expression vectors was able

to elicit a systemic immune response against the protein encoded by the vector. Unlike inoculation of DNA-based vaccines using a gene gun [3] or a needle [4], the procedure is non-invasive and requires no special skill or equipment. In contrast to NIVS using adenovirus (Ad) recombinants [1], construction and preparation of recombinant plasmid DNA is technically less demanding. Re-vaccination by DNA-based vaccines is also possible [3]. Although NIVS using protein-based vaccines has recently been demonstrated [5], DNA-based vaccines can be purified at lower costs than their protein-based counterparts, and may be able to stimulate a broader spectrum of immune responses for achieving greater efficacy similar to natural infections [6].

* Corresponding author. Tel.: +1-205-975-5603; fax: +1-205-975-0455; e-mail: tang@vaxin.com.

2. Materials and methods

2.1. Cell cultures

Human 293 cells for the propagation of AdCMV-luc [7] were cultured in RPMI medium 1640. W162 cells for the propagation of Ad dl1014 [8] were cultured in DMEM/F12 medium. All media contained 2% fetal bovine serum and 6% calf serum.

2.2. Preparation of DNA/Ad and DNA/liposome complexes

DNA/Ad complexes were prepared by mixing 100 µg plasmid DNA to 1×10^{11} particles of Ad dl1014 for each inoculation. Ad particles were chemically linked to polylysine as described [9] before reacting with DNA. The DNA/Ad complex was further condensed with polylysine. The titer of Ad was determined by absorbance as described [10]. DNA/liposome complexes were prepared by mixing 100 µg plasmid DNA with 100 µg DOTAP/DOPE (1:1; Avanti) for each inoculation. Plasmids were prepared using Qiagen Plasmid Maxi Kits.

2.3. Skin-targeted non-invasive gene delivery

Mice (C57BL/6 strain; 3–8 months old; Jackson) were anesthetized and hair covering a restricted area of neck skin was removed with a WAHL cordless trimmer (Model 8900). The shaved skin was further treated with a depilatory (e.g. Nair) which potentially may facilitate the removal of more cornified epithelium. Ad recombinants, DNA/Ad complexes or DNA/liposome complexes were pipetted into a plastic cylinder (made by drilling a hole through the cap of a Nalgene cryogenic vial) that was glued onto the pre-shaved neck of a mouse. Expression vectors were allowed to incubate with naked skin for 1–18 h. Animal care was in accordance with institutional guidelines.

2.4. Luciferase assay

A piece of excised skin was homogenized and luciferase activity in the skin extract was determined with a luminometer by measurement of integrated light emission for 2 min using the Promega's luciferase assay system.

2.5. Western blot analysis

Sera from tail bleeds were diluted 1:250 and reacted with purified human growth hormone protein (hGH) (CalBiochem) that had been separated in a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane (Millipore) as described [1].

2.6. ELISA for quantitating anti-hGH antibodies

Titers of anti-hGH IgG were determined by ELISA as described [11] using purified hGH as the capture antigen. Serum samples and peroxidase-conjugated goat anti-mouse IgG (Promega) were incubated sequentially on the plates for 1 h at RT with extensive washing between each incubation. The serum samples were diluted in 10-fold increments. The end-point titer was calculated as the dilution of serum producing the same OD₄₉₀ as a 1/100 dilution of preimmune serum.

3. Results

3.1. Skin-targeted non-invasive gene delivery

As an initial step for the development of DNA-based NIVS, we expressed exogenous genes in the skin of mice by pipetting DNA/Ad or DNA/liposome complexes onto naked skin. As shown in Fig. 1, minute amounts of luciferase could be produced in the skin after incubating naked skin with AdCMV-luc particles (an adenovirus vector encoding luciferase driven by the human cytomegalovirus (CMV) promoter) [7], pVR-1216 DNA (a plasmid expression vector encoding luciferase driven by the CMV promoter) complexed with the E4-defective Ad dl1014 [8] or pVR-1216 DNA complexed with DOTAP/DOPE liposomes. No luciferase was detectable in internal organs (e.g. muscle, liver, spleen, heart, lung and kidney) after topical application of expression vectors. The level of transgene expression from Ad recombinants in the skin was on average higher than that from DNA/Ad complexes, which was higher than that from DNA/liposome complexes. The amount of protein produced may potentially be amplified by incubating more vectors with a larger area of skin for a longer period of time. Topical application of pVR-1216 DNA alone without complexing to Ad particles or liposomes produced no measurable luciferase activity in the skin.

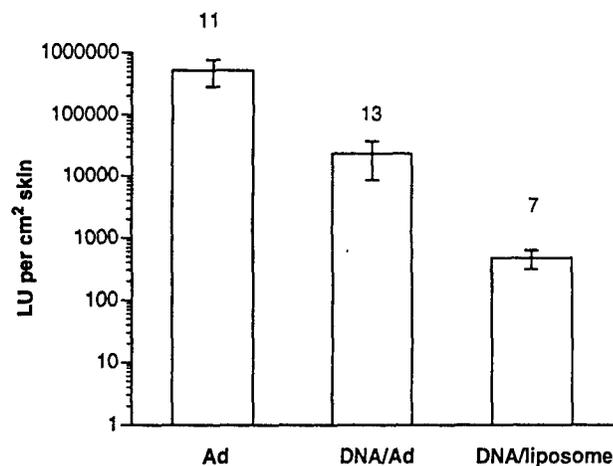


Fig. 1. Skin-targeted non-invasive gene delivery. Mice were inoculated with expression vectors encoding luciferase in a non-invasive mode as described in Section 2. The treated skin was removed at the end of the 18-h incubation period, homogenized and assayed for luciferase activity and background was subtracted from the readings. Mice mock-inoculated or inoculated with DNA alone produced no detectable luciferase activity in the skin. LU, light units; Ad, AdCMV-luc; DNA/Ad, pVR-1216 DNA complexed with Ad dl1014; DNA/liposome, pVR-1216 DNA complexed with DOTAP/DOPE. Results are the mean log[LU per cm² skin] \pm S.E. (*n* is shown on top of each column).

3.2. DNA-based NIVS

The expression of transgenes in the skin from topically-applied DNA/Ad or DNA/liposome complexes suggests that these complexes may be formulated as components in skin-targeted non-invasive vaccines. To determine whether the amount of antigen produced in the skin from a topically-applied vector was sufficient for eliciting an immune response, an expression plasmid encoding hGH (pCMV-GH) [3] was complexed with either Ad dl1014 or DOTAP/DOPE liposomes. Mice were subsequently vaccinated by incubating DNA/Ad or DNA/liposome complexes with a restricted subset of pre-shaved skin for 1–18 h. Only a small fraction of vectors could be absorbed by the skin as shown by the ability to retrieve most DNA from the skin surface an hour after incubation. No physical abrasions were found in the skin tissue after incubation, and there was no inflammation associated with the treated skin. Immunized animals were subsequently monitored for the production of antibodies against hGH by assaying sera from tail-bleeds. A month after incubating DNA with naked skin, the test sera from representative mice could react in western blots with purified hGH, but not with bovine serum albumin (BSA) (Fig. 2). Pre-immunization sera, sera from untreated animals and sera from animals vaccinated with irrelevant vectors all failed to react with hGH. Of

7 mice vaccinated by DNA/Ad complexes, all (100%) produced antibodies against hGH within 7 months (Table 1). Of 12 mice vaccinated by DNA/liposome complexes, all (100%) treated animals produced antibodies against hGH (Table 1). The possibility of oral vaccination by ingesting DNA complexes through grooming was eliminated by cleaning the neck skin after removing the DNA-containing cylinder and by mixing naive and vaccinated animals in the same cages. No cross-vaccination between naive and vaccinated mice was ever observed.

The titer of antibodies induced by topical application of DNA/Ad complexes was about 10-fold lower than that elicited by intramuscular injection (IM) of 50 μ g of pCMV-GH DNA (Table 1). ELISA showed that DNA/liposome complexes were even less potent than DNA/Ad complexes for eliciting an immune response (Table 1), probably due to the low efficiency of skin-targeted gene delivery (Fig. 1). To demonstrate the feasibility that DNA-based vaccines could be vaccinated animals in a non-invasive mode, we incubated naked skin of 3 naive mice with DNA/Ad complexes containing Ad dl1014 complexed with irrelevant plasmid DNA (e.g. pGT37 DNA [12]). As shown in Table 1, antibodies against hGH were still induced when animals with pre-exposure to Ad dl1014 were immunized 9 weeks later by topical application of pCMV-GH DNA/Ad complexes.

4. Discussion

Vaccinating animals or humans by delivering DNA-based vaccines onto the outer layer of skin in a non-invasive mode is an appealing strategy provided that extrinsic antigens can be expressed in viable cells in the authentic skin tissue environment in sufficient quantities for eliciting immunity. We have shown that the production of very small amounts of protein in the skin (Fig. 1) was sufficient for eliciting a systemic immune response (Table 1 and Fig. 2) which may have arisen as a result of antigen expression in a limited number of cells *in vivo*. The amount of DNA absorbed by the skin during incubation has not been quantitatively measured although it must be small as reflected by the amount of protein produced and the ability to retrieve most DNA from the skin surface. It has been determined that a humoral immune response can be elicited in a mouse by inoculating 40 ng of plasmid DNA into the skin using a gene gun and a cellular immune response can be induced with 0.4 ng of plasmid DNA [13]. Because a fraction of the DNA may be scraped off gold microprojectiles

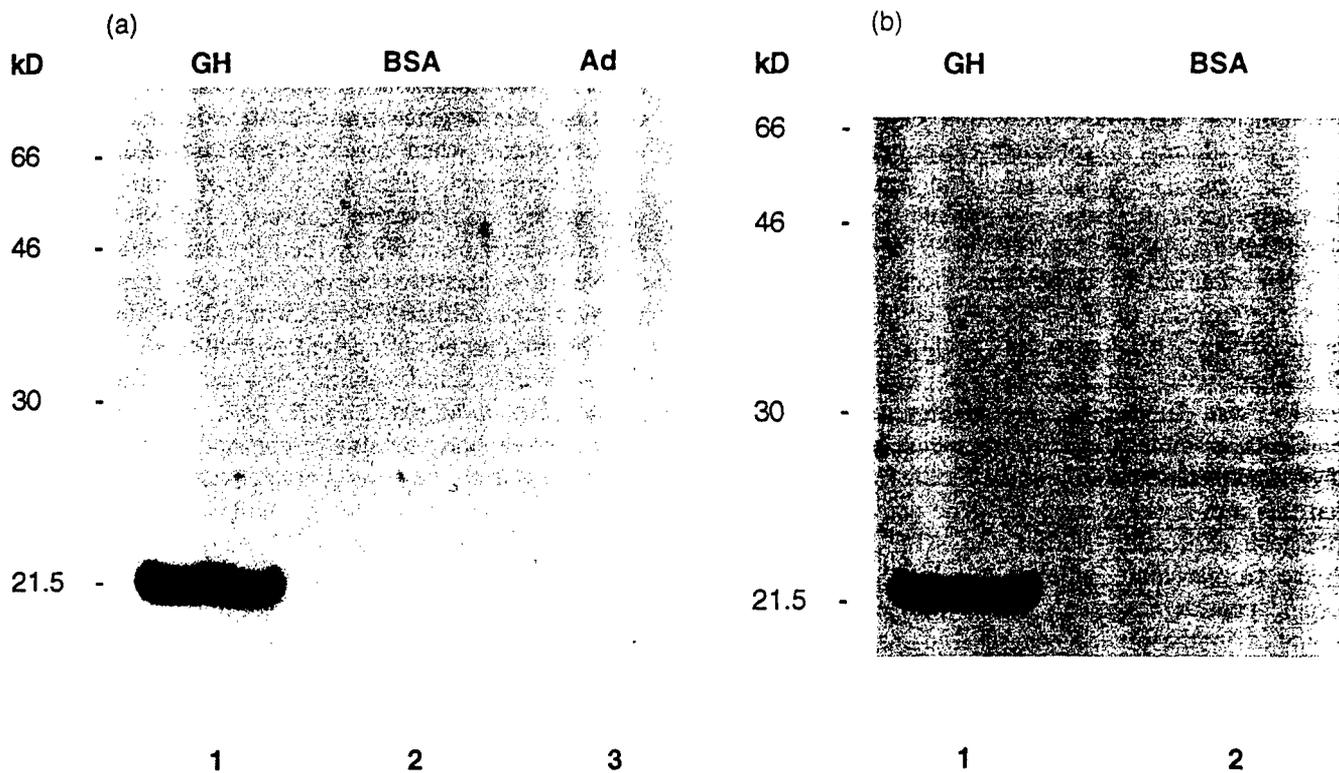


Fig. 2. (a) DNA/Ad-mediated NIVS. Serum from a mouse vaccinated by topical application of pCMV-GH DNA/Ad complexes was analyzed for the presence of anti-hGH antibodies by western blot analysis. Lane 1, hGH (1 µg); lane 2, BSA (1 µg); lane 3, Ad dl1014 (10^{10} particles). (b) DNA/liposome-mediated NIVS. Serum from a mouse vaccinated by topical application of pCMV-GH DNA/liposome complexes was analyzed for the presence of anti-hGH antibodies. Lane 1, hGH (1 µg); lane 2, BSA (1 µg). Animals were immunized by NIVS and western blots were analyzed as described in Section 2.

during a flight when DNA is inoculated into tissues with a gene gun, the minimal amount of DNA that is required for eliciting humoral and cellular immune responses by expressing antigens in the skin may be even less than 40 and 0.4 ng, respectively. The minute amount of DNA that is required for vaccinating an animal via the skin route highlights the immunocom-

petence of the outer layer of skin and the safety of DNA-based NIVS.

Expression vectors applied onto the skin in a non-invasive mode presumably could penetrate into the body via hair follicles, sweat ducts or minor breaches in the skin. The principal target cells for topically-applied vectors have been identified utilizing either an

Table 1
Summary of the immune responses in mice following DNA-based vaccination

Vector ^a	Pre-exposure to Ad	Mode	Weeks post-immunization	Number of boost	Number of mice producing anti-GH	Anti-hGH IgG serum titer
DNA/Ad	-	NIVS	28	0	2/2	1,000
DNA/Ad	-	NIVS	14	2	5/5	1,000-10 000
DNA/liposome	-	NIVS	28	0	2/2	1,000
DNA/liposome	-	NIVS	22	3	10/10	1,000
DNA	-	IM	12	0	4/4	10,000-100 000
DNA/Ad ^b	+	NIVS	37	1	3/3	10 000

^a C57 BL/6 mice were immunized by NIVS or IM injection of DNA. DNA/Ad, pCMV-GH DNA complexed with Ad dl1014; DNA/liposome, pCMV-GH DNA complexed with DOTAP/DOPE; DNA, 50 µg pCMV-GH DNA dissolved in saline (1 mg/ml) was injected into the tongue muscle of an anesthetized mouse. ^b Mice were exposed to Ad by topical application of Ad dl1014 complexed with irrelevant DNA (e.g. pGT37 DNA [12]) as described in Section 2. Nine weeks later, animals with pre-exposure to Ad were immunized by non-invasive vaccines containing pCMV-GH DNA complexed with Ad dl1014.

Ad vector encoding β -galactosidase [14] or liposome-complexed plasmid DNA encoding β -galactosidase [15, 16]. Cells within hair follicles [14-16], interfollicular keratinocytes within epidermis [14, 15], as well as dermal fibroblasts [15] appeared as target cells for topically-applied expression vectors. Although the target cells for topically-applied DNA/Ad complexes have not been studied, it is conceivable that they are identical to those transduced by Ad vectors since the tropism of the DNA/Ad complex should be mediated by Ad particles within the complex. Consistent with our finding that no luciferase expression was detected in internal organs including the muscle layer underlying the treated skin, the absence of β -galactosidase-positive muscle cells [14-16] suggests that this non-invasive mode of gene delivery may limit transgene expression within the skin. However, the ability to vaccinate animals by NIVS implies that specific peptide fragments of the exogenous antigens produced in the outer layer of skin may be acquired by professional antigen-presenting cells (APCs) that are able to relocate to lymphoid organs or other sites in the body. Alternatively, a small number of APCs may be directly transfected by topically-applied vectors. Identification and characterization of these putative APCs may provide insights into the mechanisms of NIVS.

The possibility that animals may have been immunized by orally ingesting DNA has been eliminated as described above. It is unlikely that DNA (in the format as described in this report) can immunize animals orally by resisting digestive enzymes found in the gastrointestinal tract. In contrast to the hostile environment that oral vaccines encounter before they battle against pathogens, the skin surface is less destructive to biomolecules. Absorption of DNA by the skin may thus allow epidermal vaccines to be formulated with less sophistication than their oral counterparts. In future vaccination programs, it is conceivable that NIVS and other modes of immunization may complement each other because vaccination via different routes may elicit different immune responses by different mechanisms.

The E1-defective Ad vectors may not be able to vaccinate animals repeatedly as vaccine carriers, attributed to the immunogenicity of Ad vectors which impair Ad-mediated gene expression in animals with pre-existing immunity to Ad [17]. DNA/liposome complexes which do not contain any antigenic components other than the antigen encoded by the DNA should allow continued re-vaccination. It is promising that DNA/Ad complexes containing Ad vectors with reduced immunogenicity (e.g. E4-defective Ad [8], 'gutless' Ad

with viral genes deleted [18] or UV-inactivated Ad [10]) will allow re-vaccination or the induction of immune reactivity in animals with pre-exposure to Ad. Given the high skin-targeted transfection efficiency of DNA/Ad complexes when compared to that of DNA/liposome complexes (Fig. 1), a higher antibody titer induced by non-invasive delivery of DNA/Ad complexes over that elicited by their liposome counterparts (Table 1), a persistent wave of *in vivo* transgene expression from either E4-defective Ad [19] or 'gutless' Ad [20] in immunocompetent animals and the ability to immunize animals with pre-exposure to Ad by DNA/Ad complexes (Table 1), it is likely that DNA/Ad complexes may be able to consolidate the high efficiency of Ad for *in vivo* gene delivery, the ease with plasmid manipulation and the ability to re-vaccinate animals into one formula for the development of skin-targeted non-invasive vaccines.

Although IM injection of DNA could elicit a more potent immune response than DNA-based NIVS (Table 1), the amount of DNA absorbed by the skin during NIVS was probably only a small fraction of that injected into muscle. NIVS is thus potentially safer than injection of a large dose of DNA into deep tissues. It is conceivable that the efficacy of DNA-based NIVS may be improved by (1) covering a large area of skin for a long period of time, (2) developing a new generation of skin-targeted vectors with a higher *in situ* transfection efficiency and (3) developing specific adjuvants for NIVS. In contrast to IM injection of pCMV-GH DNA which is capable of eliciting an immune response, intradermal injection of naked DNA appeared as an ineffective vaccination mode for this specific vector [3]. Although direct comparisons between topically-applied DNA/Ad or DNA/liposome complexes and their intradermally-injected counterparts have not been made, it was reported that the deeper the DNA was inoculated into the skin, the less potent the vaccine was [2]. Animals may have evolved an immune surveillance mechanism within epidermis for warding off potential infections along the skin border. We hypothesize that the expression of antigens in a small number of cells within the outer layer of skin can activate the surveillance mechanism and subsequently result in an immune response against the antigen encoded by the vector.

The immunologic competence of the skin, the ease with which genes can be targeted to defined sites on the skin, the rapid turn-over of skin cells, the efficacy of DNA-based vaccines and our finding that animals can be immunized by DNA-based NIVS, may allow for the development of a unique method for vaccination. We envision that skin-targeted non-invasive

vaccines could be delivered by a patch containing a uniform dose of DNA. Since DNA-based NIVS is simple, economical, painless and potentially safe, it may be able to boost vaccine coverages in a wide variety of disease settings.

Acknowledgements

We thank M. Alder and R. Young for their support; A. Graf, E. Sorscher and A. LoBuglio for critical reading of the manuscript; D. Marks, S. Johnston, T. Strong, A. Takashima and M. Olman for lively discussion and Vical for providing the plasmid pVR-1216. This work was supported by a UAB start-up fund, an ALA grant, a US Army idea award and a NIH SBIR grant to D.T. Z.S. was supported by the World Health Organization and the Emerging Technology Partners.

References

- [1] Tang D-c, Shi Z, Curiel DT. Vaccination onto bare skin. *Nature* 1997;388:729–30.
- [2] Eisenbraun MD, Fuller DH, Haynes JR. Examination of parameters affecting the elicitation of humoral immune responses by particle bombardment-mediated genetic immunization. *DNA Cell Biol.* 1993;12:791–7.
- [3] Tang D-c, DeVit M, Johnston SA. Genetic immunization is a simple method for eliciting an immune response. *Nature* 1992;356:152–4.
- [4] Ulmer JB, Donnelly JJ, Parker SE et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 1993;259:1745–8.
- [5] Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin. *Nature* 1998;391:851.
- [6] McDonnell WM, Askari FK. DNA vaccines. *N. Engl. J. Med.* 1996;334:42–5.
- [7] Tang D-c, Jennelle RS, Shi Z et al. Overexpression of adenovirus-encoded transgenes from the cytomegalovirus immediate early promoter in irradiated tumor cells. *Human Gene Ther.* 1997;8:2117–24.
- [8] Scaria A, Curiel DT, Kay MA. Complementation of a human adenovirus early region 4 deletion mutant in 293 cells using adenovirus-polylysine-DNA complexes. *Gene Ther.* 1995;2:295–8.
- [9] Nguyen DM, Wiehle SA, Roth JA, Cristiano RJ. Gene delivery into malignant cells in vivo by a conjugated adenovirus/DNA complex. *Cancer Gene Ther.* 1997;4:183–90.
- [10] Cotten M, Wagner E, Zatloukal K, Phillips S, Curiel DT, Birnstiel ML. High-efficiency receptor-mediated delivery of small and large (48 kilobase) gene constructs using the endosome-disruption activity of defective or chemically inactivated adenovirus particles. *Proc. Natl. Acad. Sci. USA* 1992;89:6094–8.
- [11] Deck RR, DeWitt CM, Donnelly JJ, Liu MA, Ulmer JB. Characterization of humoral immune responses induced by an influenza hemagglutinin DNA vaccine. *Vaccine* 1997;15:71–8.
- [12] Conry RM, LoBuglio AF, Loechel F et al. A carcinoembryonic antigen polynucleotide vaccine for human clinical use. *Cancer Gene Ther.* 1995;2:33–8.
- [13] Johnston SA, Barry MA. Genetic to genomic vaccination. *Vaccine* 1997;15:808–9.
- [14] Lu B, Federoff HJ, Wang Y, Goldsmith LA, Scott G. Topical application of viral vectors for epidermal gene transfer. *J Invest. Dermatol.* 1997;108:803–8.
- [15] Alexander MY, Akhurst RJ. Liposome-mediated gene transfer and expression via the skin. *Human Mol. Genet.* 1995;4:2279–85.
- [16] Li L, Hoffman RM. The feasibility of targeted selective gene therapy of the hair follicle. *Nature Med.* 1995;1:705–6.
- [17] Yang Y, Nunes FA, Berencsi K, Furth EE, Gonczol E, Wilson JM. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc. Natl. Acad. Sci. USA* 1994;91:4407–11.
- [18] Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc. Natl. Acad. Sci. USA* 1996;93:13565–70.
- [19] Wang Q, Greenburg G, Bunch D, Farson D, Finer MH. Persistent transgene expression in mouse liver following in vivo gene transfer with a delta E1/delta E4 adenovirus vector. *Gene Ther.* 1997;4:393–400.
- [20] Chen HH, Mack LM, Kelly R, Ontell M, Kochanek S, Clemens PR. Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc. Natl. Acad. Sci. USA* 1997;94:1645–50.



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)

21 Feb 03

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

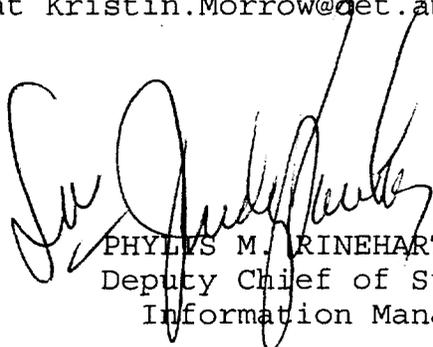
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers 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. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

ADB263458	ADB282838
ADB282174	ADB233092
ADB270704	ADB263929
ADB282196	ADB282182
ADB264903	ADB257136
ADB268484	ADB282227
ADB282253	ADB282177
ADB282115	ADB263548
ADB263413	ADB246535
ADB269109	ADB282826
ADB282106	ADB282127
ADB262514	ADB271165
ADB282264	ADB282112
ADB256789	ADB255775
ADB251569	ADB265599
ADB258878	ADB282098
ADB282275	ADB232738
ADB270822	ADB243196
ADB282207	ADB257445
ADB257105	ADB267547
ADB281673	ADB277556
ADB254429	ADB239320
ADB282110	ADB253648
ADB262549	ADB282171
ADB268358	ADB233883
ADB257359	ADB257696
ADB265810	ADB232089
ADB282111	ADB240398
ADB273020	ADB261087
ADB282185	ADB249593
ADB266340	ADB264542
ADB262490	ADB282216
ADB266385	ADB261617
ADB282181	ADB269116
ADB262451	
ADB266306	
ADB260298	
ADB269253	
ADB282119	
ADB261755	
ADB257398	
ADB267683	
ADB282231	
ADB234475	
ADB247704	
ADB258112	
ADB267627	