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Topical transfection using plasmid DNA in a water-in-oil nanoemulsion

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Abstract

Expression plasmids encoding chloramphenicol acetyltransferase (CAT) or human interferon-α2 cDNA were formulated in water-in-oil nanoemulsions and applied to murine skin. The histological location of transfected cells was assessed by in situ DNA PCR and showed that the deposition of plasmid DNA was primarily in follicular keratinocytes. Transgene expression in the skin was monitored for 24–72 h, following topical application of either single or multiple daily doses by quantitative RT-PCR and ELISA. It was found that transgene expression was optimal at 24 h following topical application of a single dose of water-in-oil nanoemulsion containing plasmid DNA. Dose–response studies using a total dose of 3, 10 or 30 μg of plasmid DNA suggested that topical transfection using nanoemulsions is subject to both threshold and saturation effects. None of the cationic liposome formulations tested as controls mediated transgenic protein expression at levels higher than background values of the ELISAs used to assay transgenic protein. Single and multiple dose experiments using human interferon-α2 as a transgene indicated that the efficiency of nanoemulsion mediated transfection was most effective in the context of normal versus atrophic hair follicles. In addition, the total amount of human interferon-α2 present in skin appeared to accumulate as a consequence of multiple dosing. Histologic evaluation of treated skin showed no overt signs of toxicity or irritation associated with the short-term application of the nanoemulsions. The results suggest that water-in-oil nanoemulsions can be used to facilitate transfection of follicular keratinocytes in vivo.

Keywords: Non-viral vectors; Water-in-oil nanoemulsions; In vivo transfection; Keratinocytes

1. Introduction

The advantages of topical delivery of gene vectors to cells within the skin include the possibility that the expression of potentially therapeutic transgenic proteins could be directed and confined

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to the site of clinically active skin lesions (Ghazizadeh et al., 1998). However, in order to efficiently deliver the gene to the target cells within the skin, it is necessary to circumvent the inherent barrier function of the skin. Although it is highly unlikely that highly charged hydrophilic macromolecules (such as plasmid DNA) can permeate intact stratum corneum, the presence of hair follicles and associated structures may allow for localized delivery to viable skin cells. In turn, expression of transgenic proteins in and around the follicles may promote diffusion of soluble proteins into the surrounding dermal tissue and/or the systemic circulation and may mediate local or systemic biological effects.

Several groups have earlier reported that topical application of liposome plasmid DNA formulations can mediate transient transfection of follicular cells (Alexander et al., 1991; Li and Hoffman, 1995; Alexander and Akhurst, 1995; Yarosh and Klein, 1996; Niemiec et al., 1997; Yu et al., 1999; Fan et al., 1999). Our own studies using hybrid non-ionic–cationic lipid containing liposomes we observed consistent levels of transgenic expression of human interleukin-1 receptor antagonist protein in hamster skin following topical application (Niemiec et al., 1997). These observations suggested that liposome-mediated delivery of expression plasmid to follicular cells for transient transfection was not only possible but might be optimized by systematic manipulation of the liposomal components.

Unfortunately attempts to improve the efficiency of transfection using this approach have proved unsuccessful, leading us to hypothesize that the limitations of topical transfection using liposomes are fundamentally related to the physical conformation of the complexes that occurs quickly after topical application. A closer examination of the liposome DNA formulations using numerous methods for determination of particle size and zeta potential, indicated that increasing the size of these complexes severely retarded follicular transport of DNA. Although initially it was believed that adsorption of positively charged complexes to the negatively charged skin surface was a major detriment, it was subsequently established that severe interparticle aggregation of the liposome DNA complexes occurred upon partial dehydration of the liposomes following topical application (unpublished observations). Such aggregation resulted in extensive micromolecular networks of complex particles that prevented intra-follicular delivery of the DNA. It became apparent that optimization of DNA delivery to hair follicles (and as a consequence transfection), would require the development of a new class of topical formulations. These formulations would need to possess the following characteristics: (1) a significant capacity to encapsulate aqueous solutions of high concentration plasmid DNA; (2) a simple method of preparation that avoided physical disruption of the plasmid DNA; (3) a profile of physical and biochemical stability upon storage for extended periods of time; (4) an acceptable profile of skin irritation and toxicity.

In our experience, the most promising type of lipid based formulations that would potentially meet these criteria are water-in-oil nanoemulsions. Nanoemulsions are thermodynamically stable liquid isotropic dispersions composed of water, oil and surfactants (Osborne et al., 1988). At defined stoichiometric ratios of the ingredients, the formation of a transparent nanoemulsion is spontaneous. Since high shear energies are not required during the preparation of nanoemulsions the likelihood of physical damage to the plasmid DNA degradation is virtually eliminated. Nanoemulsions are readily scalable and can be prepared with oil and surfactant components that are generally regarded as safe (GRAS), thus reducing the likelihood of local skin irritation or toxicity. Since they are thermodynamically stable, nanoemulsions are capable of encapsulating significant amounts of water. Water-in-oil nanoemulsions have been reported to improve absorption of water-soluble peptides following intraduodenal administration (Constantinides et al., 1995, 1996). There are relatively fewer studies that address the feasibility of topical drug delivery using nanoemulsions (Osborne et al., 1991; Bolzinger et al., 1998). In this report, we describe the preparation of water-in-oil nanoemulsions containing expression plasmid DNA that appear to facilitate follicular transfection following topical application in vivo.

2. Materials and methods

2.1. Preparation of expression plasmid DNA

The expression plasmids pCF1CAT, used in these studies have been described earlier (Raczka et al., 1998). The expression plasmid pNGVL3huINFα2 consists of the cDNA for human interferon-α2 cloned into the polylinker of pNGVL3, an expression plasmid described earlier (Sant et al., 1998). Plasmid DNA was amplified in *Escherichia coli* bacteria and then isolated by double cesium chloride gradients followed by dialysis against sterile Tris–EDTA buffer (Sambrook et al., 1989). Samples were assayed for endotoxin using quantitative kinetic limulus amoebocyte lysate assay (KQCL) (Bio-Whittaker Inc., Walkersville MD) and batches of plasmid with a endotoxin level of ≤ 20 EU/ml were used for in vivo studies.

2.2. Preparation of water-in-oil plasmid nanoemulsions

Polyoxyethylene 20 sorbitan monooleate (Tween® 80), sorbitan monooleate (Span® 80), and olive oil were purchased from Sigma Chemical Company (St. Louis, MO). 1,2-Dioleyl-3-trimethylammonium propane (DOTAP) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Eighteen parts by volume of polyoxyethylene 20 sorbitan monooleate (Tween® 80) and 30 parts by volume of sorbitan monooleate (Span® 80) were dissolved in 46 parts by volume of olive oil. The surfactant solution in olive oil was then warmed to 50°C and sterile filtered with a 0.22 μm filter into sterile microfuge tubes. Six parts by volume of aqueous plasmid solution were then added to the surfactant mixture in olive oil and mixed gently to yield a clear nanoemulsion. The concentration of pCF1CAT, or pNGVL3huINFα2 in the respective nanoemulsions was held constant at 0.2 mg/ml total volume. Blank nanoemulsions containing no DNA were also prepared, along with aqueous plasmid DNA solutions that served as controls.

The CAT plasmid nanoemulsion formulations and the blank nanoemulsion formulations were characterized by particle size distribution analysis using a Beckman Coulter N4 Plus Submicron Particle Size Analyzer utilizing photon correlation spectroscopy.

2.3. Preparation of plasmid–liposome formulations

The non-ionic lipids, glyceryl dilaurate (GDL) and polyoxyethylene-10-stearyl ether (POE-10), as well as cholesterol (CH) were supplied by IGI, Inc. (Little Falls, NJ). The water used was double-distilled and deionized using a Millipore Milli-Q® system and sterile filtered with a 0.22 μm filter. All other solvents were of chromatographic grade. The liposomal formulations used in the experiments contained GDL, CH, POE-10 and DOTAP at a weight percent ratio of 50:15:23:12. 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 (Nucleopore) 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 3-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 stored at 4°C until use. The total lipid concentration in the suspension was 100 mg/ml. The resulting liposomal suspensions were examined using a Nikon Diaphot light microscope to assure integrity and quality of the liposomal preparations. The liposomal suspensions were sonicated for 20 min at room temperature before use in the experiments. The particle size of the sonicated liposomes was then determined using a NiComp 370 Particle Sizer. The sizes of the sonicated liposomes used in the transfection studies were between 100 and 140 nm with a narrow distribution (polydispersity index between 0.2 and 0.3). If the sizes did not fall in the desired range further sonication was carried out. Appropriate amounts of an aqueous plasmid pCF1CAT solution were then added to the 100

mg/ml sonicated NC liposomes by inversion mixing to obtain formulations containing the desired DOTAP:DNA weight ratios. The liposomal formulations tested contained DOTAP:DNA weight ratios of 2:1, 4:1, 6:1, 8:1 and 10:1.

2.4. In vivo transfection studies

All animal experiments were performed in accordance with institutional guidelines and were approved by the UCUCA. For the single dose experiments male hairless mice (Skh-hr-1, 60 days old, Charles River Breeding Laboratories, Wilmington, DE) were used and in the multiple dosing experiments male hairy mice (C57 BL/6J, 60 days old, Jackson Laboratory) were used. Mice were anesthetized by intraperitoneal injection of 60 mg/kg sodium pentobarbital. A glass donor cap with an area of 1.1 cm^2 was affixed on the dorsal side of the mouse skin by application of cyanoacrylate adhesive to the perimeter of the cap. In the case of the C57 BL/6J mice, hair was removed with electric trimmers prior to adhesion of donor caps to the skin.

In the single dose experiments 20, 50 or 200 μl of the water-in-oil nanoemulsion formulation was placed on the skin circumscribed within the donor cap and spread evenly using a pipette tip to achieve complete surface coverage. These volumes corresponding to three different total doses of plasmid DNA (3, 10 and 30 μg, respectively) that were used in order to examine dose response effects. In the multiple dosing experiment, 50 μl of the test formulations was applied onto animal skin within the donor caps. After application of a unit dose, the donor cap was then occluded with Parafilm and then the entire area was tightly wrapped with Coban™ self-adherent wrap (3M Health Care, St. Paul, MN) in order to prevent removal of the donor cap by the animal. In the multiple dosing experiments, animals were retreated every 24 h and any remaining formulation was removed with Kimwipes® prior to application of a fresh 50 μl of each formulation. This procedure was carried out for four consecutive days. Six to ten animals per group were examined in the single dose experiments and four animals per group were examined in the multiple dosing experiments.

2.5. Processing of murine skin

Animals were sacrificed with a lethal dose injection of sodium pentobarbital, the occlusive dressing and donor cap were removed, and the skin was excised using sharp dissection. Subcutaneous fat was removed and the skin placed on a wooden board, epidermis up and secured with push-pins. Multiple 4 mm diameter punch biopsies (Miltex, Inc.), were then obtained from the area of skin exposed to treatment. Seven to ten biopsies were typically collected from the treated area and placed as groups of three into sterile Eppendorf tubes. One group was used for quantitative RT-PCR, a second group was used for ELISA. Both groups of samples were snap frozen in dry ice–ethanol slurry and stored at −70°C until the extraction procedures were undertaken. A third group was immersion fixed in 10% buffered formalin and processed for paraffin sectioning.

Isolation of total RNA was accomplished by homogenization of snap frozen skin biopsies in Trizol reagent (GibcoBRL Life Technologies, Bethesda, MD) followed by precipitation in isopropanol. Poly A RNA was then isolated using oligo (dT)-cellulose columns (Qiagen) and immediately converted to single stranded cDNA using oligo (dT) (Sambrook et al., 1989; Osborne et al., 1991; Nuovo, 1994; Foreman et al., 1997; Bolzinger et al., 1998; Raczka et al., 1998; Sant et al., 1998) and M-MLV reverse transcriptase (GibcoBRL Life Technologies, Bethesda, MD). Samples were ethanol precipitated, resuspended in Tris–EDTA buffer, quantified by spectrophotometry and frozen at −80°C until performance of quantitative PCR assays.

Isolation of total protein was accomplished by adding 100 μl of lysis buffer (Boehringer Mannheim, Indianapolis IN) to each tube containing the skin tissue samples and vortexing a few seconds. The tubes containing skin tissue were always kept on ice. A probe sonicator (Micro Ultrasonic Cell Disruptor, Kontes, Inc.) was used to disrupt the skin in each tube under the following conditions: 40 W and output 60 (range from 0 to 100). The samples were sonicated two times and each sonication consisted of seven pulses. The interval between the two sonications was about 10

min. The samples were then centrifuged at 15 000 rpm and 4°C for 20 min. The supernatant from each tube for the same animal sample was then combined for ELISA determinations. To extract the CAT protein from skin tissue completely, the skin samples were sonicated again for a total of four times using the conditions described above after the above process was completed. The supernatants from the additional extractions were analyzed separately.

2.6. Quantitative RT-PCR

The skin samples were analyzed using a real time quantitative RT-PCR assay (TaqMan, Applied Biosystems, Brachburg, NJ) specific for human interferon-α mRNA. The quantitative RT-PCR assay used a forward primer (5′TTTAGTGAACCGCACCGTCGTCG3′) that spanned the splice donor and splice acceptor of the CMV intron within pNGVL3 including sequences from 675 to 1066 of pNGVL3huINFα2. This was done to reduce the possibility of false positive signals arising from inadvertent priming with residual plasmid DNA present in the recovered skin samples. The reverse transcription primer (5′GAGATTCTCCTCATCTGTGCCAGG3′) was specific for sequences identified in the human interferon-α2 cDNA and had no significant overlap with cDNA sequences for any reported murine interferon-α (NCBI BLAST). The sequence of the indicator primer was 5′ FAM-CCCACAGCCTGGGTAGCAGGAGGACC-TAMRA 3′ (MegaBases, Inc., Evanston, IL). One μg of single stranded cDNA from each sample was used as a template. Known quantities of purified pNGVL3huINFα2 were used to generate standard curves with linearity from 10^3 to 10^8 sequences per μl. Negative technical controls for PCR amplifications were performed without reverse transcriptase and/or without primers.

2.7. ELISA

The quantitative determination of chloramphenicol acetyltransferase (CAT) was carried out using a colorimetric enzyme immunoassay. CAT and huINFα2 ELISA kits (CAT, Boehringer Mannheim, Indianapolis IN; huINFα2, PBL Biomedical Inc., New Brunswick, NJ) were used according to the manufacturer's instructions. Tissue homogenates were assayed in triplicate and the optical density of each sample was determined using a spectrophotometer set to 450 nm. A standard curve was also prepared using the homogenate buffer as the diluent from 0 to 1000 pg/ml. The detection limit of the CAT ELISA was 10 pg/ml, and that of the huINFα2 ELISA was 12.5 pg/ml. The test samples were compared with the standard curve to determine the concentrations of transgenic protein. Standard curves for total protein assay were obtained using standard BSA solutions. BCA™ (Bicinchoninic acid protein assay reagent, Pierce, Rockford, IL) was used for the measurement of total protein in the supernatant of each sample. Additional controls consisted of untreated skin spiked with known amounts of recombinant human interferon-α2, and in all cases the spikes were recovered by the ELISA at predicted levels. The results were expressed as means of pg transgenic protein/cm^2 of treated skin and/or pg transgenic protein/mg total protein ± standard deviations (S.D.). Differences in mean values and S.D. were analyzed using Student's *t*-test to determine statistical significance.

2.8. Histology and in situ DNA PCR

Representative paraffin sections (10 μm) were obtained from all of the treated and untreated skin, stained with hematoxylin/eosin and examined in blinded fashion by a veterinary pathologist for evidence of treatment specific irritation or inflammation. Alternate sections were analyzed using direct in situ PCR performed according to earlier published methodology (Nuovo, 1994; Foreman et al., 1997). Oligonucleotide primers were designed so that the amplified fragments would span intronic sequences within the pNGVL3huINFα2 plasmid in order to prevent inadvertent amplification of expression plasmid transcripts. The sequence of the forward primer was 5′TCCATGGGTCTTTTCAGCAGT3′ and the sequence of the reverse primer was

5′ATTCTCCTCATCTGTGCCAGG3′. The length of the expected PCR fragment specific for the pNGVL3huINFα2 plasmid was 100 bp. Controls included sections assayed in the absence of oligonucleotide primers. PCR amplifications were performed using a GeneAmp PCR 1000 System (Perkin-Elmer Cetus Instruments.) as follows: 92°C for 1 min, 65°C for 1 min, and 72°C for 1 min for a total of 40 cycles. Control reactions used for each sample included reaction mixtures without Taq polymerase, and/or primers, skin treated with aqueous DNA, skin treated with blank nanoemulsions, as well as tissue samples from untreated mouse skin. Anti-digoxigenin-alkaline phosphatase-conjugated Fab fragments (1:2000 dilution Boehringer Mannheim) were used for binding to in situ amplified PCR fragments and NBT/BCIP was used as a colorimetric detection reagent. Sections were lightly counterstained with dilute hematoxylin and examined using light photomicroscopy (Nikon Diaphot).

3. Results and discussion

The nanoemulsions both with and without expression plasmid DNA exhibited no electroconductivity at a field potential of >300 V. The particle size analysis (Fig. 1) indicated that the nanoemulsion without DNA had a mean particle size of 42.3 ± 14.6 nm. while the nanoemulsion with DNA had a mean particle size of 32.1 ± 20.2 nm ($P > 0.05$). Plasmid DNA in an aqueous solution behaves as a hydrodynamic colloidal particle with a particle size on the order of 50–100 nm. The DNA containing nanoemulsion had a slightly smaller mean particle size compared with the nanoemulsion without DNA. The smaller mean particle size suggests that a percentage of the aqueous particles do not contain DNA, however, we believe that the results of the particle size analysis suggest that most of the aqueous particles within the DNA containing nanoemulsion likely contain a single molecule of DNA. We hypothesize that the formation of a stable water in oil nanoemulsion may drive the conformation of the DNA towards a more condensed state. The absence of large particles or aggregated material detected during particle size analysis would also support this hypothesis.

In situ DNA PCR analysis of treated skin was conducted to identify intracellular plasmid DNA (pNGVL3huINFα2) 24 h following topical in vivo application of water-in-oil plasmid nanoemulsions to normal mouse skin. Fig. 2 shows representative light photomicrographs of treated (panels A, B and C) and control (panel D) sections of hairy murine skin after in situ DNA PCR using NBT/BCIP as the chromogenic substrate. Evidence of plasmid DNA (dark purple pigment) was identified within numerous hair follicles with extension of positive signals onto the perifollicular surface keratinocytes. No pigment was detected in areas of the sections that were non-follicular or in

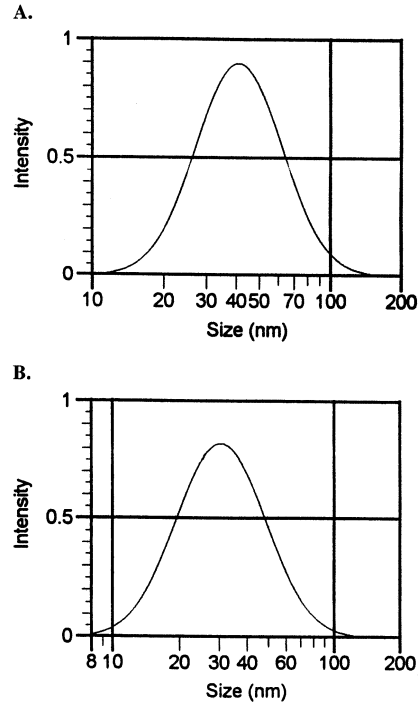

Fig. 1. Unimodal particle size distribution analyses of water-in-oil nanoemulsions both without (panel A) and with (panel B) plasmid DNA. Samples were measured with a Beckman Coulter N4 Plus submicron particle size analyzer using a scattering angle of 90° and a run time of 900 s, at a temperature of 25.0°C. Plasmid DNA was present in the aqueous phase at a concentration of 5 mg/ml. Nanoemulsions without plasmid DNA had a polydispersity index of 0.22, while those with plasmid DNA had a polydispersity index of 0.36.

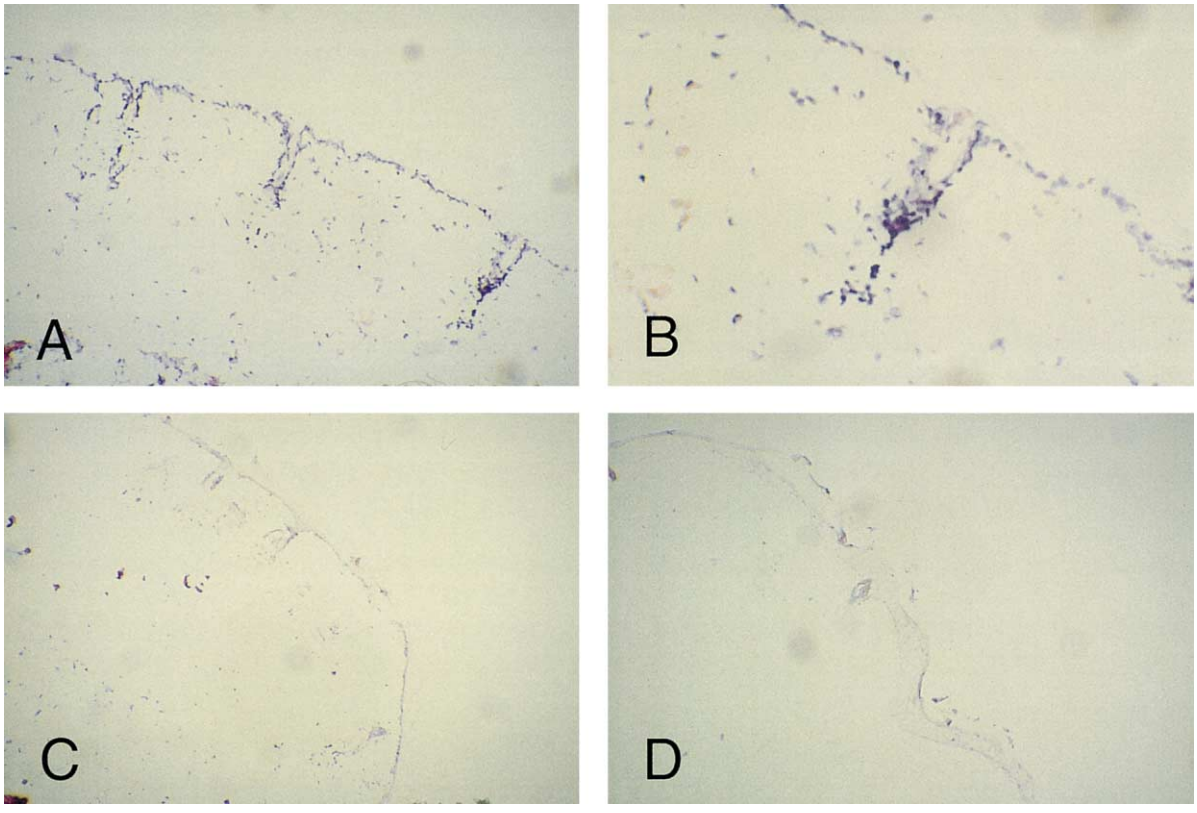

Fig. 2. Identification of transfected skin cells by in situ DNA hybridization. Paraffin sections (10 μm) of skin treated with four consecutive daily doses of nanoemulsion with expression plasmid DNA, aqueous DNA or nanoemulsion alone, were subjected to DNA PCR using primers specific for expression plasmid sequences as described in Section 2. Skin treated with nanoemulsion containing expression plasmid DNA (Panel A, 100X and panel B, 200X) exhibited evidence of plasmid containing cells primarily along the length of the hair follicles and to some extent in a histologic location consistent with surface keratinocytes. Skin treated with aqueous plasmid DNA or empty nanoemulsion exhibited undetectable levels of intracellular plasmid DNA (Panel C, 100X and panel D, 100X).

the remainder of the dermis. Skin sections from a variety of controls showed no evidence of specific plasmid DNA amplification (Fig. 2, panels C and D). Additional controls included sections from all animals assayed in the absence of oligonucleotide primers (Nuovo, 1994; Foreman et al., 1997). None of these sections exhibited any evidence of intracellular or nuclear pigment deposition (data not shown). These observations strongly imply that the delivery of plasmid from water-in-oil nanoemulsions into skin occurs predominantly via a follicular pathway. In addition, the distribution of pigment suggested that the plasmid DNA identified was present predominantly in an intracellular location.

The presence of expression plasmid transcripts in skin following application of the test formulation was determined in both hairless and normal mice using real time quantitative RT-PCR. In all cases, the results of these assays were expressed as huINFα2 transcripts per cm^2 of treated skin. The C57 BL/6J mice had a mean value of 5.0×10^5 huINFα2 transcripts per cm^2, while hairless mice had a mean value of 2.0×10^4 huINFα2 transcripts per cm^2, a difference that was not statistically significant.

We next identified specific expression of transgenic protein in samples of treated and control skin from both normal and hairless mice. The results of the dose–response studies assayed at 24

h following in vivo topical application of various pCF1CAT plasmid formulations to hairless mouse skin at DNA doses of 3, 10 and 30 μg are shown in Table 1. No significant transgene expression was observed at a total DNA dose of 3 μg regardless of the formulation used. At a total DNA dose of 10 μg, significant levels of transgene expression were observed in both the aqueous DNA and nanoemulsion DNA groups. However, the levels of CAT expression achieved using the nanoemulsion DNA formulation were significantly higher (an approximately 1 log increase) than those observed using an equivalent dose of aqueous DNA ($P < 0.001$). It was also observed that increasing the total DNA dose to 30 μg, (using either aqueous DNA or a nanoemulsion formulation), did not enhance transgene expression. In fact, at a DNA dose of 30 μg, an aqueous formulation was unable to mediate detectable levels of transgene expression. These results suggest that topical transfection regardless of the drug delivery system used is subject to both threshold and saturation effects associated with the total dose of DNA applied.

Fig. 3 illustrates the relative time course of transgene expression following application of a single dose of aqueous DNA or nanoemulsion DNA. Consistent with earlier data using both liposomal systems and aqueous DNA, the observed levels of transgene expression were highest 24 h following application of the topical formulations and had returned to baseline by 72 h (Niemiec et al., 1997; Fan et al., 1999; Yu et al., 1999). The levels of transgene expression observed using nanoemulsion DNA were significantly greater than those observed using aqueous DNA at both 24 and 48 h ($P < 0.001$ and 0.01, respectively).

None of the liposomal formulations tested (DOTAP:DNA (w/w) ratios of 2:1, 4:1, 6:1, 8:1, and 10:1) were able to mediate levels of CAT transgene expression in skin that were above the limits of detection for the ELISA employed (10 pg/ml) We believe that the failure of these liposomal formulations to elicit levels of transgene expression underlines their inability to deliver significant amounts of the intact plasmid to follicular cells under occluded conditions. This is in contrast to the earlier reports that have indicated successful topical transfection using cationic liposomes when applied to skin without the use of occlusive dressings. (Alexander et al., 1991; Li and Hoffman, 1995; Alexander and Akhurst, 1995; Yarosh and Klein, 1996; Niemiec et al., 1997; Yu et al., 1999; Fan et al., 1999).

Comparisons were made between groups of C57 BL/6J and hairless mice ($n = 4$) that were treated with a single dose of nanoemulsion containing 10 μg of pNGVL3huINFα2 plasmid DNA in a total volume of 100 μl. The treated skin was harvested 24 h following dosing and analyzed using ELISA for human interferon-α2 protein. As shown in Fig. 4, the C57 BL/6J mice expressed a mean value of 57.0 ± 10.8 pg huINFα2/cm^2 while

Table 1
Dose–response 24 h following topical application of nanoemulsions to hairless mouse skin

Topical formulation	Dose of pCF1CAT					
	3 μg		10 μg		30 μg	
	Levels of CAT protein observed in skin					
	pg/cm^2	pg/mg total	pg/cm^2	pg/mg total	pg/cm^2	pg/mg total
Nanoemulsion DNA	<10	<10	376 ± 49	285 ± 201	290 ± 144	140 ± 87
Aqueous DNA	<10	<10	31 ± 12	53 ± 46	<10	<10
Empty nanoemulsion	<10	<10	<10	<10	<10	<10

Values are expressed as mean ± S.D. Samples that exhibited mean levels of CAT that were below the limits of detection for the ELISA are expressed as <10.

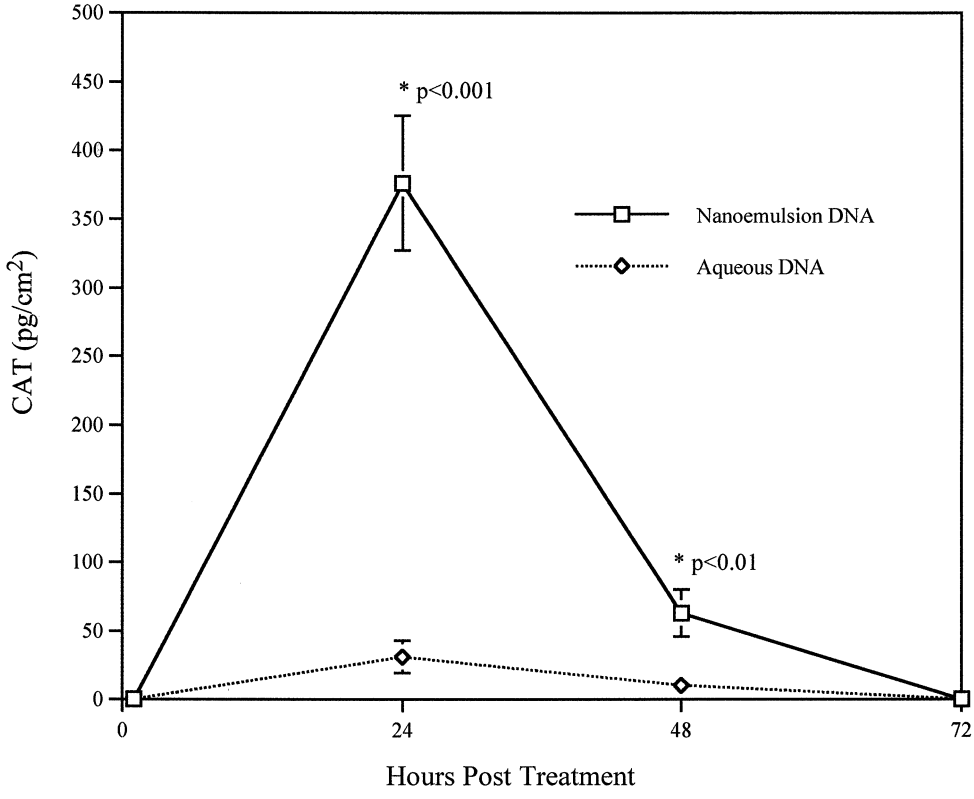

Fig. 3. Time course of transgene expression following topical application of pCF1CAT. Hairless mice ($n = 4$ per time point) were treated with a single topical application of pCF1CAT (total dose = 10 μg) in either an aqueous buffer or water-in-oil nanoemulsion. The levels of transgenic CAT protein present in whole skin lysates at 24, 48 and 72 h following treatment were determined using ELISA and expressed as pg/cm^2 of treated skin surface area. The levels of CAT expression mediated by use of the water-in-oil nanoemulsion were significantly greater than those obtained using aqueous DNA at both 24 and 48 h.

the hairless mice expressed a mean value of 15.4 ± 2.2 pg huINFα2/cm^2 of treated skin ($P = 0.01$). These experiments suggest that the level of transgene expression that can be achieved in skin with normal follicular structure is higher than that observed in the abnormal follicles present in the hairless mice.

In the C57 BL/6J mice treated with four daily doses of the pNGVL3huINFα2 containing nanoemulsions, the skin contained human interferon-α2 at an average level of 5.2 ± 0.98 pg of interferon-α2 protein per mg of total protein, or approximately 100 pg interferon-α2/cm^2 of treated skin. Control mice treated with empty nanoemulsions had mean values below the limits of detection for the ELISA ($P < 0.001$). The total amount of transgenic protein present in the treated skin also appeared to increase over the course of multiple topical applications (approximately 50 pg/cm^2, 24 h following a single application, versus 100 pg/cm^2, 24 h following four daily applications). This suggests that multiple topical applications of the nanoemulsion and expression plasmid DNA allows for continuous transgene expression during the entire treatment period.

Preliminary assessment of dermal toxicity following application of four daily doses of nanoemulsion DNA was determined by a veterinary pathologist blinded to the treatment regimen. Using a qualitative pathologic assessment, each section was evaluated for epidermal thickness, hyperkeratosis, serocellular crust, epidermal ulceration, and epidermal inflammatory infiltrates. In addition, the dermis and panniculus fat were ex-

amined for the presence of cellular infiltrates, hemorrhages and congestion. No histologic differences were appreciated between sham treated animals and those treated with aqueous DNA, blank nanoemulsions, or nanoemulsions containing pNGVL3huINFα2 DNA.

While it is evident that these novel water-in-oil nanoemulsion formulations were effective in delivering plasmid DNA into the hair follicles as judged by the results of the in situ DNA PCR studies, the mechanisms by which they mediate DNA transfection are not entirely clear. Recent reports indicate that naked DNA in aqueous formulations can effect spontaneous transfection of dermal keratinocytes, and that this process is most active within the hair follicles (Fan et al., 1999). The results of our experiments suggest that the efficiency of transfection and the dynamics of transgene expression appear to be augmented by the use of a nanoemulsion vehicle. This may be the result of simple physical protection of plasmid DNA from endogenous deoxyribonucleases present in skin. It is also possible that this effect is a consequence of alterations in cell membrane fluidity or membrane integrity that results from the non-ionic detergents present in the oil phase of the nanoemulsion. Other possibilities include undefined components of the organic plant oil that may facilitate transfection.

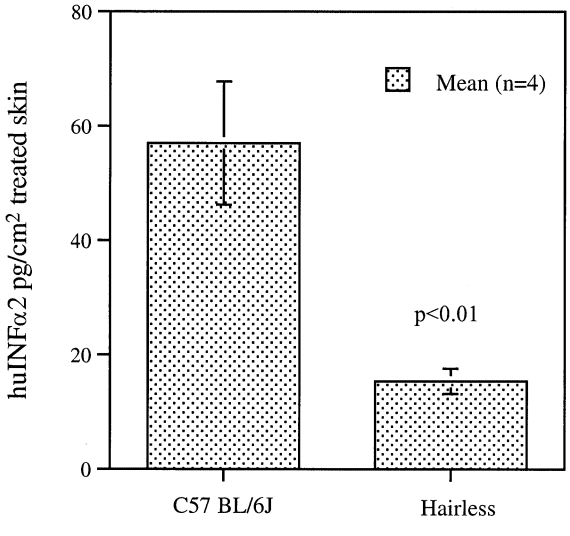

Fig. 4. Effect of follicle type on efficiency of transgene expression. Hairless or C57 BL/6J mice ($n = 4$) were treated for four consecutive days with a single topical application of pNGVL3huINFα in a nanoemulsion. Treated skin was harvested and tissue lysates were prepared. The lysates were analyzed by ELISA for interferon-α. Values are expressed as mean levels of interferon-α of treated skin surface area, pg/cm^2.

Transgene expression following topical application of nanoemulsions containing naked plasmid DNA alone was short-lived. We are currently testing the hypothesis that the duration of transgene expression may be prolonged by condensation of the DNA with hydrophilic cationic polymers. It is necessary that such complexes possess sufficiently high aqueous solubility in order to be properly encapsulated within the external oil phase of the nanoemulsion.

Short-lived transient expression of transgenes might not be disadvantageous in gene therapy of certain diseases that might be remedied by short-term gene expression (Alexander and Akhurst, 1995). The levels of gene expression required for successful therapy of specific disease states remains an unclear issue. However, it is encouraging to note that similar levels of transgene expression have been reported following ballistic particle mediated transfer of IL-12 expression plasmid into epidermal cells overlying an implanted intradermal tumor (Rakhmilevich et al., 1996). In these studies, levels of transgenic IL-12 on the order of 266.0 ± 27.8 pg/0.5 cm^2 in the skin tissue treatment site were apparently sufficient to achieve complete regression of established tumors (0.4–0.8 cm in diameter) in mice bearing Renca, MethA, SA-1, or L5178Y syngeneic tumors. By using a metastatic P815 tumor model, the authors further found that the delivery of IL-12 cDNA into the skin overlying an advanced intradermal tumor, followed by tumor excision and three additional IL-12 gene transfections, could significantly inhibit systemic metastases, resulting in extended survival of test mice. Other diseases that may be amenable to topical gene therapy include autoimmune diseases that predominantly involve the hair follicles including subacute cutaneous lupus erythematosus and discoid lupus erythematosus (Jaworsky, 1997; Chung et al., 1998). These conditions have been reported to be respon-

sive to systemic administration of recombinant human interferon-α2 and it is theoretically possible that local overexpression of interferon-α2 mediated by topical transfection may also have clinically beneficial effects (Nicolas et al., 1990; Thivolet et al., 1990).

In this report, we demonstrate that the incorporation of expression plasmid DNA into water-in-oil nanoemulsions provides a novel method for topical transfection of follicular cells. More importantly, in vivo skin transfection studies following topical application of the nanoemulsion DNA formulations indicated that the transfection efficiency of this system may be superior to naked DNA or cationic liposomes under occluded conditions. These novel systems may have several advantages over the currently used liposomal formulations for topical delivery of expression plasmid DNA including ease of manufacture and low toxicity.

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