Surfactant Sodium Lauryl Sulfate Enhances Skin Vaccination: Molecular Characterization via A Novel Technique Using Ultrafiltration Capillaries and Mass Spectrometric Proteomics

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Running title:  Surfactant Enhances Skin Vaccination: A Novel Technique

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The abbreviations used are: CUF, capillary ultrafiltration; 2-DE, two-dimensional gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FEP, fluorinated ethylene propylene; FITC, fluorescein isothiocyanate; HEL, hen egg lysozyme; H&E, hematoxylin and eosin; IgG, immunoglobulin G; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MHC, major histocompatibility complex; MWCO, molecular weight cutoff; PBS, phosphate buffer saline; PTFE, polytetrafluoroethylene; Q-TOF MS/MS, quadrupole time-of-flight tandem mass spectrometry; RuO4, ruthenium tetroxide; SLS, sodium lauryl sulfate;
The skin is a highly accessible organ and thus provides an attractive immune environment for cost-effective, simple and needle-free delivery of vaccines and immunomodulators. In this study, we pretreated mouse skin with an anionic surfactant, sodium lauryl sulfate (SLS), for a short period of time (10 min) followed by epicutaneous vaccination with hen egg lysozyme (HEL) antigen. We demonstrated for the first time that pretreatment of skin with surfactant SLS significantly enhances the production of antibody to HEL. Short-term pretreatment with SLS disorganized the stratum corneum, extracted partial lamellar lipids, induced the maturation of Langerhans cells and did not result in epidermis thickening. To reveal the mechanism underlying these changes, particularly at the molecular level, we employed a novel proteomics technique, using ultrafiltration capillaries and mass spectrometry to identify in vivo proteins/peptides secreted in the SLS pretreated skin. Two secretory proteins, named as calcium binding protein S100A9 and thymosin β4, were identified by this novel technique. These two proteins thus may provide new insight into the enhancing effect of surfactants on skin vaccination.
The skin provides an attractive immune environment for vaccine delivery. It contains many immunocompetent cells, including keratinocytes, γδ T cells and Langerhans cells, which may initiate potent immunity when skin is exposed to various antigens (1). One mechanism that has been proposed is that these antigens activate Langerhans cells in the skin which then migrate to draining lymph nodes to orchestrate robust systemic immune responses (2). The presence of both significant associated lymphoid tissue and immunocompetent cells suggests that skin might be an effective non-invasive route for vaccination. Simple access to and durability of the skin are additional advantages of applying vaccines to the external tissue. Moreover, both humoral and cellular immunity are induced after immunization via the skin (3).

Production of an immune response against a foreign antigen usually requires needle injections by medical personnel. The development of a needle-free non-invasive method for the inoculation of vaccines via the skin may reduce medical costs by allowing personnel with a lower level of medical training to administer the vaccine. Techniques for skin vaccination can be categorized into three groups, depending on which stratum of the skin is targeted (4). The first
group encompasses epidermal immunization, which includes stripping, chemical modification, and trans-epidermal immunization (5, 6, 7, and 8). The second group covers epidermal and dermal immunization, which includes gene gun technology and electroporation (9). The third group targets dermal immunization, which includes intradermal immunization and microseeding (10). Although application of above techniques can efficiently elicit immune responses at certain levels, they normally utilize toxins as adjuvants or severely damage skin by altering the epidermal structure. An alternative approach is to brush the skin before antigen application, presumably stimulating skin immunocompetent cells, with little or no harm to the epidermis (5, 6). However, brushing is difficult, if not impossible, to standardize for the future clinical use. To address this concern, we pretreated skin with various concentrations of surfactant SLS, washed away and followed by topical application of antigens. Surfactants are found in numerous product categories, from make-up to skin care, as well as being the main constituents of shower gels, bath foam and shampoos (11). Surfactants have been used for skin drug delivery and serve as penetration enhancers which are absorbed into skin to reversibly decrease barrier resistance (12, 13).
More recently, Vyas and coworkers used non-ionic surfactant based vesicles (niosomes) for topical delivery of encapsulated DNA encoding hepatitis B surface antigen and (14) and tetanus toxoid (15). A detectable antibody response was evoked in mice after niosome mediated topical immunization (14, 15). One potential drawback to the above technique is that surfactant-based vesicles remain in the host following vaccination, possibly resulting in adverse effects.

The question of whether pretreatment of skin with surfactants prior to epicutaneous application of antigens evokes sufficient antibody responses has not been explored. To test the efficiency of surfactant pretreatment on the skin vaccination, we pretreated mouse skin with the surfactant SLS followed by epicutaneous application of HEL antigen. Our results demonstrated for the first time that pretreatment of skin with surfactant SLS significantly enhances the production of antibody to HEL. In an attempt to understand the mechanism of this response, particularly at the molecular level, we applied a novel proteomics technique using ultrafiltration capillaries and mass spectrometry to detect \textit{in vivo} the proteins/peptides secreted in the pretreated skin. The novel capillary
ultrafiltration technique will allow us to obtain pure, low abundant and \textit{in vivo} secretory proteins/peptides. Secretory proteins such as S100A9 and thymosin β4 identified by this novel technique will provide new insight into the enhancement of skin vaccination by surfactants.

\section*{EXPERIMENTAL PROCEDURES}

\textit{Skin Vaccination and Enzyme-Linked Immunosorbent Assay (ELISA)}

Female ICR mice at 3-6 months old (Jackson Laboratory, Bar Harbor, Maine) were anesthetized by administrating 10 mg of ketamine and 1.5 mg of xylazine per 100 g of body weight. The abdominal skin of ICR mice was depilated with an electric trimmer. 100 µl of SLS (0.1, 1, and 5%, v/v) or phosphate buffer saline (PBS) was applied to the depilated skin for 10 min and then rinsed with water to remove unabsorbed SLS from the skin, immediately followed by epicutaneous vaccination with HEL (100 µg/100 µl; HEL/PBS) (Sigma, St. Louis, MO). For epicutaneous vaccination, the HEL was spread as a thin film over pre-shaved skin followed by the application of a Tegaderm patch (3M, St. Paul, MN)
for one hour. Unabsorbed HEL on the skin was washed away one hour following patch removal. Each animal was vaccinated with HEL for one month without booster. Naïve groups of mice were prepared by pipetting 100 μl of PBS onto the pre-shaved skin. Each group contained five mice. Three independent experiments were performed. Mice were maintained in the University of Alabama at Birmingham animal care facility in accordance with the animal protocol approved by the IACUC. Serum samples were assayed for anti-HEL antibodies one month after vaccination with HEL. Titers of anti-HEL immunoglobulin G (IgG) were determined by ELISA as described (5, 6). The HEL (0.5 µg/well) serves as the capture antigen to coat on a 96-well ELISA plate (Corning Inc., Corning, NY). Serum samples and peroxidase-conjugated goat anti-mouse IgG (1:5000 dilution) (Promega, Madison, WI) were incubated sequentially on the ELISA plates for one hour at room temperature with extensive washing between each incubation. The end-point was calculated as the dilution of serum producing the same OD$_{490}$ as a 1/100 dilution of preimmune serum. Sera negative at the lowest dilution tested were assigned endpoint titers of 100. The data was presented as geometric mean endpoint ELISA titers.
The areas of the skin treated with PBS or SLS were fixed overnight at 4°C in modified Karnovsky’s fixative, containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.06% CaCl$_2$, in 0.1M cacodylate buffer, pH 7.4. Skins were then washed in 0.1M cacodylate buffer, postfixed in 0.25% ruthenium tetroxide (RuO$_4$) (Polyscience, Warrington, PA) in 0.1M cacodylate for 45 min in the dark at room temperature. Lastly, the specimens were rinsed in buffer, dehydrated in graded ethanol solutions, and embedded in an Epon-epoxy resin mixture. Ultrathin 60-80 nm sections were examined under a Hitachi 7000 transmission electron microscope. Images were captured at 75 KV after staining with uranyl acetate/lead citrate.

For histological observation, the skins treated with PBS or SLS were cross-sectioned, stained with hematoxylin and eosin (H&E) (Sigma diagnostics) (16), and viewed on a Zeiss Axioskop2 plus microscope (San Marcos, CA). Images
were collected using an Axiocam digital camera in conjunction with AxioVision 3.1 software.

*Immunohistochemical Staining of Epidermal Langerhans Cells*

Mice were exposed to PBS or SLS on the dorsal side of ears for 10 min and killed for staining of epidermal Langerhans cells. The preparation and staining of epidermal Langerhans cells were performed as published with minor modifications (17). Briefly, after removal of ear, the ear tissue was incubated in 0.25% trypsin (Sigma, St. Louis, MO) for 60 min at 37°C, the epidermis sheets were separated from dermis, dispersed in Hank’s balanced salt solution containing 0.025% DNase and 10% fetal bovine serum, and then fixed immediately in cold acetone for 10 min. The epidermal sheets were rehydrated in PBS for 60 min and incubated in the blocking buffer (0.5% BSA in PBS) for 30 min at room temperature. After one wash, the epidermal sheets were incubated with biotinylated anti-major histocompatibility complex (MHC) class II antibody (BD Biosciences, San Jose, CA) overnight on a shaker at 4°C. The sheets were then washed three times in PBS at room temperature. Streptavidin-
TRITC (BD Biosciences, San Jose, CA) (1:100) was added and the epidermal sheets were incubated on a shaker at room temperature for 2 h. For double staining, MHC class II-stained epidermal sheets were further incubated with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD86 monoclonal antibody (BD Biosciences, San Jose, CA) (1:100) at room temperature for 2 h. After washings as described above, epidermal sheets were mounted on slides with 90% glycerol. Three photographs were taken from each epidermal sheet using a Leiz microscope connected to a digital camera and saved in a computer with the IP Spectrum software. The result was evaluated with Photoshop. The number of MHC class II-stained cells was counted on each photograph and the mean stained cell number/mm² was calculated for each experimental group with at least five mice per group.

Design and Preparation of Capillary Ultrafiltration (CUF) Probes

A real CUF probe is pictured in Fig. 5A. The probe consists of a semi-permeable hollow membrane fiber (Fig. 5A, B, a) joined to a
polytetrafluoroethylene (PTFE) tube (Fig. 5A, B, c). The semi-permeable hollow membrane fiber, with a molecular weight cutoff (MWCO) of 50 kDa, was made with polyacrylonitrile and obtained from a kidney dialyzer (AN69-HF; Hospal-Gambro, Inc.). The AN69-HP was reported to have a mean pore size of 290 nm and a maximum of 550 nm (18). One end of the semi-permeable hollow membrane fiber was attached to the PTFE tubing (ID/OD:0.102mm/0.406mm, Cole-Parmer Instrument Company, IL) through a small section of fused silica capillary (Fig. 5A, B, b) (ID/OD:0.110mm/0.170mm, SGE, Incorporated, Texas) (19), while the other end was sealed with epoxy. The inner wall of the fused silica capillary was methyl deactivated to prevent possible protein binding. A sharpened G27 needle (Fig. 5A, B, e) was attached to the end of the PTFE ultramicrobore tube (Fig. 5A, B, d) (0.794mm OD Teflon fluorinated ethylene propylene (FEP) tube; Upchurch Scientific, WA), so the CUF probe can be connected to a vacutainer (Fig. 5C, D, f) with negative pressure that will drive the ultrafiltration process to collect interstitial fluids. The negative pressure was created by vacuuming the vacutainer with a syringe (Fig. 5D, h).
Continuous Sampling in Vivo with CUF Probes

The dorsal ear skin of 2 to 3 month-old female ICR mice (Jackson Laboratory, Bar Harbor, ME) was cleaned with water before implantation with CUF probes. The CUF probe was then inserted near the head. The mice were anesthetized with 10 mg of ketamine and 1.5 mg of xylazine per 100 g of body weight during implantation. The semi-permeable membrane fiber in the front end of CUF probe was entirely covered by ear skin. Implantation of the CUF probe involved subcutaneously inserting a 22-gauge needle into the ear skin as a guide and then feeding the probe through the needle. The needle was then removed. After implantation of CUF probes, 50 µl of PBS or SLS (1.0%, v/v) was epicutaneously applied to the ear skin for 10 min and then rinsed with water to remove unabsorbed PBS or SLS from the skin. The sample collection was conducted for 24 h after implantation. During the 24 h collection, mice wore elastomer saddle tethers (Instech Solomon, Plymouth meeting, PA) and were placed in a balanced level arm (Instech Solomon, Plymouth meeting, PA). The vacutainer was replaced every hour and collected samples were maintained at -86 °C in order to avoid degradation. After the collection, approximately 15 to 20 µl
of sample was obtained from both PBS- and SLS-treated skins. The protein concentrations of collected samples were approximately 0.3 mg/ml. The protein concentration was determined by BCA™ Protein Assay Kit (PIERCE, Rockford, IL). The samples collected from PBS- and SLS-treated skin were adjusted to the same concentration by adding distilled water. They were digested with 0.04 µg/µl trypsin for 15 h. The tryptic digests of collected samples were then subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and quadrupole time-of-flight tandem mass spectrometry (Q-TOF MS/MS) analyses.

**MALDI-TOF MS**

Peptides in the tryptic digests of CUF probe-collected samples were eluted from ZipTips with 75% acetonitrile/0.1% trifluoroacetic acid and air-dried. Peptide fragments were then reconstituted in matrix solution containing α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile/0.1% trifluoroacetic acid and analyzed with a PerSeptive Voyager-DE MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA) (20). Peptides were laser-evaporated at 337 nm,
and each spectrum was the cumulative average of 50-100 laser pulses. All peptides were measured as mono-isotopic masses, and a trypsin autolytic peak at 2164.1 m/z was selected for internal calibration. Up to one missed trypsin cleavage was allowed, although most matches did not contain any missed cleavages. This procedure resulted in mass accuracies of 100 ppm. For the detection of intact proteins, samples collected by CUF probes were mixed with matrix solution containing α-cyano-4-hydroxycinnamic acid dissolved in 50% acetonitrile/0.1% trifluoroacetic acid. The proteins with m/z ranging from 12,400 - 13,700 and 4,500 - 5,500 were observed in the MALDI-TOF MS spectra.

**Q-TOF MS/MS sequencing and database searching**

Samples digested with or without trypsin were eluted from ZipTips (21) and were introduced into a nano reverse-phase column (75 µm x 15 cm, with Jupitor 4 µm Proteo bead packed in our laboratory), and gradient eluted into a Q-TOF 2 quadrupole time-of-flight tandem mass spectrometer (Micromass, Manchester, UK) through an electrospray interface for tandem mass spectral analyses. Liquid chromatography was performed using a LC Packings Ultimate LC, Switchos
RESULTS

Elicitation of Antibody to HEL by Skin Vaccination

In an attempt to investigate whether mice immunized epicutaneously with HEL could elicit antibody, we spread HEL (100 μg/μl) over pre-shaved abdominal skin of ICR mice with a piece of the 3M Tegaderm patch for one hour. After washing away the unabsorbed HEL, mice were maintained in their cages for one month. An equal volume of PBS was applied to the non-vaccinated mice in the naïve group (Fig. 1). Mice epicutaneously administered HEL had a detectable
specific IgG response (a geometric mean titer = 1,613 for HEL-vaccinated mice compared to a geometric mean titer = 120 for non-vaccinated naïve mice).

To test the effect of surfactants on skin vaccination, we applied three different doses (0.1, 1.0, and 5%) of SLS to the surface of pre-shaved skins for 10 min prior to vaccination with HEL. Pretreatment of skin with SLS significantly enhanced the HEL-specific IgG response in the immunized mice (Fig. 1). Pretreatment with 0.1, 1.0, and 5.0% solutions of SLS resulted in a 2.2-, 4.8-, and 1.6-fold higher HEL antibody titer over the HEL alone. In addition, a mixture of 100 µg HEL with 0.1, 1.0, and 5% of SLS, was prepared. Mice did not evoke significant HEL-specific IgG response when they were immunized simultaneously with HEL and SLS (data not shown). Simultaneous immunization was accomplished through epicutaneous application of the three HEL/SLS mixtures onto abdominal skins. The geometric mean of HEL antibody titer was 100, 110, 110, 132, 152 for non-vaccinated, HEL-, HEL/0.1 % SLS-, HEL/1.0% SLS, and HEL/5.0% SLS-vaccinated mice, respectively. These results suggest that pretreatment with SLS yields better immunologic potency than simultaneous administration with the antigen. Moreover, the strongest enhancement of the
HEL-specific IgG response was obtained with the 1.0% SLS strength.

**Effect of Surfactant SLS on Skin Architecture**

Previous studies indicating that SLS application resulted in disruption of stratum corneum, skin irritation, and activation of signaling transduction in the epidermal cells were utilized relatively long application periods (>24 h) (23, 24). The effects of short-term SLS application on skin architecture remain unexplored. We thus epicutaneously applied SLS (0.1, 1.0, and 5%, v/v) onto the skin for 10 min and investigated the ultrastructure of stratum corneum barrier, epidermal inflammation, and Langerhans cell morphology. Our findings are in agreement with previous evidence (24) that epicutaneous application of 5% (v/v) SLS for 24 h significantly removed the lamellar lipids in stratum corneum (data not shown) and induced a thickening of epidermis (Fig. 4F). In contrast to previous studies, our data indicated that epicutaneous application of 1% (v/v) SLS for 10 min (followed by washing with water only partially depleted lamellar lipids (Fig. 2 C and D). Application of the 1% (v/v) SLS solution also resulted in disorganization of the structure and widening of the intracellular spaces of the stratum corneum
(Fig. 2A and B). Application of 0.1 and 5.0% SLS solutions resulted in similar disorganization of stratum corneum structure and similar widening of intracellular spaces. More importantly, epicutaneous application of SLS (0.1, 1.0, and 5%, v/v) (Fig. 4 B - E) or PBS (Fig. 4A) onto the skin for 10 min did not change the thickness of epidermis or induce detectable skin inflammation.

The skin is an immunological organ. Langerhans cells form a contiguous network in the epidermis where they serve as sentinels of skin immunity by capturing and processing antigens that get through the stratum corneum and responding to cell signals activated by surrounding keratinocytes (1). It has been shown that disruption of the stratum corneum alters the maturational status of Langerhans cells (26). It has been known that ear skin is an idea model to investigate the Langerhans cell maturation (17). Epidermal sheets were prepared from ear skin and Langerhans cell maturation was examined by double staining the sheets with Langerhans cell surface marker (MHC class II) and maturation marker (CD86). Spreading 1.0% (v/v) SLS on the dorsal surface of ear skin for 10 min did not change the number of MHC-class II-positive Langerhans cells residing in the epidermis (Fig. 3 A and B). These results indicate that SLS did not
induce a migration of Langerhans cells from the epidermis. Intriguingly, in the PBS-treated skin (Fig. 3C), the expression of CD86 was undetectable. In contrast, after spreading of 1.0% (v/v) SLS on the dorsal surface of ear skin for 10 min, the expression of CD86 on the MHC-class II-positive Langerhans cells was dramatically enhanced (Fig. 3D), indicating that epicutaneous application of SLS induced Langerhans cell maturation. Application of 0.1 and 5% (v/v) SLS for 10 min produces similar effects with 1.0% (v/v) SLS on the number and maturation of epidermal Langerhans cells (data not shown).

**In Vivo Sampling of Secreted Proteins Using CUF Probes**

A novel proteomics approach was employed to characterize the molecular nature of surfactant SLS induced skin changes. Transitional proteomics approaches, primarily using 2D electrophoresis (2-DE) gels, are often successful in identifying the proteins/peptides released from cells (27). However, most of the secreted proteins/peptides identified via 2-DE gels are of high abundance. The 2-DE gel approaches generally are not sufficiently sensitive for the detection of low abundant proteins/peptides including secretory proteins. Moreover, it remains a
significant challenge to obtain pure secretory proteins/peptides \textit{in vivo}. To obtain low abundant and/or secretory proteins/peptides \textit{in vivo}, our laboratory has designed novel CUF probes (Fig. 5A, B). Capillary ultrafiltration using CUF probes involves applying a vacuum to semi-permeable membrane fibers in order to extract fluid containing the desired secretory molecules from the interstitial space. The CUF probe (Figs. 5 and 6) was implanted into the mouse ears of ICR mice to collect skin secreted proteins. A semi-permeable membrane hollow fiber with MWCO of 50 kDa was connected to the front end of the CUF probes in order to avoid collection of cell debris or contaminated proteins leaking from lysed cells (Fig. 5A, B). The semi-permeable membrane hollow fiber was entirely covered by ear skin following implantation (Fig. 6A). After implantation, a 1\% (v/v) solution of SLS was applied to the dorsal surface of the ear skin for 10 min. The ear skin was then washed extensively with distilled water to remove SLS from the skin surface. Skin secreted proteins were collected using CUF probes for 24 h following SLS application. During the 24 h collection, mice worn an elastomer saddle tether and were placed in a balanced level arm in order to avoid relocation of the CUF probes (Fig. 5C, D). Mouse ears treated with PBS served
as a control group. Histological analysis clearly demonstrated that the CUF probe was implanted into the subcutaneous space (Fig. 6B). At 24 h post implantation, a collection of 15 to 20 µl of fluid was obtained from each mouse. After digestion with trypsin, the fluid containing a complex mixture of peptides was subjected to MALDI-TOF MS and Q-TOF MS/MS analyses.

**Mass Spectrometric Identification of the CUF Probe-collected Proteins**

Comparing the MALDI-TOF MS spectra of samples collected from PBS- and SLS-treated mouse ears (Fig. 7), we found that at least four peptide peaks (1399.6, 1428.6, 1609.8, and 1681.8 m/z) were exclusively present in the sample collected from the PBS-treated group (Fig. 7A). Six peptide peaks (1179.6, 1308.6, 1694.8, 1708.8, 2395.0, and 2706.2 m/z) were exclusively detectable in SLS-treated mouse ears (Fig. 7B). Two peptide peaks (1475.7 and 1796.8 m/z) were present in samples both from PBS- and SLS-treated mouse ears. The peptide peak with 2164.1 m/z which is visible in the MALDI-TOF MS spectra of fig. 6 is derived from the auto-digestion of trypsin enzymes. To identify the
peptide peaks in the MALDI-TOF MS, tryptic peptides from PBS- and SLS-treated mouse ears were subjected to Q-TOF MS/MS for amino acid sequencing. Three peptides with parent masses of 1438.6, 1681.8 and 1694.8 were successfully sequenced via Q-TOF MS/MS. The parent masses of 1438.6 and 1681.8 were sequenced as internal peptides, SITTIIDTFHQY and SITTIIDTFHQYSR, respectively, of calcium binding protein S100A9 (accession number P31725) (Fig. 8A). The parent mass of 1694.8 was sequenced as an N-terminal peptide (Ac-SDKPDMAIEKFDK) of acetylated thymosin β4 (accession number P20065) (Fig. 8B). The two peptides with masses of 1681.8 and 1694.8 Da were also detectable in the spectra of MALDI-TOF MS (Fig. 7). Peptides with masses of 1475.7 and 1796.8 were sequenced as an internal peptide (WELLQQVDTSTR) of keratin 1 (accession number Q6IFZ6) and an internal peptide (TDTEDKGEFLSEGGV) of fibrinogen α chain (accession number Q99K47), respectively (Supplemental Fig. 1).

To confirm that S100A9 was exclusively present in the PBS-treated skin and thymosin β4 was solely found in the SLS-treated skin, we measured the intact proteins in the samples from PBS- and SLS-treated skin via mass spectrometry.
The samples were collected continuously with CUF probes for 24 h. Collected samples without trypsin digest were subjected to MALDI-TOF MS (Fig. 9 A, B) and Q-TOF MS/MS (Fig. 9 C - F) analysis. The spectra of MALDI-TOF MS illustrated that one major protein peak detected between 12,400 and 13,700 m/z was present in the PBS-, but not in the SLS-treated skin (Fig. 9A) and another major protein peak detected between 4,500 and 5,500 m/z was present in the SLS-, but not in the PBS-treated skin (Fig. 9B). The observed mass of S100A9 in mass spectrometry had been reported as 12,791 m/z (28). This protein was detected in PBS- (Fig. 9 C, E), but not in SLS-treated skin (data not shown). On the other hand, although the mass of thymosin β4 has been reported to be 5,679 Da (29), we found an observed mass of 4,963 m/z (Fig. 9F) in the CUF collected samples. This protein with an observed mass of 4,963 m/z was calculated as acetylated thymosin β4 (29). In addition, this protein was exclusively detected in SLS- (Fig. 9 D, F), but not in PBS-treated skin (data not shown). These results indicate that S100A9 and acetylated thymosin β4 were exclusively present in the PBS-, and SLS-treated skin, respectively.
DISCUSSION

Routine use of cleansers, shampoos, cosmetics, and other skin care products may result in daily exposure of our skin to surfactants (30). In addition, it is known that surfactants efficiently enhance transdermal delivery of various drugs such as testosterone (31) and antidepressant agents (32). Scientifically, it has been reported that anionic surfactant SLS can enhance skin permeability by extracting lamellar lipids from stratum corneum (25). Moreover, higher concentrations of SLS can form micelles and penetrate into the epidermis (33). It thus seems likely that SLS application may be beneficial in skin vaccination. Thus SLS may be a suitable candidate to be applied in skin vaccination. Surfactants have been designed as vesicles to encapsulate the antigens (14, 15). Mice evoked the detectable antibody response after epicutaneous application of surfactant based vesicles (niosomes) encapsulated with antigens. This device, however, is likely to have unexpected side effects since the surfactants cannot be removed. We thus pretreated mice with anionic surfactant SLS and epicutaneously applied HEL antigen after washing. Our results demonstrated that
pretreatment of mouse skin with 0.1, 1.0 and 5% (v/v) anionic surfactant SLS for 10 min significantly enhanced production of antibody to HEL (Fig. 1). Surprisingly, however, the 5% (v/v) solution exhibits the lowest enhancement. Additionally, vaccinating mice simultaneously using the mixture of SLS with HEL did not elicit detectable antibody to HEL. One possible explanation is that higher concentrations of SLS may be absorbed into epidermis and can not be removed completely after washing. Another possibility is that higher concentrations of SLS may impair the skin immunocompetent cells such as keratinocytes (34) leading to lower immune responses. Alternatively, mixing the anionic SLS with HEL may biochemically decrease the immunogenicity of HEL. It is worth investigating the efficiency of various surfactants at different concentrations and electric charges on the enhancement of skin vaccination.

A strategy involving pretreatment of skin with surfactant prior to antigen application has significant clinical promise. It is possible that the best approach with the least side effects may involve short term exposure followed by washing. It is also worth investigating whether antigens need to actually penetrate surfactant-treated skin in order to stimulate the desired immune response.
Recent evidence has shown that intestinal dendritic cells can reach invasive pathogens by either entering or extending dendrites into the epithelium and intestinal lumen (35). It is thus possible that skin dendritic cells might extend dendrites into the surfactant-induced disorganized stratum corneum, eliminating the requirement for skin penetration.

It has been reported that skin exposed to anionic SLS for 24 h impaired the skin barrier and caused inflammation as well as irritation such as allergic contact dermatitis (36, 37). However, our results demonstrated that treatment of skin with SLS for 10 min followed by extensive washing only caused a partial impairment of the integrity of lipid lamellar structure and did not result in epidermal thickening (Figs. 2 and 4). More importantly, the partial impairment of lipid lamellar structure caused by SLS is sufficient to enhance skin vaccination induced by HEL antigen. Thus short-term exposure to surfactants may be an efficient and relatively safe means to enhance skin vaccination.

Although 10 min of SLS exposure was not sufficient to cause migration of Langerhans cells from the epidermis, significant maturation of Langerhans cells was observed (Fig. 3). Acute cutaneous barrier perturbation by acetone
treatment or tape stripping induces maturation of Langerhans cells in hairless mice (26, 38). Previous studies indicated that maturation and migration of Langerhans cells can be independently regulated events (26, 39). Thus perturbation of the skin barrier by SLS may signal Langerhans cells to maintain cutaneous homeostasis against increasing exposure to external substances including antigens.

Very little is known about the molecular mechanisms mediating the effects of SLS on skin. The expression of some proteins such as vascular endothelial growth factor (40, 41) and heat shock protein 27 (42) was found to be altered in SLS-treated keratinocytes. Here we applied a novel proteomic technique using ultrafiltration capillaries and mass spectrometry to detect in vivo secreted proteins/peptides in the PBS- and SLS-treated skins. Comparing the MALDI-TOF MS spectra of sample collected from PBS- and SLS-treated skins (Fig. 6), we found that at least four and six peptide peaks were exclusively present in the sample collected from PBS- and SLS-treated skins, respectively. S100A9 was detected exclusively in the PBS-treated skin, whereas thymosin β4 was exclusively present in the MALDI-TOF MS spectrum of samples collected from
SLS-treated skin. Thymosin β4 is a 5 kDa peptide originally recognized as thymic hormone. Thymosin β4 possesses an actin-binding domain and serves as an actin buffer by binding the monomeric actin (1:1 ratio) to prevent polymerization into actin filaments. Although it has been known that thymosin β4 is expressed ubiquitously in the cytoplasm of various cell types including skin cells, it is also detected outside of cells in blood plasma or in wound fluid. However, nothing is known about the molecular mechanisms mediating the effects attributed to extracellular thymosin β4. Interestingly, thymosin β4 enhanced the antigen-presenting capacity of macrophages when macrophage monolayers were cultured in its presence. Data from the detection of intact proteins via mass spectrometry indicated that an acetylated form of thymosin β4 is present in SLS-treated skin. It is thus worth investigating the possible role of extracellular (acetylated) thymosin β4 on the enhancement of SLS mediated skin vaccination.

S100A9, a calcium-binding protein, exists in extracellular fluids and is abundantly detectable in the cytosolic fraction of keratinocytes of normal epidermis (44) and neutrophils (45). S100A9 is frequently co-expressed with S100A8 and forms S100A8/S100A9 complex which was recognized as calprotectin (45). The
complex was robustly secreted from cells in response to tissue injury, inflammation and disease. The binding of calcium induced conformational changes in S100A8/S100A9, the calcium-saturated status, which allows the binding of other proteins (46). Conformational changes in S100A9 and/or binding with other proteins may lead to the failures of collection by CUF probes and/or detection via mass spectrometry. Quantitative mass spectrometric analysis using isotope-coded affinity tag (ICAT) may help in qualifying the relative amounts of secreted S100A9 in the PBS- and SLS-treated skins (47). To further validate the differential expression of S100A9 and (acetylated) thymosin β4 in the skin treated with or without SLS, other techniques such as ELISA and immunohistochemistry will be conducted in the future when antibodies are available.

Because of differences in the ionization process between MALDI-TOF MS and Q-TOF MS/MS, eight peptides with high intensities (1399.6, 1479.8, 1609.8, 1179.6, 1308.6, 1708.8, 2385.0, and 2706.2 m/z) (Fig. 7) in the MALDI-TOF MS spectrum do not appear in the Q-TOF MS spectrum (data not shown), resulting in a failure to identify these proteins. The MALDI-TOF-TOF instrument is able to obtain a fragmentation spectrum on the same ions detected in the peptide mass
fingerprint of MALDI-TOF MS (48). Thus use of the MALDI-TOF-TOF may be an alternative method of sequencing unidentified proteins demonstrable in the MALDI-TOF MS spectrum. Identification of S100A9 and thymosin β4 as \textit{in vivo} secreted proteins was made possible by a newly designed technique of CUF probes using semi-permeable membrane hollow fibers to capture secreted proteins/peptides in PBS- and SLS-treated skins. Secreted proteins/peptides were identified by MALDI-TOF MS in combination with Q-TOF MS/MS without prior 2-DE gel separation. Our data demonstrate for the first time that \textit{in vivo} secreted proteins collected by CUF probes can be identified directly by mass spectrometry.

In summary, we demonstrated that short-term pretreatment of skin with SLS is a simple method to enhance skin vaccination. The selection and standardization of various adjuvants or immunomodulators are still obstacles to the development of non-invasive transdermal vaccines. Our method using short-term application of surfactants may offset these challenges. In parallel, we present a novel technique using ultrafiltration capillaries to capture \textit{in vivo} secreted proteins. When combined with mass spectrometry, two proteins were
identified and sequenced as S100A9 and thymosin β4. These proteins will be potentially selected as targets for understanding how surfactants enhance skin vaccination. We also believe this technique has numerous potential clinical applications involving their detection of \textit{in vivo} biomarkers.
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FIG. 1. **Pretreatment with SLS enhances skin vaccination.** The abdominal skin of ICR mice was depilated with an electric trimmer. 100 µl of SLS (0.1, 1, and 5%, v/v) or PBS was applied to the depilated skin for 10 min and then rinsed with water to remove unabsorbed liquid. Pretreatment was followed by epicutaneous immunization with HEL (100 µg/100 µl). Serum was harvested for ELISA-based anti-HEL IgG analysis at one month post immunization. The control group consists of mice exposed to (100 µl) PBS for 10 minutes (open bar). Antibody titers of HEL IgG are low but detectable in the PBS pretreated mice immunized with HEL (solid bar). Antibody titers of HEL IgG were significantly enhanced after SLS pretreatment at there different doses (hatched bars). Data represent geometric means from three independent experiments. *P < 0.01, **P < 0.001, compared to HEL immunized mice pretreated with PBS by Student’s t test.
FIG. 2. *Pretreatment with SLS disrupts the stratum corneum barrier, widens the intercellular spaces and partially removes the lipids of stratum corneum.* 100 µl of PBS (A, C) or SLS (1.0 %, v/v) (B, D) was applied onto the abdominal skin of ICR mice for 10 min. The treated area was excised and fixed with acrolein vapor and 2.5% glutaraldehyde, and then post fixed with 0.5% RuO4. Tissues were examined on a Hitachi 7000 transmission electron microscope. SLS pretreatment resulted in both disorganization of stratum corneum (*solid arrow*) and widening of intracellular spaces (*SC*) (A, B). Ultrastructural observation demonstrated that the lipid lamellar structure (*white arrows*) was partially disrupted (*white line*) in the SLS-treated epidermis (C, D). Bars: 50 nm.
FIG. 3. **Treatment with SLS does not cause Langerhans cell migration from the epidermis, but alters the maturational status of Langerhans cells.**

Confocal images of ear epidermal sheets from ICR mice were stained for MHC class II (TRITC) after epicutaneous exposure to PBS (A, C) or SLS (1.0 %, v/v) (B, D) for 10 min. The number of Langerhans cells (MHC class II-stained cells) was counted on each photograph and the mean stained cell number/mm² was calculated and expressed as stained cells ± SE. Skin exposed to SLS yielded 279 ± 41 per mm² compared to 288 ± 57 per mm² when exposed to PBS alone.

Ear epidermal sheets were double stained for MHC class II (TRITC) and CD86 (FITC) and observed by confocal microscopy (C, D) in order to examine the maturational status of Langerhans cells. The expression of CD86 on Langerhans cells (*yellow*) in the SLS-treated skin (D) indicates that considerable maturation was induced by SLS. Bars: 10 µm.
FIG. 4. **Epicutaneous treatment of SLS for 10 min does not result in inflammation.** The ear skins of ICR mice were cross-sectioned and stained with hematoxylin and eosin (H&E) after epicutaneous exposure to PBS (A, B) or SLS (C - F) at indicated concentrations (v/v) for 10 min (A, C, D, and E) or 24 h (B, F). Although 24 h treatment with 5% (v/v) SLS causes significant epidermal thickening (F), short-term exposure (10 min) has no effect as compared to a PBS control. Bar: 25 µm.
FIG. 5. **Illustration of capillary ultrafiltration (CUF) probes for continuous *in vivo* sampling from mice.** A CUF probe (A) was prepared based on the design described in the EXPERIMENTAL PROCEDURES. a, semi-permeable hollow membrane fiber. b, fused silica capillary. c, PTFE tube. d, PTFE ultramicrobore tube. e, sharpened G27 needle. The magnification of the semi-permeable hollow membrane fiber with is shown (A, *insert*). Bar: 7.5 mm. Schematics of a CUF probe with vacutainer (B, C, f) and the awake animal CUF collection system (D) are shown. After implantation of a CUF probe into the ear skin, negative pressure was created in the vacutainer, driving the collected molecules to move towards it. The negative pressure was created by vacuuming the vacutainer with a syringe (D, h). For continuous sampling, ICR mice wore elastomer saddle tethers and were placed in a balanced level arm (B, C, g) in order to avoid movement of the CUF probes. A CUF probe placed in the collection system was highlighted in blue (B, D).
FIG. 6 Subcutaneous implantation of capillary ultrafiltration (CUF) probes into ear skin. The CUF probe was implanted into the mouse ear from the end near the head (A, arrow). Damage to blood vessels in the ear skin was avoided during the probe implantation. The semi-permeable membrane hollow fiber was entirely covered by ear skin. H&E staining indicated that the CUF probe (B, arrow) was implanted into the subcutaneous space of the ear skin (B). Bar: 0.3 mm.
FIG. 7. The MALDI-TOF MS fingerprints of samples collected by CUF probes. After implantation of CUF probes, PBS or SLS (1.0% v/v) was epicutaneously applied to ear skin for 10 min and then rinsed with water to remove any residual. The samples from PBS- (A) and SLS- (B) treated skins were collected continuously with CUF probes for 24 h. After tryptic digestion, collected samples containing a mixture of tryptic peptides were subjected to the MALDI-TOF MS analysis. Five peaks of 1399.6, 1438.6, 1479.8, 1609.8, and 1681.8 m/z were detected exclusively from the PBS-treated skin and were absent in the sample collected from SLS-treated skin. Six peaks of 1179.6, 1308.6, 1694.8, 1708.8, 2385.0 and 2706.2 m/z were detected exclusively from the SLS-treated skin. Two peaks with 1475.7 and 1796.8 m/z were present in both PBS- and SLS-treated skins, and were identified as internal sequences of keratin 1 and fibrinogen alpha polypeptide, respectively. Two peaks with 1438.6 and 1681.8 m/z were identified as internal sequences of calcium binding protein S100A9 (see Fig. 7). The peak with 1694.8 m/z was identified as an internal sequence of thymosin β4 (see Fig. 7). A tryptic autodigestive peak at m/z value 2164.1 (asterisk) served as an internal calibration standard.
FIG. 8. Identification of S100A9 and thymosin β4 by Q-TOF MS/MS sequencing. An internal peptide of S100A9 with m/z value at 1681.8 analyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as SITTIIDTFHQYSR (A). An internal peptide of thymosin β4 with m/z value at 1694.8 analyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as an acetylated Ac-SDKPDMAEIEKFDK (B).
FIG. 9. Measurements of the intact proteins (S100A9 and thymosin β4) by mass spectrometry. The samples from PBS- and SLS-treated skins were collected continuously with CUF probes for 24 h. Collected samples containing a mixture of intact proteins were subjected to the MALDI-TOF MS (A, B) and Q-TOF MS/MS (C - F) analysis. A major protein peak with m/z between 12,400 and 13,700 was present in the PBS-treated skin, but not in the SLS-treated skin (A). Conversely, a major protein peak with m/z between 4,500 and 5,500 was present in the SLS-treated skin and absent in the PBS-treated skin (B). Q-TOF MS spectrum of a protein peak (12,400 - 13,700 m/z) was shown (C). The protein with an observed mass of 12,971 (E) derived from multiple charges (12+ -15+) (C) matched with the mass of S100A9. Q-TOF MS spectrum of a protein peak (4,500 - 5,500 m/z) was shown (D). The protein with an observed mass of 4,963 (F) derived from multiple charges (5+ - 7+) (D) matched with the mass of acetylated thymosin β4.
SUPPLEMENTAL FIG. 1. Identification of keratin 1 and fibrinogen α chain by Q-TOF MS/MS sequencing. An internal peptide of keratin 1 with m/z value at 1475.7 analyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as WELQQVDTSTR (A). An internal peptide of fibrinogen α chain with an m/z value at 1796.8 analyzed by MALDI-TOF MS (Fig. 7) was sequenced by Q-TOF MS/MS as TDTEDKGEFLSEGGGVR (B).
FIG. 1.

Geometric mean ELISA titer

PBS  HEL
SLS:  0.1%  1%  5%

**  **  *
FIG. 4.

A: PBS, 10 min  
B: PBS, 24 h

C: 0.1%, 10 min  
D: 1.0%, 10 min

E: 5.0%, 10 min  
F: 5.0%, 24 h
FIG. 5.

A

B

c  c
b  d

D

e  d
h
f

C

f  g

57
FIG. 7.

A: PBS

B: SLS
FIG. 8.

A

```
S T I T T I I D T F H Q Y S R
```

Relative Abundance (%)

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<tr>
<th>y11</th>
<th>y10</th>
<th>y9</th>
<th>y8</th>
<th>y7</th>
<th>y6</th>
<th>y5</th>
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<tr>
<td>1381</td>
<td>1280</td>
<td>1167</td>
<td>938</td>
<td>1053</td>
<td>837</td>
<td>690</td>
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</tbody>
</table>

m/z

B

```
Ac-S D K P D M A E I E K F D K
```

Relative Abundance (%)

<table>
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<tr>
<th>y11</th>
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<th>y8</th>
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<td>1053</td>
<td>938</td>
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<td>690</td>
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</tbody>
</table>

m/z
FIG. 9.
SUPPLEMENTAL FIG. 1.

A

Relative Abundance (%)

$\text{WELLQQVDTSTR}$

$\begin{align*}
&b_2 \quad 316 \\
&b_3 \quad 429
\end{align*}$

$\begin{align*}
&y_1 \quad 175 \\
&y_3 \quad 363
\end{align*}$

$\begin{align*}
&y_5 \quad 579 \\
&y_6 \quad 678
\end{align*}$

$\begin{align*}
&y_7 \quad 806 \\
&y_8 \quad 934
\end{align*}$

$\begin{align*}
&y_9 \quad 1047 \\
&y_{10} \quad 1161
\end{align*}$

$m/z$

B

Relative Abundance (%)

$\text{TDTEDKGFLSEGGGVR}$

$\begin{align*}
&b_2 \quad 217 \\
&b_3 \quad 562
\end{align*}$

$\begin{align*}
&y_1 \quad 175 \\
&y_3 \quad 445
\end{align*}$

$\begin{align*}
&y_5 \quad 661 \\
&y_6 \quad 921
\end{align*}$

$\begin{align*}
&y_7 \quad 1051 \\
&y_9 \quad 1108
\end{align*}$

$\begin{align*}
&y_{10} \quad 1235 \\
&y_{12} \quad 1351
\end{align*}$

$m/z$