Gene Painting as a Simple Method for Vaccinating Animals Against Breast Cancer Micro-Metastases

De-chu Tang, Ph.D.

University of Alabama at Birmingham
Birmingham, Alabama 35294-0111
E-Mail: dctang@uab.edu

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 coverage due to patient comfort.
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 and effective 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 (3, 13), and 2) genetic vaccines inoculated into the epidermis using a gene gun are more potent than those injected intramuscularly (5). 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) (16) 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 coverage 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

Task 1. Construction of a target cell line.

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 (2) with pHßAPr-1-neo (8) 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.

Task 2. Construction of a new adenovirus vector with reduced immunogenicity as carriers for cancer vaccines.

Adenovirus vectors with all viral genes deleted (gutless adenovirus vectors) were developed using the Cre-loxP recombination system (11). These vectors not only accommodate large inserts, but also may allow repeated expression of antigens in animals due to their reduced immunogenicity (1). A gutless adenovirus vector system (i.e., pRP1001, AdLC8cluc, and 293Cre4 cells) has been made available to us from the Merck Research Laboratories. However, a problem with the current gutless adenovirus system is the contamination of the viral stock by helper virus (11) because efficiency of the Cre-loxP recombination system for excising the packaging signal is not 100%. Our vector preparation contained substantial amounts of the AdLC8cluc helper virus during our preliminary studies. Although the vector/helper ratio may be improved after prolonged propagation in 294cre cells due to a selective disadvantage for the helper virus to be packaged, the procedure is time-consuming and the viral stock can hardly be helper-free. To this end, we are taking a novel approach for constructing helper-free gutless adenovirus vectors by co-transfecting pRP1001-based plasmids with a packaging signal (7)-free helper plasmid. To construct a helper plasmid without a packaging signal (AdEasy-hp), the left ITR (inverted terminal repeat) of adenovirus was amplified from the pShuttle plasmid (10) using two primers 5'-CGGGGATCGATGCGCATCATCAATAATATACCTTATTT-3' and 5'-ATATCGATACAACATCCGCCTAAAACCGCGCG-3'. pAdEasy-hp was constructed by cloning the left ITR sequence into the unique Cla I site of the pAdEasy-1 plasmid which lacks the left ITR, packaging signal, and E1 region (10). When pRP1001-based plasmids are co-transfected with pAdEasy-hp into human 293 cells, the E1 function will be provided in trans by the host whereas all other adenoviral proteins may be produced by pAdEasy-hp. Because pAdEasy-hp contains two functional ITRs as the origin of replication, it may replicate as an autonomous replicon in 293 cells (9), and late gene transcripts may be terminated correctly after DNA synthesis. Since pAdEasy-hp does not contain a packaging signal, none of its DNA molecules can be packaged into infectious particles. pAdEasy-hp may thus support the production of helper-free gutless adenovirus vectors.

Task 3. Develop DNA-based gene painting schemes.
We are the first to have demonstrated that an immune response could be elicited by topical application of adenovirus recombinants (13, 16), DNA:adenovirus complexes (12), or DNA:liposome complexes (12). Others have shown that the immune system could also be activated to some extent following topical application of cholera toxin protein (6) or naked DNA (4). However, emerging evidence suggests that the adenovirus-based vector system is more promising than the DNA-based system as a carrier for skin-targeted noninvasive vaccines because antigen expression from an adenovirus vector is more efficient than DNA-mediated gene expression following topical application (12, 13). The problem of eliciting an anti-adenovirus immune response which may interfere with subsequent cycles of immunization following topical application of the E1/E3-defective adenovirus vector may readily be circumvented by booster applications (13) and/or the development of the helper-free gutless adenovirus vector system (Task 1).

Task 4. Vaccination of animals against mammary tumors by gene painting.

1. Elicitation of anti-CEA antibodies in mice. We have experience in immunizing animals with a noninvasive vaccine patch (12, 13, 16). Figure 1 shows that antisera against CEA could be induced in mice by a vaccine patch containing AdCMV-hcea (an adenovirus vector encoding CEA) (16). 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) (2), 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 (13). 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 naive mice and mice immunized by topical application of an irrelevant vector all failed to elicit anti-CEA.

2. 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 $3 \times 10^6$ 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.

3. In vivo cytotoxicity assay. To analyze the antitumor immune response in an in vivo setting at an early stage following tumor challenge, JC-hcea cells were grown on a small disk and implanted onto muscle as described (14). Figures 3A and 3C show histologically that JC-hcea cells proliferated from a monolayer to a tumor nodule after 5 days of in vivo growth in a naïve animal. In contrast to the naïve control, the implanted JC-hcea cells were nearly eradicated after 5 days of in vivo growth in animals immunized by topical application of AdCMV-hcea (Figures 3B and 3D). Moreover, a large number of immune effectors infiltrated into the implantation bed concomitantly with the eradication of breast tumor cells (Figures 3B and 3D). Histologic evidence thus supports the hypothesis that the death of tumor cells was mediated by a potent antitumor cellular immune response.

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 (15) into neck skin. As shown in Figure 4, 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.

Task 5. Compare gene painting with other modes of genetic immunization.

We have compared gene painting with intramuscular injection of DNA-based vaccines. As shown in Figures 1 and 2, topical application of $10^8$ pfu AdCMV-hcea was more potent in eliciting an anti-CEA antibody response as well as a protective immune response against tumor challenge than intramuscular injection of 100 μg (equivalent to $10^{13}$ copies) pGT-37 DNA. We envision that only a small number of AdCMV-hcea particles could be absorbed by skin following topical application of $10^8$ pfu. Results provide solid evidence that the outer layer of skin is more immunocompetent than muscle, and the surface of skin is an effective target site for vaccine administration.
KEY RESEARCH ACCOMPLISHMENTS

- Topical application of an adenovirus vector is capable of protecting animals against a lethal dose of tumor challenge. Although several different laboratories have shown that a humoral immune response could be elicited by topical application of adenovirus vectors (13, 16), DNA:adenovirus complexes (12), DNA:liposome complexes (12), naked DNA (4), or cholera toxin protein (6), this is the first demonstration that topical application of vaccines without causing tissue damage is able to protect animals against cancers in a disease setting.

- The in vivo cytotoxicity assay (14) provided evidence that the eradication of breast tumor cells in vivo may have been mediated by a potent antitumor cellular immune response.

- We have demonstrated that the outer layer of skin is more immunocompetent than muscle (13). This observation makes biological sense because the outer layer of skin is in frequent contact with environmental pathogens, and should be the focus of immunosurveillance.

- Construction of a helper-free gutless adenovirus system is underway.

- We have demonstrated that the skin does not allow environmental DNA to persist. Subfragments of degraded vector DNA could traffic to a variety of tissues following topical application, presumably via antigen-presenting cells.
REPORTABLE OUTCOMES

• Publications:


• Oral Presentations:
Skin-targeted noninvasive vaccination against mammary tumor cells. *Era of Hope Department of Defense Breast Cancer Research Program Meeting.* Atlanta, Georgia, June 11, 2000


• Patent: Allowance of the U.S. patent "Vaccination by topical application of genetic vectors" (U.S. Serial No. 09/402,527)

• Special Award: Year 2000 Wallace H. Coulter Award for Innovation and Entrepreneurship with a stipend of $100,000

• Grant Awards:

Principal Investigator, 2001-2004, Office of Naval Research Grant (#N00014-01-1-0945): "Vaccination by topical application of recombinant vectored vaccines", $3,049,000


Co-Principal Investigator, 2001-2006, National Institutes of Health R01 grant (#1-R01-NS-43947-01): "Alzheimer vaccinations: Noninvasive vaccination by DNA-based vectors", $1,250,000

Collaborator, 2001, Department of Defense Small Business Innovation Research Program Phase I grant (#N00014-01-M-0178): "Needleless topical administration of dengue DNA vaccine", $69,000

Preceptor, 2000-2001, Dermatology Foundation Postdoctoral Fellowship: "Development of a skin-targeted vaccine patch against anthrax", $25,000

- **List of Personnel**
  De-chu C. Tang, PhD
  Zhongkai Shi, MD
  Mingtao Zeng, PhD
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 the foreseeable future because the procedure is simple, effective, economical, painless, and safe. It may also boost vaccine coverage due to patient comfort.
REFERENCES


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 μg of pGT37 DNA, intranasal inoculation (IN) with a dose of $2.5 \times 10^7$ 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 (12) 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 OD$_{490}$ 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.
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 \((3 \times 10^6)\) 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.
Figure 3. Analysis of antitumor immune responses by in vivo cytotoxicity assay. BALB/c mice (3 months old) were immunized by topical application of AdCMV-hcea as described in Figure 1 legend. One week after the last boost, 5 X 10^5 JC-hcea cells were implanted onto muscle as a monolayer using a small disk as described (14). After 5 days of in vivo growth, the implantation bed was cross sectioned, stained with hematoxylin and eosin, and examined under a light microscope. A: tissue section from the site of implantation of JC-hcea cells in a naïve mouse 5 days after implantation. Note the presence of a tumor layer on top of muscle (X33). B: tissue section from the site of implantation of JC-hcea cells in an AdCMV-hcea-based-vaccine-patch immunized mouse 5 days after implantation. Note the eradication of tumor cells and the infiltration of immune effectors into the implantation bed (X33). C: tissue section as shown in A was visualized at a higher magnification. Note the dominance of JC-hcea cells in the target cell layer with little immune intervention (X132). D: tissue section as shown in B was visualized at a higher magnification. Note the eradication of tumor cells and evidence for a potent immune intervention (X132).
Figure 4. 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 (12). DNA was extracted by DNAZOL (GIBCOBRL), and amplified by the following sets of primers:

Luc5.1: GCGCCATTCTATCCTCTAGA
Luc3.1: ACAATTTGGGACTTTCCC GCC
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 1.7Kb full-length luciferase gene; Luc5.2 and Luc3.2 amplifies an 0.52Kb subfragment encompassing the central portion of the luciferase gene; Fb5.1 and Fb3.1 amplifies the 1.7Kb full-length adenovirus type 5 fiber gene; Fb5.2 and Fb3.2 amplifies an 0.55Kb 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.
Protection against Tetanus by Needle-Free Inoculation of Adenovirus-Vectored Nasal and Epicutaneous Vaccines

ZHONGKAI SHI, MINGTAO ZENG, GUANG YANG, FELIX SIEGEL, LAURA J. CAIN, KENT R. VAN KAMPEN, CRAIG A. ELMETS, and DE-CHU C. TANG

Vaxin, Inc., and Department of Dermatology and Gene Therapy Center, University of Alabama at Birmingham, Birmingham, Alabama

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Tetanus continues to be a threat to public health with more than half a million fatalities each year being associated with infection with Clostridium tetani (23). Due to the ubiquitous distribution of the causal agent, vaccination is the most effective medical intervention for protection of the public against this deadly disease. The effectiveness of the vaccine is due, at least in part, to the fact that the sequences of the neurotoxin molecules are conserved among different strains of C. tetani, which permits elicitation of a protective immune response against all C. tetani strains through the use of a single vaccine (23). The effectiveness of the vaccine is limited, however, by the needle-based delivery method currently in use. Effective protection requires injection of three consecutive doses of the tetanus toxoid vaccine (23). The effectiveness of the vaccine is limited, however, by the needle-based delivery method currently in use. Effective protection requires injection of three consecutive doses of the tetanus toxoid vaccine (23). Moreover, booster injections must be administered periodically during adulthood to compensate for the age-related decline in antitoxin levels (16). In developing countries, vaccine coverage against this disease is generally low due to failure to follow up as well as a lack of the trained medical personnel and facilities required for administration of the vaccine. In developed countries, although vaccine coverage in childhood is high, there is a general lack of compliance of adults with recommended schedules for booster injections (16). These factors have led to the realization that tetanus vaccination programs would be improved significantly worldwide through the development of low-cost, needle-free vaccines.

Needle-free vaccination requires the development of novel vaccines that can be administered simply and, preferably, painlessly without the need for timed booster injections. Tetanus is a prime example of a disease that is readily preventable by vaccination but remains a major threat to public health due to the problems associated with administration of the present vaccine. Here we show that a protective immune response against live Clostridium tetani infection in mice can be elicited by an adenovirus vector encoding the tetanus toxin C fragment when administered as a nasal or epicutaneous vaccine. The results suggest that these vaccination modalities would be effective needle-free alternatives. This is the first demonstration that absorption of a small number of vectored vaccines into the skin following topical application of a patch can provide protection against live bacteria in a disease setting.

The effectiveness of vaccination programs would be enhanced greatly through the availability of vaccines that can be administered simply and, preferably, painlessly without the need for timed booster injections. Tetanus is a prime example of a disease that is readily preventable by vaccination but remains a major threat to public health due to the problems associated with administration of the present vaccine. Here we show that a protective immune response against live Clostridium tetani infection in mice can be elicited by an adenovirus vector encoding the tetanus toxin C fragment when administered as a nasal or epicutaneous vaccine. The results suggest that these vaccination modalities would be effective needle-free alternatives. This is the first demonstration that absorption of a small number of vectored vaccines into the skin following topical application of a patch can provide protection against live bacteria in a disease setting.

Materials and Methods

Construction of expression vectors. The TetC fragment was amplified by PCR from plasmid pTET-nir (2) (provided by J. VanCott and J. McGhee, University of Alabama at Birmingham) using the primers StetC (5'CGCGGATCCACATTTGTCCATCCTTCATC3') and ST3C (5'CCCGGATCTCATCATTTGTCCATCCTTCATC3'). These DNA primers created unique BamHI sites at the ends of the 1.35-kb TetC fragment with a synthetic eukaryotic ribosomal binding site (13) inserted in frame. The PCR-amplified fragment was subsequently cloned into the BamHI site of the pACCMV.PLP shuttle plasmid (12) under the transcriptional control of the human cytomegalovirus (CMV) early promoter to create the plasmid pACCMV.TetC. A replication-incompetent E1/E3-defective human Ad serotype 5-derived vector encoding TetC was constructed by homologous recombination between cotransfected pAC-tetC and pJM17 in human 293 cells as described (13). High-titer Ad stocks were prepared by ultracentrifugation over a cesium chloride gradient as described (20). A plasmid expression vector encoding TetC (pCMV-tetC) was constructed by cloning the same TetC fragment into the BamHI site of the plasmid pVR1012 (provided by Vical, Inc., San Diego, Calif.) in the correct orientation under transcriptional control of the human CMV early promoter. The AdCMV-PR8.ha vector encoding the influenza A/PR/8/34 hemagglutinin (HA) was constructed as a control by excising the 1.8-kb BamHI fragment containing the entire coding sequence of HA from the plasmid pDPI221 (Amer...
American Type Culture Collection [ATCC], Manassas, Va.), followed by cloning the HA gene into the BamHI site of pACCMV.PLPA and subsequent homologous recombination with pM17 in human 293 cells.

Immunization of mice. Young (2 to 3 months old) female BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were immunized by intranasal inoculation, topical application, intradermal injection, and intramuscular injection of vaccines (Ad-vectored or DNA-based).

NIVS was carried out by pipetting 10^7 to 10^9 particles (the number of particles was determined by quantitative PCR as described below). 10^9 particles were equivalent to approximately 10^6 PFU of a recombinant Ad vector or 100 μg of pACCMV-tetC DNA as a thin film onto the preshaved abdominal skin of a mouse followed by coverage with a piece of the Tegaderm patch (3M). The skin was prepared by depilation with an electric trimmer in conjunction with gentle brushing using a soft-bristle brush without inducing erythema (Draize scores [15] of ≤1). Unabsorbed vectors were washed away after 1 h. The possibility of nasal or oral immunization by grooming was eliminated as described (18). Moreover, we have also demonstrated that the patch failed to immunize mice when vectors were removed from the skin a few seconds after topical application. The incompetence of a brief contact with respect to eliciting an immune response provides firm evidence that the immunization was mediated by skin transudation rather than inhalation of airborne vectors.

Intranasal inoculation was performed by pipetting 10^7 to 10^9 particles of a recombinant Ad vector, 10^8 particles of Ad5 wild-type adenovirus serotype 5, ATCC number VR-5; ATCC), or 100 μg of pCMV-tetC DNA into one of the nostrils of an anesthetized mouse. DNA-based vaccines (100 μg of pCMV-tetC DNA) and AdCMV-tetC particles were inoculated by intranasal injection into the hind-leg quadriceps as described (24). AdCMV-tetC particles were also injected intradermally into the abdominal skin of mice. Animals in some groups were given booster doses at weeks 3 and 6 after the primary immunization, whereas others were immunized only once. All experiments in mice were performed according to institutional guidelines.

Quantitative PCR. Two PCR primer sets were designed to selectively amplify subfragments of the Ad5 gene. The primers Pd5.3 (5′CTGTAACCTTTGGAGAGCCC3′) and Pd5.3 (5′AGGAAGACCATAGGCTATTGTCTG) encompassed a 560-bp fragment of the fiber gene, whereas the primers Pf5.4 (5′TGACAGGAGGACTAAGGAT3′) and Pf5.4 (5′TATCCAAGGTTGGGCTG) encompass an 193-bp subfragment within the 560-bp fragment. The sensitivity of a nested PCR procedure was determined as follows. A PCR mix containing 1 μg of genomic DNA extracted from the abdominal skin of a naive mouse was spiked with dilutions of purified AdCMV-tetC vector DNA ranging from 1 to 10,000 copies, followed by 40 cycles of amplification with the Pf5.3/Pf5.4 primer pair. After purifying the PCR products with the Qiagen Quick PCR purification kit (Qiagen, Inc., Valencia, Calif.), DNA was subjected to another 40 cycles of amplification with the Pd5.3/Pd5.3 primer pair. Native mouse skin DNA without the addition of Ad DNA templates was amplified with identical primer sets as a negative control. Following this amplification protocol, we consistently detected 10 or more copies 100% of the time in several independent experiments. It was therefore concluded that the sensitivity of the assay was 10 copies or less.

To quantitatively determine the number of vectors that were absorbed by the skin following topical application of an Ad-vectored vaccine, total DNA was extracted from the skin tissue underneath the patch at 1 and 24 h postimmunization. The skin was washed under tap water and blotted dry to remove any unabsorbed vectors. Purified DNA was diluted in 10-fold increments and subjected to amplification with the nested PCR protocol. The amount of DNA per PCR was kept constant by adding native mouse skin DNA to a final quantity of 1 μg. The highest dilution of a DNA sample that generated a detectable signal should contain the AdCMV-tetC vector in the range of 10^3 particles, under the assumption that each vector contains a single copy of the fiber gene. DNA was extracted using the DNeasy kit (Qiagen, Inc.). Purified DNA fragments were fractionated on a 1.5% agarose gel and visualized as described (28). Samples were scored as positive if the diagnostic band was detected.

ELISA. Titers of anti-TetC immunoglobulin G (IgG) were determined by enzyme-linked immunosorbent assay (ELISA) as described (18) using purified TetC protein (CalBiochem, San Diego, Calif.) as the capture antigen. Briefly, serum samples and peroxidase-conjugated goat anti-mouse IgG (Promega Corp., Madison, Wis.) were incubated sequentially on the plates with extensive washing between incubations. The endpoint was calculated as the dilution of serum producing the same optical density at 490 nm as a 1/100 dilution of preimmune serum. Serum negative at the lowest dilution tested were assigned endpoint titers of 1.

RESULTS

Elicitation of an anti-TetC humoral immune response. The AdCMV-tetC vector was used in these studies to permit administration of a vectored tetanus vaccine in a needle-free manner. The needle-free routes of administration included topical and intranasal inoculations. A DNA-based vaccine (pCMV-tetC) also was administered by these routes as well as by intramuscular injection as another control. We report here that the efficiency of vector absorption into the skin was very low, with approximately 1 in 4,000 particles being taken within 1 h of the topical application (Fig. 1A). Most of the vectors applied to the surface failed to bind to the skin and were subsequently washed away. No adverse effects associated with inoculation were observed in any of the immunized mice during the course of these studies. Absorption of AdCMV-tetC particles following topical application was more effective in eliciting an anti-TetC antibody response than intradermal or intramuscular injection of the same vector at equivalent doses, as determined by seroconversion, which was monitored by analysis of sera from tail bleeds for the production of IgG antibody directed against TetC using an ELISA (Fig. 1B).

Seroconversion was observed in all of the mice immunized intranasally with >10^6 particles (groups B to E, M, and P to R) (Table 1) or topically with >10^6 particles (groups I, J, N, T, and V) of the AdCMV-tetC vector. The anti-TetC geometric mean titer (GMT) increased as the dose of AdCMV-tetC escalated (groups B to E and O to I) (Table 1). The level of anti-TetC reached a plateau when >10^9 particles of AdCMV-tetC were applied topically (groups I and J) (Table 1).

The TetC-specific IgG response of the mice that were administered AdCMV-tetC through application of an epicutaneous patch could be boosted to a high-titer response by three consecutive applications of the patch (GMT = 29,406 for group U compared to GMT = 5,971 for group T) (Table 1). The highest titers of antibody were observed in the mice administered AdCMV-tetC by the intranasal route (GMT ≥ 409,600; groups E, P, and Q) (Table 1). Intranasal boosting did not result in a significant increase in the anti-TetC titer (group
ADENOVIRUS AS A CARRIER FOR NEEDLE-FREE VACCINATION

P compared to group Q) (Table 1). Intramuscular injection of 100 µg (equivalent to approximately 10^13 copies) of pCMV-tetC DNA induced seroconversion in all animals; however, only a weak immune response was produced (GMT = 1,055; group W) (Table 1). Intranasal inoculation (group A) (Table 1) and topical application (group F) (Table 1) of pCMV-tetC DNA were ineffective in eliciting any detectable anti-TetC antibodies.

Protection against tetanus following live bacterial challenge. Seroconversion does not necessarily confer protection. Therefore, the protective effects of the vaccination regimens were determined by challenge with a toxigenic strain of C. tetani injected in the footpad at a dose of 6 x 10^3 cells (LD_{50}, 12.5). On such challenge, unprotected mice inevitably succumbed to tetanus with paralysis of the inoculated paw followed by distortion of the backbone and death. Administration of AdCMV-tetC as a nasal vaccine at a dose of ≥10^7 particles conferred complete protection in all animals after a single inoculation or three consecutive inoculations (P < 0.001) (Table 1; Fig. 2A and 3A). Full protection was also achieved after three consecutive applications of 10^11 particles of AdCMV-tetC in the absence of tetanus (P < 0.001), with 80% of the mice being protected (P < 0.001) by a single application (Table 1; Fig. 2B and 3B). All naive animals that were not immunized (groups K and X), as well as mice immunized with an irrelevant Ad vector (i.e., AdCMV-PR8.8a in groups O and S), were paralyzed severely or succumbed to tetanus within 4 days (Table 1; Fig. 3). Some mice that survived the challenge have been kept alive for up to 4 months and no symptoms of tetanus developed in any of the animals after day 10. Analysis of the anti-TetC IgG levels in the mice indicated that 100% survival was correlated with an anti-TetC titer of >6,400, although some animals with a lower titer could still survive the challenge.

Only 10% of the mice (group W) were protected by a single intramuscular injection of 100 µg of pCMV-tetC DNA despite a low-level seroconversion that occurred in all animals (Table 1). This low potency is consistent with a report that intramuscular injection of DNA is not very effective in protecting mice against tetanus (16).

We have defined the limit of the protective capacity of current Ad-vectored vaccines. A single inoculation of epicutaneous vaccines by topical application of 10^10 AdCMV-tetC particles conferred no protection when animals were challenged with 6 x 10^3 C. tetani cells (LD_{50}, 125; group N), whereas a single intranasal inoculation of 10^9 AdCMV-tetC particles protected only 20% of the immunized mice when challenged at this high dose (group M) (Table 1; Fig. 4). It is conceivable that larger animals with anti-TetC of the same titer may be able to counteract a higher number of invading bacteria because there are more neutralizing antibody molecules in the reservoir, and more toxin molecules may be required to paralyze a larger animal.
Protection in the context of preexisting immunity to Ad. The possibility that preexisting immunity to Ad could interfere with the ability of an Ad-vectored vaccine to elicit a protective immune response was considered. We investigated this possibility by inoculating the mice intranasally with Ad5 2 weeks prior to the primary administration of the vaccine. Anti-Ad neutralizing antibodies were subsequently detected in all mice with exposure to Ad5 (CPEI GMT = 171 for group R and CPEI GMT = 197 for group V) (Table 1).

Even though the animals had preexisting immunity to Ad, administration of AdCMV-tetC either intranasally or through an epicutaneous patch induced an IgG response to TetC (GMT = 382,170 for group R; GMT = 1,866 for group V) (Table 1). Protection against C. tetani challenge was unaffected by preexisting immunity to Ad in the mice that were administered AdCMV-tetC intranasally (group R, P < 0.001) (Fig. 5A). Although preexisting immunity did affect the level of protection conferred by the vaccine administered through the epicutaneous patch (group V, P = 0.003) (Fig. 5B), protection was positively demonstrated. Therefore, the immune repertoire can be mobilized toward a protective response against live C. tetani-mediated pathogenesis either by intranasal inoculation or topical application of an Ad-vectored vaccine in animals that have preexisting immunity to Ad.

**DISCUSSION**

A new generation of vaccines is required if the effectiveness of the global vaccine programs is to be enhanced. This demonstration that intranasal inoculation or topical application of an Ad-vectored vaccine results in statistically and preclinically significant protection against tetanus in mice ushers in two new modalities for the needle-free administration of tetanus vaccines. The vaccine used in these studies was a recombinant Ad vector encoding the toxic C fragment of the tetanus toxin. The use of Ad-vectored vaccines is an attractive strategy due to its efficacy in eliciting an immune response. Another positive aspect of this strategy is the possible negation of the current requirement for an unbroken cold chain, since lyophilized Ad vectors can be reconstituted into active infectious particles.

Although all naive mice and those immunized with irrelevant control vectors were paralyzed within 4 days of challenge, the partially protected animals exhibited symptoms of tetanus at a later date. Death occurred in some partially protected

### TABLE 1. Summary of immune responses in mice

<table>
<thead>
<tr>
<th>Vector (dose)</th>
<th>Route</th>
<th>Numb. of mice producing anti-TetC IgG on 7th week postimmunization</th>
<th>GMT (range)</th>
<th>C. tetani challenge survival</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCMV-tetC (100 µg)</td>
<td>i.n.</td>
<td>0/10</td>
<td>4,222 (1,600-25,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^6 particles)</td>
<td>i.n.</td>
<td>0/10</td>
<td>117,627 (25,600-409,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^7 particles)</td>
<td>i.n.</td>
<td>0/10</td>
<td>235,253 (25,600-409,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^8 particles)</td>
<td>i.n.</td>
<td>0/10</td>
<td>429,300 (163,800-1,638,400)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>pCMV-tetC (100 µg)</td>
<td>NIVS</td>
<td>0/10</td>
<td>100 (10/10)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^6 particles)</td>
<td>NIVS</td>
<td>0/10</td>
<td>765 (100-25,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^7 particles)</td>
<td>NIVS</td>
<td>0/10</td>
<td>16,890 (640-25,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^8 particles)</td>
<td>NIVS</td>
<td>0/10</td>
<td>16,890 (640-25,600)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>AdCMV-tetC (10^9 particles)</td>
<td>i.m.</td>
<td>0/10</td>
<td>470,507 (102,400-1,638,400)</td>
<td>&lt;0.001</td>
<td>0 (0/10)</td>
</tr>
</tbody>
</table>

---

* Mice were immunized by intranasal (i.n.) inoculation, topical application (NIVS), or intramuscular (i.m.) injection of vaccines (Ad-vectored or DNA-based). To determine the optimal dose of vectored vaccines, escalating amounts of AdCMV-tetC were inoculated as nasal or epicutaneous vaccines. To determine a booster effect, mice were challenged with 6 × 10^6 C. tetani cells; other groups were all challenged with 6 × 10^7 C. tetani cells. The vaccine used in these studies was a recombinant Ad vector encoding the toxic C fragment of the tetanus toxin.

**DISCUSSION**

A new generation of vaccines is required if the effectiveness of the global vaccine programs is to be enhanced. This demonstration that intranasal inoculation or topical application of an Ad-vectored vaccine results in statistically and preclinically significant protection against tetanus in mice ushers in two new modalities for the needle-free administration of tetanus vaccines. The vaccine used in these studies was a recombinant Ad vector encoding the toxic C fragment of the tetanus toxin. The use of Ad-vectored vaccines is an attractive strategy due to its efficacy in eliciting an immune response. Another positive aspect of this strategy is the possible negation of the current requirement for an unbroken cold chain, since lyophilized Ad vectors can be reconstituted into active infectious particles.

Although all naive mice and those immunized with irrelevant control vectors were paralyzed within 4 days of challenge, the partially protected animals exhibited symptoms of tetanus at a later date. Death occurred in some partially protected
animals as late as 10 days postchallenge (Fig. 5B). This suggests that there is a critical initial time period during infection with *C. tetani* in which the host immune system is undergoing mobilization prior to achieving a response sufficient to eradicate the pathogen. Although the effectiveness of a tetanus vaccine may depend on its ability to rapidly induce adequate levels of antitoxin, it remains to be seen whether an anti-TetC cellular immune response (16) is involved in the eradication of live *C. tetani* cells in vivo.

Ad-vected nasal vaccine elicited high titers of anti-TetC antibodies after a single administration, and these levels were not increased by short-term booster inoculations (groups P and Q). Moreover, as reported previously for rabies (27), the high titers were not suppressed appreciably by preexposure to Ad (groups Q and R). The potency of the intranasal inoculation may reflect the natural tropism of the Ad vector for the airway, where it can transduce a large number of cells and possibly activate the mucosal immune machinery to its full potential.

Although administration of the Ad-vected vaccine through an epicutaneous patch was capable of causing seroconversion and was effective in protecting the animals from challenge with live *C. tetani* particularly after boosting, the titers of anti-TetC antibodies were relatively low when compared to those elicited by intranasal inoculations. Several strategies for optimization...
FIG. 3. Effects of booster applications in protecting mice against tetanus after bacterial challenge. Mice were immunized either once or three times at intervals of 3 weeks. Eight weeks after the primary immunization, mice were challenged by footpad injection of $6 \times 10^9$ C. tetani cells and monitored daily for survival for 14 days. (A) Mice were immunized by intranasal inoculation with Ad vectors. Group O, intranasal inoculation with AdCMV-PR8.ha three times; group P, intranasal inoculation with AdCMV-tetC once; group Q, intranasal inoculation with AdCMV-tetC three times. (B) Mice were immunized by topical application of Ad vectors. Group S, topical application of AdCMV-PR8.ha three times; group T, topical application of AdCMV-tetC once; group U, topical application of AdCMV-tetC three times; group X, naive control mice. The data were plotted as described in the legend to Fig. 2. Numbers in parentheses represent the number of animals for each treatment.

of the response can be envisioned. As boosting was effective in these studies, the immune response could be enhanced by applying the vaccine patch multiple times or over a large area. The efficacy of epicutaneous administration also may be enhanced by increasing the affinity of the Ad vector for specific cell types within the outer layer of skin (e.g., terminally differentiated keratinocytes, antigen-presenting cells that traffic to the surface, or other cell types along the skin barrier), which may be achieved by inserting a preselected ligand into the adenoviral fiber as previously described (6,25) in an attempt to augment transduction efficiency in a targeted manner. Such a “skin-binding” vector could be a more effective carrier for epicutaneous vaccines than the current Ad vector due to the overexpression of antigens in specific cell types that are potent immunostimulators.

Emerging evidence suggests that the outer layer of skin may be more immunocompetent than deep tissues (7,9) (Fig. 1B). It is also conceivable that the immune system may focus its surveillance on an interface that is in frequent contact with environmental pathogens. Overexpression of immunogens from a small number of vectored vaccines in the outer layer of skin may thus elicit a more potent immune response than the inoculation of an equivalent dose into deep tissues. This is borne out by the magnitude of the immune response that was elicited (Table 1 and Fig. 1B) by a relatively low number of vectors absorbed following topical application (Fig. 1A). However, it is unclear whether the leveling off of vaccine potency following topical application of AdCMV-tetC at a dose of $\geq 10^9$ particles (groups G to J) (Table 1) was due to saturation of the antigen expression machinery or the antigen presenting capacity within a restricted subset of skin. A skin-targeted vector may amplify antigen expression in the outer layer of skin; however, strategies to mobilize antigen-presenting cells will have to be exploited if antigen presentation should appear to be the limiting step.

The suppression of the efficacy of the Ad-vectored epicutaneous vaccine by preexisting immunity to Ad (Table 1 and Fig. 5) is a problem that must be circumvented during the development of an Ad-vectored vaccine patch. It is likely that the suppression may not be attributed to anti-Ad antibodies that prevent Ad vectors from entering target cells, because a reporter gene (i.e., luciferase) can be effectively expressed in skin
ADENOVIRUS AS A CARRIER FOR NEEDLE-FREE VACCINATION

The rapid loss of vector DNA observed in these studies after topical application (Fig. 1A) may be beneficial in preventing the persistence of exogenous DNA. Presumably, the mechanisms that have evolved to degrade or expel skin-associated environmental DNA in order to protect the genomic integrity of the host may contribute to the elimination of the vector DNA following topical application. In contrast to the short half-life of vectored epicutaneous vaccines, DNA-based vaccines have been reported to persist in animals for up to a year after intramuscular injections (26). Conceivably, the degradation of vectored epicutaneous vaccines may contribute to not only a safer vaccine but also a more effective one, as the transient expression of antigens in vivo could foster the longevity of memory T cells by minimizing antigen-induced apoptosis of T lymphocytes (19, 29). If efficacy should be limited by overdegradation, the problem potentially can be circumvented by booster applications as shown in this report (Table 1 and Fig. 3B).

It has been demonstrated previously that topical application of TetC protein in conjunction with cholera toxin is capable of eliciting anti-TetC antibodies in mice (10). It is unclear, however, whether a protein-based epicutaneous vaccine is able to protect animals against bacterial infections. The profile of the immune response elicited by vectored vaccines may be quite different from that induced by their protein-based counterparts since the vector DNA dictates the synthesis of exogenous proteins in animals' own cells after inoculation, and antigens produced in situ can often induce a more solid immunity than inoculation of protein-based vaccines (14). Evidence suggests that an Ad vector encoding TetC could be more effective than the TetC protein as an epicutaneous vaccine since the latter required the coadministration of cholera toxin as an adjuvant (10), whereas topical application of the AdCMV-TetC vector alone was able to protect all of the recipients against challenge with live C. tetani (Fig. 3B). Although topical application of plasmid DNA also has been shown capable of eliciting an antibody response (1, 8, 18), it has not been demonstrated that this modality can evoke any protective immunity. Topical application of naked plasmid DNA, in the absence of an association with Ad or liposome, produced no detectable gene expression in the skin (4, 18), and we report here that naked plasmid DNA was ineffective in eliciting an anti-TetC antibody response or a protective immune response against tetanus following either topical or intranasal inoculations (Table 1 and Fig. 2). The potency of an Ad-vectored vaccine may be attributed to an efficient gene delivery in conjunction with robust transgene expression when compared to its plasmid counterpart (21).

In these studies, intranasal inoculation was more effective than topical application with respect to eliciting an immune response, although this difference may be attributed, in part, to the low absorption efficiency of the skin (Fig. 1A). There are, however, some issues that detract from the practicality of intranasal administration of vaccines. A major concern is the possibility of the elicitation of a severe adverse pulmonary reaction in those individuals who are allergic to components of the vaccine or in individuals with underlying pulmonary disease. Moreover, intranasal administration can irritate the nose, causing responses that result in expulsion of an unpredictable amount of the vaccine.

The recent report that a humoral immune response could be...
elicited in humans by topical application of a vaccine patch (11) suggests that the outer layer of human skin may be as immunocompetent as that of their animal counterparts. Although an experimental murine tumor could be arrested following topical application of tumor epitope peptides (17), it has not been demonstrated, prior to our studies, that a pathogen of human relevance could be arrested following topical application of any vaccines. We have shown, for the first time, that topical application of a vectored vaccine patch in a non-invasive manner could protect animals against infection by live bacteria in a disease setting.

By expressing antigens in an immunocompetent area without causing tissue damage, nasal and epicutaneous vaccines may emerge as preferred modalities for the inoculation of future vaccines in a simple, effective, economical, painless, and safe manner.

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REFERENCES


American Heart Association. Transgenic mice were generated and maintained at the University of Iowa Transgenic Animal Facility, which is supported in part by the College of Medicine and the Diabetes and Endocrinology Research Center. DNA sequencing was performed at the University of Iowa DNA Core Facility. Requests for materials should be addressed to Curt D. Sigmund at curt-sigmund@uiowa.edu. Address correspondence to Dr. Curt D. Sigmund, Chair, Molecular Biology Interdisciplinary Program, Director, Transgenic and Gene Targeting Facility Department of Internal Medicine and Physiology & Biophysics, 2191 Medical Laboratory, The University of Iowa College of Medicine, Iowa City, IA 52242, USA. e-mail: curt-sigmund@uiowa.edu

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David E. Stec, Satoshi Morimoto, and Curt D. Sigmund
The University of Iowa
College of Medicine
Iowa City, IA, USA

AdEasy System Made Easier by Selecting the Viral Backbone Plasmid Preceding Homologous Recombination

BioTechniques 31:260-262 (August 2001)

Recombinant adenovirus is widely used as a mammalian cell expression vector for multiple purposes including gene therapy (1) and noninvasive vaccination (5). It is urgent at this juncture to develop an efficient protocol for constructing adenovirus recombinants in a timely manner. The conventional approach to construct a replication-defective recombinant adenoviral vector requires a series of time-consuming and labor-intensive steps involving homologous recombination between two transfected plasmids in mammalian packaging cells (3). The finding that homologous recombination can be carried out in E. coli (2,4) streamlined the procedure by allowing recombination to occur overnight in bacterial cells and obviated the need for plaque purification. The AdEasy system (4) is one of the fast-track systems for generating recombinant adenovirus by homologous recombination in E. coli. Typically, a linearized shuttle vector plasmid encoding kanamycin resistance is mixed with an adenoviral backbone plasmid (pAdEasy-1 or pAdEasy-2) encoding ampicillin resistance, followed by co-transformation into competent E. coli BJS183 cells. Recombinants are subsequently selected for kanamycin resistance and identified by size in conjunction with restriction endonuclease analysis. Finally, recombinant adenoviral vectors are generated by transfecting the recombinant plasmid into a mammalian packaging cell line (e.g., 293 or 911 cells).

We report here that the key step in producing a recombinant plasmid in E. coli can be enhanced by pre-selecting the adenoviral backbone plasmid before the delivery of the shuttle vector plasmid. In this two-step transformation protocol, we electroporated an adenoviral backbone plasmid (i.e., pAdEasy-1) into E. coli BJS183 cells, followed by growing transformants on LB agar plates containing 50 μg/mL ampicillin. A single colony containing pAdEasy-1 (BJS183pAdEasy-1) was subsequently characterized by restriction analysis of the plasmid. BJS183pAdEasy-1 cells were made electrocompetent as described (4) and kept in aliquots at -80°C. To trigger a recombination reaction, a shuttle vector plasmid encoding the Bacillus anthracis protective antigen (pShuttle-CMV.PA63) was digested with restriction endonuclease Pmel, and 25 ng linearized plasmid DNA, without enzyme inactivation and purification, was directly electroporated into BJS183pAdEasy-1 cells. Transformants were selected on LB agar plates containing 50 μg/mL kanamycin. Plasmid DNA was prepared from individual colonies using the QIAprep® spin miniprep kit (Qiagen, Valencia, CA, USA). The 9.2-kb pShuttle-CMV.PA63 plasmid and the 35-kb recombinant plasmid, both selectable by kanamycin resistance, could be clearly identified by size after fractionating supercoiled DNA in agarose gel.

As shown in Figure 1, transformation by mixing pShuttle-CMV.PA63 with pAdEasy-1 before electroporation as described (4) resulted in a success rate of 12% (2/17), whereas the new two-step transformation protocol generated recombinant plasmids at a frequency of 94% (16/17). The difference in outcomes between the two protocols was highly significant (P < 0.001 by Fisher's exact test). BJS183pAdEasy-1 competent cells that had been frozen at -80°C for up to eight months were as efficient as their freshly prepared counterparts in generating recombinant plasmids. We envision that the enhanced selection of recombinants in antibiotics following two-step transformation may be attributed to productive recombination between incoming shuttle vector plasmids and pre-selected pAdEasy-1 plasmids that existed as autonomous replicons in E. coli cells. It is conceivable that only a small fraction of the pAdEasy-1 plasmid pool may be allowed to persist in E. coli cells following transformation because there is a high chance for a large plasmid [pAdEasy-1 is 33 kb in size (4)] to be defective (e.g., nicks along its long DNA strands), and/or the efficiency for
Benchmarks

connecting a large plasmid to the cellular replication machinery may be low. Therefore, homologous recombination between a shuttle vector plasmid and an adenoviral backbone plasmid unable to exist as a replicon in E. coli cells is counterproductive for generating selectable recombinant plasmids because such recombinants are abortive. The two-step transformation protocol ensures that homologous recombination occurs in a productive manner by eliminating defective and non-replicative adenoviral backbone plasmids in advance, thereby allowing a higher success rate during the selection for recombinants. Moreover, the step to inactivate PmeI with phenol/chloroform and purify the linearized shuttle vector plasmid as described (4) is no longer required because the need to mix the linearized shuttle vector plasmid with the supercoiled adenoviral backbone plasmid before transformation is negated. Overall, this two-step transformation protocol may have broad utility in systems that involve homologous recombination in bacteria.

REFERENCES


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M. Zeng1,2, S.K. Smith1, F. Siegel1, Z. Shi1, K.R. Van Kampen1, C.A. Elmets2, and D.C. Tang1,2
1Vaxin, Inc.
2University of Alabama at Birmingham
Birmingham, AL, USA

Figure 1. Enhanced generation of selectable plasmid recombinants following homologous recombination in E. coli cells by pre-selecting the adenoviral backbone plasmid. Homologous recombination between the pShuttle-CMVPA63 plasmid (9.2 kb) and the pAdEasy-1 plasmid (33 kb) in E. coli BJ5183 cells was triggered by two different protocols: (i) a one-step transformation protocol by which the two plasmids were mixed before electroporation as described (4) and (ii) a two-step transformation protocol that allows the linearized pShuttle-CMVPA63 plasmid to be transformed into E. coli BJ5183/pAdEasy-1 cells harboring the pAdEasy-1 plasmid as a pre-selected autonomous replicon. Lane M, molecular weight marker (HindIII-cleaved λ DNA) (Life Technologies, Rockville, MD, USA); lane A, supercoiled pAdEasy-1 DNA control; lane B, supercoiled pShuttle-CMVPA63 DNA control; lanes 1–34, supercoiled plasmid DNA from individual kanamycin-resistant colonies following homologous recombination using either the one-step transformation protocol (lanes 1–17) or the two-step transformation protocol (lanes 18–34). DNA samples were fractionated in 0.8% agarose gel, stained with ethidium bromide, and visualized with the Gel-Doc™ 2000 (Bio-Rad Laboratories, Hercules, CA, USA).
DNA-based non-invasive vaccination onto the skin

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

\textsuperscript{a}Vaxin Pharmaceuticals, Inc., Birmingham, AL 35209, USA  
\textsuperscript{b}Gene Therapy Center, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA  
\textsuperscript{c}Department of Medicine, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA  
\textsuperscript{d}Division of Pulmonary and Critical Care Medicine, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294, USA  
\textsuperscript{e}Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, THT 215, 1900 University Blvd., Birmingham, AL 35294-0006, USA

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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 \cite{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 \cite{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 \cite{3} or a needle \cite{4}, the procedure is non-invasive and requires no special skill or equipment. In contrast to NIVS using adenovirus (Ad) recombinants \cite{1}, construction and preparation of recombinant plasmid DNA is technically less demanding. Re-vaccination by DNA-based vaccines is also possible \cite{3}. Although NIVS using protein-based vaccines has recently been demonstrated \cite{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 \cite{6}.
2. Materials and methods

2.1. Cell cultures

Human 293 cells for the propagation of AdCMV-luc [7] were cultured in RPMI medium 1640. WI62 cells for the propagation of Ad dll014 [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 x 10¹¹ particles of Ad dll014 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 epidermis. 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 dll014 [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.
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 dll1014 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 μg 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 re-vaccinate animals in a non-invasive mode, we incubated naked skin of 3 naïve mice with DNA/Ad complexes containing Ad dll1014 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 dll1014 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...
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 immunocompetence 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

![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 dll014 (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.](image)

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

<table>
<thead>
<tr>
<th>Vector*</th>
<th>Pre-exposure to Ad</th>
<th>Mode</th>
<th>Weeks post-immunization</th>
<th>Number of boost</th>
<th>Number of mice producing anti-GH</th>
<th>Anti-hGH IgG serum titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA/Ad</td>
<td>–</td>
<td>NIVS</td>
<td>28</td>
<td>0</td>
<td>2/2</td>
<td>1,000</td>
</tr>
<tr>
<td>DNA/Ad</td>
<td>–</td>
<td>NIVS</td>
<td>14</td>
<td>2</td>
<td>5/5</td>
<td>1,000–10,000</td>
</tr>
<tr>
<td>DNA/liposome</td>
<td>–</td>
<td>NIVS</td>
<td>28</td>
<td>0</td>
<td>2/2</td>
<td>1,000</td>
</tr>
<tr>
<td>DNA/liposome</td>
<td>–</td>
<td>NIVS</td>
<td>22</td>
<td>3</td>
<td>10/10</td>
<td>1,000</td>
</tr>
<tr>
<td>DNA</td>
<td>–</td>
<td>IM</td>
<td>12</td>
<td>0</td>
<td>4/4</td>
<td>10,000–100,000</td>
</tr>
<tr>
<td>DNA/Ad*</td>
<td>+</td>
<td>NIVS</td>
<td>37</td>
<td>1</td>
<td>3/3</td>
<td>10,000</td>
</tr>
</tbody>
</table>

* C57 BL/6 mice were immunized by NIVS or IM injection of DNA. DNA/Ad, pCMV-GH DNA complexed with Ad dll104; 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. Mice were exposed to Ad by topical application of Ad dll104 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 dll104.
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.

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References

De-chu Christopher Tang, Ph.D.
Scientific Founder, Vice President and Chief Technical Officer
Vaxin, Inc.

Research Associate Professor
University of Alabama at Birmingham
Department of Dermatology
Gene Therapy Center
Comprehensive Cancer Center

VH-501
1670 University Boulevard
Birmingham, Alabama 35294-0019

Phone: 205-975-5603
Fax: 205-975-0455
Email: dctang@uab.edu

Professional Employment

2001-Present  Research Associate Professor
University of Alabama at Birmingham
Birmingham, Alabama

1997-Present  Vice President and Chief Technical Officer
Vaxin, Inc.
Birmingham, Alabama

1994-2001  Research Assistant Professor
University of Alabama at Birmingham
Birmingham, Alabama

1992-1994  Instructor
Dr. David P. Carbone's laboratory
University of Texas Southwestern Medical Center
Dallas, Texas

1991-1992  Assistant Instructor
Dr. Stephen A. Johnston's laboratory
University of Texas Southwestern Medical Center
Dallas, Texas
1990-1991 Research Associate
Dr. Stephen A. Johnston's laboratory
Duke University
Durham, North Carolina

1988-1990 Research Associate
Dr. Gretchen J. Darlington's laboratory
Baylor College of Medicine
Houston, Texas

Education

1989 Ph.D. in Microbiology and Molecular Biology
Dr. Milton W. Taylor's laboratory
Indiana University
Bloomington, Indiana

1976 M.S. in Animal Physiology
Tunghai University
Taichung, Taiwan

1974 B.S. in Biology
Tunghai University
Taichung, Taiwan

Memberships

American Society of Gene Therapy (ASGT #: fCy40XC)
American Association for Cancer Research (AACR #: 10889)
American Association for the Advancement of Science (AAAS#: 02231980)
Society of Chinese Bioscientists in America

Invited and Selected Lectures


Gene painting as a simple method for eliciting an antitumor immune response. *Sixth International Conference on Gene Therapy of Cancer*. San Diego, California, November 22, 1997

Vaccination by painting genetic vaccines onto the skin. *Second National Symposium on Basic Aspects of Vaccines*. Bethesda, Maryland, April 30, 1997

Vaccination by painting genetic vectors onto skin. *Fifth International Conference on Gene Therapy of Cancer*. San Diego, California, November 15, 1996

Cancer immunotherapy by the B7-1 gene in conjunction with radiotherapy. *Fifth International Conference on Gene Therapy of Cancer*. San Diego, California, November 14, 1996


**Awards**

Principal Investigator, 2001-2002, National Institutes of Health Small Business Innovation Research Program Phase I grant (#1-R43-Al-47558-01A2): "Noninvasive delivery of skin-targeted anthrax vaccines", $100,000

Principal Investigator, 2001-2004, Office of Naval Research Grant (#N00014-01-1-0945): "Vaccination by topical application of recombinant vectored vaccines", $3,049,000


Co-Principal Investigator, 2001-2006, National Institutes of Health R01 grant (#1-R01-NS-43947-01): "Alzheimer vaccinations: Noninvasive vaccination by DNA-based vectors", $1,250,000

Collaborator, 2001, Department of Defense Small Business Innovation Research Program Phase I grant (#N00014-01-M-0178): "Needleless topical administration of dengue DNA vaccine", $69,000

Award Winner, Year 2000 Wallace H. Coulter Award for Innovation and Entrepreneurship (http://www.coulter.gatech.edu/2000recip.html), $100,000

Preceptor, 2000-2001, Dermatology Foundation Postdoctoral Fellowship: "Development of a skin-targeted vaccine patch against anthrax", $25,000

Investment to Vaxin, Inc., 1999-2000, Paradigm Venture Partners I, L.L.C., $1,000,000

Principal Investigator, 1999-2000, National Institutes of Health Small Business Technology Transfer Program Phase I grant (#1-R41-Al-44520-01): "Non-invasive delivery of skin-targeted tetanus vaccines", $100,000

Principal Investigator, 1998-1999, National Institutes of Health Small Business Innovation Research Program Phase I grant (#1-R43-Al-43802): "Non-invasive delivery of skin-targeted flu vaccines", $100,000


Investment to Vaxin, Inc., 1997-1999, Emerging Technology Partners, $733,347

Principal Investigator, 1994, *University of Alabama at Birmingham Start-Up Fund*, $300,000

Award Winner, *Year 1994 American Association for Cancer Research Young Investigator Travel Grant*, $500

**Reviewer for Scientific Journals**

Ad Hoc Reviewer, *BioTechniques*
Ad Hoc Reviewer, *Gene Therapy*
Ad Hoc Reviewer, *Human Gene Therapy*

**Patents**

Allowance of the U.S. patent "Vaccination by topical application of genetic vectors" (Filed on 08/13/97; U.S. Serial No. 09/402,527)

"Noninvasive genetic immunization, expression products therefrom, and uses thereof" (Filed on 05/03/99; PCT publication No. WO 00/66179)

**Publications**


Lees CY, Briggs DJ, Davis RD, Moore SM, Gordon C, Xiang Z, Ertl HCJ, Tang DC, and Fu ZF. Mice immunized on the skin with a recombinant adenovirus expressing rabies virus glycoprotein developed high titers of neutralizing antibodies and were protected against lethal challenge (submitted).