

Immunization by Application of DNA Vaccine onto a Skin Area Wherein the Hair Follicles Have Been Induced into Anagen-onset Stage

Dalia S Shaker¹, Brian R Sloat¹, Uyen M Le¹, Christiane V Löhner², Nijaporn Yanasarn¹, Kay A Fischer² and Zhengrong Cui¹

¹Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon, USA; ²Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon, USA

An attractive approach to immunization is to apply DNA vaccine topically onto the skin. However, it is important to ensure that a strong immune response is induced without disrupting the skin stratum corneum. The hair follicles have been shown to be the major portal of entry for DNA applied onto the skin, and it has been reported that the transfection of hair follicle cells occurs mainly at the onset of a new growing stage of the hair cycle. Using an anthrax protective antigen (PA) protein-encoding plasmid in mice, we demonstrated that the anti-PA immune responses were significantly stronger when the hair follicles in the application area were induced into anagen-onset stage than when in telogen stage. The anti-PA antibodies enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge. The enhanced immune responses can be partially attributed to the enhanced antigen gene expression and plasmid DNA uptake in the skin area wherein the hair follicles were induced into anagen-onset stage. Moreover, the moderate dermal inflammation associated with the anagen induction may also have contributed to the enhancement of the resultant immune response. This represents a novel approach to enhancing the immune response induced by a topically applied DNA vaccine.

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INTRODUCTION

The feasibility of topical immunization onto the skin using a DNA vaccine was confirmed in the 1990s.^{1,2} Unfortunately, the immune response induced by applying unformulated DNA onto intact skin was generally weak, and efforts to enhance the immune response without disrupting the skin stratum corneum had only limited success.³ In order to induce an immune response, the topically applied DNA has to have access to the viable cells in the skin. The cornified skin stratum corneum layer presents a barrier preventing the DNA from entering into the viable skin layers.⁴ Data from several recent studies have shown that the hair follicles, being

natural entry points for foreign objects, are the main portals of entry for topically applied DNA.^{5,6} When plasmid was applied onto the skin, the expression of the encoded gene was largely confined to the hair follicles.⁵⁻⁹ We therefore reasoned that the immune responses induced by a topically applied DNA vaccine can be enhanced by modifying the hair follicle cycle in the application area. Data from recent studies have shown that the hair follicles are open for penetration by foreign objects only when in the anagen (growth) stage, and closed when in the telogen (resting) stage.¹⁰ In addition, the stage of the cycle at the time of DNA application had a critically important bearing on the resultant gene expression in the hair follicles.⁹ In mouse skin, as well as in human skin grafted onto nude mice, the expression of a topically applied reporter gene was highest when the hair follicles were induced into anagen-onset stage.⁹ Apart from the fact that the anagen-stage hair follicles are open for penetration, hair follicles usually proliferate only during the anagen stage, and proliferating cells express DNA more efficiently than quiescent cells.¹¹ In addition, the anagen stage is associated with alterations in cutaneous immunity (e.g., the immuno-suppression of the skin immune system).¹² Although the proximal region of hair follicles becomes immune-privileged in certain stages of anagen, the distal region of hair follicles is rich in Langerhans cells, T cells, major histocompatibility complex I⁺ and II⁺ macrophages, and other immune cells.¹³ Moreover, it was found that hair follicles were essential for achieving successful DNA immunization by topical application onto the skin.⁶ On the basis of all these findings, we hypothesized that applying a DNA vaccine onto a skin area wherein the hair follicles are in the anagen-onset stage will induce a stronger immune response than when the hair follicles in the application area are in the telogen stage.

In order to test this hypothesis, we used a plasmid, pGPA, which encodes the anthrax PA63 protein, in a mouse model.¹⁴ An anthrax vaccine that can be applied topically onto the skin is expected to be amenable for self-administration. Anthrax is a toxin-mediated disease.¹⁵ The protective antigen (PA) is required for transporting anthrax lethal factor (LF) and edema factor into the cytosol of host cells.¹⁶ LF and edema factor are toxic only when they are inside cells.¹⁶ Anti-PA antibodies (Abs) were thus shown

Correspondence: Zhengrong Cui, Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon 97331, USA. E-mail: Zhengrong.cui@oregonstate.edu

to protect hosts against an anthrax challenge.¹⁷ Mouse hair follicles go through synchronous cycles during the first 2–3 months of life.¹⁸ The second telogen phase begins between days 30 and 35 and lasts for at least 40 days. Because of this, the hair follicles on the backs of mice of 40–70 days of age are predominately in their second telogen phase.¹⁸ We induced the hair follicles on the backs of 6-week-old mice into anagen-onset stage by hair-plucking. Hair-plucking is known to cause resting hair follicles to enter anagen stage in a predictable and synchronous manner.¹⁸ We then applied the pGPA plasmid, complexed with Lipofectamine, onto the prepared skin area.⁹ Cholera toxin (CT) was used as the adjuvant.^{19,20} We found that the PA-specific antibody responses induced by the topical pGPA were significantly enhanced by inducing the hair follicles in the application area into anagen-onset stage. The integrity of the skin in the application area was not compromised at the time of DNA application. It is likely that the enhanced antigen gene expression in the skin and the dermal inflammation associated with the anagen induction were both responsible for the enhancement of the resultant immune responses.

RESULTS

Topical immunization by application of pGPA onto a skin area wherein the hair follicles had been induced into anagen-onset stage led to a strong anti-PA immune response

The hair on the back of mice was trimmed and then plucked in an area of ~1 cm². After 48 hours, the skin color in the plucked area turned from pink to white, indicating the start of anagen-onset stage. The pGPA vaccine was then applied onto the prepared area. As shown in **Figure 1**, by inducing the hair follicles into anagen-onset stage in the application area on the mouse skin, the resultant anti-PA immunoglobulin G (IgG) titer was significantly higher than when the hair follicles were left in the telogen stage. The level of the anti-PA IgG found in the anagen-onset stage group was comparable to that in mice intramuscularly (IM) injected with the “naked” pGPA alone ($P = 0.11$). This experiment was repeated, and similar results were obtained. Similar results were

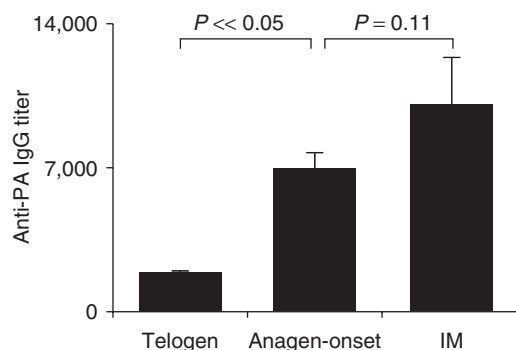


Figure 1 Immunization by applying pGPA topically onto a skin area wherein the hair follicles had been induced into anagen-onset stage generated a stronger anti-PA immunoglobulin G (IgG) titer than when the hair follicles were in the telogen stage. The IgG titers were measured 35 days after the first immunization. The values of the anagen-onset group and the telogen group differed from each other ($P < 0.05$). There was no difference in titer values between the anagen-onset samples and those from intramuscular (IM) delivery of the “naked” pGPA ($P = 0.11$).

also obtained when the hair follicles in the application area were induced into anagen-onset stage by pre-treatment with tretinoin (0.05%) (data not shown). Therefore subsequent experiments evaluated the immune responses only in skin application areas with anagen-onset stage hair follicles.

The anti-PA IgG was IgG1-biased, whereas IM injection of the “naked” pGPA induced an IgG2a-biased Ab response (**Figure 2a**). **Figure 2b** depicts the kinetics of the anti-PA IgG level induced by the topical pGPA. The splenocytes isolated from these mice also proliferated significantly after *in vitro* re-stimulation (**Figure 2c**).

In order to evaluate the protective activity of the induced anti-PA Abs, the anthrax lethal toxin neutralization activity was evaluated both *in vitro* and *in vivo*. The anti-PA Abs in mice topically immunized with the pGPA were able to protect macrophages (J774A.1) against anthrax lethal toxin *in vitro* (**Figure 2d**), and enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge. When intravenously injected with the lethal toxin at a dose equivalent to 7.5 times the LD₅₀, all unimmunized mice ($n = 5$) died within 48 hours, while all mice ($n = 4$) immunized with pGPA by topical application onto the skin or by IM injection survived.

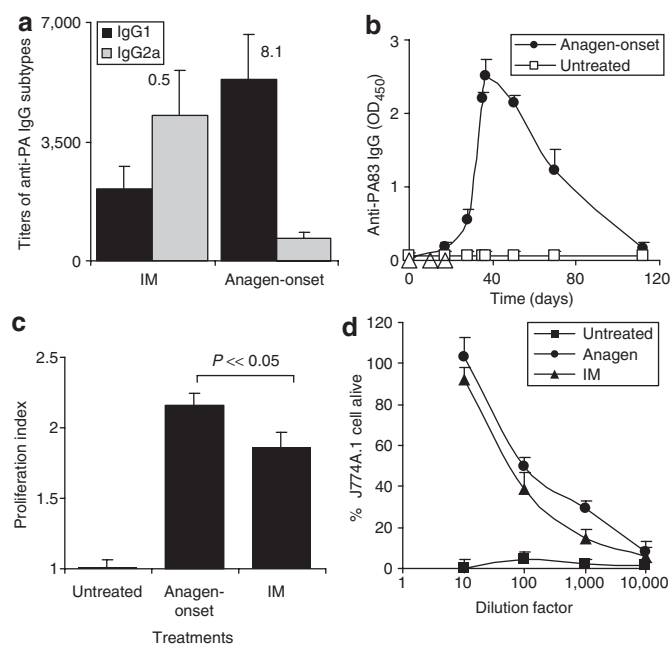


Figure 2 Immunization by applying pGPA topically onto a skin area wherein the hair follicles had been induced into the anagen-onset stage generated strong anti-PA immune responses. **(a)** Serum anti-PA immunoglobulin G (IgG) subtypes. The IgG1 and IgG2a titers were determined 35 days after the first immunization. The numbers above the bars are the ratio of the IgG1 titer to the IgG2a titer (IgG1/IgG2a). **(b)** Kinetics of the anti-PA IgG response. The anti-PA IgG level was reported as the OD₄₅₀ values after the serum samples were diluted 40-fold. The (open triangle) symbols indicate the days on which the mice were immunized. **(c)** Splenocyte proliferation. The values of anagen-onset samples and intramuscular (IM) samples were significantly different from each other ($P < 0.05$), and both were higher than those of the untreated control. **(d)** The anti-PA Abs protected macrophages against anthrax lethal toxin. The serum samples were taken from mice topically immunized with pGPA-Lipofectamine lipoplexes admixed with cholera toxin. Data reported are mean \pm SD ($n = 7$). PA, protective antigen.

CT was required for the pGPA to induce anti-PA Abs

Because bacterial plasmid DNA is immuno-stimulatory, it was unclear whether CT was essential for the pGPA to induce anti-PA responses. We therefore evaluated the levels of the anti-PA IgG induced by the pGPA in lipoplexes, with and without CT. Again, the hair follicles in the skin application area were induced into anagen-onset stage prior to the application. The data in **Figure 3a** show that CT is needed for the topical pGPA to induce a detectable level of anti-PA immune response.

A relatively large dose of pGPA was required to induce a detectable level of anti-PA Abs

Figure 3b shows the relationship between the dose of pGPA applied topically onto the skin and the level of serum anti-PA IgG induced. It is evident that a relatively large amount (or high concentration) of the pGPA (50 µg) was required in order to induce anti-PA Abs.

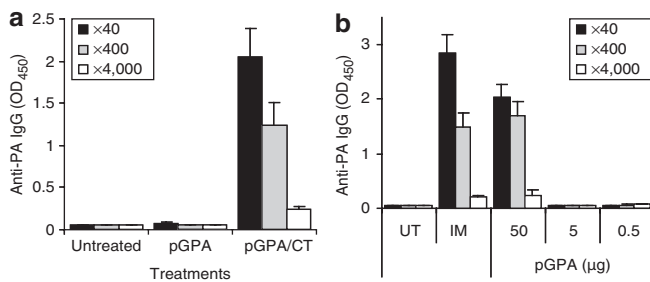


Figure 3 Cholera toxin (CT) was necessary for the pGPA to induce anti-PA antibodies (Abs), and a relatively large amount of pGPA was required in order to induce a detectable level of anti-PA Abs. **(a)** Mice ($n = 7$) received topical applications of pGPA-Lipofectamine lipoplexes, with or without CT, on a skin area wherein the hair follicles had been induced into anagen-onset stage. **(b)** Mice ($n = 7$) received topical applications of pGPA-Lipofectamine lipoplexes admixed with CT on a skin area wherein the hair follicles had been induced into anagen-onset stage. The dose of pGPA was 0.5, 5, or 50 µg per mouse. The anti-PA immunoglobulin G (IgG) levels were determined 35 days after the first immunization and reported as the OD₄₅₀ values. UT denotes “untreated”.

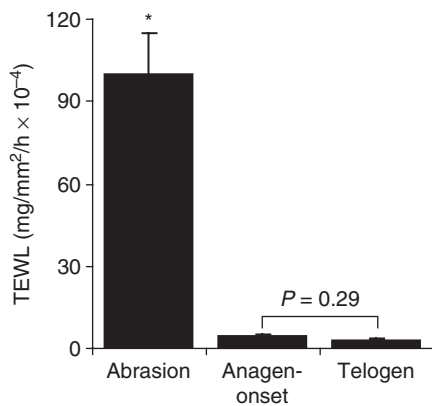


Figure 4 The integrity of the skin at the time of DNA application was not compromised. Hair on the skin was trimmed (telogen) or trimmed and plucked (anagen-onset). The positive control consisted of mice with skin abraded. The trans-epidermal water loss (TEWL) values ($n = 3$) were measured 48 hours later. The value of the control (abrasion) group was higher than that of the others. The values of the anagen-onset and the telogen groups were comparable ($P = 0.29$).

The integrity of the skin at the time of DNA application was not compromised

In order to evaluate the extent to which any potential physical damage to the skin by the plucking procedure might be responsible for the enhancement of the resultant immune responses, the trans-epidermal water loss from the skin samples was measured 48 hours after the hair was plucked, and compared to the corresponding value 48 hours after the hair was simply trimmed. As shown in **Figure 4**, the trans-epidermal water loss values in the plucked and un-plucked areas were not significantly different ($P = 0.29$), thereby indicating that the integrity of the skin area

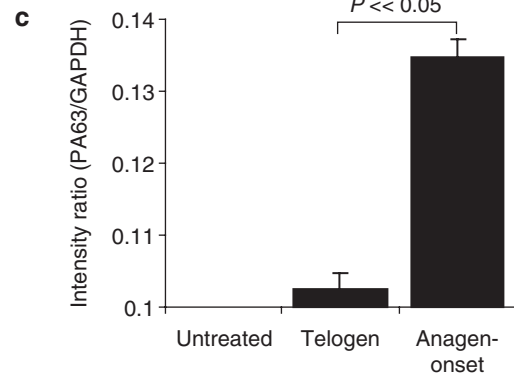
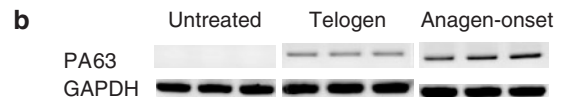
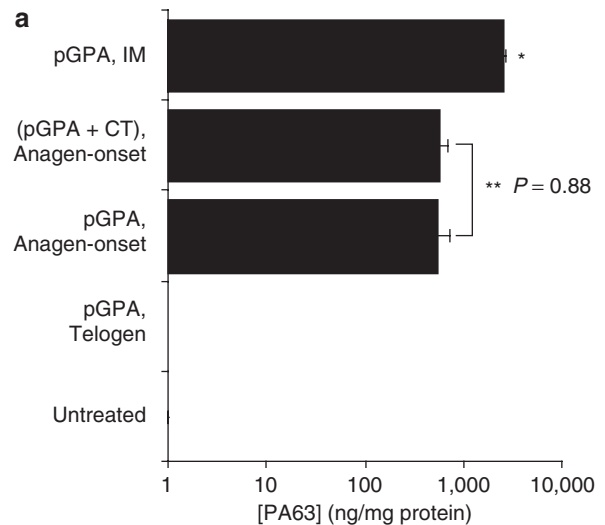


Figure 5 Induction of hair follicles into anagen-onset stage enhanced the expression of the antigen gene (PA63) applied onto the skin and increased the retention of the pGPA plasmid in the skin. **(a)** PA63 expression was not detectable in the untreated mice and in mice whose hair follicles in the application area were in telogen stage. Values shown within the same bracket were not different from each other ($P = 0.88$). Values labeled with different asterisks (*, **) were significantly different from one another ($P << 0.05$). **(b and c)** More pGPA plasmid was recovered from the skin area wherein the hair follicles had been induced into anagen-onset stage. **(b)** The polymerase chain reaction (PCR) products of a protective antigen (PA) gene fragment (PA_{336-1,060}) and a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene fragment (GAPDH₆₁₋₅₆₁). **(c)** Band intensity ratios (PA/GAPDH). The values of the telogen and the anagen-onset groups were significantly different from each other ($P = 0.00027$). IM, intramuscular.

was not significantly compromised at the time when the pGPA vaccine was applied.

Induction of hair follicles into anagen-onset stage enhanced the expression of the antigen gene applied topically onto the skin and increased the retention of the plasmid in the skin

Figure 5a shows that the skin area wherein the hair follicles were induced into anagen-onset stage had a significantly higher level of PA63 expression than the skin area wherein the hair follicles were in the telogen phase ($P = 0.005$). The inclusion of the CT had no significant effect on the level of PA63 expression.

Polymerase chain reaction was carried out for relative quantification of the amount of pGPA retained in the skin area 24 hours after the application of the pGPA. As shown in **Figure 5b** and **c**, relatively more PA63 gene fragments were recovered from the skin samples in which the hair follicles were induced into anagen-onset stage than from skin samples in which the hair follicles were in the telogen stage.

The anagen induction procedure induced moderate dermal inflammation

As shown in **Figure 6**, simple trimming of the hair did not result in any significant dermal inflammation immediately or 48 hours after the trimming (**Figure 6a** and **c**). In contrast, just after the plucking, we observed signs of early inflammation (edema and endothelial swelling in the superficial dermis) (**Figure 6b**). Forty-eight hours after the plucking, moderate epidermal hyperplasia and mild hyperkeratosis were observed. There was also mild proliferation of the hair follicle epithelium. In addition, increased cellularity was apparent in the dermis and the subcutaneous tissue, evidenced by moderate mid-dermal or full-thickness periadnexal or interstitial infiltration of inflammatory cells (*e.g.*, lymphocytes, neutrophils, macrophages, and mast cells) (**Figure 6d**).

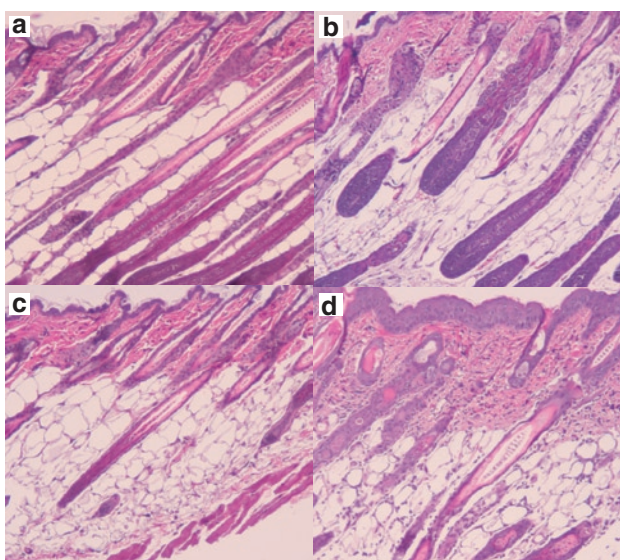


Figure 6 Hair-plucking induced an acute dermal inflammation. Skin samples were collected (**a**, **b**) immediately, or (**c**, **d**) 48 hours later after the hair on mice was (**a**, **c**) trimmed, or (**b**, **d**) trimmed and plucked. The photographs were taken at a $\times 10$ magnification.

DISCUSSION

In this study we showed that the application of an anthrax PA63-encoding plasmid onto a mouse skin area wherein the hair follicles had been induced into anagen-onset stage, led to stronger anti-PA immune responses than when the plasmid was applied onto a skin area wherein the hair follicles were predominantly in the telogen stage. The anti-PA Abs induced had anthrax lethal toxin neutralization activity and enabled the immunized mice to survive a lethal dose of anthrax lethal toxin challenge. Moreover, our data indicated that the enhanced immune responses may be attributed partly to the enhanced antigen gene expression and plasmid uptake in the skin area wherein the hair follicles had been induced into anagen-onset stage, and partly to the acute dermal inflammation associated with the anagen induction.

In mice with induced anagen-onset-stage hair follicles in the application area, the anti-PA IgG response was relatively long-lasting (**Figure 2b**). The anti-PA IgG level peaked about 40 days after the first immunization. Four months after the first immunization, anti-PA IgG was still detectable in the immunized mice. The anti-PA Abs in the topically immunized mice were confirmed to be functional in both *in vitro* and *in vivo* lethal toxin challenge studies. The finding that the immune responses induced by a topically applied DNA vaccine can be significantly enhanced by simply modifying the cycle of hair follicles in the application area is significant, considering that the wax plucking approach is commonly used by humans to remove unwanted hair. Moreover, human hair follicles are much larger than mouse hair follicles, and the majority of the follicles in a normal human scalp are in the anagen stage.^{21,22} Also, it was shown that the transfection of hair follicles in human skin grafted onto nude mice was significantly enhanced by inducing the hair follicles into anagen-onset stage.⁹ Therefore this mode of topical DNA immunization may hold great promise for non-invasive administration of DNA vaccines to humans, although more experiments will have to be completed to confirm this. In humans, a topical DNA vaccine of interest may be dosed onto the scalp in a liquid dosage form (*e.g.*, lotion) if anagen-onset stage induction is found to be unnecessary. Otherwise, if an anagen induction step (*e.g.*, plucking) is required, a vaccine patch may be applied to an area such as the axilla.

The anti-PA IgG induced by the topically applied pGPA was IgG1-biased, whereas the anti-PA IgG in mice IM injected with the “naked” pGPA alone was IgG2a-biased (**Figure 2a**). This agreed well with previous findings that the immune responses induced by IM injected plasmid DNA were usually T helper (Th1)-biased,^{23,24} while the immune responses induced when the plasmid was applied through the skin usually trended toward Th2.^{6,24} Moreover, the splenocytes isolated from mice topically dosed with the pGPA in lipoplexes also proliferated significantly when re-stimulated *in vitro* with the PA83, thereby suggesting that T cells had been activated (**Figure 2c**).

Because bacterial plasmid DNA has an inherent immunostimulatory activity, and data from a previous study demonstrated that unmethylated CpG motifs in bacterial plasmid enhanced the immune responses induced by topically applied protein subunit vaccines, we tested whether CT was essential for the topical pGPA to induce anti-PA Abs.²⁵ Surprisingly, no detectable level

of anti-PA IgG was induced in the absence of CT (**Figure 3a**). It is likely that, when dosed topically, the CpG motifs in the pGPA plasmid were not sufficiently immunostimulatory as to promote the expressed PA63 to induce a detectable level of anti-PA immune response. The precise mechanism of the adjuvant effects of topically applied CT is not well understood yet. It was previously shown that CT and other heat-labile enterotoxins from *Escherichia coli* (LT) were usually necessary for topically applied protein subunit vaccines to induce immune responses.¹⁹ Both CT and LT are adenosine diphosphate–ribosylating exotoxins. They are known to be mucosal vaccine adjuvants. It was shown that topical LTs activated Langerhans cells and promoted them to migrate to the regional lymph nodes for antigen presentation.²⁶

Our data also showed that a relatively high dose or high concentration of DNA was required for the topically applied pGPA to induce a detectable level of anti-PA Abs (**Figure 3b**). Yu *et al.*²⁷ investigated the effect of the concentration of DNA applied onto the skin on the resultant gene expression in the application area. The highest gene expression was observed when the concentration of the plasmid applied was in the range of 0.25–1.0 mg/ml.²⁷ The concentrations of pGPA applied onto the skin in the present study ranged from 0.00625, 0.0625, to 0.625 mg/ml, and only the 0.625 mg/ml concentration (50 µg per mouse) induced a detectable level of anti-PA Abs (**Figure 3b**). Therefore, future studies should be directed toward maintaining the concentration of the DNA on the skin application area at a sufficiently high level.

We have also preliminarily investigated the mechanisms by which the immune responses induced by the topical pGPA were enhanced when the hair follicles in the application area were induced into anagen-onset stage by hair-plucking. The possible effects of wax-based hair depilation are many: (i) It is well-known that depilation induces hair follicles into anagen stage.²⁸ Hair follicles in the anagen stage are open and amenable to penetration, whereas hair follicles in the telogen stage are closed.¹⁰ (ii) Plucking removes hair from the follicles; it may also remove layers of the skin stratum corneum, thereby compromising the integrity of the skin. (iii) Depilation increases cellularity in the skin, and induces local skin inflammation characterized by epidermal hyperplasia.^{18,28} (iv) Depilation, especially over a large skin area (e.g., the whole dorsal surface), induces hair follicles into anagen stage and leads to immuno-suppression of the skin immune system.^{29,30} (v) Depilation induces hair follicles into anagen stage, and the proximal region of the anagen-stage hair follicles are immune privileged.^{12,13,31}

Of the five possible outcomes listed above, the last two are expected to be detrimental to the induction of immune response by a topically applied DNA vaccine. However, because we depilated only a very small area (~1 cm²), the immuno-suppression associated with the resultant hair growth was expected to be weak.¹² The first three outcomes listed above are all potentially helpful in enhancing the immune response induced by a topical DNA vaccine. However, our data in **Figure 4** clearly show that the integrity of the skin in the application area at the time of the DNA application was not compromised. Therefore, potential physical disruption of the skin, if any, by the plucking was unlikely to be a factor in enhancing the immune responses.

Our data clearly showed that, when the PA63-encoding plasmid pGPA was used in mice, skin areas wherein the hair follicles

had been induced into anagen-onset stage expressed a significantly higher level of PA63 than the skin areas wherein the hair follicles were predominantly in the telogen stage (**Figure 5a**). In addition, we found that more pGPA plasmid was retained in the skin areas having anagen-onset-stage follicles than in those with telogen-stage follicles (**Figure 5b** and **c**). These data suggest that the enhanced anti-PA immune response after application onto a skin area with anagen-onset-stage hair follicles can be attributed to enhancement of antigen gene expression and plasmid DNA uptake in the application area.

However, these were apparently not the only factors responsible for the enhancement of the resultant immune responses. As shown in **Figure 6d**, the hair-plucking procedure clearly generated an acute dermal inflammation. The migration of immune cells such as lymphocytes, neutrophils, macrophages, and mast cells into the depilated skin area also probably contributed to the enhancement of the resultant anti-PA immune responses. This observed dermal inflammation was also consistent with previous findings that anagen-onset-stage hair follicles are associated with significant alterations in the cutaneous immunity and the redistribution of immune cells in the treated area.^{13,21,32,33} In future studies we plan to identify the extent to which each of these two potential mechanisms, enhanced antigen gene expression and acute dermal inflammation, contribute to the enhancement of the resultant immune response.

Taken together, our findings suggest that strong and functional immune responses can be induced by applying a DNA vaccine topically onto a skin area wherein the hair follicles have been induced into the anagen-onset stage. The ability of hair follicles in the anagen-onset stage to retain more of the applied DNA and to express an increased level of the applied antigen gene is likely to be partially responsible for the enhancement of the resultant immune response. In addition, the dermal inflammation and the alteration of the skin immune system associated with the anagen induction are also likely to have contributed to the enhancement of immune response.

MATERIALS AND METHODS

Materials. Female Balb/C mice (6-weeks old) were obtained from Simonsen Laboratories (Gilroy, CA). Plasmid pGPA, which encodes a PA fragment (amino acids 173–764), was kindly provided by Dr. Dennis M. Klinman (National Institutes of Health).¹⁴ The plasmid was purified using a Perfectprep Plasmid Mini Kit from Eppendorf (Westbury, NY). PA83 and LF were obtained from the BEI Resources (Manassas, VA). PA63 and CT were from the List Biological Laboratories (Campbell, CA). Horseradish peroxidase–labeled goat anti-mouse secondary Abs (anti-mouse IgG, IgG1, and IgG2a) were obtained from Southern Biotechnology Associates (Birmingham, AL). 3,3',5,5'-Tetramethylbenzidine solution and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution kits were from Sigma-Aldrich (St. Louis, MO). The pGPA was complexed with the Lipofectamine reagent (Invitrogen, Carlsbad, CA) to prepare lipoplexes as previously described.⁹

Application of DNA formulations onto the mouse skin. National Institutes of Health guidelines for animal use and care were followed in all animal studies. Mice ($n = 5-7$) were lightly anesthetized with pentobarbital (6 mg/100 g body weight intraperitoneally). The hair on the mid-dorsum of mice was trimmed and then plucked using a Veet wax strip (Reckitt Benckiser, Parsippany, NJ) in ~1 cm² area. The onset of anagen was also confirmed by observing the skin color and by staining the plucked area

with a black hair dye (Clairol) diluted 2:1 with 3% H₂O₂ 24 hours after the plucking.¹⁸ The plucked area was hydrated for 15 minutes using gauze drenched with normal saline, cleaned with an alcohol swab, and dried. A plastic donor cap (1.12 cm² area) was fixed onto the skin using glue (Permapond, Pottstown, PA).⁸ The DNA vaccine formulation (80 µl) was then carefully applied into the cap through a small opening, which was sealed after the application. The formulation comprised the pGPA (50 µg per mouse)-Lipofectamine lipoplexes admixed with CT (7 µg per mouse). Doses were applied to the mice on days 0, 10, and 17 on three different areas on the mid-dorsal skin. On all three application days, the hair follicles in the application area were induced into anagen-onset stage by hair-plucking. The hair follicles were predominately in the telogen stage before the induction because the mice were 6–10 weeks old.¹⁸ On day 35, mice were killed and bled. The mice used as controls were treated similarly, except that the hair was only trimmed and not plucked. Control mice were left untreated or injected in the gastrocnemius muscles of their hind legs with “naked” pGPA (25 µg per leg on days 0, 10, and 17). In order to evaluate the kinetics of the anti-PA IgG induced, this experiment was repeated, but this time the mice were bled on days 17, 28, 35, 37, 50, 60, 70, and 112 via the tail vein. In order to identify the role of CT, mice ($n = 7$) whose hair follicles in the application area had been induced into anagen-onset stage were dosed with the pGPA-Lipofectamine lipoplexes with or without CT. In order to evaluate the effects of different doses of the DNA, mice ($n = 7$) received topical applications of 0.5, 5, or 50 µg of pGPA in the lipoplexes, admixed with CT (7 µg per mouse). Positive control mice ($n = 5$) were injected (IM) with pGPA in phosphate-buffered saline (PBS) (25 µg per leg).

Enzyme-linked immunosorbent assay, splenocyte proliferation assay, and in vitro lethal toxin challenge. The levels of anti-PA Abs in serum samples were determined using enzyme-linked immunosorbent assay as previously described.³⁴ The plates were coated with PA83. The Ab titers were derived by comparing the OD₄₅₀ values of the samples with twice the average OD₄₅₀ values of the untreated mice. The kinetics of the anti-PA IgG was reported as the OD₄₅₀ after the serum samples were diluted 40-fold. The splenocyte proliferation assay and the *in vitro* lethal toxin neutralization assay were also completed as previously described.³⁴

In vivo lethal toxin challenge. Two weeks after the final immunization, mice ($n = 4–5$) were injected via the tail vein with a mixture of rPA83 (90 µg per mouse) and LF (45 µg per mouse), equivalent to 7.5 times the LD₅₀ value of anthrax lethal toxin. The mice were monitored multiple times a day for 2 weeks.

Determination of trans-epidermal water loss. The hair on the dorsal skin of mice (6–7 weeks, $n = 3$) was carefully trimmed, or trimmed and plucked. The mice were killed 48 hours later, and the skin in the prepared area was collected in order to determine the trans-epidermal water loss values, as previously described.³⁵ As a positive control, hair was trimmed off the backs of another group of deeply anesthetized mice, and the skin was abraded with sandpaper. Mice were killed immediately, and the skin samples were collected. An S-Plus software (Insightful Corporation) was used for analyzing the data.

In vivo gene transfection. pGPA-Lipofectamine lipoplexes, with and without CT, were applied to the skin on the dorsal area of mice ($n = 7$). The hair follicles in the application area were either in the telogen stage or induced into the anagen-onset stage. The mice were killed 24 hours later, and the skin in the application area was collected. Total proteins were extracted from the samples as previously described.³⁶ The total protein content in the extract was quantified using a Bradford dye reagent protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Quantitative analysis of PA63 expression by enzyme-linked immunosorbent assay. Briefly, 96-well plates were coated with 100 µl of the supernatant of the skin extract overnight at 4°C. The plates were washed with

PBS/Tween 20 (10 mmol/l, pH 7.4, 0.05% Tween 20) and blocked with 4% (wt/vol) of bovine serum albumin in PBS/Tween 20 for 1 hour at 37°C. An anti-PA83 anti-serum sample was added to the wells following the removal of the blocking solution. The plates were incubated for an additional 3 hours at 37°C and washed with PBS/Tween 20. Horseradish peroxidase-labeled goat anti-mouse IgG (5,000-fold dilution) was added to the wells, followed by another 1 hour of incubation at 37°C. The plates were washed again and incubated in the presence of 3,3',5,5'-tetramethylbenzidine solution for 30 minutes at room temperature. The reaction was stopped by the addition of 0.2 mol/l of sulfuric acid. The absorbance was read at 450 nm. The OD₄₅₀ values were converted to PA63 concentrations using a standard curve established on the basis of known concentrations of rPA63 diluted in the total protein extract of skin samples free of PA, and their corresponding OD₄₅₀ values. From 100 to 1,000 ng/ml, a linear regression resulted in a R² value of 0.9913. The PA63 expression was expressed as the amount of PA63 in unit amount of total proteins.

Relative quantification of the pGPA plasmid remaining in skin samples.

Plasmid pGPA (50 µg) in lipoplexes was applied onto the skin of mice ($n = 3$) whose hair follicles in the application area were in the telogen stage or induced into the anagen-onset stage. As a control, another group of mice received application of PBS only. Mice were killed 24 hours after application. The skin in the application area was thoroughly washed with tap water for 15 minutes, dried, dissected, and weighed. Total DNA was extracted from the skin samples using the DNAzol reagent (Invitrogen). Polymerase chain reaction was carried out to semi-quantify the amount of pGPA plasmid remaining in the skin samples. The polymerase chain reaction was completed using 1 µg of the DNA template. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified as an internal control. The GAPDH primers were GAPDH5 (5'-GGTGAAGGTCGGTGTCAACGGATT-3') and GAPDH3 (5'-GATGCCAAAGTTGTCATGGATGACC-3'), which amplified a 501 base pair fragment (61–561) of the mouse GAPDH gene. The PA primers were PA5 (5'-CCTACGGTTCAGACCGTGACAATG-3') and PA3 (5'-AGCCCAAGTTCTTCCCTGCTAGAGATAG-3'), which amplified a 525 base pair fragment (536–1,060) of the PA gene. The GAPDH was amplified for a total of 38 cycles, and the PA for a total of 35 cycles. The polymerase chain reaction products (20 µl) were analyzed using 1% agarose gel containing ethidium bromide, and quantified by measuring their band intensity using Kodak 1 D Scientific Imaging Systems. The relative amount of pGPA remaining in each skin sample was arrived at by dividing the band intensity of the PA_{536–1,060} gene fragment by that of the GAPDH_{61–561} gene fragment.

Histological study. Hair on the backs of mice (6–7 weeks) was carefully trimmed, or trimmed and plucked. Mice were killed either immediately (0 hour) or 48 hours later. Skin samples ($n = 3$) were taken from the prepared area, fixed in formaldehyde, embedded in paraffin, sectioned vertically, and stained using hematoxylin and eosin. The slides were examined under a light microscope.

Statistical analysis. Except where mentioned, statistical analyses were completed by performing analysis of variance followed by pair-wise comparisons using Fisher's protected least significant difference procedure. A *P*-value of ≤0.05 (two-tail) was considered to be significant.

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