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Note

Topical immunization onto mouse skin using a microemulsion incorporated with an anthrax protective antigen protein-encoding plasmid

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Abstract

The current anthrax vaccine in the U.S., the anthrax vaccine adsorbed, has several serious drawbacks, most notably the very lengthy and complicated dosing schedule. Thus, there is a critical need to develop an alternative anthrax vaccine with a simplified immunization schedule. To address this need, we evaluated the feasibility of topically priming or boosting onto the skin using an anthrax protective antigen (PA) protein-encoding DNA vaccine. To this end, we have shown that topical immunization of mice onto their skin with a perflubron-based microemulsion incorporated with a PA63-encoding plasmid, pGPA, led to significant PA-specific antibody responses, which have anthrax lethal toxin-neutralization activity. Moreover, topical boosting of mice primed with PA protein with the pGPA-incorporated, perflubron-based microemulsion significantly enhanced the anti-PA antibody responses induced. This topical anthrax DNA vaccine has the potential to be combined with a vaccine, such as the current AVA, to produce a simplified and more convenient dosing schedule.

Keywords: Vaccine; Transcutaneous; Ab response; Lethal toxin neutralization

Anthrax is an often fatal bacterial infection caused by *Bacillus anthracis*. Only on rare occasions, can anthrax be transmitted to humans. Although *B. anthracis* strains are sensitive to many antibiotics such as ciprofloxacin, the lack of early symptoms and the rapid course of the disease make antibiotic therapy ineffective, especially in a systemic anthrax infection (Leppla et al., 2002). Vaccination is an ideal approach in preventing an anthrax infection. Interest in developing new anthrax vaccines was recently heightened by the deliberate contamination of the U.S. mail with anthrax spores shortly after 11 September 2001 (Inglesby et al., 2002). The current anthrax vaccine licensed in the U.S., the anthrax vaccine adsorbed (AVA), is an aluminum hydroxide (Alum)-adsorbed, formalin-treated culture supernatant of a toxigenic *B. anthracis* strain (Puziss et al., 1963). Although efficacious, AVA has many limitations including its difficulty in maintaining consistency, a relatively high rate of local and systemic side-effects, and most importantly, a very lengthy and complicated dosing schedule (i.e., an initial series of multiple injections on 0, 2, 4 weeks and 6, 12, and 18 months with subsequent annual boosters) (Leppla et al., 2002; Puziss et al., 1963; Wang and Roehrl, 2005), which might limit its feasibility for the vaccination of a large population of people in case of an emergency. Therefore, there is a critical need to develop an alternative anthrax vaccine. Recently, a growing body of evidence has confirmed the feasibility of non-invasive, topical immunization onto the skin using either protein subunit or DNA vaccines (Babiuk et al., 2002; Baca-Estrada et al., 2000; Beignon et al., 2001; Cui and Mumper, 2001, 2002; Fan et al., 1999; Glenn et al., 1998, 2000; Hammond et al., 2000; Heckert et al., 2002; Liu et al., 2001; Shi et al., 1999, 2001; Tang et al., 1997; Watabe et al., 2001).

An anthrax vaccine that can be applied topically onto the skin could be an attractive alternative. In fact, the feasibility of topical immunization on the skin using the anthrax protective antigen (PA) protein had been confirmed (Kenney et al., 2004; Matyas et al., 2004). DNA vaccine is generally thought to be advantageous over protein subunit vaccines because the CpG motifs in plasmid DNA have adjuvant activities, and plasmid DNA has relatively superior stability (Cui, 2005). Unfortunately, due to the stratum corneum barrier on the skin surface, application of protein or DNA alone directly on intact skin usually induces no or only a very weak immune response, necessitating the need

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for special treatment of the skin prior to antigen application or for effective vaccine adjuvants and/or delivery systems (Cui et al., 2003; Fan et al., 1999; Glenn et al., 1998; Kenney et al., 2004; Matyas et al., 2004; Ozaki et al., 2005; Tang et al., 1997; Van Kampen et al., 2005). Previously, we have reported a novel plasmid DNA-incorporated, ethanol/water-in-perflubron microemulsion, which, when applied onto shaved mouse skin, significantly enhanced the specific immune responses against a model antigen encoded by the plasmid DNA (Cui et al., 2003). The microemulsion was shown to be stable in a 3-month stability study in ambient condition (Cui et al., 2003). In the present study, we sought to evaluate the feasibility of using this microemulsion to topically dose an anthrax DNA vaccine. We chose to use the anthrax protective antigen (PA) protein as the antigen because anthrax is considered a toxin-mediated disease (Klein et al., 1962). The PA binds to the anthrax toxin receptor on the surface of host cells and mediates the entry of other components of the anthrax toxin, the lethal factor (LF) and the edema factor (EF), into the cytosol of host cells (Bradley et al., 2001), only in where the LF and EF are toxic (Ascenzi et al., 2002). Thus, anti-PA antibodies that block the entrance of the LF and EF into the cells block anthrax infection (Pitt et al., 2001; Turnbull et al., 1986).

The plasmid used in this study was the pGPA, a PA63-encoding plasmid kindly provided by Dr. Dennis Klinman in the FDA (Gu et al., 1999). The pGPA was amplified, purified using an endo-free plasmid purification kit (Qiagen, Valencia, CA), and incorporated into the ethanol/water-in-perflubron microemulsion as previously described (Cui et al., 2003). Briefly, 350 mg of perflubron (Sigma, St. Louis, MO) and 150 mg of the fluorosurfactant FSN-100 (Sigma) were weighed and mixed in a glass vial. While stirring, a combination of 35 μL of ethanol and 10 μL of pGPA in water were added into the mixture. The system was stirred for about 30 min until it became a clear, yellowish monophase. The final pGPA concentration in the microemulsion was about 160 μg/mL. The pGPA-incorporated microemulsion was then used to immunize mice on their skin. Female Balb/C mice (8–10 weeks old, 18–20 g) were from Simenson Lab (Gilroy, CA). NIH guidelines for the care and use of laboratory animals were observed. To topically immunize mice on their skin, mice were anesthetized using pentobarbital (i.p.). The hair covering their back was removed with a clipper. After the shaved area was brushed to remove cornified epithelium and cleaned with an alcohol swab (Shi et al., 2001), 40 μg of pGPA in PBS (10 mM, pH 7.4) or in the perflubron-based microemulsion was dripped onto the hair-removed area. Subcutaneous immunization was completed by simply injecting mice in their flank with recombinant PA protein (rPA, List Biological Laboratories, Inc., Campbell, CA, 5 μg/mouse) adsorbed on aluminum hydroxide gel (Alum, USP grade, Spectrum, New Brunswick, NJ, 15 μg/mouse). Mice were euthanized with CO_2 and bled by cardiac puncture. PA-specific antibody responses were measured using a previously described enzyme-linked immunosorbent assay (ELISA) (Sloat and Cui, 2006). The ability of the anti-PA Abs to neutralize anthrax lethal toxin was measured using an in vitro macrophage (J774A.1) protection assay (Boyaka et al., 2003; Sloat and Cui, 2006). Briefly, confluent J774A.1 cells were plated (5.0×10^4 cells/well) in sterile, 96-well clean-bottom plates (Corning Costar, Corning, NY) and incubated at 37 °C, 5% CO_2 for 16 h. A fresh solution (50 μL) containing rPA (400 ng/mL) and LF (40 ng/mL) was mixed with 50 μL of the diluted serum or BAL samples in triplicate and incubated for 1 h at 37 °C. The cell culture medium was removed, and 100 μL of the serum/lethal toxin mixture was added to each well and incubated at 37 °C, 5% CO_2 for 3 h. Cell viability was determined using an MTT assay kit (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide, Sigma–Aldrich) with untreated or lethal toxin alone treated cells as controls.

To evaluate the feasibility of using the pGPA-incorporated, perflubron-based microemulsion to induce PA-specific immune responses, mice were dosed topically on their skin with the pGPA-incorporated microemulsion, and the resulted anti-PA immune responses were measured. As shown in Fig. 1, pGPA alone did not induce any significant anti-PA IgG responses, only two of the six mice produced some very weak anti-PA IgG. In contrast, immunization of mice with the pGPA-incorporated microemulsion induced a significant level of anti-PA IgG in the serum of all mice, although the anti-PA IgG titer in this group of mice was still lower than that in mice subcutaneously injected with rPA adsorbed onto Alum (Fig. 1). As expected, anthrax lethal toxin-neutralization activity was not detectable in any of the mice topically immunized with the pGPA alone on their skin (Table 1). However, at the same dilution (10-fold), lethal toxin-neutralization activity was detectable in the serum

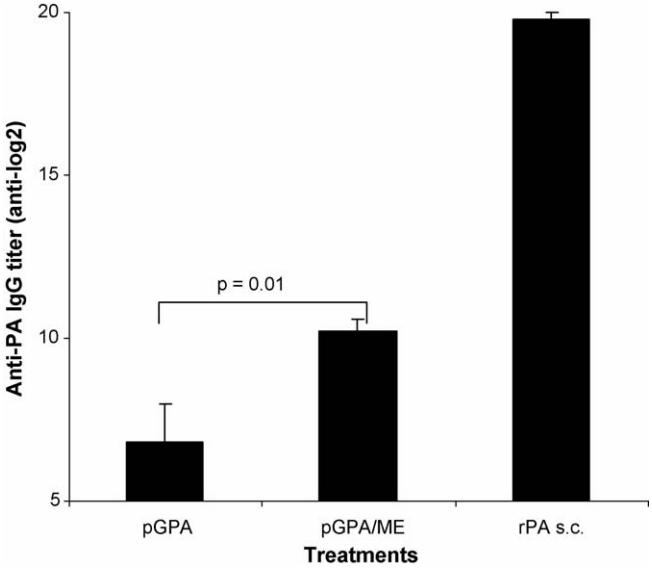

Fig. 1. Mice ($n = 6$) were topically immunized with pGPA alone (pGPA) or pGPA-incorporated microemulsion (pGPA/ME). As controls, one group of mice ($n = 6$) were left untreated, and another group of mice ($n = 5$) were s.c. injected with rPA (5 μg/mouse) adsorbed on Alum (15 μg/mouse) (rPA s.c.) on days 0, 7, and 14. On day 30, mice were euthanized, and their serum samples were collected. Anti-PA IgG titers in them were determined using ELISA. ANOVA revealed significant differences among the values for pGPA, pGPA/ME, and rPA (s.c.) ($p \ll 0.05$). The values for pGPA and pGPA/ME were significantly different from each other ($p = 0.01$, two-tail). Also, the value for rPA (s.c.) was significantly higher than that of the others. Data shown were mean ± S.D. The experiment was repeated independently three times with similar results. One representative was shown.

Table 1
Lethal toxin-neutralization activity measured using an in vitro macrophage (J774A.1) protection assay

	Serum dilution factor				
	10×	100×	1000×	10,000×	100,000×
pGPA	0/6[a]				
pGPA/ME	6/6	3/6	2/6	0/6	
(% live J774A.1 cells)	45.5 ± 4.9[b]	32.7 ± 7.0	32.2 ± 1.0		
rPA s.c.	N/D	5/5	5/5	4/5	1/5
(% live J774A.1 cells)	N/D	109.6 ± 8.4	40.7 ± 19.1	23.6 ± 16.8	8.6

Mice ($n = 6$) were topically immunized with pGPA alone (pGPA) or pGPA-incorporated microemulsion (pGPA/ME). As controls, one group of mice ($n = 6$) were left untreated, and another group of mice ($n = 5$) were s.c. injected with rPA (5 μg/mouse) adsorbed on Alum (15 μg /mouse) (rPA s.c.) on days 0, 7, and 14. On day 30, mice were euthanized, and their serum samples were collected. The serum samples from individual mice were diluted 10-fold serially, and the lethal toxin-neutralization activity in them was determined using an in vitro macrophage (J774A.1) protection assay (i.e., J774A.1, 5.0×10^5 cells/mL; PA, 400 ng/mL; LF from List Biological, 40 ng/mL). No lethal toxin-neutralization activity was detected in un-immunized mice.

[a] The number of mice who had lethal toxin-neutralization activity.

[b] The % of J774A.1 cells still alive after the lethal toxin challenge. Data shown were mean ± S.D.

of all mice topically immunized with the pGPA-incorporated microemulsion (Table 1). Even after 1000-fold dilution, LeTx neutralization activity was still detectable in two out of six of the immunized mice. Although more experiments have to be completed to elucidate the mechanisms of immune enhancement by the perflubron-based microemulsion, we speculate that the microemulsion enhanced the anti-PA immune responses mainly be enhancing the expression of the PA63 genes in the application area on the skin or the local draining lymph nodes. The possibility of immunostimulatory activity from the fluorinated compounds, the perflubron and the FSN-100, was unlikely because they are generally considered to be biologically and immunologically inert.

Although topical immunization with the pGPA-incorporated, perflubron-based microemulsion induced significantly stronger anti-PA immune responses when compared to immunization with the pGPA alone, the immune responses induced were still rather weak. More experiments will have to be completed to further improve the potency of the anti-PA immune response, but we believe that the immune responses would have been stronger if the codons on the plasmid pGPA had been optimized because the PA gene from bacillus is very AT rich (Gu et al., 1999; Hermanson et al., 2004). In addition, induction of the hair follicles in the application sites into anagen-onset stage is expected to further enhance the anti-PA immune responses induced (Domashenko et al., 2000). Thus, without further improvements, topical immunization with the current pGPA-incorporated microemulsion alone might not provide a sufficient protection against an anthrax challenge.

To better use this pGPA-incorporated, perflubron-based microemulsion, we tested the feasibility of using it to boost the anti-PA immune responses induced by the s.c. injected, Alum-adjuvanted rPA. As shown in Fig. 2, boosting with the pGPA-incorporated microemulsion significantly enhanced the serum anti-PA IgG titer in mice pre-immunized once with the Alum-adjuvanted rPA ($p = 0.04$, t-test, two-tail). The lethal toxin-neutralization activities in mice boosted or not boosted with the pGPA-incorporated microemulsion were also measured, but were found not different from each other (data not shown). It is possible that the three-fold enhancement in the anti-PA IgG titer generated by boosting with the pGPA-incorporated microemulsion was not large enough to lead to a significant enhancement in the lethal toxin-neutralization activity, because the lethal toxin-neutralization assay was a cell-based assay, which might be less sensitive than the ELISA in detecting the differences in the anti-PA immune responses. The anti-PA Ab response induced was biased towards anti-PA IgG1 regardless of whether the mice were topically boosted with the pGPA-incorporated microemulsion or not (Fig. 3). However, boosting the mice by topically immunizing them with the pGPA-incorporated microemulsion tended to make the Ab response less IgG1-biased because, at

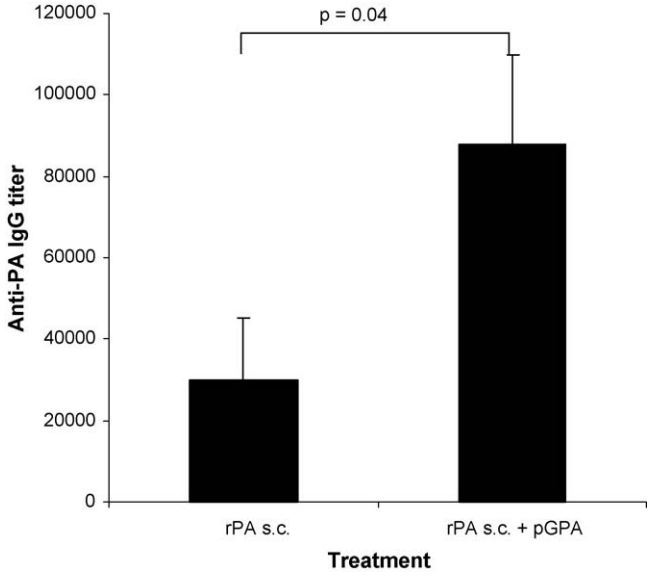

Fig. 2. Mice were either left untreated or s.c. injected with rPA (5 μg/mouse) adsorbed on Alum (15 μg/mouse) on day 0. On days 14, 21, and 28, half of the mice that were injected with the rPA/Alum were topically dosed onto their skin with the pGPA-incorporated microemulsion (rPA s.c. + pGPA), while the others were left untreated. On day 35, mice were euthanized, and anti-PA IgG titers in the serum samples were determined using ELISA. A t-test revealed that the values for rPA (s.c.) and rPA (s.c.) + pGPA were significantly different from each other ($p = 0.04$, two-tail). Data shown were mean ± S.D. ($n = 6$). The anti-PA IgG levels in the boosted and non-boosted groups were comparable on the day (day 14) when the boosting immunization started (data not shown).

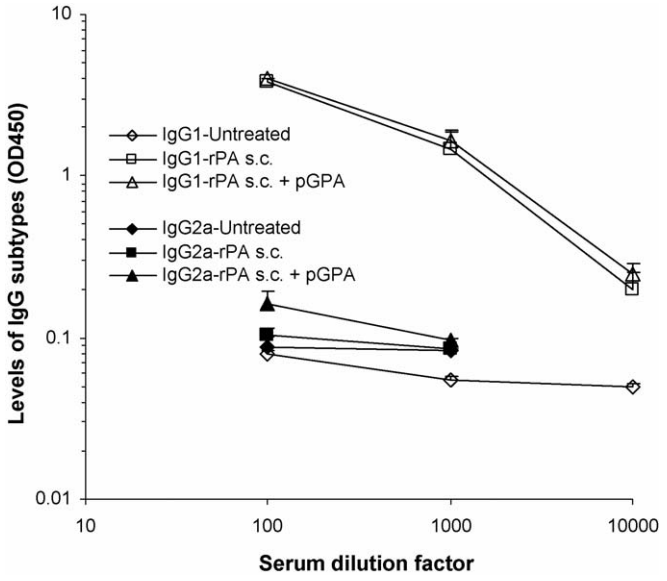

Fig. 3. Mice were either left untreated or s.c. injected with rPA (5 μg/mouse) adjuvanted with Alum (15 μg/mouse) on day 0. On days 14, 21, and 28, half of the mice that were injected with the rPA/Alum were topically dosed onto their skin with the pGPA-incorporated microemulsion (rPA s.c. + pGPA), while the others were left untreated. On day 35, mice were euthanized, and the levels of anti-PA IgG subtypes (IgG1 and IgG2a) in the serum samples were determined using ELISA. Data shown were mean ± S.D. ($n = 6$) of the OD450 values obtained in serum samples diluted 10-fold serially.

the 100-fold dilution, the $OD450_{IgG1}/OD450_{IgG2a}$ value for the non-boosted mice (37.1 ± 2.9) was significantly higher than that for the boosted mice (25.7 ± 2.0) ($p \ll 0.05$). This was understandable because the immune response from DNA vaccine is generally Th1-biased (Cui, 2005).

In conclusion, we have shown that topical immunization of mice onto their skin with a perflubron-based microemulsion, incorporated with an anthrax protective antigen protein (PA63)-encoding plasmid led to PA-specific Ab responses, which have anthrax lethal toxin-neutralization activity. Moreover, topical immunization of mice previously immunized with PA protein with the pGPA-incorporated, perflubron-based microemulsion significantly boosted the anti-PA Ab responses induced. This topical anthrax DNA vaccine has the potential to be combined with the current AVA to significantly simplify the dosing schedule of the current AVA.

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