

Protective Immunization against Inhalational Anthrax: A Comparison of Minimally Invasive Delivery Platforms

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A new anthrax vaccine under clinical investigation is based on recombinant *Bacillus anthracis* protective antigen (rPA). Here, we investigated microneedle-based cutaneous and nasal mucosal delivery of rPA in mice and rabbits. In mice, intradermal (id) delivery achieved up to 90% seroconversion after a single dose, compared with 20% after intramuscular (im) injection. Intranasal (inl) delivery of a liquid formulation required 3 doses to achieve responses that were comparable with those achieved via the id or im routes. In rabbits, id delivery provided complete protection against aerosol challenge with anthrax spores; in addition, novel powder formulations administered inl provided complete protection, whereas a liquid formulation provided only partial protection. These results demonstrate, for the first time, that cutaneous or nasal mucosal administration of rPA provides complete protection against inhalational anthrax in rabbits. The novel vaccine/device combinations described here have the potential to improve the efficacy of rPA and other biodefense vaccines.

Fatal infection with *Bacillus anthracis* has occurred sporadically, most notably as an occupational hazard of the textile and tanning industries of the 19th and early 20th centuries. Although the cutaneous and gastrointestinal routes of infection have produced documented fatalities, the fatality rate of inhalational anthrax is nearly 100% without antibiotic intervention. Inhalational anthrax is difficult to diagnose, often abruptly manifesting

itself as respiratory failure or hemodynamic collapse 2–4 days after the initial onset of symptoms [1].

The largest documented outbreak of human inhalational anthrax occurred near a Soviet military facility in 1979 and resulted in 42 deaths [2, 3]. The intentional release of anthrax spores in 2001 resulted in 23 confirmed or suspected cases of inhalational and cutaneous anthrax. These events have heightened concerns about intentional mass exposure to agents such as *B. anthracis* [4] and have resulted in the creation of the Centers for Disease Control and Prevention's Category A list, which catalogs diseases that are considered to pose the greatest risks. Prophylactic vaccination is the first line of defense against anthrax, and therapeutic vaccination combined with antibiotics is the favored postexposure treatment.

Anthrax toxin is a binary A-B toxin composed of a combination of protective antigen (PA) and either lethal factor (LF) or edema factor (EF) [5–7]. PA mediates the entry of LF and EF into the cytosol. LF is a zinc metalloprotease that inactivates mitogen-activated protein kinase kinases, which rapidly induces cell death [8–11]. EF is a calmodulin-dependent adenylcyclase that inhibits mitogen-activated protein kinase [12]. Antibodies that neutralize PA block the transport of both

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EF and LF to the cytosol and, in so doing, block the course of infection.

Although the combination of PA and either LF or EF constitutes a lethal toxin, PA alone is nontoxic and is the protective component of the current anthrax vaccine licensed for use in the United States, anthrax vaccine adsorbed (AVA). AVA consists of a culture filtrate from a toxigenic, nonencapsulated strain of *B. anthracis* adsorbed onto aluminum hydroxide [13–15]. A recombinant form of PA (rPA) is being considered as a replacement for AVA.

In clinical trials, rPA is currently administered via intramuscular (im) injection [16], although no data are available that suggest that this route is optimal. A number of studies have demonstrated that vaccination via the skin can improve efficacy [17–20]. For example, compared with im delivery, intradermal (id) delivery provides a 10-fold dose-sparing benefit for rabies and hepatitis B vaccines [17–19]. Given the dose-sparing effect produced by id delivery of these vaccines, it is reasonable to suggest that immunological benefits might also be realized for rPA; however, no studies have investigated id delivery of rPA or other biodefense vaccines. New approaches are needed to replace the standard Mantoux method for id injection by use of conventional needles, because of its variability in execution from one clinician to the next, the time required per injection, and the discomfort that is often associated with the technique.

Intranasal (inl) delivery is another alternative to im delivery. It has the potential to induce mucosal immunity [21–26] and offers the advantage of easy, noninvasive delivery, potentially reducing the need for administration by highly trained personnel. Typically, inl delivery has employed liquid formulations that require refrigerated or frozen storage [27]. Recently, there has been growing interest in powder formulations of vaccine, because of the increased storage stability of powders [28–31]. Nonetheless, few studies of inl vaccination with powder [30, 31]—and no studies of the application of such technology to biodefense—have been conducted.

Here, we investigated cutaneous and nasal mucosal delivery of anthrax rPA in mice and rabbits, compared with im delivery. These experiments were facilitated by (1) novel, disposable, minimally invasive devices that were based on microneedles and were designed for id injection of vaccine, (2) microenhancer array (MEA) devices [32] that were designed for topical administration of vaccine to the epidermis via microabrasion, and (3) an rPA powder formulation and device platform that was designed for inl delivery of vaccine. The results of the present study demonstrate the feasibility of cutaneous and nasal mucosal delivery of rPA and show, for the first time, that such novel vaccine/device combinations provide complete protection against inhalational anthrax in rabbits.

MATERIALS AND METHODS

Animals and immunizations. The animal experiments described here were conducted in accordance with US Department of Agriculture and National Institutes of Health guidelines for the care and use of animals and under Institutional Animal Care and Use Committee–approved protocols. Mice were housed at BD Technologies (Research Triangle Park, NC), and rabbits were housed at Genassess (Doylestown, PA) for immunizations and at the US Army Medical Research Institute of Infectious Diseases (USAMRIID; Frederick, MD) for spore challenge. Female BALB/c mice (Charles River Laboratories), 6–8 weeks old (10 mice/group), were immunized with 10 μ g of rPA (USAMRIID) on days 0, 21, and 42. rPA either was formulated in PBS without adjuvant, was adsorbed onto aluminum hydroxide (70 μ g of aluminum hydroxide/10 μ g of rPA) (Alhydrogel; Superfos Biosector) as adjuvant, or was formulated with unmethylated, phosphorothioate-linked, CpG-containing oligonucleotides (hereafter, “CpG”; 10 μ g of CpG/10 μ g of rPA) (number 1826, 5'-TCCATGACGTTCCCTGACGTT-3'; Proligo) as adjuvant. Two injections of 25 μ L each were administered for the im, id, epidermal, and topical groups. For inl delivery, liquid was instilled (15 μ L in each nostril) into the nasal cavities of anesthetized mice. A 30-gauge needle and 1-mL syringe (BD) was used for im delivery into the quadriceps; id delivery was performed on the shaved lower back by use of a 1-mm-long stainless-steel microneedle and a 1-mL syringe. Epidermal delivery was accomplished by use of plastic MEA devices, the design specifications of which are described elsewhere [32]. Shaved skin was preabraded with an MEA device [32] before topical application of the vaccine to the treated site. Topical delivery was performed in accordance with the same protocol as for epidermal delivery, except that vaccine was applied directly to shaved skin without abrasion. Anesthetized mice were bled via their retroorbital sinuses on days 21, 42, and 56.

Female New Zealand White rabbits (Myrtles Rabbitry) (6–9 rabbits/group) were immunized with 50 μ g of rPA on days 0, 21, and 42. For liquid administration, rPA was formulated in PBS without adjuvant or with Alhydrogel (350 μ g of aluminum hydroxide/50 μ g of rPA) or CpG (50 μ g of CpG/50 μ g of rPA). Two injections of 50 μ L, each at a different site, were administered for the id, epidermal, and topical groups; im delivery consisted of a single injection of 100 μ L into the quadriceps. A Penn-Century nasal sprayer was used for inl delivery of liquid vaccine (100 μ L into a single nostril); the delivery device described in Results was used for inl delivery of powder. Rabbits were bled via their marginal ear veins on days 21, 42, and 56.

Preparation of powder formulations. Formulations containing rPA and CpG were prepared as either freeze-dried (FD) or spray-freeze-dried (SFD) powder. Formulations were prepared by mixing trehalose with an aqueous solution of rPA and

CpG at a 1:1 wt/wt ratio. FD samples were prepared by rapidly freezing solutions in dry ice and then subjecting them to lyophilization under vacuum at 50 mTorr for 72 h. SFD samples were prepared by spraying solutions into liquid nitrogen as described elsewhere [33], followed by evaporation and subjecting them to lyophilization as described above.

The FD rPA/CpG/trehalose powder was milled by use of a microball mill (Reflex Analytical) for 30 min and was then loaded into polyethylene capsules at a fill weight of 10.3 mg, to create a 50- μ g rPA dose. Capsules were heat sealed with polyethylene film.

A total of 36.2 mg of SFD chitosan/trehalose (20% chitosan and 80% trehalose; prepared from an aqueous solution by SFD as described above) was added to 205.3 mg of FD rPA/CpG/trehalose powder, to obtain 3% wt/wt chitosan in the powder. The resultant SFD rPA/CpG/trehalose powder was milled and loaded into capsules as described above, except that, to remove agglomerates, the powder was passed through a 425- μ m sieve before being loaded into capsules. A total of 33.4 mg of SFD chitosan was added to 189.5 mg of SFD rPA/CpG/trehalose powder; the powder was blended for 4 min by use of the microball mill as described above, but without the ball. The resultant powder blend was then passed through a 425- μ m sieve and loaded into capsules as described above.

ELISA. Maxisorp 96-well plates (Nunc) were coated with 1 μ g/mL rPA in 0.05 mol/L carbonate coating buffer (pH 9.6) at 4°C overnight. Plates were blocked for 1.5 h at 37°C in blocking buffer (5% skim-milk powder in PBS–Tween 20) and then were washed 3 times with PBS–Tween 20. Samples were serially diluted 2-fold in duplicate across the plate in blocking buffer and were incubated for 1 h at 37°C. After 3 washings, plates were incubated with horseradish peroxidase (HRP)–conjugated goat anti-mouse or anti-rabbit IgG (Southern Biotechnology) for 45 min at 37°C. After washing, plates were developed for 30 min at room temperature with 3,3',5,5'-tetramethylbenzidine substrate (Sigma) and were stopped by the addition of 0.5 mol/L H₂SO₄; optical densities were read at 450 nm. End-point titers were defined as the highest dilution of a sample yielding an OD_{450 nm} value at least 3 times the background obtained for serum samples from unimmunized control animals. Antibody isotypes were determined by use of HRP-labeled goat anti-mouse IgG1 or IgG2a antibodies and mouse reference standards (Bethyl Laboratories).

Toxin-neutralizing antibody (TNA) assay. TNA titers were determined by use of a modified version of a method described elsewhere [34]. Confluent J774A.1 cells were plated (7×10^4 cells/well) in sterile, 96-well, clear-bottom, black plates (Corning Costar) at 37°C and in 5% CO₂. A fresh solution containing 100 ng/mL LF (List Biological Laboratories) and 200 ng/mL rPA was mixed with an equal volume of diluted samples in triplicate and was incubated for 1 h at 37°C. Medium was then

replaced with 100 μ L of diluted solution of LF, PA, and test sample, which was incubated for 4 h at 37°C in 5% CO₂. Cell viability was determined by ATP content (ViaLight HS; Cambrex Bio Sciences Rockland), with untreated cells used as a reference control. End-point TNA titers were defined as the reciprocal of the highest serum dilutions producing a significant neutralization (by *t* test) of PA-LT binary toxin cytotoxicity that was 3-fold greater than that of control serum samples.

B. anthracis aerosol challenge. Aerosol challenge was performed as described elsewhere [35]. The determination of the presented aerosol dose was calculated by use of respiratory minute volume (V_m) estimates that were derived from the respiratory function measurements performed before the exposures. The presented aerosol dose was then calculated by multiplying the total volume (V_t) of experimental atmosphere inhaled by each animal ($V_t/V_m \times$ length of exposure) and the empirically determined exposure concentration from chamber sampling (C_e) (presented dose = $C_e \times V_t$). Dose is expressed as a multiple of lethal doses, on the basis of 1.1×10^5 cfu equaling 1 lethal dose [35]. Rabbits received a mean $\pm$ SD inhaled dose of 103 ± 45 LD₅₀. Survival rates were compared by use of Fisher's exact test, with bootstrap adjustments for multiple comparisons. Time to death comparisons were made by use of a *t* test, with bootstrap adjustments for multiple comparisons.

RESULTS

Novel cutaneous-delivery technologies were developed to overcome the shortcomings of the current ones. Our first approach investigated stainless-steel microneedles that have the approximate dimensions of a strand of hair (figure 1A) and that are integrated into a depth-limiting hub attached to a standard syringe [36]. The microneedles are designed to be inserted perpendicular to the skin surface; thus, the depth of id delivery is precisely controlled by the length of the microneedle. In addition, we investigated MEA devices consisting of solid, plastic microprojections designed to breach the stratum corneum barrier when abraded over the skin surface (figure 1B), thus accessing the epidermal Langerhans cells. These MEA devices, originally fabricated from silicon, have been shown to effectively breach the skin-barrier function in humans with very little discomfort or skin irritation and to enable epidermal administration of DNA vaccines in mice [32].

A specialized device, shown in figure 1C, was developed for inl delivery of powder to rabbits. The device incorporates a powder-filled capsule sealed with rupturable film. By depressing a syringe plunger, the user generates air pressure, thus bursting the film and pushing powder through the nosepiece. This device enables inl administration of powder vaccines in a simple, disposable delivery system.

In the present study, rPA was formulated either as a liquid or as FD or SFD powder. The SFD process results in high-

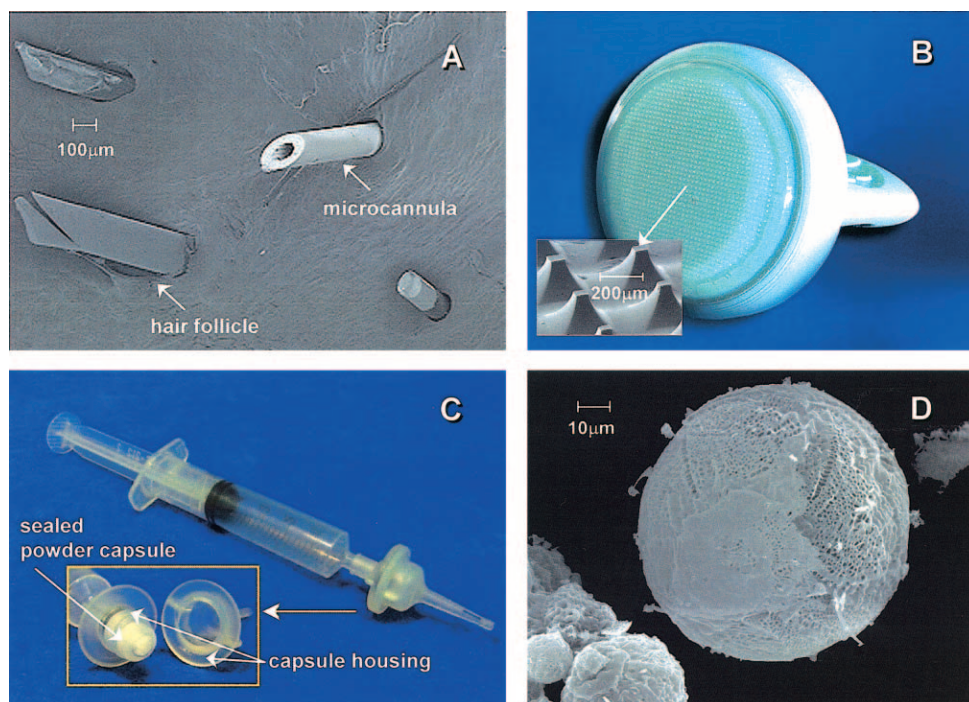


Figure 1. Vaccine delivery devices and spray-freeze-dried (SFD) powder formulation of recombinant *Bacillus anthracis* protective antigen (rPA) vaccine. *A*, Scanning electron micrograph (SEM) of a 1-mm-long stainless-steel microcannula (inner diameter, 76 μm ; outer diameter, 178 μm) penetrating swine skin (original magnification, $\times 94$). The microcannula has the approximate dimensions of a strand of hair. *B*, Microenhancer array device used for skin abrasion. The device consists of an abrading surface composed of a series of plastic microprojections (length, ~ 150 – $200\ \mu\text{m}$) affixed to a plastic handheld applicator. The inset shows an SEM of the abrading surface (original magnification, $\times 103$). *C*, Device used for intranasal delivery of powder formulations of vaccine. The device incorporates a housing that contains a powder-storage capsule with rupturable film at each end. After the exit diffuser is placed in the nasal cavity, the user depresses the plunger, bursting the film to deliver a powder plume. *D*, SEM of the SFD powder formulation of anthrax rPA vaccine (original magnification, $\times 1320$).

porosity vaccine particles (figure 1*D*), which are readily aerosolized and delivered to the nasal mucosa [37]. FD particles had a mean diameter of $\sim 50\ \mu\text{m}$ (with spans of 3–5 μm), whereas SFD particles had a mean diameter of $\sim 70\ \mu\text{m}$ (with spans of 2–3 μm).

Two animal models were evaluated. The mouse is a convenient model for an initial comparison of immunogenicity, whereas aerosol anthrax-spore challenge in the rabbit is an accepted animal correlate of humans [35, 38, 39]. Because of the small size of the murine nasal cavity, it was impractical to evaluate inl delivery of powder in mice. Thus, in our initial immunogenicity studies, we evaluated inl delivery using a conventional liquid formulation; inl delivery of powder was performed only in rabbits.

For id and epidermal administration, mice were immunized either with rPA alone, with rPA plus CpG as adjuvant, or with rPA adsorbed onto aluminum hydroxide as adjuvant. Aluminum hydroxide was chosen because only aluminum-containing adjuvants are licensed for human use in the United States and it is under clinical investigation in ongoing rPA trials [16]. CpG was investigated because this adjuvant has shown promise in preclinical trials as an alternative to aluminum hydroxide [40].

For inl administration, mice were immunized with rPA alone or with rPA plus CpG. The highest seroconversion rates after 1 dose were provided by id delivery, up to 90% (figure 2*A*). Even without adjuvant, 60% of mice immunized id were seropositive by ELISA after 1 dose was administered. In contrast, only 20% of mice immunized im with rPA plus aluminum hydroxide generated a detectable response after the first dose was administered (figure 2*A*). After the second and third doses were administered, all groups of mice immunized by the id, epidermal, and im routes displayed 100% seroconversion (figure 2*B* and 2*C*). On completion of the 3-dose series, ELISA titers induced by id delivery of rPA plus aluminum hydroxide were 4-fold greater than those induced by im delivery (figure 2*C*). Seroconversion rates after primary vaccination (figure 2*A*) and ELISA titers after each dose (figure 2) for MEA device-based epidermal delivery were similar to those for im delivery. Topical delivery without skin abrasion stimulated a weak response in a subset of mice after the second and third doses (figure 2*B* and 2*C*). Unlike cutaneous-based delivery, inl administration required an adjuvant and 3 doses to achieve responses that were comparable to those obtained by other routes (figure 2).

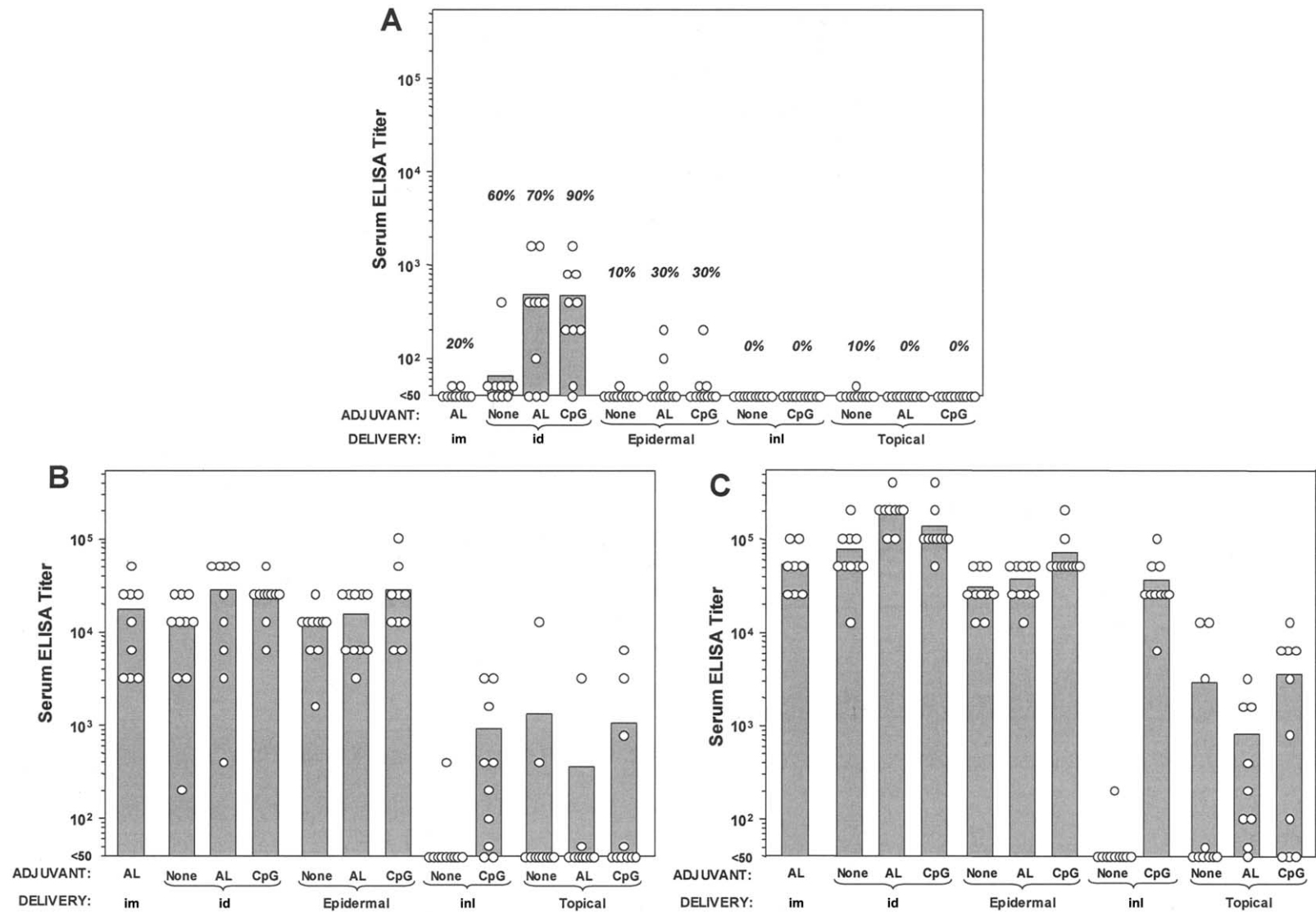


Figure 2. *Bacillus anthracis* protective antigen (PA)-specific serum ELISA titers in mice. BALB/c mice (10 mice/group) were immunized 3 times (on days 0, 21, and 42) with 10 μ g of recombinant *B. anthracis* PA either alone or with aluminum hydroxide (AL) or CpG-containing oligonucleotides (CpG) as adjuvant. The bars represent group means, and the white circles represent titers for individual mice. A, ELISA titers on day 21, after administration of 1 dose of vaccine. Percentages indicate seroconversion rates. B, ELISA titers on day 42, after administration of 2 doses of vaccine. C, ELISA titers on day 56, after administration of 3 doses of vaccine. id, intradermal; im, intramuscular; inl, intranasal.

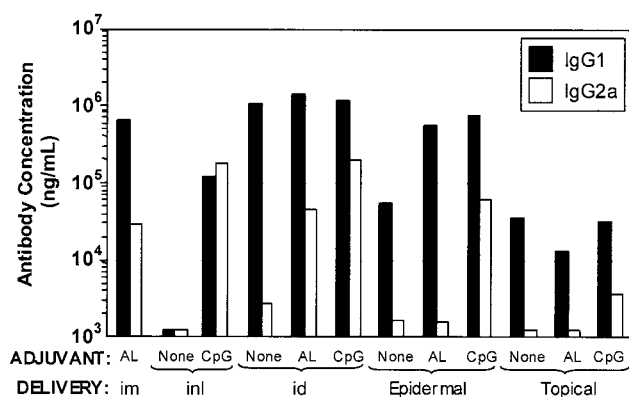


Figure 3. Serum *Bacillus anthracis* protective antigen-specific IgG1 and IgG2a concentrations in mice. Shown are the group mean concentrations from pooled serum samples obtained on day 56, after administration of 3 doses of vaccine, as determined by quantitative ELISA. AL, aluminum hydroxide; CpG, CpG-containing oligonucleotides; id, intradermal; im, intramuscular; inl, intranasal.

All groups of mice receiving rPA alone or rPA plus aluminum hydroxide generated responses consisting of higher IgG1:IgG2a ratios than did corresponding groups of mice receiving rPA plus CpG (figure 3), as has been predicted for these types of adjuvants [40, 41]. Notably, IgG2a levels surpassed IgG1 levels in mice immunized inl with rPA plus CpG (figure 3), suggesting that inl delivery may induce greater Th1 activity than the other routes.

TNA responses were evaluated from pooled serum samples. It was found that id delivery of rPA induced TNA titers that were equivalent to those induced by im delivery (figure 4). The inclusion of adjuvant increased TNA titers after id delivery, although significant titers were observed without adjuvant. After 3 doses were administered, TNA titers induced by id delivery of rPA without adjuvant were equivalent to those induced by im delivery of rPA with adjuvant. Epidermal delivery by use of MEA devices required the administration of 3 doses to achieve the same level of response as id or im delivery (figure 4). Low TNA titers were elicited by id and im delivery after 1 dose was administered, whereas inl and epidermal delivery required at least 2 doses (figure 4). In addition, after 3 doses were administered, PA-specific antibody was present in the bronchioalveolar lavage fluid of all mice immunized by the im, id, and epidermal routes and in all mice immunized inl with rPA plus CpG (data not shown).

To determine the protective efficacy of cutaneous and nasal delivery, a lethal challenge study was performed in rabbits. In addition, the rabbits provided a suitable model to evaluate the powder formulations of vaccine and inl delivery device described above (figure 1C and 1D). For inl formulations, CpG was used alone or in combination with chitosan, a biocompatible mucoadhesive polymer derived from crustacean shells that boosts mucosal immune responses [31]. After adminis-

tration of a single dose of rPA plus aluminum hydroxide by the id route, rabbits had a mean ELISA titer that was (1) 20-fold greater than that of groups receiving rPA alone or rPA plus CpG and (2) 5-fold greater than the corresponding im group (figure 5A). Among the groups immunized inl, the SFD formulation containing chitosan and CpG provided the strongest response after the administration of 1 dose, producing group mean ELISA titers that were 2–7-fold greater than those produced by the other inl formulations (figure 5A). A strong booster effect was observed across all responding groups after the second dose of vaccine was administered (figure 5B), whereas the third dose generally provided little additional increase (figure 5C). After 3 doses were administered, the highest overall ELISA titers were observed for im and id delivery, with similar titers among these groups (figure 5C). Also after 3 doses were administered, ELISA titers were similar across all groups immunized inl with powder formulations, with titers comparable with those achieved by im delivery. Powder formulations produced up to 5-fold higher group mean ELISA titers than did liquid administered inl (figure 5C). Responses induced by MEA device-based epidermal delivery were considerably weaker than those induced by other immunization routes, although, relative to passive topical delivery, a >10-fold increase in ELISA titers and a higher seroconversion rate were observed (figure 5C).

Six weeks after the last dose was administered, rabbits were aerosol challenged with ~100 LD₅₀ of anthrax spores (Ames strain), and TNA titers for individual rabbits were determined. The results demonstrate that id and inl delivery, like im delivery, provide complete protection against lethal inhalational anthrax (figure 6). The groups immunized im, id, and inl displayed

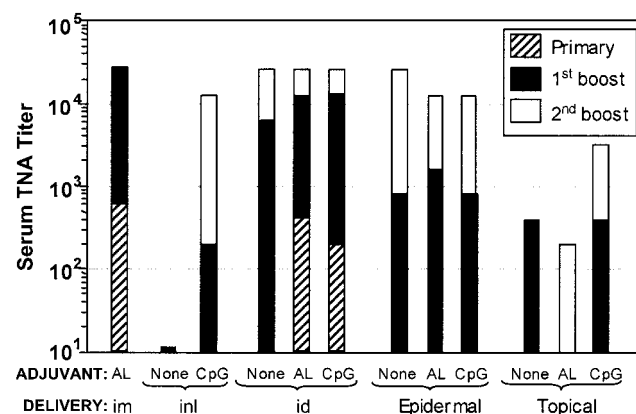


Figure 4. Serum toxin-neutralizing antibody (TNA) titers in mice. Shown are the group mean titers from pooled serum samples obtained (A) on day 21, after administration of 1 dose of vaccine (primary immunization); (B) on day 42, after administration of 2 doses of vaccine (first boost); and (C) on day 56, after administration of 3 doses of vaccine (second boost). AL, aluminum hydroxide; CpG, CpG-containing oligonucleotides; id, intradermal; im, intramuscular; inl, intranasal.

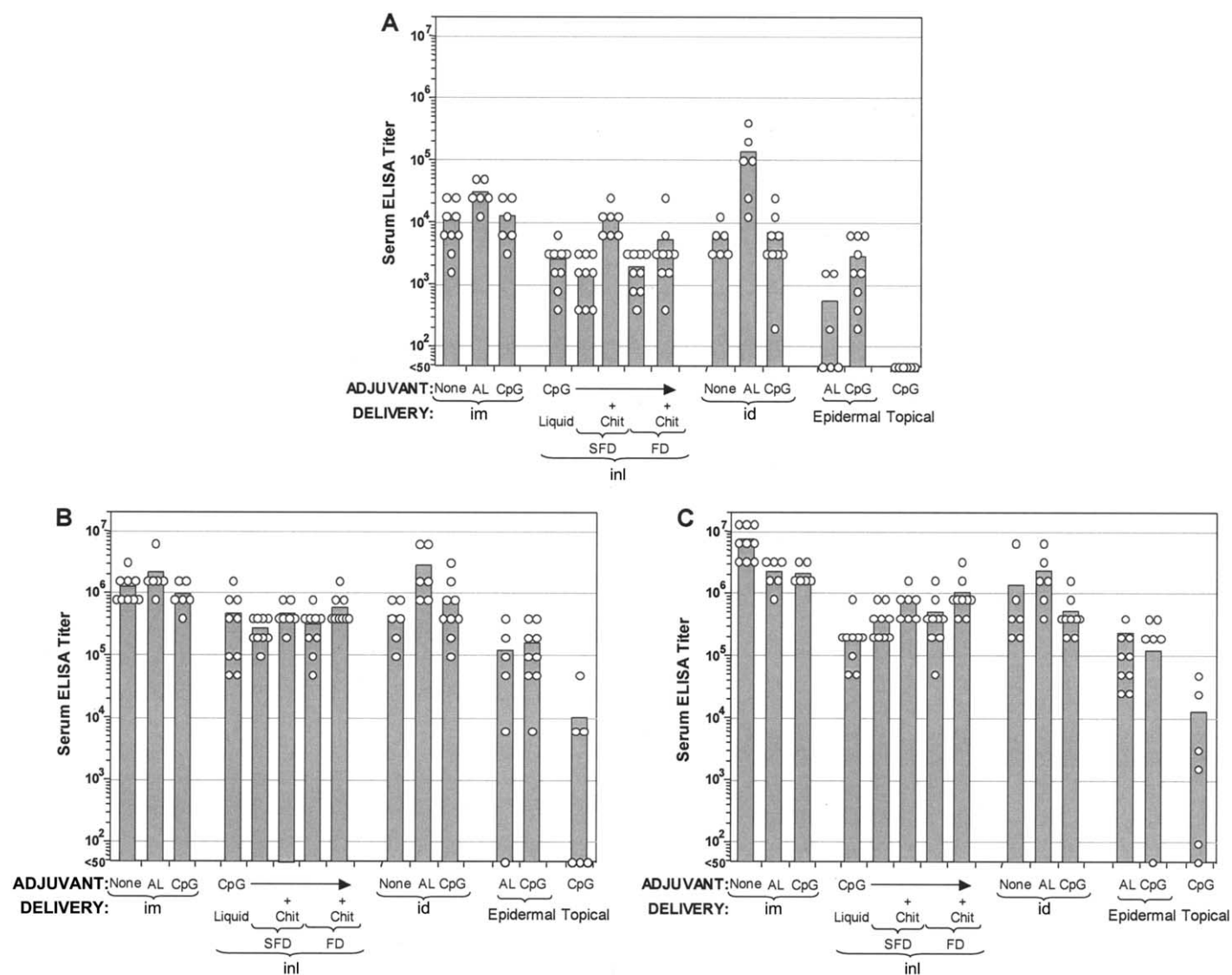


Figure 5. *Bacillus anthracis* protective antigen (PA)-specific serum ELISA titers in rabbits. New Zealand White rabbits (6–9 rabbits/group) were immunized 3 times (on days 0, 21, and 42) with 50 μ g of recombinant *B. anthracis* PA either alone or with aluminum hydroxide (AL) or CpG-containing oligonucleotides (CpG) as adjuvant. For intranasal delivery, vaccine was formulated as a liquid, freeze-dried (FD) powder, and spray-freeze-dried (SFD) powder. Powder rPA formulations contained CpG either alone or with chitosan (chit). The bars represent group means, and the white circles represent titers for individual rabbits. A, ELISA titers on day 21, after administration of 1 dose of vaccine. B, ELISA titers on day 42, after administration of 2 doses of vaccine. C, ELISA titers on day 56, after administration of 3 doses of vaccine. id, intradermal; im, intramuscular; inl, intranasal.

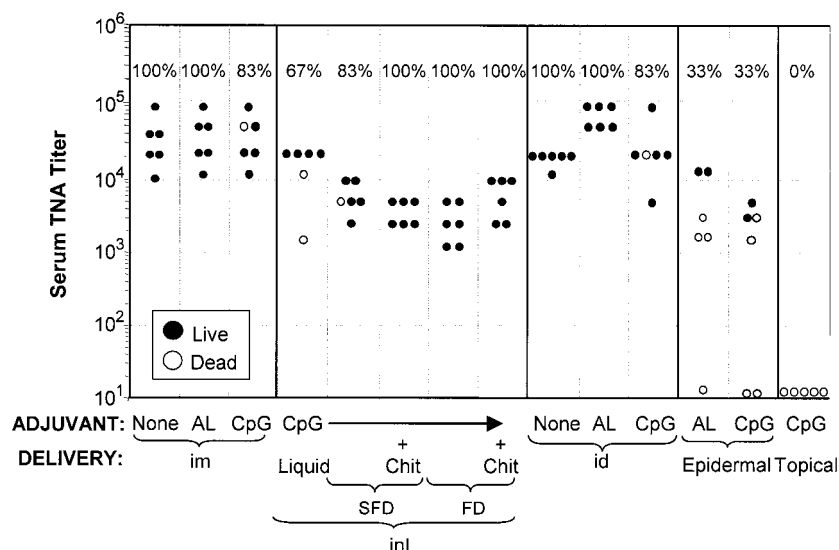


Figure 6. Relationship between toxin-neutralizing antibody (TNA) titers and survival after aerosol challenge with *Bacillus anthracis* spores (Ames strain). Six weeks after the last dose of vaccine was administered, a subset of immunized rabbits (6 rabbits/group for all except the topical group, in which there were 5 rabbits) were challenged with ~ 100 LD₅₀ of inhaled *B. anthracis* spores. Shown are serum TNA titers on day 56 for individual rabbits. Percentages indicate survival rates for each group. AL, aluminum hydroxide; chit, chitosan; CpG, CpG-containing oligonucleotides; FD, freeze-dried powder; id, intradermal; im, intramuscular; inl, intranasal; SFD, spray-freeze-dried powder.

significantly higher survival rates ($P \leq .0005$, in all cases) than did the group of unimmunized control rabbits. For rabbits immunized id and im, rPA alone and rPA plus aluminum hydroxide provided 100% (6/6) protection. Although not dramatic, there was a slight reduction in survival (83% [5/6]) for rabbits immunized id or im with rPA plus CpG (figure 6).

One-hundred percent protection (6/6) was observed in rabbits immunized inl with powder, except in the group immunized with the SFD formulation lacking chitosan, in which a single rabbit died (83% [5/6]). There was a further slight reduction in survival (67% [4/6]) in rabbits immunized inl with the liquid formulation (figure 6). Notably, the nonsurviving rabbit in the group immunized inl with powder survived for a significantly ($P = .006$) longer time (6 days) after challenge than did the unimmunized control rabbits, which died within 2–3 days (mean, 2.4 days). In contrast, there was not a significant difference ($P = .3$) in mean time to death between nonsurviving rabbits immunized inl with liquid and unimmunized control rabbits. In general, the method of powder formulation (SFD or FD) or the inclusion of adjuvant or mucoadhesive (chitosan) did not have a major effect on survival.

MEA device-based epidermal delivery provided partial protection (33% [2/6]), regardless of whether CpG or aluminum hydroxide was used as adjuvant (figure 6). In contrast, topical application without abrasion did not provide protection (0/5). Although epidermal delivery provided only partial protection, nonsurviving rabbits immunized by this route displayed significantly longer ($P = .002$) times to death (mean, 4.75 days) than did unimmunized control rabbits. Overall, these results

demonstrate the feasibility of vaccination against inhalational anthrax by use of a simple skin-abrasion system, although improvements in the device and/or formulation will be required to achieve complete protection.

For most individual rabbits, higher serum TNA titers correlated with survival (figure 6). Serum TNA titers for surviving rabbits were generally $\geq 10^4$, except for rabbits immunized inl with powder. Among these rabbits, the ones that survived had serum TNA titers that were consistently $\leq 10^4$. Notably, surviving rabbits immunized inl with liquid did not demonstrate this trend (figure 6). The highest TNA titers among all rabbits were produced by id vaccination with rPA plus aluminum hydroxide. Although nominal ELISA titers were measured for rabbits receiving vaccine topically (figure 5), no significant TNA titers were measured, and all of these rabbits died (figure 6).

DISCUSSION

Skin is a tissue with strong immunostimulatory properties that contains an abundance of antigen-presenting cells (APCs); thus, it is an attractive site for vaccine delivery [42]. Unfortunately, current methods of id administration with conventional needles are difficult to master and subject to clinical variability. Needle-free alternatives—including jet injection, iontophoresis, laser induction, sonophoresis, electroporation, and microporation—have been attempted [42, 43]; however, with the exception of jet injection, they are in very early stages of development and have yet to be proven clinically. Other researchers have developed silicon microneedles that have dimensions similar to those

of the stainless-steel microneedles described here [43–45]. Although elegant structures can be obtained from microfabricated silicon, it is uncertain whether such microneedles will be structurally robust enough to withstand routine clinical use without breaking. Furthermore, unlike stainless-steel needles, which have been a mainstay of drug and vaccine delivery during the past century, silicon microneedles are not yet approved for medical use and have not been integrated into large-scale manufacturing processes for medical devices.

Numerous laboratories are developing specialized formulations that are designed to breach the skin barrier. Bacterial enterotoxins [46–48], viral vectors [49, 50], liposomes [51, 52], and nanoparticles coupled to APC-targeting molecules [53, 54] have been described. Recently, Matyas et al. [48] have shown that, in mice, complete protection can be provided against subcutaneous anthrax challenge by immunization with rPA via a skin patch that contains *Escherichia coli* heat-labile enterotoxin, although it is not yet clear whether this approach will also protect against aerosol challenge in rabbits. Despite recent questions regarding the potential safety of applying bacterial enterotoxins to skin [55], this approach offers considerable promise, especially if used in combination with direct, mechanical skin-targeting devices, such as those described here and elsewhere [32, 43, 56].

Vaccination via the nasal mucosa has been widely studied in animal models and is now in clinical use [27, 57]. The inl route may be suited to mass vaccination, given the simplicity of the delivery systems, their ease of use, and the advantage that delivery is noninvasive. Many pathogens infect via the respiratory mucosa, and immunization at these mucosal sites can be more efficacious than immunization by injection. Jones et al. [58] have shown that inl delivery of recombinant influenza hemagglutinin (rHA) plus proteosome adjuvant produces superior protection against lethal challenge than does conventional im delivery of unformulated rHA. In addition, Lowell et al. [59] have shown that, in nonhuman primates, staphylococcal enterotoxin B toxoid plus proteosome adjuvant produces robust systemic and mucosal immune responses and 100% protection from aerosol challenge. Anthrax rPA has been administered inl as a liquid in mice [23, 24, 26] and has provided complete protection against aerosol challenge in murine models [23]. Until now, however, the inl route has not been shown to provide protection in rabbits. Furthermore, the present study is the first to demonstrate that a powder formulation provides protective immunity against anthrax. Notably, our data (figure 6) suggest that, in rabbits immunized inl with powder, serum TNA titers alone may not be predictive of survival. This is in contrast to the correlation reported previously for AVA or rPA injected im [35, 60]. The mechanism for this difference is not clear, although it is possible that inl delivery may provide stronger local responses at mucosal surfaces, thus enabling protection from

aerosol challenge, despite the lower serum TNA titers. Additional studies are required to further address this issue.

It is common for new vaccines and new delivery modalities to be tested in 2 animal species before human clinical trials are conducted. It is for this reason that we tested immunogenicity in both mice and rabbits. The rabbits generally appeared to respond more strongly to a single dose of rPA than the mice, although it is difficult to draw meaningful conclusions from such cross-species comparisons. Of all the delivery routes investigated, epidermal delivery via skin abrasion yielded the most disparate results between these species. This was likely due to differences in skin composition. We noted that rabbit skin was much more sensitive to abrasion than mouse skin, which may have affected the efficiency of vaccine uptake and immunization. Regardless, lethal challenge was performed only in rabbits, because this species provides a more robust anthrax-challenge model that closely resembles the human infection [38].

Recently, Little et al. [60] demonstrated protective efficacy against aerosol challenge in rabbits after 2 im injections of rPA plus aluminum hydroxide at low antigen doses; 83% survival was achieved with as little as 0.2 μ g of rPA. We chose a high priming dose, because the present study was the first to evaluate immunization of rabbits via skin and nasal tissue. Given the observed dose-sparing benefits provided by id delivery of vaccine for other antigens [17–19], it is possible that similar benefits may be achieved for rPA. By use of the novel delivery approaches described here, it may also be possible to reduce or eliminate adjuvant and to reduce the number of immunizations required to provide complete protection. Additional dose-reduction studies that further address these issues are in progress.

In summary, the present study provides the first systematic comparison of 5 direct-delivery modalities for anthrax vaccine: im injection, microneedle-based id injection, inl delivery of liquid and powder, and epidermal delivery by microabrasion. This comparison was based on inhalational challenge in rabbits and, for the first time, demonstrates that complete protection may be obtained by vaccination via the skin and nasal mucosa. Minimally invasive delivery of vaccine to skin or nasal tissue may significantly improve the viability of mass biodefense immunization campaigns by allowing for rapid vaccination by trained personnel in the clinic and may enable self-administration during times of emergency. If packaged as unit-dose disposables, such platforms may significantly improve vaccine-distribution logistics.

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References

- Friedlander AM. Clinical aspects, diagnosis and treatment of anthrax. *J Appl Microbiol* **1999**; 87:303.
- Abramova FA, Grinberg LM, Yampolskaya OV, Walker DH. Pathology of inhalational anthrax in 42 cases from the Sverdlovsk outbreak of 1979. *Proc Natl Acad Sci USA* **1993**; 90:2291–4.
- Meselson M, Guillemin J, Hugh-Jones M, et al. The Sverdlovsk anthrax outbreak of 1979. *Science* **1994**; 266:1202–8.
- Wein LM, Craft DL, Kaplan EH. Emergency response to an anthrax attack. *Proc Natl Acad Sci USA* **2003**; 100:4346–51.
- Klimpel KR, Molloy SS, Thomas G, Leppla SH. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. *Proc Natl Acad Sci USA* **1992**; 89:10277–81.
- Milne JC, Furlong D, Hanna PC, Wall JS, Collier RJ. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. *J Biol Chem* **1994**; 269:20607–12.
- Bradley KA, Mogridge J, Mourez M, Collier RJ, Young JA. Identification of the cellular receptor for anthrax toxin. *Nature* **2001**; 414:225–9.
- Klimpel KR, Arora N, Leppla SH. Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Mol Microbiol* **1994**; 13:1093–100.
- Kochi SK, Schiavo G, Mock M, Montecucco C. Zinc content of the *Bacillus anthracis* lethal factor. *FEMS Microbiol Lett* **1994**; 124:343–8.
- Duesbery NS, Webb CP, Leppla SH, et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **1998**; 280:734–7.
- Pellizzari R, Guidi-Rontani C, Vitale G, Mock M, Montecucco C. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFN γ -induced release of NO and TNF α . *FEBS Lett* **1999**; 462:199–204.
- Leppla SH. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. *Proc Natl Acad Sci USA* **1982**; 79:3162–6.
- Friedlander AM, Pittman PR, Parker GW. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. *JAMA* **1999**; 282:2104–6.
- Pittman PR, Kim-Ahn G, Pifat DY, et al. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. *Vaccine* **2002**; 20:1412–20.
- Sever JL, Brenner AI, Gale AD, Lyle JM, Moulton LH, West DJ. Safety of anthrax vaccine: a review by the Anthrax Vaccine Expert Committee (AVEC) of adverse events reported to the Vaccine Adverse Event Reporting System (VAERS). *Pharmacoepidemiol Drug Saf* **2002**; 11:189–202.
- National Institutes of Health. A new anthrax vaccine administered by the intramuscular (IM) route in healthy adults. Available at: <http://www.clinicaltrials.gov/ct/show/NCT00057525>. Accessed 20 February 2004.
- Warrell MJ, Warrell DA, Suntharasamai P, et al. An economical regimen of human diploid cell strain anti-rabies vaccine for post-exposure prophylaxis. *Lancet* **1983**; 2:301–4.
- Redfield RR, Innis BL, Scott RM, Cannon HG, Bancroft WH. Clinical evaluation of low-dose intradermally administered hepatitis B virus vaccine: a cost reduction strategy. *JAMA* **1985**; 254:3203–6.
- Bryan JP, Sjogren MH, Macarthy P, Cox E, Legters LJ, Perine PL. Persistence of antibody to hepatitis B surface antigen after low-dose, intradermal hepatitis B immunization and response to a booster dose. *Vaccine* **1992**; 10:33–8.
- Propst T, Propst A, Lhotta K, Vogel W, Konig P. Reinforced intradermal hepatitis B vaccination in hemodialysis patients is superior in antibody response to intramuscular or subcutaneous vaccination. *Am J Kidney Dis* **1998**; 32:1041–5.
- Staats HF, Bradley CP, Gwinn WM, et al. Cytokine requirements for induction of systemic and mucosal CTL after nasal immunization. *J Immunol* **2001**; 167:5386–94.
- Debin A, Kravtsoff R, Santiago JV, et al. Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. *Vaccine* **2002**; 20:2752–63.
- Flick-Smith HC, Eyles JE, Hebdon R, et al. Mucosal or parenteral administration of microsphere-associated *Bacillus anthracis* protective antigen protects against anthrax infection in mice. *Infect Immun* **2002**; 70:2022–8.
- Gaur R, Gupta PK, Banerjee AC, Singh Y. Effect of nasal immunization with protective antigen of *Bacillus anthracis* on protective immune response against anthrax toxin. *Vaccine* **2002**; 20:2836–9.
- Rharbaoui F, Drabner B, Borsutzky S, et al. The mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant. *Eur J Immunol* **2002**; 32:2857–65.
- Boyaka PN, Tafaro A, Fischer R, Leppla SH, Fujihashi K, McGhee JR. Effective mucosal immunity to anthrax: neutralizing antibodies and Th cell responses following nasal immunization with protective antigen. *J Immunol* **2003**; 170:5636–43.
- Davis SS. Nasal vaccines. *Adv Drug Deliv Rev* **2001**; 51:21–42.
- LiCalsi C, Maniaci MJ, Christensen T, Phillips E, Ward GH, Witham C. A powder formulation of measles vaccine for aerosol delivery. *Vaccine* **2001**; 19:2629–36.
- Rexroad J, Wiethoff CM, Jones LS, et al. Lyophilization and the thermostability of vaccines. *Cell Preservat Technol* **2002**; 1:91–104.
- Anderson J, Fishbourne E, Corteyn A, Donaldson AI. Protection of cattle against rinderpest by intranasal immunisation with a dry powder tissue culture vaccine. *Vaccine* **2000**; 19:840–3.
- Illum L, Jabbal-Gill I, Hinchcliffe M, Fisher AN, Davis SS. Chitosan as a novel nasal delivery system for vaccines. *Adv Drug Deliv Rev* **2001**; 51:81–96.
- Mikszta JA, Alarcon JB, Brittingham JM, Sutter DE, Pettis RJ, Harvey NG. Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. *Nat Med* **2002**; 8:415–9.
- Maa YF, Nguyen PA, Sweeney T, Shire SJ, Hsu CC. Protein inhalation powders: spray drying vs spray freeze drying. *Pharm Res* **1999**; 16:249–54.
- Little SF, Leppla SH, Friedlander AM. Production and characterization of monoclonal antibodies against the lethal factor component of *Bacillus anthracis* lethal toxin. *Infect Immun* **1990**; 58:1606–13.
- Pitt ML, Little SF, Ivins BE, et al. In vitro correlate of immunity in a rabbit model of inhalational anthrax. *Vaccine* **2001**; 19:4768–73.
- Alchas PG, Laurent P. Method of intradermally injecting substances [US patent 6,569,143]. **2003**.
- Hwang R, Sullivan V, Huang J, et al. Pharmaceutical compositions in particulate form [US patent application 186,271]. **2003**.
- Zauch G, Pitt LM, Estep J, Ivins BE, Friedlander AM. The pathology of experimental anthrax in rabbits exposed by inhalation and subcutaneous inoculation. *Arch Pathol Lab Med* **1998**; 122:982–92.
- Fellows PF, Linscott MK, Ivins BE, et al. Efficacy of a human anthrax vaccine in guinea pigs, rabbits, and rhesus macaques against challenge by *Bacillus anthracis* isolates of diverse geographical origin. *Vaccine* **2001**; 19:3241–7.
- Klinman DM. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* **2003**; 2:305–15.
- Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol Med Microbiol* **2001**; 32:65–71.
- Babiuk S, Baca-Estrada M, Babiuk LA, Ewen C, Foldvari M. Cutaneous vaccination: the skin as an immunologically active tissue and the challenge of antigen delivery. *J Control Release* **2000**; 66:199–214.
- Down JA, Harvey NG. Minimally invasive systems for transdermal drug delivery. In: Guy RH and Hadgraft J, eds. *Transdermal drug delivery*. 2nd ed. Vol. 123. New York: Marcel Dekker, **2002**:327–59.
- Henry S, McAllister DV, Allen MG, Prausnitz MR. Microfabricated microneedles: a novel approach to transdermal drug delivery. *J Pharm Sci* **1998**; 87:922–5.
- McAllister DV, Wang PM, Davis SP, et al. Microfabricated needles for transdermal delivery of macromolecules and nanoparticles: fabrication methods and transport studies. *Proc Natl Acad Sci USA* **2003**; 100:13755–60.
- Glenn GM, Taylor DN, Li X, Frankel S, Montemarano A, Alving CR.

- Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* **2000**;6:1403–6.
47. Guarena-Burgueno F, Hall ER, Taylor DN, et al. Safety and immunogenicity of a prototype enterotoxigenic *Escherichia coli* vaccine administered transcutaneously. *Infect Immun* **2002**;70:1874–80.
 48. Matyas GR, Friedlander AM, Glenn GM, Little S, Yu J, Alving CR. Needle-free skin patch vaccination method for anthrax. *Infect Immun* **2004**;72:1181–3.
 49. 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.
 50. Tang DC, Shi Z, Curiel DT. Vaccination onto bare skin. *Nature* **1997**;388:729–30.
 51. Alexander MY, Akhurst RJ. Liposome-mediated gene transfer and expression via the skin. *Hum Mol Genet* **1995**;4:2279–85.
 52. Li L, Hoffman RM. The feasibility of targeted selective gene therapy of the hair follicle. *Nat Med* **1995**;1:705–6.
 53. Cui Z, Mumper RJ. Topical immunization using nanoengineered genetic vaccines. *J Control Release* **2002**;81:173–84.
 54. Lisiewicz J, Bakare N, Lori F. Therapeutic vaccination for future management of HIV/AIDS. *Vaccine* **2003**;21:620–3.
 55. Riminton DS, Kandasamy R, Dravec D, Basten A, Baxter AG. Dermal enhancement: bacterial products on intact skin induce and augment organ-specific autoimmune disease. *J Immunol* **2004**;172:302–9.
 56. Matriano JA, Cormier M, Johnson J, et al. Macroflux microprojection array patch technology: a new and efficient approach for intracutaneous immunization. *Pharm Res* **2002**;19:63–70.
 57. Kemble G, Greenberg H. Novel generations of influenza vaccines. *Vaccine* **2003**;21:1789–95.
 58. Jones T, Allard F, Cyr SL, et al. A nasal proteosome influenza vaccine containing baculovirus-derived hemagglutinin induces protective mucosal and systemic immunity. *Vaccine* **2003**;21:3706–12.
 59. Lowell GH, Kaminski RW, Grate S, et al. Intranasal and intramuscular proteosome-staphylococcal enterotoxin B (SEB) toxoid vaccines: immunogenicity and efficacy against lethal SEB intoxication in mice. *Infect Immun* **1996**;64:1706–13.
 60. Little SF, Ivins BE, Fellows PF, Pitt ML, Norris SL, Andrews GP. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. *Vaccine* **2004**;22:422–30.

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