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Transcutaneous immunization and immunostimulant strategies: capitalizing on the immunocompetence of the skin

Gregory M Glenn†, Richard T Kenney, Larry R Ellingsworth, Sarah A Frech, Scott A Hammond and J Paul Zoeteweij

The skin is an attractive target for vaccine delivery. Topical application of adjuvants results in potent immune responses and good safety profiles. Adjuvants can be coadministered in a patch with vaccine antigens (transcutaneous immunization) or similar delivery format, or administered separately with an injection or IS™ patch (Iomai), leading to enhanced immune responses. These observations have moved into the clinic, highlighting the likelihood that skin delivery of vaccines will play an important future role in vaccine applications.

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The skin, especially the epidermal layer, is an accessible and competent immune environment that is an attractive target for vaccine delivery. Recent work focused on vaccine delivery into the skin using a patch or similar means has established skin delivery as a new vaccine paradigm. The skin as a noninvasive route for vaccine delivery has great potential for safe use of potent immunostimulatory compounds that can target the dense population of immune cells found in the skin. The basic insights gained through initial studies have led to several efforts to formulate and test human use vaccines. Predictably, the path to commercialization has many challenges and vaccines delivered to the skin have a unique set of hurdles. However, it seems almost certain that skin delivery techniques will play an important future role. Looking back at the experience with the smallpox vaccine, the most successful vaccine in history and one delivered to the skin, it seems that this presumption can be held with confidence.

In this review we consider the basic makeup of the skin from a vaccinologist's point of view, discuss the use of adjuvants in the context of the epidermis and provide data supporting the hypothesis that Langerhans cells are the principal targets of immunogens in the skin. We then turn to aspects of immunization focusing on immune responses to topical immunization. Data on both transcutaneous immunization and a new application, the immunostimulant patch are presented. Finally, clinical data from recent trials are discussed and the way forward for product development is outlined.

Background

Issues related to skin structure and the skin immune system have been thoroughly discussed elsewhere and are briefly reviewed here [1]. Mammalian skin is composed of three primary layers. The stratum corneum (SC) is the outermost layer of the skin. It is composed of 10–20 layers of quiescent, cornified epidermal skin cells called keratinocytes that are continuously shed. During the formation of the SC, the keratinocytes secrete lipids that form a type of lipid mortar that encases the dead and dying keratinocytes. The human SC is 10–20 µm thick in a 'bricks and mortar' format and represents an effective but fragile barrier to the hostile microbial world. The epidermis underlies the SC and is composed

of epidermal keratinocytes and other skin elements in a continuous growing layer of epithelium. The epidermis is a dynamic environment, with continuous growth that pushes out keratinocytes into the SC, secretion of the lipid mortar and traffic of immune cells in and out of the epidermis. The primary antigen-presenting cell (APC) found in the epidermis is the Langerhans cell (LC), a dendritic cell (DC) derived from bone marrow that migrates from the bone marrow into the skin and plays the dual role of immune surveillance and antigen presentation [2]. Confocal microscopy of human skin demonstrated that LCs cover 25% of the total skin surface area, although they account for approximately only 3% of the epidermis cells [3]. Their density, accessibility and antigen presentation function create an ideal target for vaccine delivery. The skin, like the mucosa, represents a logical target for vaccine delivery because the immune challenge of vaccine antigens replicates common daily events and the normal route of invasion by microbes to which the skin is exquisitely well equipped to respond. Finally, the underlying layer of the skin, the dermis, undergirds the epidermis with connective tissue, blood vessels (generally the target for transdermal drug delivery) and lymphatics and provides a foundation for the epidermal appendages, such as hair and sweat glands. The dermis contains DCs and LCs in transit but the density of APCs in the dermis does not match that of the epidermis [4]. Clearly, the normal practice of parenteral immunization by needle will perforate the skin and thus bypass the layers that should be of most interest to vaccinologists.

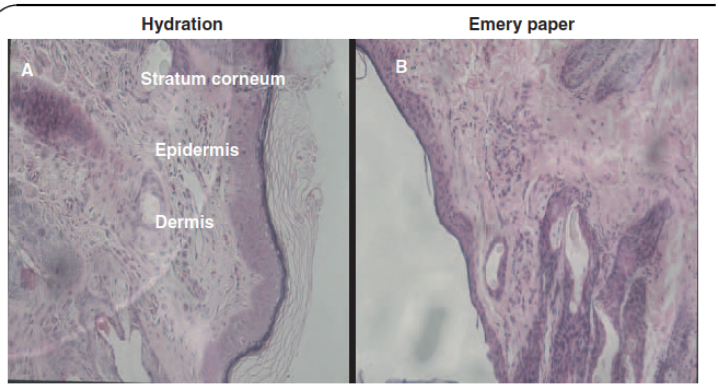

Figure 1. Normal hairless guinea pig skin hydrated with 10% glycerol/70% isopropyl alcohol (panel A) and hydrated skin pretreated with emery paper (20 strokes) to disrupt the stratum corneum.

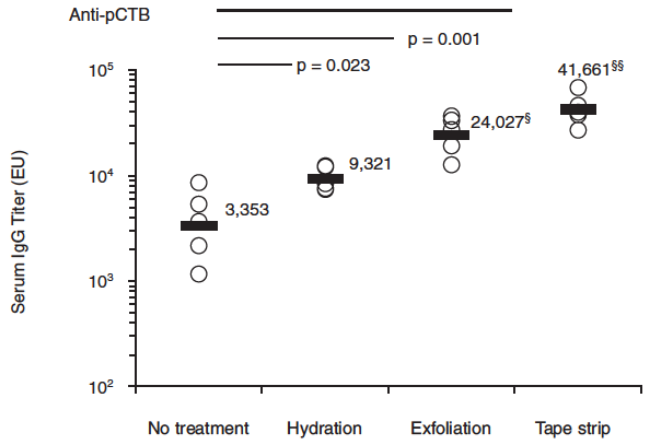

Figure 2. Enhanced topical delivery of purified cholera toxin B-subunit (pCTB) on skin pretreated to disrupt the stratum corneum. Groups of five C57Bl/6 mice were shaved 2 days prior to immunization on the dorsal caudal surface. Immediately prior to immunization, the shaved skin was pretreated by hydration with a saline saturated gauze swab (20 strokes), application (15 minutes) of a commercial exfoliating cream followed by rinsing, or by tape stripping (10 times) with 3M tape. pCTB (10 µg) was loaded onto a gauze pad (1 cm diameter) affixed to an adhesive backing. Patches were removed the next day and the skin rinsed with water. Serum was collected two weeks after a single immunization and serum antibody titers to pCTB were determined by an ELISA method. The results are reported as ELISA Units (EU), which is the serum dilution equal to 1 OD unit at 405 nm. The geometric mean titer for each group is indicated. Significance compared with skin hydration: $^{\S}p = 0.003$, $^{\S\S}p = 0.00004$.

Penetration of the stratum corneum

Under normal circumstances, the stratum corneum is an effective barrier to the penetration of fluids, large molecules, particles and microbes. Long held maxims of skin penetration have stated that even with use of skin penetration techniques, the delivery of drugs and bioactive molecules greater than 500 Da was not possible. However, it has become clear that these maxims are restricted to transdermal delivery of small drugs usually to the blood vessels in the dermis. Vaccine antigens and adjuvants targeting the skin, defined as transcutaneous immunization (TCI), merely require delivery to the epidermis. Thus, upon reconsideration of the barriers to penetration, it has become clear that new rules for antigen size come into play in the context of vaccine delivery. In human skin, which is the most relevant setting, we have shown that with crude patches and minimal SC disruption, very large recombinant antigens of the order of 1 MDa can be delivered to elicit systemic immune responses [5].

Investigations have led our group to use several strategies for penetration of the

SC. Occlusion, wetting of the skin and other techniques lead to hydration of the SC. Hydration of the SC results in swelling of the keratinocytes and pooling of fluid in the intercellular spaces, leading to dramatic microscopic changes in the SC structure that have no lasting effect once the skin is allowed to dry [6]. Hydrated SC clearly allows antigens to pass through the skin, although the transit pathways utilized by antigens to traverse the stratum corneum are unknown at this time. Transdermal drug delivery of polar small molecule drugs is thought to occur through aqueous intercellular channels formed between the keratinocytes in hydrated skin and it is possible that similar pathways are engaged for antigen delivery by TCI [6].

Physical and chemical penetration enhancement techniques that disrupt the integrity of the SC have also been investigated and described [101]. Concepts with clinical relevance have been tested in several model systems and were finally applied to human skin where penetration enhancement appears to represent an improvement over simple hydration of intact skin. Guiding principles for product development include the use of simple, inexpensive materials with clinical utility in other settings and simple methods of use/application that lead to consistent, heightened immune responses.

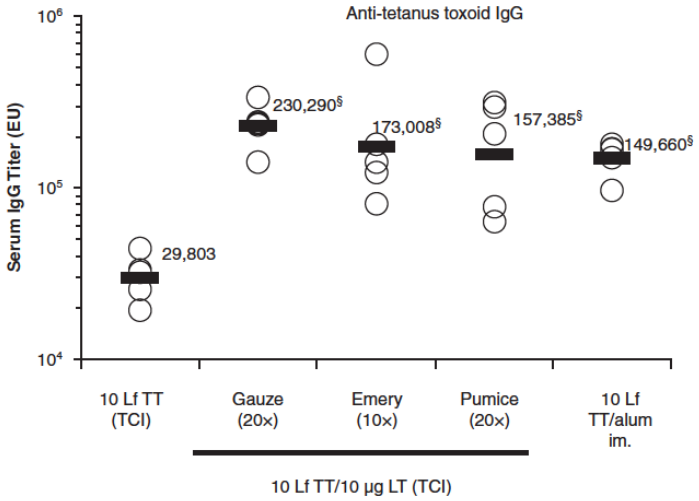

Figure 3. Skin pretreatment methods enhance the topical delivery and immune response to tetanus toxoid vaccine. Mice were prepared by shaving the dorsal caudal surface near the base of the tail two days prior to immunization. The shaved skin was hydrated with 70% isopropyl alcohol and 10% glycerol. The skin was pretreated to disrupt the stratum corneum by rubbing 20 strokes with a wet gauze sponge, 10 strokes with emery paper (GE Medical Systems, E9001CK), or 10 strokes with a pumice swab (Electrode prep pad, PDI B59800). Patches were constructed by affixing a 1 cm diameter Nu-gauze pad (Johnson & Johnson, 7632) to an adhesive backing. Patches were loaded with 25 µl of tetanus toxoid (10 Lf) alone or mixed with 10 µg LT and the patches applied over the pretreated skin. The group topically immunized with TT alone received three immunizations (day 0, 14 and 28) and the groups topically immunized with TT and LT were immunized twice (day 0 and 14). A separate group was immunized twice (day 0 and 14) by intramuscular injection (thigh muscle) of 10 Lf TT adjuvanted with 10 µg aluminum hydroxide (alum). Serum was collected two weeks after the last immunization and evaluated by ELISA for antibody titers to TT. The geometric mean titer for each group is indicated.

Hairless guinea-pigs were used to examine the effect of mildly abrasive emery paper used to disrupt the stratum corneum, the results of which are shown at the cellular level in FIGURE 1. Hairless guinea-pigs have an epidermis and SC that is similar in thickness to humans and have been widely used for studying penetration enhancement techniques. In this study, animals were biopsied post-treatment and the dermis, epidermis and SC are shown using hematoxylin–eosin staining. In panel A, the effect of SC hydration can clearly be seen, whereas in panel B, the effective removal of the SC has occurred using emery paper. Clinically, the removal of the outer layer of dead skin cells can be accomplished in a tolerable fashion, leaving little or no local irritation. The histology results suggest that SC disruption might aid in efficiency of antigen delivery, yet topical immunization of hairless guinea-pigs is ineffectual in our labs and others [7], illustrating the limitations of animal models for optimizing penetration enhancement techniques. By contrast, less invasive hydration techniques allow antigen delivery and penetration enhancement in human skin and further improve the results, suggesting that characteristics such as the dense lipid layer found in hairless guinea-pigs make them unsuitable as an animal model. It has been suggested that hair follicles represent an important route for topical administration but this hypothesis would not explain the enhancement seen with SC removal. This is supported by the observation (unpublished) that outbred CD1 mice with normal hair follicle development and hairless SKH mice with sparse vestigial follicles respond equally well to topical immunization with tetanus toxoid (TTx) adjuvanted with heat-labile enterotoxin from *Escherichia coli* (LT). CD1 mice develop high titer (EU = 652,000) serum immunoglobulin (Ig)G to tetanus that is not significantly different from the immune response elicited by hairless SKH mice (EU = 795,000).

Although the SC is the most significant barrier to topical immunization, it is a fragile barrier that can be easily disrupted, possibly allowing antigens to more readily diffuse to the target cell population. Despite the differences in mouse SC and epidermis, murine studies have been remarkably predictive for clinical studies. Using cholera toxin B (CTB), which has previously been shown to be immunogenic on the skin with hydration techniques, we compared a commercial exfoliating cream and tape stripping to test whether enhanced delivery, illustrated

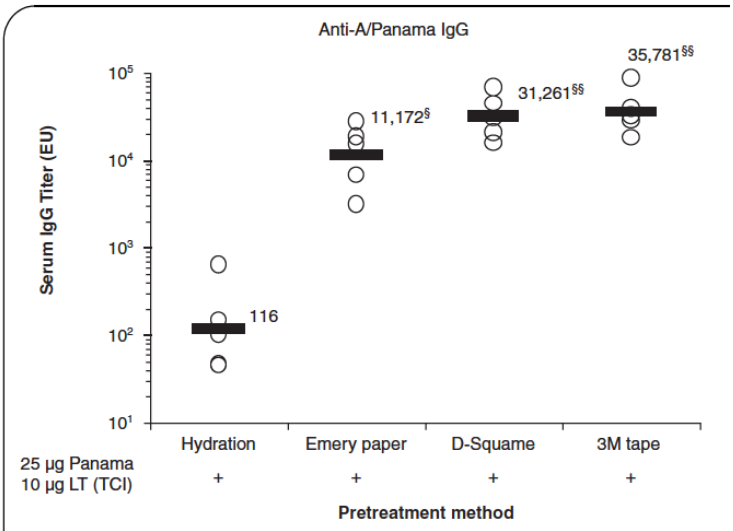

Figure 4. Improved topical delivery of influenza split-virus vaccine by disruption of the stratum corneum. C57Bl/6 mice were shaved on the dorsal caudal surface 2 days prior to topical immunization. Exposed skin was hydrated by gently rubbing (20 strokes) with a saline saturated gauze sponge. The SC was disrupted by mild abrasion with emery paper (10 strokes) or tape stripping with D-Squame (10×) or 3M™ tape (10×). A solution consisting of 25 µg A/Panama and 10 µg LT was applied to the pretreated skin for 1 h and then washed off with warm water. Groups were immunized three times (day 0, 14 and 28) and serum collected 2 weeks after the third immunization on day 42. Serum antibody titers to A/Panama were determined by ELISA and titers were reported as ELISA Units (EU). The geometric mean titer for each group is indicated. Serum elicited by hydration pretreatment was compared with antibody titers elicited by skin pretreatment with emery paper or by tape stripping. §p = 0.012; §§p = 0.006. ELISA: Enzyme-linked immunosorbent assay; Ig: Immunoglobulin; LT: Heat-labile toxin of *Escherichia coli*; TCI: Transcutaneous immunization.

by an increased magnitude of immune responses, might be seen. As shown in FIGURE 2, a tenfold increase in the immune response to CTB was seen after a single immunization compared with hydration alone, exceeding the enhanced response seen by use of an exfoliating cream as well.

Many studies in our lab have extended this observation to the delivery of both an antigen and adjuvant and enhancement of the adjuvant effect. Using TTx as antigen and LT as adjuvant, mice were immunized topically using hydration compared with tape stripping, vigorous gauze rubbing and a commercial pumice pad to disrupt the SC. As shown in FIGURE 3, an almost tenfold greater immune response to the coadministered antigen was seen by adding a physical penetration enhancement step compared with hydration alone. Notably, these data confirm that the adjuvant plays an important role in enhancing immune responses even when the SC is disrupted or penetrated by other means [8]. These and other studies suggested that a technique such as tape stripping or a more simple technique employing swabbing the skin with a mild abrasive could enhance delivery. The use of a mild abrasive swab using LT and TTx as adjuvant and antigen was compared with the same dose of TTx injected using alum. As shown in FIGURE 3, two immunizations with TTx led to similar levels of TTx antibodies when TTx was given by the intramuscular route using alum.

The effect of pretreatment is also illustrated using split-virus influenza vaccine (A/Panama) adjuvanted with LT applied to intact skin that has been pretreated to disrupt the SC. As illustrated in FIGURE 4, shaved intact mouse skin was hydrated with saline, or the skin was pretreated with emery paper (10 strokes) to disrupt the SC, or the SC was disrupted with D-Squame tape (10×) or 3M™ tape (10×). Immediately following the pretreatment, a solution containing split virus A/Panama influenza vaccine and LT was applied to the pretreated skin for 1 h and rinsed to remove excess vaccine. After three immunizations every other week, serum was collected and evaluated for antibody titers to A/Panama. The serum antibody response to topical immunization was increased 100- to 300-fold by disrupting the SC immediately prior to topical application of the influenza vaccine with the LT adjuvant. The enhancement of the immune response is also highly dependent upon coadministration of LT with the antigen, even in the context of SC disruption which, skillfully done, is not normally accompanied by clinically apparent local reactions except for some mild local and self-limiting rashes. The combination of hydration and SC disruption, using a swab or similar simple step, improves the efficiency and therefore enhances the immune responses to antigens delivered by this method, while continuing to be well-tolerated and practical. These preclinical studies have led to optimization (see below) of clinically acceptable and simple use, consistent skin pretreatment materials and protocols.

Adjuvants & the skin

TCI, the delivery of antigens and adjuvants to the skin for the purpose of immunization, is consistently dependent on the presence of an adjuvant in the formulation for the induction of robust immune responses [5,8–12,102]. The bacterial ADP-ribosylating exotoxins (bAREs) are potent adjuvants in the context of the skin and include cholera toxin (CT), LT and their mutants and subunits. bAREs have had extensive use as adjuvants via intranasal and oral routes and are found in many natural disease settings, suggesting that their topical use would not be accompanied by long-term side effects [11,12–18]. The safety of these adjuvants is reviewed elsewhere in this journal issue. TCI is similar to intranasal or oral immunization, as the simple admixture of CT or LT with a coadministered antigen, such as TTx or influenza hemagglutinin, results in markedly higher antibody levels compared with the administration of antigens alone, which can themselves elicit immune responses

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