Novel Ethanol-in-Fluorocarbon Microemulsions for Topical Genetic Immunization

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Received August 27, 2002; accepted September 23, 2002

Purpose. Traditionally, vaccines have been administered by needle injection. Topical immunization through the intact skin with either protein- or DNA-based vaccines has attracted much attention recently. We sought to enhance the immune responses induced by DNA-based vaccines after topical application by developing novel ethanol-in-fluorocarbon (E/F) microemulsion systems to aid in the delivery of plasmid DNA (pDNA).

Methods. Ten different fluorosurfactants were selected or synthesized and screened by pseudo-phase-diagram construction for their ability to form E/F microemulsions. Plasmid DNA was successfully incorporated into E/F microemulsions using several different fluorosurfactants and perfluorooctyl bromide as the continuous fluorocarbon phase. For several reasons, Zonyl® FSN-100 (an ethoxylated nonionic fluorosurfactant) was selected for further studies. *In vivo* studies were performed in mice to assess pDNA expression in skin and immunologic responses after topical application of this system using a luciferase-encoding plasmid (CMV-luciferase) and a CMV-β-galactosidase-encoding plasmid, respectively.

Results. Plasmid DNA incorporated into E/F microemulsion using FSN-100 as the surfactant was found to be stable. After topical application of this E/F microemulsion system, significant enhancements in luciferase expression and antibody and T-helper type-1 biased immune responses were observed relative to those of "naked" pDNA in saline or ethanol. For example, with the E/F microemulsion system, the specific serum IgG and IgA titers were increased by 45-fold and over 1000-fold, respectively.

Conclusion. A novel fluorocarbon-based microemulsion system for potential DNA vaccine delivery was developed.

KEY WORDS: skin; perflubron; DNA vaccine; topical; β-galactosidase.

INTRODUCTION

Traditionally, vaccines have been comprised of proteins, live attenuated viruses, or killed bacteria. However, DNA vaccines have attracted much attention recently. Besides being able to elicit humoral immune responses, protein antigens encoded on plasmids expressed by the transfected cells can be presented endogenously through the MHC class-I pathway and thus lead to the generation of a cytotoxic T-lymphocyte (CTL) response or cellular immune response (1). Other potential advantages of DNA vaccines include stability, cost-effectiveness for manufacture and storage, safety, and the potential to present multiple antigens on one plasmid (2). Until recently, intramuscular injection (i.m.) has been the primary method to administer DNA vaccines. The i.m. route has proven to be very effective in eliciting protective and therapeutic immune responses in small animal models. However, recent studies involving primates and humans have shown that the potency of these DNA vaccines was very limited and variable (2). Consequently, there exists a clear need to improve the potency of DNA vaccines for human immunization and/or to explore alternative routes for administration.

Skin, as the body's front line for immunosurvelliance, may be an ideal site for immunization. For example, the viable epidermis of skin contains Langerhans cells (LC), immature dendritic cells (DC) believed to be the most potent antigen-presenting cell (APC). Although LCs account for only 1–4% of the total cells in the epidermis, they cover over 25% of the skin area because of their "dendritic" structure (3). In fact, as an alternative to i.m. administration, researchers have investigated the administration of DNA vaccines to the skin using intradermal needle injection (i.d.) (4), needle-free jet injection (5), and the gene gun (6). Although all of these methods have shown promise to some extent, a more robust, cost-effective, and convenient means to deliver DNA vaccines to the skin is still needed.

Recently, a growing body of evidence has pointed out the advantages and feasibility of topical immunization on skin (7,8). Skin is available and readily accessible, which makes topical application convenient. Moreover, topical immunization could be much less expensive because the strict sterility requirement for parenteral vaccines is unnecessary for topical administration. The feasibility of topical immunization with protein-based antigen was first demonstrated by Glenn *et al.* (7). Subsequent work by this group and others showed that this means of immunization is viable for a variety of protein antigens in animal models ranging from mouse to sheep, dog, and even human (9–11). The first successful DNA immunization on skin by the topical application was reported in 1997 by Tang *et al.* and Niemiec *et al.* (8,12). Thereafter, several reports have been published that demonstrated improved efficiency and the elucidation of possible mechanisms for the induction of immune responses (13–18). The potency of immunization using "naked" pDNA alone by the topical route has proven to be quite low (12–18). Consequently, pDNA has been complexed with cationic liposomes and polymers, and/or coated on the surface of preformed cationic nanoparticles to elicit stronger immune responses.

For example, Shi *et al.* (13), by complexing hGH-encoding pDNA with liposomes made of DOTAP/DOPE and applying the complexes on shaved and Nair®-treated skin, and Fan *et al.* (1999), by complexing HBsAg-encoding pDNA with Lipofectin® and applying the complexes on shaved or unshaved skin, demonstrated little or no enhancement of immune responses in mice over "naked" pDNA alone. Fan *et al.*, using skin graft transplantation, demonstrated that the presence of normal hair follicles was required to elicit an immune response to the expressed antigen, indicating a possible mechanism for topical DNA immunization (14). However, Watabe *et al.* showed that, when applied topically to Balb/C mice, an influenza virus A/PR/8/34 matrix (M) gene containing plasmid complexed with DC-Chol/DOPE liposomes, produced a stronger immune response and enhanced protection against influenza virus challenge, relative to the "naked" pDNA alone (17). For these studies, the keratinocytic layer of the skin was artificially removed before dosing,

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0724-8741/03/0100-0016/0 © 2003 Plenum Publishing Corporation

making the immunization no longer noninvasive. By coating pDNA on preformed chitosan-based nanoparticles, Cui and Mumper found that these chitosan-based systems produced both detectable and quantifiable levels of luciferase expression in mouse skin 24 h after topical application to shaved skin and significant enhancements in antigen-specific IgG titer to expressed β-galactosidase at day 28 (15). The IgG titer with selected chitosan-based nanoparticles was up to 32-fold greater than that in mice immunized with "naked" pDNA alone. Further, when pDNA was coated on the surface of novel cationic nanoparticles engineered from microemulsion precursors, the resulting IgG titer was enhanced 16-fold over that produced by "naked" pDNA alone. These pDNA-coated nanoparticles were also found to elicit significantly enhanced splenocyte proliferative immune responses (16).

Microemulsions, especially the water-in-oil (W/O) type, have shown promising potential in enhancing absorption and delivery of water-soluble macromolecules, such as peptides and pDNA. For example, W/O microemulsions have been reported to improve the absorption of RGD peptides following intraduodenal administration (19,20). Wu *et al*., using plasmids encoding chloramphenicol acetyltransferase (CAT) or human interferon-α_2, showed that W/O microemulsions prepared from Tween 80, Span 80, and olive oil can be used to facilitate transfection of murine follicular keratinocytes by about 20-fold (21).

Fluorocarbons are carbon-based molecules with some or all of the hydrogen atoms replaced with fluorine. They have unique properties including chemical and biologic inertness, low surface tension, high density, and the ability to dissolve large amounts of gases. Fluorocarbons, such as perfluorooctyl bromide (perflubron), have well-documented safety profiles and have seen growing use in (micro)emulsion-based delivery systems for drugs and oxygen (22–25). Moreover, these systems have been explored as a delivery vehicle for pDNA and viruses (22–25).

It was hypothesized that a fluorocarbon-based microemulsion system could be used to deliver pDNA topically on the skin. Experiments in our laboratories have been exploring the use of ethanol-in-fluorocarbon (E/F) microemulsion systems to delivery pDNA in the ethanol phase or nanoparticle-encapsulated pDNA made from the ethanol droplets. The overall goal of this present work was to develop stable E/F microemulsions containing pDNA and to test both the expression and immune responses after topical application to mice.

MATERIALS AND METHODS

Materials

Plasmids containing a CMV promoter and β-galactosidase reporter gene (CMV-β-gal) or CMV promoter and luciferase gene (CMV-luc) were gifts from the Valentis, Inc. (Woodlands, TX). Normal goat serum (NGS) and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). PBS/Tween 20 buffer (20×) was from Scytek Laboratories (Logan, UT). Antimouse IgG peroxidase-linked species-specific $F(ab')_2$ fragment (from sheep) was purchased from Amersham Pharmacia Biotech Inc. (Piscataway, NJ). Goat antimouse IgA, IgG1, and IgG2a peroxidase-linked were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL). Tetramethylbenzidine (TMB) soluble reagent was from Pierce (Rockford, IL). Luciferase assay kits were purchased from Promega (Madison, WI). Absolute ethanol and perfluorooctyl bromide [$CF_3(CF_2)_6CF_2Br$, perflubron] were from Spectrum Chemical Manufacturing Corporation. (New Brunswick, NJ).

Methods

Selection of Fluorosurfactants

For initial testing as potential surfactants to form ethanol-in-perflubron (E/F) microemulsions, a series of fluorinated surfactants, listed in Table I, were selected. Most of these were commercially available; two of them were synthesized. Tridecafluorooctyloxypropionitrile (D2) was prepared using standard synthetic methodology by treating tridecafluoro-1-octanol with sodium hydride and reacting the subsequent alkoxide with acrylonitrile (26). The product was characterized by elemental analysis, NMR, and mass spectrometry (data not shown). In a like manner, tridecafluorooctyloxypropionitrile was oxidized to tridecafluorooctyloxypropionic acid (D4) by refluxing in concentrated sulfuric acid and extracting the product from a water–ether mixture. This product was similarly characterized by elemental analysis, NMR, and mass spectrometry (data not shown).

Fluorosurfactant Screen: Microemulsion Pseudo-Phase-Diagram Construction

Classic pseudo–phase diagrams for microemulsions using a matrix-screening approach were constructed for each fluo-

Table I. The Structure of Fluorosurfactants Used

(Fluoro)surfactants	Structure	Source
FSN-100 (Zonyl®)	$F(CF_2CF_2)_{1-9}CH_2CH_2O(CH_2CH_2O)_{0-25}H$	DuPont
FSO-100 (Zonyl®)	$F(CF_2CF_2)_{1-7}CH_2CH_2O(CH_2CH_2O)_{0-15}H$	DuPont
Pentadecafluorooctanoic acid (PDFOA)	$CF_3(CF_2)_6COOH$	Aldrich
Tridecafluorooctyloxypropionitrile (D2)	$CF_3(CF_2)_5(CH_2)_2O(CH_2)_2CN$	Synthesized
Tridecafluoro-1-octanol (D3)	$CF_3(CF_2)_5(CH_2)_2OH$	Aldrich
Tridecafluorooctyloxypropionic acid (D4)	$CF_3(CF_2)_5(CH_2)_2O(CH_2)_2COOH$	Synthesized
Tridecafluoroheptanoic acid (D5)	$CF_3(CF_2)_5COOH$	Aldrich
Perfluorotetradecanoic acid (D6)	$CF_3(CF_2)_{12}COOH$	Aldrich
Perfluorododecanoic acid (D7)	$CF_3(CF_2)_{10}COOH$	Aldrich
Pentadecafluoromethyloctanoate (D8)	$CF_3(CF_2)_6COOCH_3$	Oakwood
Octanoic acid (control)	$CH_3(CH_2)_5CH_2COOH$	Sigma

rosurfactant candidate listed in Table I. Briefly, a 0.5-g mixture of perflubron (70–95%, w/w) and fluorosurfactant (5–30%, w/w) was prepared in glass vials. While the mixture was stirring, ethanol was added in 5-μL (4.1-μg) aliquots. The clarity (transparency) of the systems as a function of the percentages of the three phases was plotted in order to define the microemulsion window, if any. This process was repeated for each of the fluorosurfactant candidates. Octanoic acid was used as a control surfactant.

Incorporation of Plasmid DNA into the Ethanol-in-Perflubron (E/F) Microemulsion Systems

Based on the results of the fluorosurfactant screen, plasmid DNA was incorporated into two microemulsion systems containing either pentadecafluorooctanoic acid (PDFOA) or FSN-100 (Zonyl®). For the preparation of the PDFOA-containing system, exactly 300 mg of perflubron (60%, w/w) and 200 mg of PDFOA (40%, w/w) were weighed and mixed in a glass vial. Five microliters of absolute ethanol was added into the mixture, which was stirred for about 5 min. A combination of 25 μL ethanol and 5–6 μL of pDNA solution (3.4–4 μg/μL in water) was then added to the perflubron/PDFOA mixture while stirring. After another short period of stirring (2–5 min), a clear transparent system was obtained. The total volume of this preparation was 400 μL, which led to a final pDNA concentration of 50 μg/mL. For the preparation of the system containing FSN-100, exactly 350 mg of perflubron (70%, w/w) and 150 mg of FSN-100 (30%, w/w) were weighed and mixed in a glass vial. While this was stirred, a combination of 35 μL ethanol and 5–6 μL of pDNA solution (3.4–4 μg/μL in water) were added to the mixture. The system was stirred for 2–5 min until it became clear. Again, the final pDNA concentration was 50 μg/mL. To prepare larger volumes of each microemulsion system, all the components were increased proportionally.

Characterization of the Microemulsion Systems

The droplet sizes of the microemulsion systems were measured at 25°C by photon correlation spectroscopy (PCS) using a Coulter N4 Plus Submicron Particle Sizer (Coulter Corporation, Miami, FL) at 30°, 60°, and 90° angles, each for 90 s. The viscosities of the systems, with and without pDNA incorporation, were measured using a Model DV-III Plus Programmable Rheometer (Brookfield Engineering Laboratories, Middleboro, MA). Finally, the clarity of the pDNA-incorporated systems at ambient condition was checked daily to monitor the stability of the microemulsions.

Stability of the Incorporated Plasmid DNA in the Microemulsion System

To determine the short-term stability of the pDNA incorporated in the microemulsion, we extracted the plasmid from the microemulsion and performed gel electrophoresis. Briefly, 400 μL of the pDNA-incorporated microemulsion was mixed with 200 μL deionized water and 500 μL chloroform. The mixture was gently shaken and allowed to stay on the laboratory bench at room temperature for 15 min. The mixture was then spun down at 14,000 rpm for 5 min, and the top water phase (about 200 μL) was collected. After the top phase was combined with 20 μL of sodium acetate (3 M) and 600 μL ice-cold absolute ethanol, the mixture was kept on dry ice for an additional 15 min. If there was further phase separation, the top phase was withdrawn into an Eppendorf tube and spun down at 14,000 rpm for 5 min. A small white pDNA pellet remained in the bottom of the tube after decanting of the liquid phase. The pellet was air-dried and resuspended in 20 μL Tris-EDTA (TE) buffer (pH 7.4). The extracted pDNA was applied on a 1% Seakem® Gold Agarose Gel for electrophoresis (BioWhittaker Molecular Applications, Rockland, ME).

Gene Expression in Mouse Skin after Topical Application

Ten- to 12-week-old female mice (Balb/C) from Harlan Sprague-Dawley Laboratories were used for all animal studies. NIH guidelines for the care and use of laboratory animals were observed. Various pDNA formulations were applied directly to shaved mouse skin as described previously with some modifications (15). The final plasmid (CMV-Luc) concentration in all of the formulations was 50 μg/mL. Balb/C mice (n = 5 per group) were anesthetized with pentobarbital (i.p.). The hair covering the back of the mouse was shaved with an A5® Single-Speed Clipper (Oster Professional Products, McMinnville, TN). The skin was wiped with an alcohol swab and allowed to air dry for 5 min. One hundred microliters of each formulation (pDNA, 5 μg) was dripped onto the skin with a pipette and subsequently spread, if necessary, with the pipette tip to cover an area within a circle having a diameter of 2.3-2.5 cm. Care was taken to disperse the formulations over the skin without applying pressure. After 24 h, mice were killed, and the skin was collected and immediately frozen to –20°C in 2-mL conical screw cap microtubes (Quality Scientific Plastics) filled with zirconia/silica beads (10–20, 1 mm diameter, Biospec Products, Bartlesville, OK). The skin samples were cut into small pieces (~2 × 2 mm) and crushed three times using a Minibead Beater (Biospec Products) for 1 min each at 5000 rpm. After each minute, the samples were cooled down on ice to minimize protein damage from the heat created during bead beating. Then 100 μL of 1× reporter lysis buffer (Promega) was added to the homogenized samples. After brief vortexing at room temperature, the samples were frozen and thawed for three cycles and subsequently spun down at 14,000 rpm for 10 min, and the supernatant fluid from each sample was collected. Luciferase expression in each sample was assayed by adding 100 μL of reconstituted luciferase assay solution (Promega) to 20 μL of each sample and counting in Dynatech Microlite® 1 plates in an ML2250 Dynatech Luminometer (Dynatech Laboratories, Chantilly, CA). The peak values for each sample and negative control as reported from the Luminometer were recorded. Protein concentration in the samples (20 μL) was determined using the Coomassie® Plus protein assay reagent (Pierce). As recommended by Dynatech, luciferase expression data were reported as the ratio of the peak values of the samples to that of the negative control, divided by the total amount of protein in the 20 μL of samples assayed. A one-way ANOVA followed by Fisher's protected least significant difference procedure (PLSD) was used to analyze the data. A result of $p \leq 0.05$ was considered to be statistically significant.

Topical Genetic Immunization

Balb/C mice (n = 5/group) were anesthetized and dosed on days 0, 7, and 14 using a CMV-β-gal plasmid as described

above. The hair was shaved again for the day-7 and day-14 applications. As a positive control, 50 μL pDNA in saline (50 μg/mL) was injected into the gastrocnemius muscles of both legs on days 0, 7, and 14. On day 28, all mice were anesthetized using pentobarbital (i.p.), and blood was collected by cardiac puncture. The blood was transferred into a Vacutainer brand blood collection tube (Becton Dickinson, Franklin Lakes, NJ). Serum was isolated by centrifugation and stored at –20°C until analyzed.

Immunoassays

The β-galactosidase-specific IgG, IgA, IgG1, and IgG2a levels in sera were quantified by ELISA (15). Briefly, Costar high-binding 96-well assay plates were coated with 50 μL of β-galactosidase antigen (8 μg/mL) overnight at 4°C. The plates were then blocked with 100 μL/well of 4% BSA/4% normal goat serum (NGS) solution made in 10 mM PBS/Tween 20 (Scytek Laboratories, Logan, UT) for 1 h at 37°C. Mouse serum samples (50 μL/well; starting dilution of 20:100 in 4% BSA/4% NGS/PBS/Tween 20) were serially diluted and then incubated for 2 h at 37°C. Wells were washed with 10 mM PBS/Tween 20 buffer four or five times, and then, either antimouse IgG HRP $F(ab')_2$ fragment from sheep (diluted 1:3000 in 1% BSA) or goat anti-mouse IgA (1:5000) was added (50 μL/well) and incubated for 1 h at 37°C. Plates were washed 4-5 additional times with 10 mM PBS/Tween 20 buffer. Next, 100 μL of tetramethybenzidine (TMB) solution reagent (Pierce) was added to each well and incubated at room temperature for 30 min followed by the addition of 50 μL of 0.2 M H_2SO_4. The optic density (OD) of each sample was measured by a Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT) at 450 nm. The IgA and total IgG titers were determined by comparing the OD_{450} of the samples with that of the negative controls. For the determination of IgG1 and IgG2a levels, the OD_{450} of the fivefold-diluted serum samples after 30 min of development with TMB solution reagent was recorded. In addition, the goat anti-mouse IgG1 and IgG2a were diluted 1:5000 in 1% BSA. The data were reported as the percentage increase in the OD_{450} of the immunized mice over that of the negative controls, which were defined as $100 \times (OD_{450,samples} - OD_{450,control})/OD_{450,control}$.

RESULTS

Selection of Fluorosurfactants

Ten different fluorosurfactants were selected or synthesized and screened using pseudo-phase-diagram construction for their ability to form E/F microemulsions (Table I). One of the fluorosurfactants was an alcohol, two were ethoxylated alcohols, five were acids, one was an oxyproprionitrile, and one was an ester.

Fluorosurfactants Screen: Microemulsion Pseudo-Phase-Diagram Construction

Initial studies were focused on the selection of ethanol-in-perflubron (E/F) microemulsions that allowed for maximum solubilization of ethanol. After careful screening of the fluorosurfactants listed in Table I by pseudo-phase-diagram construction, six of the surfactants were found to be able to form microemulsions. These six fluorosurfactants and the maximum amount of ethanol incorporated into each stable clear microemulsion using a perfluorooctyl bromide/fluorosurfactant ratio of 70:30 w/w ratio are listed in Table II. As expected, no E/F microemulsion could be formed with the octanoic acid because this molecule is not fluorinated and has very limited solubility or miscibility with perflubron. The microemulsions shown in Table II were clear and stable throughout the microemulsion window, except for the system made with D4. The use of D4 produced a slightly opaque system. The use of D6 and D7 resulted in microemulsions that apparently solubilized a very high amount of ethanol in perflubron. However, it is very likely that these were actually reversed fluorocarbon-in-ethanol (F/E) systems. For some reason, no microemulsion windows were formed using D8, an ester of PDFOA. Finally, D2, D3, D5, and D9 were highly soluble in perflubron and, therefore, were not used for microemulsion construction.

The amount of ethanol solubilized in various microemulsions using the fluorosurfactants listed in Table II is shown in Fig. 1. The plots showed the amount of final ethanol solubilized in the microemulsions versus the final fluorosurfactant concentrations in different mixtures of perflubron and each fluorosurfactant. Each data point represents the final concentration of ethanol and individual fluorosurfactant in a clear microemulsion. Each line represents the microemulsion window for a specific perflubron/fluorosurfactant mixture. Generally, as the amount of fluorosurfactant increased in the perflubron/fluorosurfactant mixtures (5–30%), the final ethanol concentration that could be solubilized in the systems increased, as did the width of the microemulsion window.

Although the systems within the microemulsion windows were clear and transparent, particle size analysis using photon correlation spectroscopy (PCS) failed to reveal spherical droplets in all ethanol-in-fluorocarbon microemulsions. Therefore, these systems were likely to be bicontinuous mi-

Table II. Maximum Amount of Ethanol Incorporated in Ethanol-in-Perflubron (E/F) Microemulsions Using Different Fluorosurfactants

Fluorosurfactants	Final [ethanol] % w/w solubilized in perflubron/fluorosurfactant microemulsion systems (70/30, w/w)
FSN-100	10.1
FSO-100	18.8
Pentadecafluorooctanoic acid (PDFOA)	37.2
Tridecafluorooctyloxypropionic acid (D4)	32.0
Perfluorotetradecanoic acid (D6)	66.6
Perfluorododecanoic acid (D7)	59.2

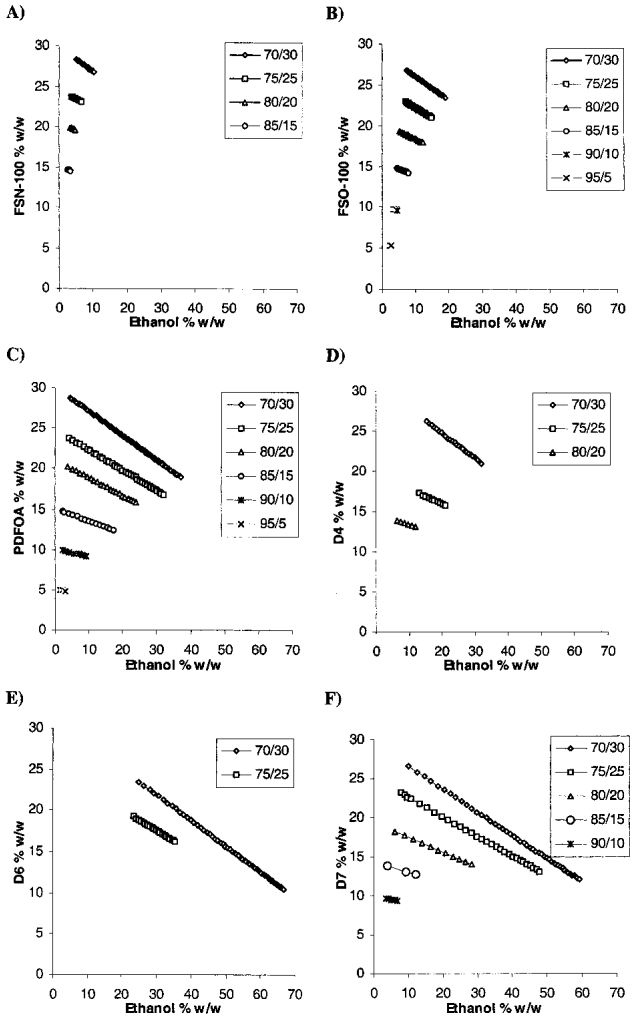

Fig. 1. Pseudo–phase diagrams of ethanol-in-fluorocarbon (E/F) microemulsions. The fluorosurfactants used were FSN-100 (A), FSO-100 (B), PDFOA (C), D4 (D), D6 (E), and D7 (F).

croemulsions, although no additional experiments were carried out to confirm this.

Incorporation of Plasmid DNA into the Ethanol-in-Fluorocarbon Microemulsion Systems

Plasmid DNA was successfully incorporated into two of the microemulsion systems—those prepared with PDFOA and with FSN-100 as the fluorosurfactant. Commercially available, PDFOA and FSN-100 were chosen as representative negatively charged and nonionic surfactants, respectively. The pDNA-incorporated systems were clear and transparent, with a final pDNA concentration of about 50 μg/mL. The system prepared with FSN-100 as the surfactant was yellowish because of the slightly yellow/tan color of the FSN-100. As shown in Table III, pDNA incorporation significantly increased the viscosity of the E/F microemulsion systems. In addition, the vicosities of the ethanol/perflubron/PDFOA (E/F/PDFOA, 32:300:200, w/w/w) microemulsion systems, with or without pDNA, were lower than that of the ethanol/perflubron/FSN-100 (E/F/FSN-100, 24:350:150, w/w/w) microemulsion system. Under ambient conditions, the pDNA-incorporated E/F/PDFOA microemulsion system became turbid within 12 h after preparation. However, the pDNA-incorporated E/F/FSN-100 microemulsion system remained clear and transparent even after 1 month of storage at room temperature. Moreover, as demonstrated in Fig. 2, pDNA incorporated into the E/F/FSN-100 microemulsion was stable, even after 24 h of storage at room temperature. The majority of the plasmid remained in the supercoiled (S.C.) form.

Gene Expression in Mouse Skin after Topical Application

As shown in Fig. 3, 24 h after topical application of the pDNA (5 μg)-incorporated E/F/FSN-100 microemulsion, the expression of the luciferase gene was relatively low and variable. However, it was significantly higher than that in the naïve mice or in mice treated with pDNA in saline or pDNA in ethanol ($p < 0.05$).

Immune Responses in Mice after Topical Application

Antigen-specific total IgG and IgA to expressed β-galactosidase after topical application of the pDNA-incorporated E/F/FSN-100 microemulsion are shown in Fig. 4A and 4B, respectively. Twenty-eight days after the initial application to mouse skin, the pDNA-incorporated E/F/FSN-100 microemulsion elicited specific total IgG titer to the level of around 6000, which was 45-fold ($p = 0.02$) greater than that after topical immunization with pDNA in saline (Fig. 4A). In fact, the IgG titer after intramuscular immunization with pDNA in saline was only 2.3-fold greater than that after topical immunization with E/F/FSN-100 microemulsion. However, this difference was not significant ($p > 0.05$).

Topical immunization with the pDNA-incorporated E/F/FSN-100 microemulsion led to an average serum specific IgA titer of about 1600, while specific IgA was virtually undetectable in mice immunized topically with either pDNA in saline or in ethanol (Fig. 4B). Conversely, in mice immunized by the intramuscular (i.m.) route with pDNA in saline, a very low level of specific IgA (titer of 160) was detected in only one of the five mice.

The antigen-specific IgG subtypes (IgG1 and IgG2a) in the serum were also determined. Shown in Fig. 5 is the rela-

Table III. Viscosities of Selected E/F Microemulsions Systems

Microemulsions	Viscosity (centiPoise) (n = 3)		p value[a]
	Without pDNA	With pDNA	
E/F/FSN-100 (24:350:150, w/w/w)	11.6 ± 0.8	13.3 ± 0.7	0.028
E/F/PDFOA (32:300:200, w/w/w)	2.59 ± 0.09	3.85 ± 0.01	0.001

[a] A two-sample t-test assuming equal variances was used to compare the viscosities of the microemulsions before and after pDNA incorporation.

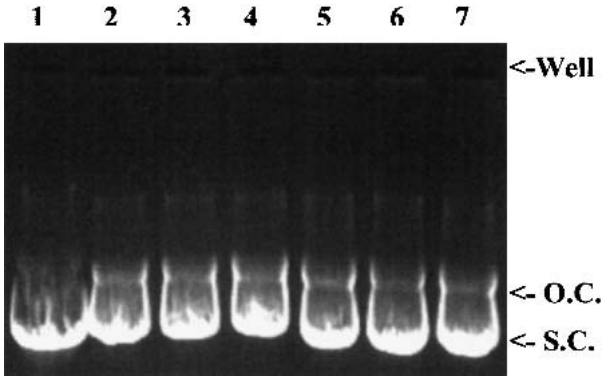

Fig. 2. Physical stability of pDNA incorporated into an ethanol/fluorocarbon/FSN-100 (E/F/FSN-100 = 24:350:150, w/w/w) microemulsion. Six E/F/FSN-100 microemulsions were prepared and stored on the laboratory bench at room temperature. After 0 h (lane 2), 0.5 h (lane 3), 1.25 h (lane 4), 4 h (lane 5), 7 h (lane 6), and 24 h (lane 7), plasmid was extracted from each preparation and analyzed by gel electrophoresis. Loaded in lane 1 was the original plasmid as a control. O.C. indicates open-circular plasmid; S.C. indicates supercoiled plasmid.

tive percentage increase in the OD_{450} of the IgG subtypes in the serum (after fivefold dilution) of the immunized mice, compared to that of the naïve mice. The percentage increase in the IgG2a was greater than the percentage increase in the IgG1 after both intramuscular immunization with pDNA in saline and topical immunization with the pDNA-incorporated E/F/FSN-100 microemulsion. Although the actual values of the percentage increase for both IgG1 and IgG2a titer in mice immunized by the intramuscular route with pDNA in saline

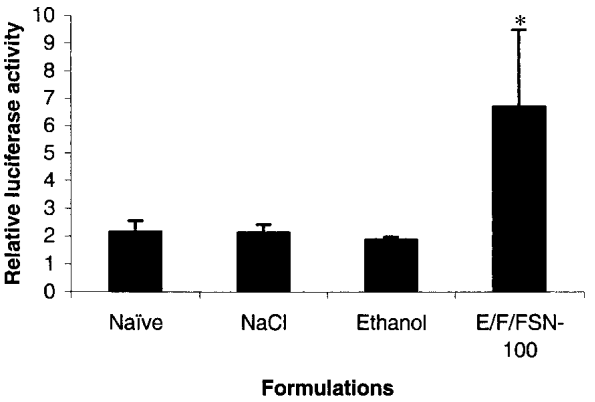

Fig. 3. Gene expression in mouse skin after topical application of pDNA in different formulations. One hundred microliters (5 μg CMV-luc) of each formulation was applied to the shaved skin of Balb/C mice (10–12 weeks). Luciferase expression in skin (n = 5/group) was determined 24 h after application. The following groups were used: Naïve, undosed negative control; NaCl, pDNA in saline; Ethanol, pDNA in absolute ethanol; E/F/FSN-100, pDNA-incorporated ethanol/fluorocarbon/FSN-100 (24:350:150, w/w/w) microemulsion. A one-way ANOVA followed by Fisher's protected least-significant-difference procedure (PLSD) was used to analyze the data. A p value ≤ 0.05 was considered to be significant. * Indicates that the expression from the E/F microemulsion group was significantly different from those of all other groups.

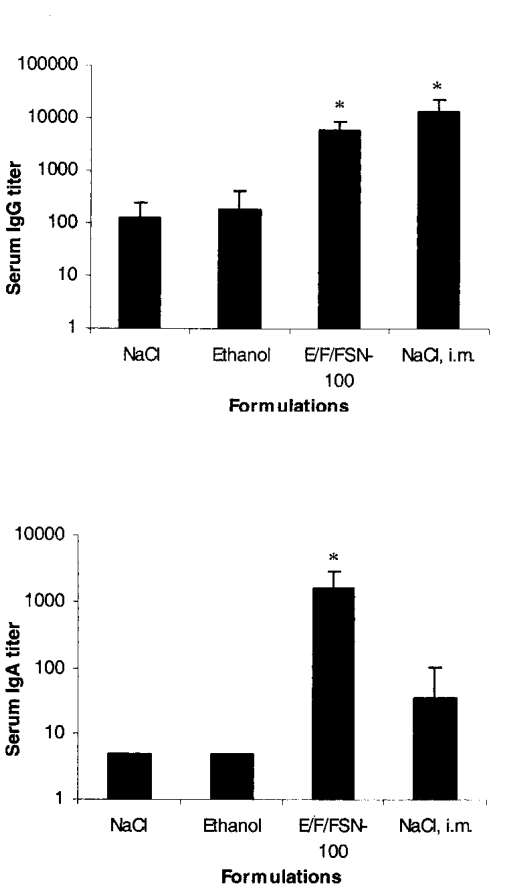

Fig. 4. Antigen-specific IgG (A) and IgA (B) titers in serum to expressed β-galactosidase 28 days after topical application of different pDNA formulations to mice (n = 5/group). One hundred microliters (5 μg CMV-β-gal) of each formulation was applied topically to the shaved skin of Balb/C mice (10–12 wks) on day 0, 7, and 14. The mouse groups were the following: NaCl, pDNA in saline; Ethanol, pDNA in absolute ethanol; E/F/FSN-100, pDNA-incorporated ethanol/fluorocarbon/FSN-100 (24:350:150, w/w/w) microemulsion; NaCl, i.m., intramuscular injection of pDNA in saline. A one-way ANOVA followed by Fisher's protected least-significant-difference procedure (PLSD) was used to analyze the data. A p value ≤ 0.05 was considered to be significant. * Indicates that these groups were significantly different from all other groups.

were greater than those after topical immunization with the pDNA-incorporated E/F/FSN-100 microemulsion, the ratio of the percentage increase for IgG2a over IgG1 (IgG2a/IgG1) for the topically immunized mice was 3.52, which was greater than that for the mice immunized by the intramuscular route (1.65).

DISCUSSION

Noninvasive topical immunization has proven to be a feasible strategy to administer DNA vaccines, although the effectiveness of applying "naked" pDNA alone to induce strong immune responses was found to be rather low (8,15,16). "Incorporating pDNA into a novel ethanol-in-fluorocarbon (E/F) microemulsion system with FSN-100 as the fluorosurfactant and applying topically to shaved mouse

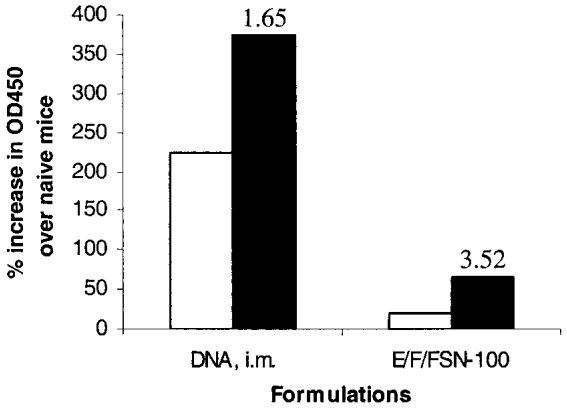

Fig. 5. Antigen-specific IgG1 and IgG2a levels in serum to expressed β-galactosidase 28 days after topical application of different pDNA formulations to mice (n = 5/group). Mice were immunized as described in the Methods. One hundred microliters (5 μg CMV-β-gal) of each formulation was applied topically to the shaved skin of Balb/C mice (10–12 weeks) on day 0, 7, and 14. The mouse groups were: E/F/FSN-100, pDNA-incorporated ethanol/fluorocarbon/FSN-100 (24:350:150, w/w/w) microemulsion; DNA, i.m., intramuscular injection of pDNA in saline. *White bars* indicate IgG1 level; *black bars* indicate IgG2a level. The numbers over the bars are the ratio of IgG2a to IgG1.

skin led to enhanced specific total IgG titer to an expressed model antigen, b-galactosidase, in serum by 45-fold over 'naked' pDNA alone." Moreover, the antigen-specific IgA titer in serum of the pDNA-incorporated microemulsion-immunized mice was over 1600, although it was practically undetectable in mice immunized with "naked" pDNA in saline or ethanol by the same route. Taken together, these data suggest the potential of this novel fluorocarbon-based microemulsion system for noninvasive topical genetic immunization.

IgA, the most abundant antibody produced by the immune system, is present mostly in the dimeric form and is secreted onto the surface of the epithelia that communicate with the external environment. These epithelia include the linings of the gastrointestinal tract, the eyes, the nose, the throat, the respiratory, urinary, and genital tracts, and the mammary glands. The high level of specific IgA titer in the serum of mice immunized topically with the pDNA-incorporated fluorocarbon-based microemulsion is expected to be mostly monomeric IgA. A significant level of high-affinity specific IgA secreted on the mucosal surface is required to neutralize microbial toxins and animal venoms. It is also required to prevent viruses and bacteria from colonizing and thereafter infecting cells. Although it would be of interest to measure the IgA levels in local mucosal epithelial fluid (e.g., nasal fluid and vaginal fluid), the high IgA titer in the serum suggests that the local mucosal IgA level should also be enhanced (27). Nevertheless, future investigations in our laboratories with this system will include the measurement of local mucosal IgA.

Both i.m. injection with "naked" pDNA in saline and topical application of pDNA-incorporated fluorocarbon-based microemulsion resulted in immune responses that biased the Th1-type (Fig. 5). Although the Th1-biased immune responses with the i.m. route agreed well with other previous reports (28,29), results from the topical application of the pDNA-incorporated fluorocarbon-based microemulsion system did not agree with a previous report. Fan *et al.* reported that topical application of liposome-complexed pDNA on C57/BL6 mouse skin led to specific immune responses that displayed Th2-biased features (14). Interestingly, in contrast to all other published reports, the same group also reported that topical application of "naked" CMV-lacZ pDNA alone resulted in statistically similar level of LacZ IgG induction as did intramuscular injection of the same "naked" pDNA.

The mechanisms of the immune response induction by the noninvasive topical immunization remain largely unknown. However, Fan *et al.*, using skin graft transplantation, demonstrated that the presence of intact hair follicles was required for the elicitation of immune responses (14). It is believed that hair-follicle transport of this E/F microemulsion system is the most probable mechanism because it is unlikely that highly charged hydrophilic macromolecules such as pDNA could penetrate the intact stratum corneum (21). It was found that the expression of luciferase in mouse skin was enhanced when the pDNA was incorporated into the fluorocarbon-based microemulsion system, as compared to pDNA in normal saline or ethanol. Therefore, enhancement of antigen expression in localized skin may be related to the enhancement in the observed immune responses. However, it should be cautioned that, in general, no direct correlation has been observed between the levels of gene expression and immune responses (6,30). Plasmid DNA may be picked up by Langerhans cells (LC) in the skin epidermis, which, after activation, migrate to the local lymph nodes for antigen expression and presentation (2). No information is available about what cell type was transfected (i.e., the follicular keratinocytes, local LCs) or the mechanism(s) of antigen presentation. It is not known whether it was an MHC class II-restricted presentation of antigens that were secreted by transfected somatic cells or a direct presentation of antigens that are produced endogenously in the transfected dendritic cells (DC). Studies are under way in our laboratories to answer these questions. However, it is apparent that the immune response enhancement was related to some effects caused by a combination of the fluorocarbon and the fluorosurfactant, FSN-100. Unlike any previous topical application of pDNA formulations in aqueous media (15,16), the perflubron-based microemulsion system, when applied to the skin, without any assistance, spread immediately and completely covered the shaved skin area. Thus, better coverage of the E/F microemulsion over the skin area may have contributed, in part, to the immune response enhancement reported in these studies. Further, the presence of a fluorosurfactant may have enhanced the uptake of pDNA into the hair follicles.

Perflubron, as mentioned above, is extremely inert (chemically and biologically) and has been widely used as an artificial blood substitute and in acute respiratory distress syndrome (ARDS) therapy to deliver oxygen (24). Topical application of perflubron should, therefore, not cause safety concerns. Ethanol is generally regarded as safe (GRAS) and is extensively used in pharmaceutical formulations. Moreover, less than 10 μL of ethanol was applied topically per application in these current studies. FSN-100 is a water-soluble, ethoxylated nonionic fluorosurfactant. To our knowledge, its effect on the skin has not been tested before. However, in the present studies, no adverse effects or gross in-

flammatory reactions were observed after topical application (~40 mg) to shaved mouse skin, even after repeated applications.

Although many of the other fluorosurfactants we screened were able to form E/F microemulsions, FSN-100 was chosen because it is commercially available and nonionic and thus is potentially less toxic than other ionic fluorosurfactants. Actually, as mentioned earlier, pDNA was also successfully incorporated into the PDFOA-containing microemulsion system. However, this system was not investigated further. PDFOA is an acidic fluorosurfactant, which may be deleterious to the incorporated pDNA. In addition, in a preliminary mouse study to assess the topical application of E/F microemulsions using PDFOA as the surfactant, severe toxic effects were observed including death.

In conclusion, we reported that noninvasive topical immunization of mice with a novel pDNA-incorporated fluorocarbon-based microemulsion system elicited significantly enhanced immune responses over "naked" pDNA alone.

ACKNOWLEDGMENTS

This work was supported, in part, by NSF grant BES-9986441, and from the AFPE and the Burroughs Wellcome Fund through the AACP New Investigators Program for Pharmacy Faculty.

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