Perifollicular Transgenic Expression of Human Interleukin-1 Receptor Antagonist Protein following Topical Application of Novel Liposome-Plasmid DNA Formulations *In Vivo*

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Abstract □ Expression plasmid DNA for the human interleukin-1 receptor antagonist (IL-1ra) protein was formulated with nonionic:cationic (NC) liposomes or phosphatidylcholine:cationic (PC) liposomes and applied to the auricular skin of hamsters in single- and multiple-dose protocols. Confocal microscopy identified delivery of plasmid DNA proximal to perifollicular cells, and successful transfection of perifollicular cells was identified by immunohistochemistry and ELISA. Skin treated for 3 days with the NC liposomes had statistically significant levels of transgenic IL-1ra present for 5 days post-treatment. Expression of transgenic IL-1ra was specific to areas of skin treated with NC liposomes but not PC liposomes. The results indicate that the NC liposomes can deliver expression plasmid DNA to perifollicular cells and mediate transient transfection *in vivo*.

Introduction

Topical delivery of gene vectors to cells within the skin is an attractive strategy for gene therapy of many human diseases, including a number of dermatological conditions thought to be mediated by abnormal regulation of soluble cytokines.[1−5] Although it is highly unlikely that macromolecules can permeate the stratum corneum, the presence of follicles and associated structures may not only allow localized delivery to viable skin cells, but may also promote diffusion of transgenic soluble proteins into the surrounding tissue and/or the systemic circulation. The development of pharmaceutical reagents that can mediate transfection of epidermal cells would have far reaching experimental and therapeutic applications. For topical gene therapy to be successful, it will be necessary to optimize delivery of recombinant DNA to accessible target cells within living skin strata with vehicles that can overcome the formidable permeability barriers of the skin and its appendages.

Initial attempts at gene delivery to living skin have been hampered by problems associated with the delivery of charged macromolecules beyond the stratum corneum and have focused mainly on parenteral methods. These methods have included direct injection of plasmid DNA into the upper epidermis as well as pneumatic acceleration of metallic microparticles coated with plasmid DNA into the skin.[6,7] Although some degree of skin cell transfection has been achieved by these methods, neither appears likely to allow for the delivery of plasmid DNA to a large number of target cells on a cost-effective, therapeutic basis. Although recent studies have reported promising initial results with cationic liposomes for topical transfection, questions remain regarding the feasibility of these methods for the delivery of potentially therapeutic transgenes.[8,9] Further, the effects of composition and preparation methods of the liposomal formulations on the integrity and stability of expression plasmid DNA have not been addressed. Current knowledge regarding the types of cells that may be transfected and the kinetics of transgene expression following topical application of liposomal gene vectors is also limited. Finally, given the ubiquitous existence of lysosomal galactosidase as an inducible enzyme within many types of skin cells, the reliability of *E. coli* β-galactosidase as a definitive reporter gene for *in vivo* transfection studies into skin needs to be re-examined.

We previously reported that topical application of a novel nonionic liposomal formulation developed in our laboratories enhanced the delivery of macromolecules such as interferons, cyclosporin-A, and growth factors into the pilosebaceous units of the hamster ear and into dermal tissues of hairless mice.[10] More efficient delivery of these macromolecules was achieved compared with those obtainable with conventional phospholipid-based liposomes and conventional solutions and gels.[11−14] Further, it was found that clinically significant levels of macromolecular proteins could be delivered to the vicinity of target sites by our nonionic formulations.[15,16]

Based on these studies we hypothesized that expression plasmid DNA could be substituted as the charged macromolecule in nonionic liposomal formulations. The goal of this substitution was the development of a topical formulation with two essential physicochemical properties required for transfection of perifollicular skin cells *in vivo*; they are (1) transdermal delivery of large amounts of plasmid DNA proximal to perifollicular cells, and (2) intracellular delivery of the DNA into the target cells. Because successful gene delivery *in vivo* is best assessed with theoretically relevant and biologically active transgenes (as opposed to marker transgenes), the cDNA for human interleukin-1 receptor antagonist protein was used as a transgene in our studies.[17−19] In this report, we show that an expression plasmid encoding the cDNA for human IL-1ra protein formulated with nonionic and cationic lipid components can be used as a topical pharmaceutical reagent for the transient transfection of skin cells *in vivo*.

Materials and Methods

Preparation and Purification of Plasmid DNA—Expression plasmids used in these studies were derived from the eukaryotic expression vector pSG5 (Stratagene), and utilize the SV40 LTR as a promoter and the SV40 polyadenylation sequence. The cDNA for human interleukin-1 receptor antagonist protein (gift of Immunex Inc.) was cloned into the BamHI site of pSG5 to yield pSG5IL-1ra. The gene for *Escherichia coli* (*E. coli*) β-galactosidase was cloned into the BamHI site of pSG5 to yield pSG5lacZ. The orientation of the transgene within recombinant plasmids was confirmed by a combination of restriction endonuclease mapping and dideoxynucleotide sequencing. Plasmid DNA was prepared from the DH5-a strain of

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E. coli transformed with the recombinant plasmids and grown in LB broth containing ampicillin (50 μg/mL). Plasmid DNA was purified by a modification of the alkaline lysis method followed by two separate bandings in cesium chloride equilibrium gradients.[20] Ethidium bromide was extracted from the DNA with butanol, and the batches were then dialyzed extensively against purified water (Millipore) and precipitated with ethanol. Aliquots of the plasmids were then resuspended in purified water, filter sterilized through 0.22-μm filters (Millipore) and stored at −20 °C until use. The purity and concentration of all the plasmid preparations were confirmed by electrophoresis in 1% agarose gels containing ethidium bromide, and UV spectroscopy at 260 and 280 nm (Beckman Spectrometer). DNA was also screened for the presence of bacterial endotoxin (Sigma), and batches with an endotoxin concentration exceeding 15 endotoxin units per milliliter were discarded. In the case of pSG5IL-1ra, all batches of purified DNA were tested for the presence of recombinant human IL-1ra protein by ELISA. None of the DNA used for the *in vivo* studies contained detectable levels of human IL-1ra (detection limits <29 pg/mL). Fluorescently labeled pSG5IL-1ra plasmid was produced in an identical fashion except that ethidium bromide intercalated into the DNA during the purification process was retained and excess non-incorporated ethidium bromide was removed by extensive dialysis in Tris-EDTA buffer.

Preparation of Nonionic/Cationic-Based Liposomal Formulations—The nonionic/cationic (NC) liposomal formulations used in the experiments contained glyceryl dilaurate (GDL; IGI, Inc.; >90% 1,3-isomer), cholesterol (CH; IGI, Inc.), polyoxyethylene-10-stearyl ether (POE-10; IGI, Inc.), and 1,2-dioleoyloxy-3-(trimethylammonio)-propane (DOTAP; Avanti Polar Lipids) at a weight percent ratio of 50:15:23:12. The lipid mixture also contained α-tocopherol (1% by weight of total lipids; Eastman Kodak). Appropriate amounts of the lipids were mixed and melted at 70 °C in a sterile polystyrene centrifuge tube. The lipid melt was then filtered through a 0.22-μm filter (Nuclepore), and the filtrate was reheated in a water bath at 70 °C prior to being drawn into a sterile syringe. A second syringe containing sterile, autoclaved, double-distilled water was preheated to 65 °C and connected *via* a three-way sterile stopcock to the lipid phase syringe. The aqueous phase was then slowly injected into the lipid phase syringe. The mixture was rapidly passed back and forth between the two syringes while being cooled under cold tap water until the mixture was at room temperature and then it was stored at 4 °C until use. The total lipid concentration in the suspension was 100 mg/mL.

The resulting NC liposomal suspensions were examined with a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations. Immediately before use in the *in vivo* experiments, the liposomal suspension was sonicated for 20 min at room temperature, and an equal volume of aqueous pSG5IL-1ra or pSG5lacZ−DNA solution (7 mg DNA/mL) was added by inversion mixing followed by incubation at room temperature for 45 min. Control formulations contained no DNA.

Preparation of Phospholipid-Based Liposomal Formulations—Large unilamellar liposomes (LUV) were prepared containing egg phosphatidylcholine (PC; Avanti Polar Lipids):CH:DOTAP and α-tocopherol (1% by weight of total lipids; Eastman Kodak) in a 1:0.5:0.1 molar ratio. A reverse-phase evaporation method based on that reported by Szoka and Papahadjopoulos [21] was used in the preparation of the LUVs. The lipids were dissolved in a chloroform:methanol mixture [2:1 (v/v)]. Isotonic 0.05 M HEPES buffer (Sigma Chemical Company), pH 7.4, was added to form a clear mixture. The solvent-to-buffer ratio was 6:1 (v/v). The solvents were removed with a rotoevaporator (Buchii) maintained at 45 °C. The total lipid concentration in the suspension was 75 mg/mL. The resulting liposomal suspensions were examined with a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations. The liposomes were then sonicated for 20 min at room temperature before the addition of an equal volume of aqueous pSG5IL-1ra DNA solution (7 mg/mL). The liposomal DNA mixture (liposome−DNA formulation) was then allowed to incubate at room temperature for 45 min prior to topical application to the ventral side of hamster ears.

In Vivo Experiments—Animal experiments were conducted under institutional guidelines with approval by the University Committee on the Use and Care of Animals (UCUCA). Male golden Syrian hamsters, 10 weeks old, were purchased from Charles River Breeding Laboratories (Wilmington, MA) and were maintained for 2 weeks at a photoperiod of 14 h of light and 10 h of darkness to maximize androgen-dependent sebaceous gland activity and thus control their size.[22] The ventral sides of the hamster ears were carefully shaved 1 day prior to the experiments. The hamsters were anesthetized with a single dose of sodium pentobarbital (0.25 cc of 40 mg/kg intraperitoneal injection). Following anesthetization, 50 μL of the test formulation containing the pSG5IL-1ra plasmid DNA were applied to the ventral surface of one ear, twice daily for 3 days. The contralateral ear was treated with an equivalent amount of liposomes without plasmid DNA (control). Additionally, a set of control animals ($n = 4$) were treated as just described with NC liposomes containing pSG5lacZ plasmid DNA. The total amount of lipid applied per ear of NC-based liposomes was 15 mg (2.5 mg/dose), and the total lipid applied per ear with the PC-based liposomes was 11.25 mg (1.875 mg/dose). For both NC- and PC-based liposome−DNA formulations, the total amount of DNA applied was 1.05 mg (0.175 mg/dose). One day later (15 h after the last application of the test formulations), the hamsters were sacrificed and the ears were excised by dissection across the base. The kinetics of transgene expression following topical application of NC liposomal pSG5IL-1ra−plasmid DNA and blank NC liposomes were studied by sacrificing treated animals at 1, 3, 5, and 8 days after the last application. For each given time point, at least three animals were used per formulation tested. Ears of untreated animals were also used as negative controls. All experiments were carried out under non-occluded conditions. At the time of sacrifice, the ears were isolated by sharp dissection, weighed, and measured along each border (to calculate the surface area exposed to treatment), then processed as described next. One milliliter of blood was also collected from each animal immediately following sacrifice and centrifuged at 14 000 rpm (Beckman) for 10 min at 4 °C. The serum was collected and assayed for IL-1ra content.

Confocal Laser Scanning Microscopy—Ear tissue was embedded in OCT (optimum cutting temperature compound) (Miles Laboratories, Elkhart Lake, IN) and frozen in isopentane-containing dry ice. The tissue blocks were stored at −80 °C before sectioning. Blocks were sectioned at 20 μm with a Tissue-Tec II model 4553 cryostat (Miles Laboratories, Inc., Naperville, IL) and placed on poly-L-lysine double-coated slides. Immediately before viewing, the slides were mounted with Vecta-Shield mounting media (Vector Laboratories, Burlingame, CA). The sections were examined with a Bio-Rad MRC 600 laser scanning confocal microscope (Bio-Rad Microscience Div., Cambridge, MA), and analyzed with the CoMOS program. The imaging procedure employed has already been described in detail.[23] The light source was argon-krypton laser equipped with a 520-nm filter wheel and cubes for detection of emitted light. Images were resized with Adobe Photoshop Version 3.0 (Adobe Systems, Inc., Mountain View, CA). Prints of the digital images were made with a Kodak XLS 8600 PS printer (Eastman Kodak Company, Rochester, NY).

Southern Analysis—Animals treated with the NC liposomal pSG5IL-1ra−plasmid DNA formulations (50 mg/mL NC liposomes, 3.5 mg/mL DNA) were sacrificed at 1, 4, 6, 8, 12, and 24 h after application. Those treated with the aqueous DNA solutions (3.5 mg/mL) were examined at 12 and 24 h after application. Following sacrifice, the ears were cut at the base and pinned to a board with the ventral side facing up. The ventral side was swabbed several times with PBS-soaked tissue paper and stripped with tape until all hair was removed and the surface looked shiny indicating complete removal of the stratum corneum. The surface of the ventral ear was swabbed several times again with PBS-soaked tissue paper before the ventral ear was separated from the underlying cartilage. The ventral ear was placed on a 60-mm polystyrene dish with the epidermal side facing down. Then, 500 μL of a 0.05 M isotonic HEPES solution (pH 7.4) was placed on the ear, and the sebaceous glands were carefully scraped with a sterile, dull scalpel. This procedure was carried out twice. Tissues were then minced in 1 mL of homogenate buffer and collected in sterile Eppendorf tubes along with the gland scrapings. Genomic DNA was isolated from the tissue homogenates with DNAzol reagent (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. The concentration of DNA isolated from each sample was determined spectrophotometrically.

Samples (10 mg of total DNA) were loaded onto 0.8% agarose gels, electrophoresed at 20 V for 21 h, and transferred to a Nytran membrane. Controls included lanes containing known amounts of pSG5IL-1ra (15 or 150 pg), both undigested and linearized by digestion with EcoR1. The membrane was probed with [^{32}P]-labeled

oligonucleotide probes specific for human IL-1ra generated from a 650 bp fragment of human IL-1ra cDNA that was isolated from pSG5IL-1ra with random hexanucleotide primers, ^{32}P-dCTP (Amersham), and Klenow fragment (BRL). Hybridization buffer included 10% dextran sulfate, 35% formamide, 5X SSC, 0.1% SDS, and 100 μg/mL of salmon sperm DNA, and hybridizations were performed overnight at 42 °C. Membranes were washed twice for 10 min at room temperature in 2X SSC/0.1% SDS, twice for 10 min at 65 °C in 1X SSC/0.1% SDS, and once for 10 min at 65 °C in 0.1X SSC/0.1% SDS. Membranes were wrapped in plastic and exposed to film (Kodak X-AR5) at −70 °C, using intensifying screens.

Detection of IL-1ra by Immunohistochemistry—Treated ears were embedded in OCT medium (Miles, Inc., Elkhart, IN) and frozen in isopropyl alcohol containing dry ice. The frozen samples were stored at −80 °C before sectioning. Serial sections (5 μm) were obtained with a cryostat (Tissue-Tec II model 4553, Lab-Tec Products, Miles Laboratories, Inc., Naperville, IL) and placed on poly-L-lysine double-coated slides. The tissue sections were then immediately fixed in 100% acetone for 10 min at 4 °C and processed with a Histostain-SP AEC kit (Zymed Lab, Inc., South San Francisco, CA) according to the manufacturer's recommendations. The sections were reacted with primary murine anti-human IL-1ra (29 ng/mL, Bachem Bioscience, Inc., King of Prussia, PA) for 30 min. After completion of the protocol, slides were counterstained with hematoxylin, rinsed a final time, and mounted with 100 μL of GVA-mount before being examined and photographed with a Nikon Optiphot microscope (Nikon, Tokyo, Japan).

Isolation of Soluble Proteins from Various Strata of the Hamster Ear—Each ear strata (ventral skin, auricular cartilage and dorsal skin) was isolated from the ear by sharp dissection as follows: Following sacrifice, the ears were cut at the base and pinned to a board with the ventral side facing up. The ventral side was swabbed several times with PBS-soaked tissue paper and stripped with tape until all hair was removed and the surface looked shiny, indicating complete removal of the stratum corneum. The surface of the ventral ear was then swabbed several times again with PBS-soaked tissue paper before the ventral ear was separated from the underlying cartilage. The ventral ear was then placed on a silanized glass slide with the epidermal side facing down. Then, 500 μL of a homogenate buffer (0.05 M isotonic HEPES at pH 7.4, 0.1% PMSF, and 0.1% HSA) was placed on the ear, and the sebaceous glands were carefully scraped with a sterile, dull scalpel. This procedure was carried out twice. The ventral skin strata was then minced in 1 mL of homogenate buffer and collected in sterile Eppendorf tubes along with the gland scrapings. The cartilage and dorsal skin were minced in 1 mL of homogenate buffer each. The samples were then sonicated on ice for 2 min and centrifuged at 15 000 rpm for 10 min at 4 °C. The supernatants were collected immediately and analyzed for human IL-1ra protein.

Detection of IL-1ra Protein—Human IL-1ra protein was measured by a human specific IL-1ra protein immunoassay (Research and Diagnostics Systems, Minneapolis, MN). Tissue homogenates were assayed in triplicate, and the optical density of each sample was determined with a spectrophotometer set at 450 nm and with the correction wavelength set at 570 nm. A standard curve was also prepared, using the homogenate buffer as the diluent from 0 to 2000 pg/mL. The detection limit of this ELISA was 29 pg/mL. The test samples were compared with the standard curve to determine IL-1ra concentration. The results are expressed as 10^{-9} g of human IL-1ra/cm^2 and/or 10^{-9} g of human IL-1ra/g tissue.

Results

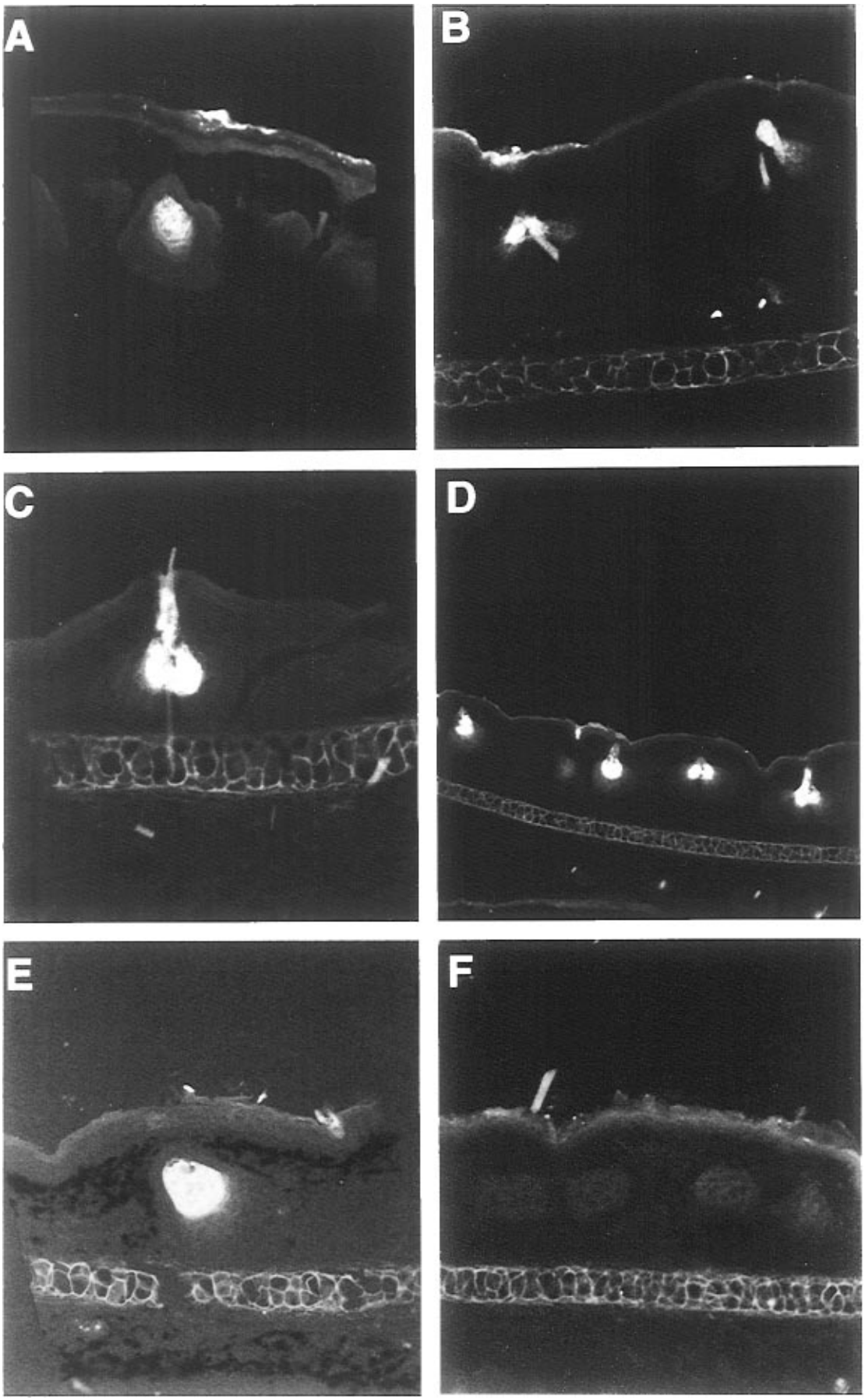
The total lipid concentration of 50 mg/mL for the NC liposomes was arrived at following toxicity screening with cultured cells (NIH 3T3) *in vitro*. No toxic effects were evident below a total lipid concentration of ~100 mg/mL; higher concentrations resulted in proportionally increasing cell death indices. In comparison, commercially available cationic liposomes (Lipofectin) induced cell death at much lower total lipid concentrations.

The amount of cationic lipid used in the NC liposome−plasmid DNA formulations was based on two factors: (a) the maximum cationic lipid concentration that could be used without inducing toxic effects, and (b) the amount that would be required for near optimum complexation of DNA based on estimates derived from a review of the literature.[24−28] Optima in lipid mixing and *in vitro* transfections were reported for DNA-to-cationic lipid ratios of 0.25 to 0.5 μg/nmol.[26] Several others have also reported optimal transfection behavior at a DNA-to-lipid charge ratio of 1.3 to 1.6.[24,25,27,28] We chose a concentration of 6 mg/mL as the maximum charged lipid concentration. In preliminary transfection studies with 293 cells with a lacZ-containing expression plasmid (pSG5lacZ), it was found that a NC liposome−plasmid DNA formulation containing 12 weight % DOTAP gave the best results when DNA concentration was ~8 mg/mL. This translated to a DNA-to-DOTAP ratio of 0.4 μg/nmol. It was also found that ratios lower than this were not as efficient. Further, dioleoyloxydimethylammoniopropane (DODAP) was not effective as a replacement for DOTAP.

We then determined how different methods for the preparation of a topical liposome-DNA formulation affected the integrity of the expression plasmid DNA. Liposome-DNA formulations were initially prepared by simple mixing followed by sonication according to previously described methods.[8] Examination of the resulting formulations by electrophoresis in agarose gels revealed that sonication of the liposome-DNA mixture results in physical fragmentation of the plasmid DNA (data not shown). We then developed a preparation method that employed sonication of the liposomes prior to addition of the plasmid DNA. This method yielded formulations that were more uniform in size (mean particle size = 100 ± 20 nm compared with a mean particle size of 500 ± 100 nm for the unsonicated NC liposomes). Addition of plasmid DNA to the sonicated liposomes did not reveal evidence of fragmentation or degradation of the plasmid DNA, as determined by electrophoresis of the topical formulations in agarose gels. The particle size of the NC liposome−plasmid DNA formulation could not be quantitatively determined; however, the mean particle size was >2 μm.

The ability of these novel topical formulations to deliver plasmid DNA into the hair follicles of intact hamster ear skin was then studied with fluorescently labeled plasmid DNA and laser scanning confocal microscopy (LCSM) to determine the location of the plasmid DNA. Plasmid DNA was visualized within the perifollicular regions of the skin 6 h (Figure 1, panels A and B) after topical application of a single dose of the NC liposomal−DNA formulation. Although some of the plasmid DNA continued to be visualized on the skin surface 6 h after application (Figure 1, panel A), the delivery of the labeled DNA into the hair follicles and perifollicular glands appeared to be complete by 24 h post administration (Figure 1, panels C, D, and E). Control animals treated with an aqueous formulation containing an equivalent dose of the fluorescently labeled plasmid DNA failed to show evidence of DNA beyond the superficial epidermis 24 h after topical application (Figure 1, panel F). This result indicates that perifollicular delivery is a physicochemical property specific to NC liposomal formulations. Because the intensity of ethidium bromide fluorescence is directly proportional to the amount of double-stranded DNA present in the formulation, these qualitative results also suggest that some of the plasmid DNA was delivered to the region of the pilosebaceous unit in the form of nondegraded plasmid.[29]

The qualitative results identified in Figure 1 were confirmed by molecular analysis of the treated skin for the presence of intact plasmid DNA. Whole cellular DNA was isolated from the treated skin and subjected to Southern hybridization with radiolabeled probes specific for the human IL-1ra cDNA. Bands specific for pSG5IL-1ra (arrows) were observed in the NC liposome-treated skin beginning 6 h after administration (Figure 2). Qualitatively, the amount of pSG5IL-1ra present


A
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within the skin remained constant from 12 to 24 h after treatment. From 12 to 24 h post treatment, some of the plasmid DNA was in the form of closed circular (cc) or linearized plasmid (linear) that was present within a broad band of plasmid degradation products. No distinct bands corresponding to supercoiled (sc) plasmid DNA were resolved by these techniques. No bands were observed in the genomic DNA obtained from animals treated with liposomes alone (C). The results of these experiments (Figure 2) indicate that most of the nondegraded plasmid DNA present within the skin continued to exist in the form of closed circular or linearized plasmid. Analysis of skin samples obtained at various times after the topical application of a single dose showed similar amounts of expression plasmid DNA present within the skin from 12 to 24 h after treatment. These results indicate that for the first 24 h post-administration, some of the plasmid was delivered intracellularly and may have been protected from digestion by extracellular nucleases.

We next tested the ability of the NC liposomal−plasmid DNA formulation to mediate transfection of the perifollicular cells proximal to the *in vivo* location of the delivered expression plasmid DNA. The *in vivo* expression of transgenic human IL-1ra was initially detected by *in situ* immunohistochemical staining with a monoclonal antibody specific for the human IL-1ra protein. As shown in Figure 3, the NC liposomal formulation also functioned as a transfecting reagent. Transfected human Il-1ra expressing cells, stained red, were identified within the follicles in the proximal third of the hair shaft (Figure 3A) and occasionally at the base of the hair shaft (Figure 3B). Negative controls treated with aqueous formulations of expression plasmid DNA, or with liposomes alone, failed to show evidence for IL-1ra expressing perifollicular cells. Analysis of serial sections obtained from multiple animals indicated that the efficiency of transfection as measured by our immunohistochemical methods was consistently <1% of the total perifollicular cells examined.

We then examined the kinetics of hIL-1ra expression within treated skin over an 8 day period following a multiple-dose (twice daily for three days), topical application protocol. Transgenic expression of human IL-1ra in the skin of the ventral ear was detected at its highest levels on day 1 after application of the final topical dose. The levels of transgene expression remained significantly above control values ($p < 0.005$) on days 1−5, and had returned progressively to baseline levels by day 8 (Figure 4A). Ear cartilage and dorsal skin were also assayed for transgenic human IL-1ra; however, all of these values were at or below the detection limits of the ELISA (29 pg/mL) and no significant differences were observed between animals treated with NC liposomal−DNA formulation and those treated with NC liposomes alone (Figures 4B and 4C). It was also found that samples of ventral ear, glands, cartilage, and dorsal skin obtained from control animals treated with NC liposomes + pSG5lacZ plasmid DNA exhibited transgenic human IL-1ra levels that were below the detection limits of the assay. In addition, no transgenic human IL-1ra was detected in the serum of the treated or control animals (data not shown). These results suggest that expression of transgenic protein is confined to tissues locally targeted by the NC liposomal pSG5IL-1ra plasmid DNA formulation, and that the diffusion of transgenic IL-1ra− protein is largely confined to the microenvironment proximal to the point of topical application. These results corroborate

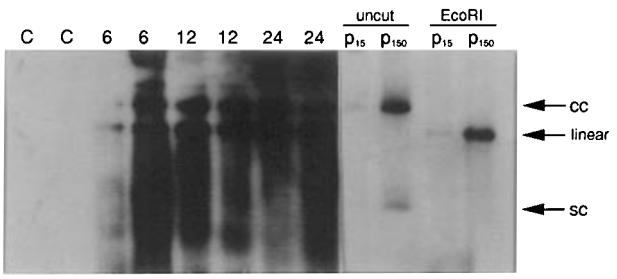

Figure 2—Hybridization analysis of total cellular DNA obtained from skin treated with a single dose of NC liposomes with or without pSG5IL-1ra DNA. Key: (numbers above lanes) hours post treatment; (C) control animals; (sc) supercoiled (cc) closed circular.

immunohistochemical analysis of treated skin (Figure 3) showing expression of transgenic hIL-1ra *in vivo*.

The dynamics of plasmid−mediated hIL-1ra expression obtained *in vivo* from the NC liposome−plasmid DNA formulation were then compared with those associated with a conventional PC liposome carrier system. Although the charged lipid-to-DNA ratios were different for the two formulations, the total dose of DNA applied to the hamster ears were identical for the two systems. Under these conditions, it was found that the NC liposome−plasmid DNA formulation mediated levels of transgene expression that were approximately four times higher than those obtained with the PC liposome−plasmid DNA formulation ($p < 0.001$, Figure 5). Of note is the fact that levels of human IL-1ra protein detected in animals treated with PC liposome−plasmid DNA formulations were not significantly different from the levels detected in animals treated with PC liposomes alone ($p = 0.991$) or NC liposomes alone ($p = 0.915$).

Discussion

Although the immunopathological pathways that are associated with human skin diseases are complex, interleukin-1 has been implicated as an important pathophysiological cytokine in seborrheic eczema, psoriasis, and alopecia areata.[1−5] IL-1ra protein is a well characterized competitive inhibitor of IL-1 that is produced by several types of skin cells, including monocytes, macrophages, fibroblasts, keratinocytes, and polymorphonuclear leukocytes.[23,30−35] Previous studies have suggested that introduction of excess IL-1ra (10−100-fold) proximal to IL-1-responsive skin cells may inhibit the pathophysiological effects of endogenous IL-1. Preclinical and clinical studies are being carried out to determine therapeutic uses of recombinant IL-1ra protein for the treatment of a wide variety of human diseases, including those with a primary manifestation in skin.[36]

Transfection of the IL-1ra transgene directly into cells at the site of disease activity promises to be a much more effective method for delivery of IL-1ra protein than the repeated application of purified recombinant protein. If the efficiency of skin cell transfection could be improved, the ability to deliver the gene vector *via* topical application would be advantageous in terms of both feasibility and total costs. The successful development of topical gene therapy represents a two-step drug delivery process; the first step involves the delivery of biologically functional (nondegraded) nucleic acids

Figure 1—Histologic localization of plasmid DNA in hamster skin following single-dose topical application of nonionic-cationic (NC) liposomes containing pSG5IL-1ra DNA or aqueous pSG5IL-1ra DNA. At 6 h following application, labeled plasmid was observed along the epidermal surface as well as within the follicles (Panels A and B, 40X). At 24 h following application, the plasmid was present within multiple follicles (Panels C, 40X; D, 10X; and E, 40X). At 24 h following application of aqueous control solution, no evidence of plasmid was found within the follicles or elsewhere in the skin (Panel F, 40X). Auricular cartilage separating ventral and dorsal skin surfaces appears as a longitudinal band across Panels B–F.

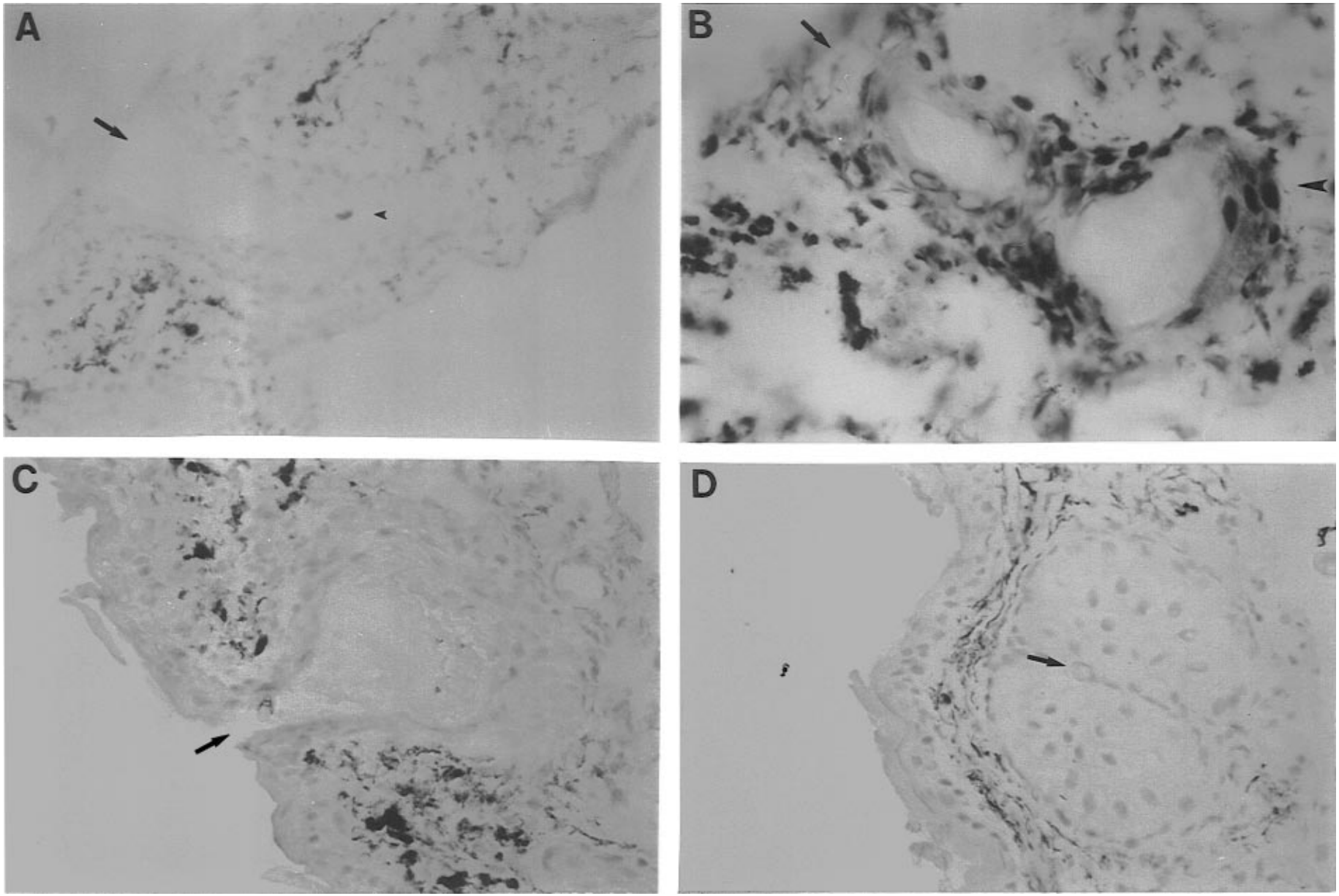

Figure 3—Immunohistochemical localization of transfected cells following topical application of NC liposomes with or without pSG5IL-1ra DNA. Cells that are stained red are a positive indication of IL-1ra expressing cells. Arrows represent location of follicular openings. NC liposome + DNA-treated skin showed transfected perifollicular cells (arrowheads) present along the length of the hair shafts (Panel A, 400X) as well as in a location at the base of the hair shaft (Panel B, 1000X). Sections of NC liposome + DNA-treated skin reacted with an irrelevant control IgG1 showed no evidence of red staining perifollicular cells (Panel C, 400X). Controls treated with NC liposomes without DNA and reacted with anti-human IL-1ra antibodies also failed to show evidence of red staining perifollicular cells (Panel D, 400X).

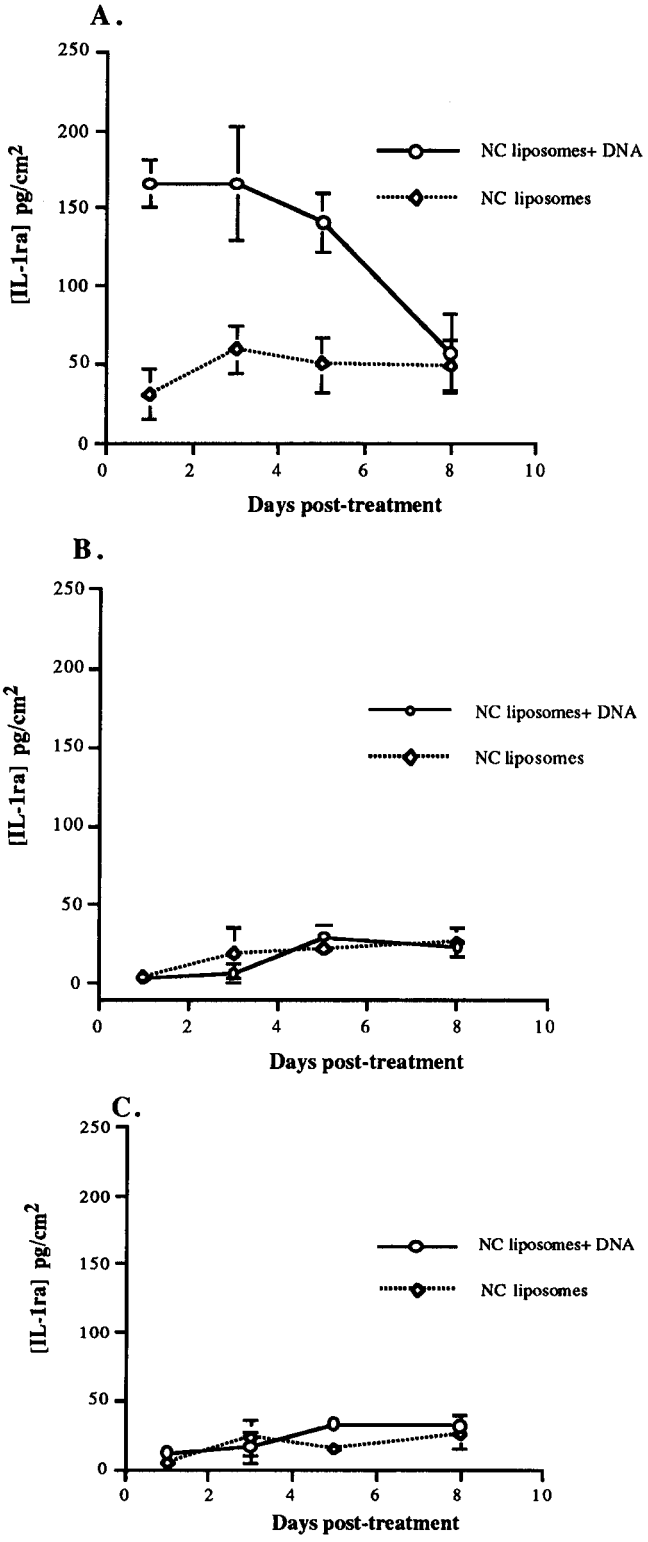

Figure 4—Expression of human IL-1ra in various strata of the hamster ear following topical *in vivo* application of nonionic/cationic (NC) liposomes with and without plasmid DNA. Assays were performed in triplicate and results are expressed as picograms of IL-1ra/cm^2 of treated skin surface area. Data points represent means ± standard error (n = 4). Levels of human IL-1ra recovered from NC liposome + DNA-treated animals were significantly above levels of IL-1ra present in controls treated with NC liposomes alone on days 1–5 ($p < 0.005$) and had returned to baseline levels by day 8. No significant differences between NC liposome + DNA-treated and NC liposome-treated controls were identified in any other skin strata analyzed: Panel A = ventral skin; Panel B = auricular cartilage; and Panel C = dorsal skin.

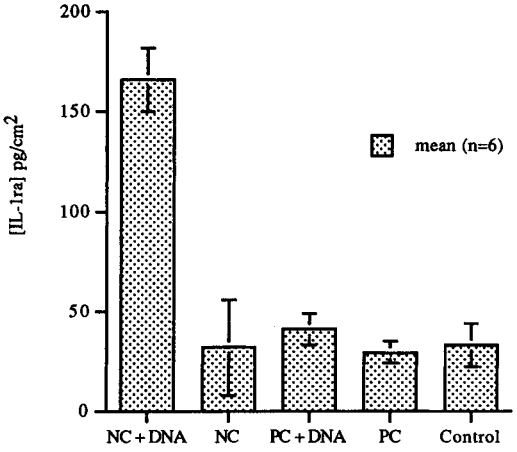

Figure 5—Expression of transgenic human IL-1ra in the ventral ear of the hamster following topical *in vivo* application of nonionic/cationic (NC) and phospholipid/cationic (PC) liposome formulations with and without pSG5IL-1ra plasmid DNA. Assays were performed in triplicate and the results are expressed as picograms of IL-1ra/cm^2 of treated skin surface area. Ears treated with the NC liposomes + DNA and NC liposomes alone resulted in expression of 165.5 ± 15.6 and 31.4 ± 24.0 pg/cm^2 of human IL-1ra, respectively. Ears treated with PC liposomes + DNA resulted in 40.8 ± 8.1 pg/cm^2 of human IL-1ra. The mean levels of human IL-1ra obtained with PC liposomes alone were below the limits of detection of the ELISA (<29 pg/cm^2). Controls treated with DNA alone expressed a mean of 32.5 ± 11.0 pg/cm^2 of human IL-1ra. Vertical bars indicate mean values ± standard error obtained from multiple animals (n = 6).

to anatomical regions proximal to skin cells, and the second step involves delivery of nucleic acids across the cell membrane of these cells. Together, these processes effect transfection of these cells *in vivo* and result in observable biological responses. This study was designed to test the hypothesis that novel NC liposome–plasmid DNA formulations could be developed that would enhance delivery of expression plasmid DNA to perifollicular cells and mediate transfection *in vivo*. Because focal overexpression of transgenic IL-1ra in areas of skin affected with these diseases might be of therapeutic value, we chose to examine the IL-1ra cDNA as a functional transgene.

In this report, we present data showing that hybrid nonionic/cationic liposomes combined with an eukaryotic expression plasmid encoding the cDNA for human IL-1ra can mediate overexpression of the IL-1ra protein after topical application to the hamster ear *in vivo*. To test the first phase of the drug delivery process, we performed an *in vivo* targeting experiment. It is clear from the results of this experiment (Figure 1) that the novel NC liposomal–plasmid DNA formulations are able to deliver plasmid DNA into the proximal third of the hair shaft within the follicles and the sebaceous glands present within these follicles. This delivery process was temporally progressive over a period of ~1 day and suggests that it is neither energy dependent nor kinetically saturable. These results are consistent with our previous studies with NC liposomes for the delivery of recombinant proteins into skin and support the hypothesis that the ability of the NC liposomes to deliver plasmid DNA into the follicles is an inherent physicochemical property of the liposomes themselves.

The ability of the NC liposomes to also facilitate transfection *in vivo* is supported by the results of the molecular (Figure 2) and immunohistochemical (Figure 3) analyses of treated skin. Because the treated skin was stripped of hair and washed prior to these analyses, we believe it is unlikely that a significant amount of the DNA identified by Southern analysis represents plasmid bound to follicular proteins. Although retention of plasmid DNA in an extracellular compartment cannot be excluded on the basis of this experiment, we believe

that the results indicate that an appreciable amount of the expression plasmid DNA is delivered intracellularly. Intracellular expression of transgenic protein by perifollicular fibroblasts *in vivo* was identified by a monoclonal antibody specific for the human IL-1ra protein.

The dynamics of hIL-1ra expression *in vivo* were determined by a multidose topical application protocol (Figure 4). Levels of hIL-1ra transgene expression achieved within the ventral skin were significant and statistically equivalent at 1, 3, and 5 days post treatment. This result suggests that it may be possible to achieve a steady-state level of transgene expression *in vivo* with a daily application schedule. Twice or thrice daily application schedules are a well-established standard for the use of topical formulations that contain conventional pharmaceutical drugs.

In summary, we believe that our results confirm the ability of hybrid NC liposomes to facilitate the delivery of plasmid DNA to skin cells beyond the superficial epidermis and mediate transient transfection of these cells *in vivo*. Our results are qualitatively and quantitatively different (Figure 5) from previous data suggesting that determinations of the utility of conventional cationic liposome-mediated topical gene therapy based on observations with a marker transgene[8,9] may be limited. Importantly, the dynamics and kinetics of regional transgenic soluble protein expression were quantitatively determined for a biologically active and potentially therapeutic immunomodulatory transgene, human IL-1ra. Alternative explanations for the observed levels of immunoreactive IL-1ra present within the NC liposome−plasma DNA-treated skin were explored and excluded. Further refinement of NC liposome formulations combined with improved expression plasmids and extensive *in vivo* studies promises to allow the development of topical gene therapy systems that may be appropriate for the initiation of human clinical trials.

References and Notes

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