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Topical delivery of plasmid DNA using biphasic lipid vesicles (Biphasix)

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Abstract

The development of non-invasive methods for the delivery of vaccines through the skin will greatly improve the safety and the administration of human and veterinary vaccines. In this study we examined the efficiency of topical delivery of plasmids by assessing the localization of gene expression using luciferase as a reporter gene and induction of immune responses using a plasmid encoding for the bovine herpesvirus type-1 glycoprotein D (pgD). Topical administration of plasmids in a lipid-based delivery system (biphasic lipid vesicles – Biphasix) resulted in gene expression in the lymph node, whereas with intradermal injection, antigen expression was found in the skin. Following administration of plasmid with the gene gun, antigen expression was observed in both the skin as well as in the draining lymph nodes. Transcutaneous immunization with pgD formulated in biphasic lipid vesicles elicited gD-specific antibody responses and a Th2-type cellular response. In contrast, immunization by the intradermal route resulted in the stimulation of a Th1-type response. These findings have implications for both vaccine design and tailoring of specific immune responses.

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Introduction

Delivery issues in vaccine development have been recognized as one of the important factors in achieving the desired efficacy. Most of the current vaccines are administered by injection, but there is now evidence that transmucosal or transdermal delivery of antigens, including DNA, can elicit an immune response (Tang et al 1997).

Similarly to the more traditional vaccines (single-shot subunit, therapeutic vaccines), genetic immunization also requires the presentation of the antigen in the most advantageous form at the required location. The success of DNA vaccines is dependent on the delivery of the DNA molecule through several membraneous barriers (i.e. the stratum corneum, cell and nuclear membranes) and expression of the antigen. Topical plasmid transfection, to date, could only be achieved by initially compromising the skin barrier properties such as by intense brushing with a toothbrush (Yu et al 1999), adhesive glue stripping (Watabe et al 2001) or chemical depilation (Alexander & Akhurst 1995; Shi et al 1999). New delivery approaches such as nanoparticles (Cui & Mumper 2001, 2002) and lipid vesicles (Baca-Estrada et al 2000a, b) will be important to increase both the efficiency and reliability of gene delivery into the skin.

The epidermis is an ideal site for DNA immunization since the skin is equipped with a network of antigen-presenting cells (APCs) that mediate the induction of immune responses. Immunization by gold particle bombardment of the skin using the gene gun has demonstrated that a small number of APCs are transfected (Condon et al 1996). APCs directly transfected may be more effective in inducing immune responses as compared with a large amount of antigen expressed by cells of non-lymphoid lineage (Fynan et al 1993; Heiser et al 2001; O'Hagan et al 2001).

Improving delivery of plasmid through the skin will likely enhance the efficacy of transcutaneous immunization with plasmid DNA. We evaluated the feasibility of a novel lipid-based delivery system (Biphasix) as a pharmaceutical delivery approach for the delivery of two model plasmids. Previously we have demonstrated that these formulations are effective in mediating the induction of immune responses to protein

antigens following transcutaneous delivery (Baca-Estrada et al 2000a). Unlike conventional liposomes, the Biphasix vesicles are multi-compartmental lipid vesicles consisting of multiple, concentric mixed-lipid bilayers entrapping lipophilic, micellar and aqueous sub-unit compartments. Our results demonstrated that topical delivery of plasmids formulated in biphasic vesicles resulted in gene expression in the draining lymph node and in the induction of cellular and humoral immune responses.

Materials and Methods

Animals

Six-week-old female BALB/c mice purchased from the Animal Resource Center at the University of Saskatchewan were used for all experiments. Mice were treated in compliance with the regulations of the Canadian Council for Animal Care.

Plasmids

Plasmid encoding the luciferase enzyme under the cytomegalovirus (CMV) promoter (pluc), was a gift from Dr Heather Davis (University of Ottawa, ON, Canada) (Weeratna et al 1998). A plasmid expressing bovine herpesvirus glycoprotein D (pgD) was constructed by inserting the gD gene into vector pCAN1 under the CMV promoter (Uwiera et al 2001). Plasmids were purified using Qiagen (Mississauga, ON) endotoxin-free plasmid kits.

Preparation of DNA delivery system

Plasmids were formulated in a lipid-based delivery system (biphasic vesicles) at a dose of 100 μg plasmid in 125 mg formulation. The three different formulation compositions were prepared based on soya phosphatidylcholine (Natterman GmBH, Germany)–cholesterol (Sigma Chemical Co., St Louis, MO) (10:4 weight ratio) and the following lipids: formula 1 – cholesteryl 3β-*N*-(dimethylaminoethyl) carbamate) (DC-chol, Sigma) at a 1:1 weight ratio; formula 2 – dimyristoylphosphatidylcholine (DMPC, Sigma) at a 1:4 weight ratio and formula 3 – dioleoylphosphatidylethanolamine (DOPE, Sigma) at a 1:4 weight ratio. Each lipid phase (vesicle forming lipids) was hydrated with a sub-micron emulsion aqueous phase, containing linoleamidopropyl-PG-dimonium chloride phosphate (Mona, Paterson, NJ) 4% and olive oil 4% w/w, by mixing until a homogeneous cream was achieved (Foldvari, US Patent no. 5, 853, 755). The appropriate amount of DNA was hand-mixed at room temperature with the Biphasix cream to achieve a homogeneous mixture at the DNA concentration required for immunization.

Immunization with gD encoding plasmid

For topical delivery, mice were carefully shaved with an electric razor 1 day before administration of plasmids to ascertain an intact skin surface (normal skin). The Biphasix formulation was placed on the back of the mouse using a patch construction as previously described (Baca-Estrada et al 2000a). Each round patch (1 cm diameter) contained 125 mg formulation with 100 μg DNA. For topical immunization with DNA in solution (naked DNA), 100 μg of pgD in 30 μL of water was placed on the back of anaesthetized mice until the solution appeared to absorb into the skin. For intradermal administration, mice were injected in the back with 1.5 μg pgD in 10 μL phosphate-buffered saline (PBS). For gene-gun immunizations, gold beads (1.6 μm) with 0.05 μg DNA were prepared according to the manufacturer's instructions for the Helios Gene Gun System (Bio-Rad, Hercules, CA) as previously described (Loehr et al 2000). For topical immunization all groups were boosted twice at two-week intervals. For intradermal and gene-gun immunizations mice were boosted once two weeks after primary immunization. Cells were harvested from spleens and draining lymph nodes two weeks after the final immunization.

Determination of luciferase expression

To determine the level of gene expression, a plasmid encoding the luciferase gene was used in the same manner as for pgD delivery. The doses of pluc were the same as those used for immunization with the different delivery methods. Testing of pluc delivery was carried out on both intact skin and tape-stripped skin. An area of approximately 4 cm^2 was shaved. Removal of the stratum corneum, but not Langerhans cells, was achieved using tape stripping ($\times$20) with Scotch tape (3M) (Proksch et al 1996). Treated skin samples (about 0.8 cm^2) as well as the axillary and inguinal draining lymph nodes were removed from mice and homogenized in 500 μL of lysis buffer (Promega, Madison, WI) with a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON) to produce protein extracts. Luciferase activity of the protein extracts was determined using Promega's luciferase assay system. Samples were counted for 30 s on a Packard Picolite Luminometer (Packard Instruments Canada Ltd, Mississauga, ON). Tissues from untreated mice were used to calculate the background levels of light units (LU).

Characterization of humoral and cellular immune responses

BHV-1 gD-specific antibody responses were determined using ELISA as previously described (Baca-Estrada et al 1996). Briefly, Immunlon 2 (DYNEX, Chantilly, VA) ELISA plates were coated with gD (1 μg mL^{-1}) or IgG standards (Serotec, Hornby, ON) overnight at 4°C. Glycoprotein D-specific IgG and IgG subclasses were determined by biotinylated antibodies specific to IgG and IgG subclasses (Caltag, Toronto, ON) followed by streptavidin-alkaline phosphatase staining (Jackson Immuno-research Labs, West Grove, PA). The alkaline phosphatase activity was determined by *p*-nitrophenol phosphate (PNPP) (Sigma). The absorbance was read after 15–20 min at 405 nm (Bio-Rad).

ELISPOT assays for interferon (IFN)-γ and interleukin (IL)-4 were performed using cells isolated from spleens or draining lymph nodes (axillary and inguinal) as previously described (Baca-Estrada et al 1997). Briefly, 1×10^6 cells/well were placed in 96-well culture plates with and without antigen (gD 0.4 μg mL^{-1}) in AIM-V media (Gibco, Life Technologies, Burlington, ON) and incubated at 37°C and 5% CO_2 for 24 h. Cells were resuspended in fresh media and seeded on nitrocellulose plates (Millipore, Nepean, ON) coated with either IFN-γ or IL-4 specific mouse cytokine capture antibody 2 μg mL^{-1} (PharMingen, San Diego, CA). For staining, biotinylated anti mouse IFN-γ or IL-4 specific antibodies were used at 2 μg mL^{-1} (PharMingen) followed by streptavidin-alkaline phosphatase (Jackson Immuno-research Labs) and developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate tablets (Sigma). Spots representing gD-specific cytokine secreting cells were counted and expressed as number of cytokine secreting cells per 1×10^6 cells. Concanavalin-A-stimulated cells were used as a positive control, with greater then 400 cytokine secreting cells per well.

Statistics

Differences in immune responses among vaccine groups were analyzed with Prism graphpad statistical software (GraphPad Software, Inc.) using a one-way analysis of variance or an unpaired t-test for the immunization experiments or an unpaired t-test for the gene expression experiments.

Results and Discussion

Evaluation of the delivery of antigen by measuring antigen-specific immune response

Plasmid delivery into the skin was evaluated by measuring the induced anti-gD specific antibodies following topical treatment with three different compositions of Biphasix-pgD patches. All three formulations induced five times higher anti-gD IgG levels than either the naked DNA or untreated mice (formulations no. 1 and no. 3 $P < 0.05$) (Figure 1A). Topical delivery of plasmid in Biphasix system also induced cellular immune responses. Induction of gD-specific cellular response was only observed in spleen cells from mice immunized with formulation no. 3 (Figure 1B). This response was characterized by the predominant secretion of IL-4 by cells isolated from spleen and draining lymph nodes (Figure 1B, C). No IFN-γ secreting cells were observed in the spleen or draining lymph nodes (data not shown). Administration of Biphasix formulations without antigen (protein or plasmid) did not elicit any specific immune responses that are significantly different from naive. However, lipids in the formulation may be able to exert an adjuvant effect (in addition to the delivery effect) in combination with antigens.

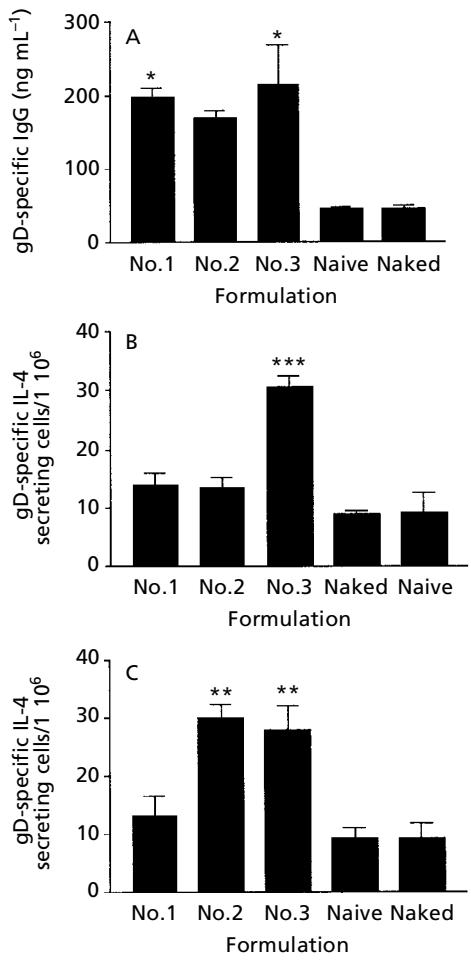

Figure 1 Immune responses in mice following topical application of pgD in 3 different lipid-based delivery systems. All groups (n = 3) were immunized three times at two-week intervals with pgD (100 μg) in three different lipid-based formulations (nos 1–3) or naked pgD. Two weeks following the final immunization, gD-specific IgG was determined using ELISA (A) and gD-specific cellular immune responses were assessed using isolated cells from the spleen stimulated with gD antigen (B) or the draining lymph nodes stimulated with gD antigen (C). Error bars represent standard error of the mean; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs naïve group.

To compare the magnitude of immune response elicited by topical immunization with other methods of delivery, mice were immunized with the gene gun or by intradermal injection with varying doses of gD-encoding plasmid. Our results demonstrated that both of these methods of delivery resulted in the induction of similar gD-specific IgG_1 antibody titers (Figure 2A), which were significantly different from the untreated animals ($P < 0.05$ and $P < 0.01$, respectively). The immune responses observed were similar to those achieved by topical administration of the Biphasix-pgD/100 μg patch (i.e. about 200 ng mL^{-1} IgG by ELISA).

Further characterization of intradermal and gene-gun-mediated plasmid delivery indicated the absence of gD-specific IgG2a antibodies (data not shown). The cellular immune responses elicited by the gene gun were charac-

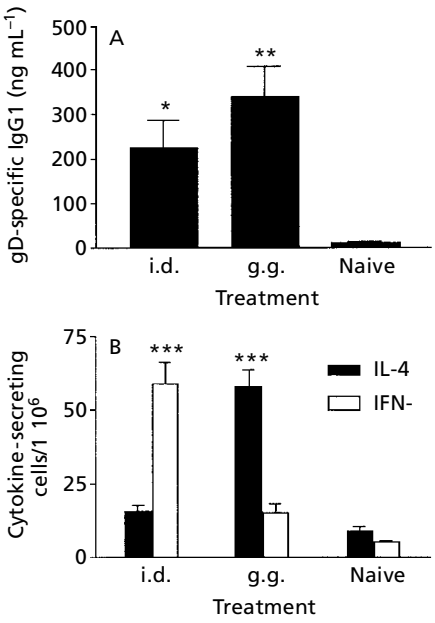

Figure 2 Immune responses induced by immunization of mice with pgD either by intradermal injection (i.d.) or by particle bombardment using the gene gun (g.g.). Groups of mice (n = 5) were immunized with 1.5 μg of pgD (i.d.) or with 0.05 μg of pgD (g.g.). gD-specific IgG1 was determined using ELISA (A) and gD-specific IFN-γ and IL-4 secreting cells in the spleen stimulated with gD antigen (B). Error bars represent standard error of the mean of five mice per group; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs naïve group.

terized by a Th2-type response in the spleen (Figure 2B) and mixed Th1/Th2 in the lymph nodes (data not shown). In addition, gene-gun immunization with 1 μg pgD still elicited a predominant Th2 response (data not shown). In contrast, the cellular responses elicited by intradermal injection were predominantly Th1 in both spleen (Figure 2B) and lymph nodes (data not shown).

Although there was no difference between the gD-specific antibody isotypes induced following immunization by a number of delivery methods, the cytokine profile generated varied depending on the type of delivery method used. Therefore, the route of delivery influences the type of cellular immune response induced. Intradermal injection of pgD elicited a strong Th1 response, whereas topical and gene-gun administration elicited a predominant Th2 response. Antigen produced in the lymph node may result in increased antigen presentation by APCs compared with antigen produced in non-lymphoid tissue. These differences in antigen presentation may explain the differences in the immune responses elicited by the epidermal and intradermal routes.

Although the doses of pgD used in these immunizations were quite different, depending on the method of delivery, the total amount of plasmid was identical between the gene-gun and intradermal immunizations, suggesting that the differences in the type of immune responses generated was not due to the amount of immunostimulatory molecules present in the plasmid.

The biphasic vesicles facilitate the penetration through the stratum corneum into the viable epidermis. Previous studies using liposomes suggest that hair follicles are important for transfection (Niemiec et al 1997; Fan et al 1999). However, we do not know which pathway(s) results in transfection from topical application of plasmid formulated in biphasic vesicles. From the three formulations tested in this study, formulation no. 3, containing PC/DOPE lipid combination, showed somewhat higher efficiency of transfection and immune response. This may indicate either a better lipid vesicle–skin interaction in vesicles containing unsaturated lipids or greater transfection level of APCs, hence the expression in the lymph node.

Evaluation of luciferase expression after topical delivery of pluc

Plasmid delivery into the skin was also evaluated by measuring the expression of luciferase activity 48 h following topical, gene-gun or intradermal administration. There was no increase in levels of luminescence in the skin after topical treatment with Biphasix-pluc/100 μg patch (formulation no. 3) (Figure 3A). Even when examined without the

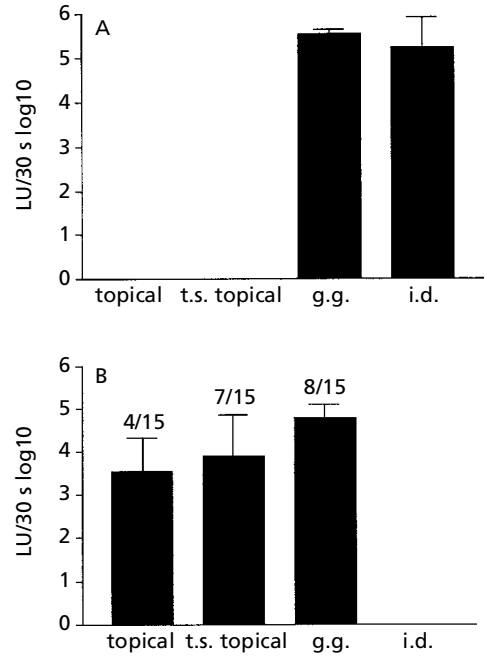

Figure 3 Gene expression following different delivery methods. Luciferase activity was determined in the skin (A) and in the draining lymph nodes (B) of mice 48 h after topical administration of 100 μg of pluc formulated in lipid-based delivery system on normal and tape-stripped (t.s.) skin, 0.05 μg of pluc administered by the gene gun (g.g.) or 1.5 μg pluc injected intradermally (i.d.). Luciferase activity was expressed in light units (LU) per 30 s. Numbers over bars represent the number of positive responses detected. Error bars represent standard deviation of the mean of 5–15 mice per group.

stratum corneum present (removed by tape stripping), expression of luciferase was not found in the skin following topical application of pluc (Figure 3A). However, there was an increase in luminescence in the lymph node homogenates after topical treatment on both intact and stripped skin. Topically applied naked pluc did not elicit any luciferase activity on normal or tape-stripped skin (data not shown).

Intradermal injection (1.5 μg) resulted in luciferase expression only in the skin, whereas administration of pluc with the gene gun (0.05 μg/dose) resulted in gene expression in both skin and lymph nodes (Figure 3A, B).

Conclusions

Immunization by topical application of vaccine antigens is a new concept that has great potential. Non-invasive strategies for vaccine administration onto the skin have recently been reported for protein (Glenn et al 1998) and for plasmid DNA vaccines (Fan et al 1999; Shi et al 1999). In this study, we assessed whether the use of biphasic lipid vesicles (Biphasix) enhances the delivery of plasmid DNA through intact skin and, subsequently, the induction of immune responses to the encoded gene. Our results demonstrate that incorporating the plasmid in biphasic lipid vesicles enhances the immune responses induced following topical application. Furthermore, we extended previous studies by demonstrating that after topical administration, plasmid expression can be achieved in the draining lymph nodes. This observation may be important to our understanding of how such low doses of antigen, produced by DNA immunization, can elicit immune responses.

Collectively, this study demonstrated the feasibility of using biphasic vesicles to enhance topical delivery of plasmid DNA into the skin. Furthermore, these results also suggest that the type of immune responses induced by various methods of immunization into the skin may be influenced by the location of gene expression. It is unknown why different modes of application result in different types of immune responses; however dendritic cells may influence the type of immune response elicited (Rissoan et al 1999). Our results indicated that gene expression in the draining lymph nodes likely results from directly transfected dendritic cells, which corresponds with induction of a Th2 response with the gene-gun and topical administration. The ability to induce different types of immune responses is significant because different diseases require different types of immunity to provide protection (Sjolander et al 1998). This information is relevant to the development of non-invasive vaccine delivery strategies.

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