Optimization of DNA-based Vaccination in Cows Using Green Fluorescent Protein and Protein A as a Prelude to Immunization Against Staphylococcal Mastitis

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ABSTRACT

Staphylococcus aureus is a contagious pathogen that often results in chronic intramammary infections in dairy cows. Current vaccine formulations are ineffective in preventing this infection. The objective of this study was to stimulate an immune response in dairy cows through injection of plasmid DNA designed to express staphylococcal Protein A in transfected cells. Intramuscular and intradermal vaccination sites were evaluated using a plasmid containing the human cytomegalovirus (CMV) promoter/enhancer directing expression of green fluorescent protein (pcDNA3/GFP). DNA was delivered by needle and syringe, or by high-, intermediate-, or low-pressure jet injections (Ped-o-Jet and LectraJet). Five cows per treatment were injected with 0.5 mg of plasmid DNA at 6, 4, and 2 wk prepartum. Serum antibody levels determined by ELISA indicated that intradermal high-pressure jet injection elicited a greater immune response compared to needle and syringe injection. Differences in antibody production among low-pressure and needle and syringe treatment groups were not significant. An expression plasmid containing the CMV promoter/enhancer driving expression of the Fc-binding domain of *S. aureus* Protein A was coinjected into cows by vulvamucosal vaccination using the high-pressure Ped-o-Jet. Beginning 6 wk prepartum, groups of cows ($n = 5$) were injected three times at 2-wk intervals with DNA in saline, DNA in aluminum phosphate adjuvant, or served as noninjected controls. A cellular immune response to Protein A was detected in 4 of 10 animals, while cellular responses to GFP were not detected. Humoral responses to Protein A were observed in 6 of 10 animals and to GFP in 2 of 10 animals. Aluminum phosphate adjuvant appeared to enhance antibody production in response to Protein A. In experiment 3, a protein boost injection of Protein A

was given to six animals approximately 5 mo postpartum. Three animals were nonvaccinated controls, and three were among those stimulated to produce antibody in response to the DNA-based vaccine. These results showed that Protein A specific antibodies remained elevated as compared to nonvaccinated controls and were stimulated in response to the protein boost. However, the magnitude of the response in animals previously vaccinated with DNA was not different than that observed in the nonvaccinated controls. We have shown that a humoral and cellular immune response to abbreviated Protein A can be raised in dairy cows using intravulvamucosal jet injection of a DNA-based vaccine.

(**Key words:** Protein A, in vivo transfection, vulva mucosa, jet-injection)

Abbreviation key: CMV = human cytomegalovirus, GFP = green fluorescent protein, HRP = horseradish peroxidase, MHC = major histocompatibility complex, PBS-T = PBS containing 0.1% Tween 20, PBS-T-G = PBS containing 0.1% Tween 20 and 0.1% gelatin.

INTRODUCTION

Staphylococcus aureus is a contagious pathogen that causes chronic, subclinical mastitis in dairy cattle. Staphylococcal mastitis has proven to be difficult to treat and has not been effectively eliminated from many herds (Bramley and Dodd, 1984; Sutra, 1994). Vaccines aimed at enhancing the animals' own defenses against staphylococcal mastitis have had limited success. This may be due to a dilution in milk of immune components, including lymphocytes, immunoglobulins, and phagocytic cells (Guidry et al., 1994; Yancey, 1999), as well as interactions between milk components and leukocytes (Russell et al., 1977) that reduce their effectiveness. *Staphylococcus aureus* has the ability to invade the mammary gland by utilizing these deficiencies as well as employing many virulence factors, including α - and β -toxin and Protein A. Ultimately, this combination of conditions allows the pathogen to attach to and survive within the epithelial cells of the mammary gland lead-

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ing to apoptosis and irreversible tissue damage (Bayles et al., 1998; Menzies and Kourteva, 1998). A Protein A-based vaccine has been the focus of previous research in an effort to prevent staphylococcal mastitis. Pankey et al. (1985) vaccinated dairy cows with either purified Protein A or a bacterin derived from *S. aureus*. The conclusion from this research was that although incidence of infection was not reduced under experimental challenge, spontaneous cure rates were significantly increased by vaccination with both Protein A and the *S. aureus* bacterin.

Wolff et al. (1990) were among the first to report that genes in a plasmid vector could be injected into the muscles of mice with the subsequent expression of the encoded gene product. Later, it was demonstrated that an immune response could be induced to the proteins expressed as a result of DNA injection (Robinson and Webster, 1993; Ulmer et al., 1993). Kerr et al. (1996) successfully used intradermal jet injection of DNA encoding a green fluorescent protein (GFP) gene to stimulate serum antibody production to GFP in pigs. In addition, it has been shown that DNA-based vulvamucosal immunization in cattle can stimulate both cellular and humoral immune responses; a unique attribute of DNA vaccination (Tuting et al., 1999; Loehr et al., 2000). Loehr et al. (2000) also reported that the circulating antigen presenting cells of the dermis, known as Langerhans cells, migrated near to the surface of the vaginal mucosal tissue, whereas in the hip skin, which was not as effective as an injection site, they remained near the basal membrane. They hypothesized that these cells are themselves transfected or migrate to the site of injection, where they process protein from other transfected cells.

A variety of compounds have also been investigated as potential components of a DNA-based vaccine to further enhance the immune response, among these are adjuvants. When comparing mineral salts, such as calcium or aluminum as potential adjuvants, aluminum phosphate proved to be superior (Wang et al., 2000). Wang et al. (2000) also investigated the effect on the immune response when booster injections of protein are administered in combination with DNA. They showed that a booster vaccination of protein enhances the immune response fivefold over DNA injection alone. Other reports supporting these findings noted that DNA priming followed by a recombinant protein boost was most effective at protecting monkeys against malaria (Doonan and Hoffman, 2001; Jones et al., 2001).

The focus of the present research was to combine the potential immune stimulating effects of Protein A with the novel DNA-based vaccine approach as an initial step in the development of a DNA-based vaccine against *S. aureus*.

MATERIALS AND METHODS

Construction of the GFP and Protein A Expression Plasmids

The GFP expression plasmid was previously constructed in this laboratory (Kerr et al., 1996) using the pcDNA3 expression plasmid (Invitrogen, Carlsbad, CA). It contained an enhanced green fluorescent protein gene (Clontech) inserted into the polylinker of pcDNA3 downstream from the human cytomegalovirus (CMV) promoter/enhancer. To construct a Protein A expression vector, a plasmid (pGX2907), containing the Protein A gene from *S. aureus* strain Cowan 1 was obtained in *Escherichia coli* from American Type Culture Collection (ATCC; 39344). A fragment encoding the four repeating Fc-binding regions of the Protein A protein was amplified from the plasmid by PCR, using the Expand High Fidelity System (Boehringer Mannheim). The 5' primer contained a Kozak site (GCCACCATGG) to enhance eukaryotic transcription and an *Eco*R1 restriction endonuclease recognition sequence to facilitate cloning. The 3' primer contained an *Apa*1 endonuclease site. The PCR fragment was ligated into the pcDNA3 vector to generate pcDNA3/Protein A. The sequence of the Protein A gene was confirmed at the University of Vermont DNA analysis facility. Plasmids for injection were isolated from *E. coli* by alkaline-lysis followed by PEG (polyethylene glycol 8000) precipitation, according to a protocol adapted from Yeung and Lau (1992) and subsequently purified by phenol, phenol/chloroform, and two chloroform extractions.

Protein Expression in Mammalian Cells

Expression of Protein A and GFP in mammalian cells was evaluated following cotransfection of the constructed plasmids into COS-7 kidney cells (ATCC, #CRL-1651). The cells were grown in Dulbecco's Modified Eagle's medium (Sigma; D5648) plus 10% fetal bovine serum. Cotransfection of COS-7 mammalian cells with the pcDNA3/Protein A and pcDNA/GFP plasmids in a ratio of 9:1 was performed by CaPO_4 precipitation. Transfection was confirmed by visualization of GFP 48 h posttransfection with fluorescence microscopy. Media and cell extracts were then evaluated for expression of Protein A by Western blot analysis. Control samples included purified Protein A (Sigma; P7837) and cell extract and media from a transfection with a lysostaphin expression vector (Kerr et al., 2001) as a negative control. Proteins were separated on a 12% SDS-PAGE gel then transferred to nitrocellulose membranes using the Trans-blot semidry electrophoretic cell (Bio-Rad, Hercules, CA). Membranes were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 1.0% BSA for

30 min and washed with PBS-T. Each membrane was then incubated for 30 min with the primary antibody, anti-Protein A (Sigma; P-3775) at a dilution of 1:1000 in PBS-T. After washing, the membranes were incubated for 2 h with anti-rabbit IgG alkaline phosphatase conjugate (Sigma; A3687) diluted 1:10,000 in PBS-T. The membranes were developed using a BCIP (Bio-Rad)/NTB (Sigma) solution as per manufacturer's directions.

Animals

All animals involved in these investigations were housed at the University of Vermont research facility and cared for in accordance with the Institutional Animal Care and Use Committee guidelines. To reduce potential variation in immune responses between groups due to age, animals were distributed such that each group contained heifers and older animals with the cumulative number of lactations per group being equal. Animals were vaccinated 6, 4, and 2 wk prepartum. Blood samples were obtained on the first day of vaccination previous to injection and every 2 wk thereafter.

Intramuscular Vaccination with the GFP Plasmid

Three groups of five animals were assigned to treatment groups receiving intramuscular injections of DNA into the deltoid muscle using three different vaccination methods. The three treatment groups included needle (20 g $\times$ 1.5 cm) and syringe or jet injection with multichannel LectraJet injectors (D'Antonio Consultants International, East Syracuse, NY) designed to deliver at low (600 psi) or intermediate-pressures (1200 psi). The multichannel injectors were equipped with 12-mm long needle-like (20 g) perforators to ensure intramuscular delivery. The pcDNA3/GFP vector was diluted to a concentration of 1.0 mg/ml in a 0.15 M saline. On each vaccination day, all animals were injected with three 0.166-ml volumes of DNA solution for a total of 0.5 mg of DNA per animal per vaccination day.

Intradermal Vaccination with the GFP Plasmid

The three treatment groups, selected as previously described, included needle (20 g $\times$ 0.5 cm) and syringe, needleless high-pressure (3000 psi) jet injection (Ped-o-Jet; Keystone, Cherry Hill, NJ), and a low-pressure (600 psi) multichannel LectraJet injector with 2-mm perforators (20 g). The Ped-o-Jet is a hand held device that propels a pressurized solution into the skin through a sapphire jewel tip with a 0.13-mm orifice. To provide close contact between the injection device and

the skin, a hair removal lotion was used to dissolve hair at the site of injection. Animals were injected intradermally in the region of the deltoid muscle.

Intravulvamucosal Vaccination with the Protein A Plasmid

Covaccination with pcDNA3/Protein A and pcDNA3/GFP into the posterior mucosal tissue of the labia was undertaken using the needleless high-pressure Ped-o-Jet injection device. Three groups of five animals were assigned to treatment groups, including nonvaccinated controls or animals receiving either a 1:1 mixture of pcDNA3/GFP and pcDNA3/Protein A dissolved in 0.15 M saline or dissolved in aluminum phosphate adjuvant (Adju-phos; E. M. Sergeant Pulp & Chemical Co., Inc., Clifton, NJ).

The pcDNA3/GFP and pcDNA3/Protein A vectors were initially diluted to a concentration of 4.0 mg/ml in a 0.15 M saline solution. One volume of each vector was combined with either two volumes of saline or Adju-Phos such that the final injection solution contained both plasmids at an equal concentration of 1.0 mg/ml, the total DNA concentration being 2.0 mg/ml. Animals were distributed such that each group contained heifers and older animals with the cumulative number of lactations per group being equal. Animals were vaccinated at 6, 4, and 2 wk prepartum as previously described.

Intravulvamucosal Booster Vaccination with Purified Protein A Protein

Six animals that had participated in the intravulvamucosal DNA vaccination trial were chosen to receive a booster injection of purified Protein A protein at approximately 5 mo postparturition. Three cows previously vaccinated with DNA were chosen on the basis of the elevated anti-Protein A antibody production as measured by ELISA. The three additional animals were nonvaccinated controls that showed no measurable anti-Protein A antibody production. Each animal was injected in the posterior mucosal tissue of the labia with purified Protein A (Sigma; P-7837), using the needleless Ped-o-Jet injector. Protein A was first suspended in 0.15 M saline, then diluted to a final concentration of 5.0 mg/ml in Adju-Phos. Each animal received four 0.250-ml doses for a total of 5.0 mg on one vaccination day. Blood samples were taken before vaccination and 2 wk postvaccination.

Determination of GFP Specific Serum Antibody by ELISA

An ELISA was performed to determine the level of anti-GFP IgG produced in response to immunization

with the pcDNA3/GFP vector. The 96-well flat bottom plates (Corning Costar) were coated with 100 μ l of 1 μ g/ml solution of GFP (Clontech) in coating buffer (0.05 M bicarbonate buffer). An equivalent number of wells per plate were coated with buffer not containing GFP. These plates were incubated overnight at 4°C. The next day, plates were washed three times with PBS-T and then blocked for 1 hr with PBS-T containing 0.1% gelatin (**PBS-T-G**). Serum samples were diluted 1:100 in PBS-T-G and plated in triplicate in both the GFP coated wells and those without GFP for a total of six wells per serum sample. A positive control of rabbit anti-GFP (Clontech) was also plated in triplicate in wells with and without GFP at a dilution of 1:2500 in PBS-T. An affinity purified horseradish peroxidase (**HRP**)-linked anti-bovine IgG₁ and IgG₂ antibody (VMRD, Pullman, WA) was diluted 1:1000 in PBS-T and 100 μ l/well was plated, while a secondary antibody (HRP-linked anti-rabbit IgG, Sigma; A-0545) was added to the positive controls wells at a 1:20,000 dilution. An HRP/3,3',5',5'-tetramethylbenzidine (Sigma) substrate was prepared and added to the wells. The reaction proceeded for 30 min and was terminated with 1 M H₂SO₄. Optical density at 450 nm was determined using a microplate reader (Bio-tek Instruments, Winooski, VT). Optical density values from triplicate wells were averaged. To account for nonspecific binding of antibody to the plate, the average values of wells without GFP were subtracted from the average values of triplicate wells with GFP. To determine the increase in GFP antibody over time in response to vaccination, the preimmune serum sample value was subtracted from each of the postvaccination serum sample values.

Determination of Protein A Specific Serum Antibody by ELISA

The level of anti-Protein A antibody produced in response to immunization with the pcDNA3/Protein A vector was determined by ELISA. Flat bottom plates (Corning Costar, 96 well) were coated with 100 μ l of a Protein A (Sigma) solution at a concentration of 0.1 μ g/ml in coating buffer. An equivalent number of wells per plate were coated with buffer not containing Protein A. These plates were incubated overnight at 4°C. The next day, plates were washed with PBS-T and then blocked for 2 hr at 37°C with 100 μ l of a solution containing human Fc fragments (Calbiochem; 401104) at a concentration of 2.0 μ g/ml in PBS-T. Plates were washed, and then a second blocking step was performed using PBS-T-G for 1 hr at room temperature. Simultaneously, serum samples were diluted 1:100 in PBS-T-G-blocking solution. Samples were plated in triplicate in both the Protein A-coated wells and those without for a total of

six wells per serum sample. An HRP conjugated affinity purified F(ab')₂ fragment of anti-bovine IgG (Jackson ImmunoResearch Lab Inc.) was diluted in PBS-T and 100 μ l/well was added to wells containing serum. The plates were developed with substrate as previously described. To determine the increase in Protein A antibody over time in response to vaccination, the preimmune value was subtracted from each of the postvaccination serum sample values.

Lymphocyte Proliferation Assay

A blood lymphocyte, antigen-specific, proliferation assay was performed to determine the cellular immune response stimulated by immunization with pcDNA3/GFP and pcDNA3/Protein. The assay, which is based on thymidine [Methyl-³H] incorporation due to cell proliferation, was performed on lymphocytes obtained 2 wk after the final vaccination and was performed essentially as described by van Drunen Little-van den Hurk et al. (2000). For each animal, quadruplicate wells were assigned containing either Protein A (1.0 μ g/ml), GFP (1.0 μ g/ml) or no antigen. Lymphocyte proliferation was reported as the stimulation index representing counts per minute in the presence of antigen divided by counts per minute in the absence of antigen.

Statistical Analysis

The Student's *t*-test was used to determine the statistical significance ($P \leq 0.05$) of the differences observed for the various parameters studied using intradermal and intravulvamucosal routes of injection for vaccination with pcDNA3/GFP, pcDNA3/Protein A, and Protein A protein.

RESULTS

Intramuscular and Intradermal Vaccination with the GFP Plasmid

The efficacy of intramuscular and intradermal injection sites was investigated using four injection methods, including needle and syringe, and low-, intermediate-, or high-pressure jet injection for delivery of pcDNA3/GFP. The development of serum antibodies to GFP in response to intramuscular vaccination is presented in Figure 1. In general, animals that responded to vaccination showed a characteristic rise in antibody production up to 8 wk postprimary vaccination followed by a gradual decline. Humoral immune responses were clearly observed in two animals per group. The magnitude of the response did not appear to differ between the methods of injection.

Figure 1. Humoral immune response as stimulated by intramuscular injection of cattle with plasmid DNA encoding green fluorescent protein (GFP). Antibodies to GFP in diluted (1:100) serum are represented as optical density measured by ELISA. All animals in all groups ($n = 5$) were vaccinated with the pcDNA3/GFP vector in 0.15 M saline on d 0, 14, and 28 as indicated by a down arrow. Each line represents a different animal within the same treatment group. Animals with undetectable responses are not represented. Animals were vaccinated using one of three methods, including; A) needle and syringe, B) low pressure jet injection (LectraJet), or C) intermediate pressure jet injection (LectraJet).

In a similar second experiment, intradermal routes of injection were investigated. In this experiment, animals were vaccinated with pcDNA3/GFP in saline by needle and syringe, low-pressure jet injection (LectraJet), or high-pressure jet injection (Ped-o-Jet). Production of serum antibody to GFP in response to intradermal vaccination is presented in Figure 2. One of five individuals showed antibody production with needle and syringe injection, whereas two in five animals produced a detectable level of GFP antibody in response to the low-pressure LectraJet injections. In contrast, the Ped-o-Jet injection elicited a response superior in magnitude and duration to that stimulated in either

the needle and syringe or the low-pressure LectraJet intradermal injections in three of four animals.

In comparison of the mean ($\pm$ SE) maximum responses observed in the intradermal and intramuscular treatment groups, intradermal delivery by the high-pressure Ped-o-Jet produced the numerically greatest response. Within the intradermally injected animals, this response was significantly ($P \leq 0.05$) greater than the needle and syringe technique but not the low-pressure LectraJet technique. Consequently, the high-pressure, intradermal injection method was selected to vaccinate animals with the pcDNA3/Protein A vector in a subsequent experiment.

Figure 2. Humoral immune response as stimulated by intradermal injection of cattle with DNA encoding green fluorescent protein (GFP). Antibodies to GFP in diluted (1:100) serum are represented as optical density measured by ELISA. All animals in all groups ($n = 5^*$) were vaccinated with the pcDNA3/GFP vector in 0.15 M saline on d 0, 14, and 28 as indicated by a down arrow. Animals with undetectable responses are not represented. Each line represents a different animal within the same treatment group. Animals were vaccinated using one of three methods, including; A) needle and syringe, B) low pressure jet injection (LectraJet), or C) high pressure jet injection (Ped-o-Jet). *One animal in Ped-O-Jet group was prematurely removed from the herd due to complications at parturition unrelated to this study.

Figure 3. Antibody production against green fluorescent protein (GFP) and Protein A in response to intravulvamucosal vaccination. Each value is the average $\pm$ the standard error of the mean of the highest optical densities observed for each animal ($n = 5$) in three treatment groups including nonvaccinated controls (Controls), vaccinated with a 1:1 mixture of pcDNA3/GFP and pcDNA3/Protein A vectors in saline (Saline), or vaccinated with a 1:1 mixture of pcDNA3/GFP and pcDNA3/Protein A vectors in aluminum phosphate adjuvant (Adjuvant). ^{a,b}Optical densities with different superscripts within a treatment differ ($P \leq 0.05$).

Transfection of COS-7 Mammalian Cells with the Protein A Plasmid

The pcDNA3/Protein A vector, containing a 903-bp fragment of the Protein A gene from *S. aureus* strain Cowan 1 under the control of the CMV promoter was constructed, and the sequence was verified. Confirmation that the plasmid would direct production of the Protein A fragment was obtained by transfection of COS-7 mammalian cells followed by Western blot analysis. The predicted 32-kDa fragment of Protein A was observed in the cell lysate, and although there was no secretion signal encoded by the plasmid, the protein was also found in the media (data not shown). This likely occurred because of cell lysis during culture. Samples obtained from cells transfected with an irrelevant lysostaphin expression plasmid as a negative control did not contain the Protein A fragment.

Intravulvamucosal Jet Injection of the Protein A and GFP Plasmids

Intravulvamucosal vaccination of the experimental animals using the Ped-o-Jet occurred without incident and appeared to be well tolerated even on the third injection day. No signs of injection trauma were observed. Overall, immune response to the intravulvamucosal injection of the GFP-encoding plasmid were low (Figure 3). Only one of five animals in each of the groups were stimulated to produce antibodies to GFP. In the nonvaccinated group, one of the five animals showed a low, unexplained anti-GFP response.

Antibody response to intravulvamucosal injection of the Protein A encoding plasmid was also quite low but clearly detectable (Figure 3). At a serum dilution of 1:100, mean ($\pm$ SE) Protein A antibody levels were significantly greater in the vaccinated animals than in the nonvaccinated control animals. In the control animals, two individuals demonstrated unexplained Protein A antibody production. Since there were no cases of *S. aureus* mastitis detected in these individuals as a result of monthly surveillance through culture before and during the vaccination trial, this unexplained antibody production is considered variability within the assay. Taking this variability into consideration, three of five animals for each vaccinated group responded to the vaccine. In responding animals, the Protein A antibody response was typified by an increase 4 to 6 wk postprimary injection with a subsequent decline. In general, injection of the DNA vectors in adjuvant resulted in increased antibody production that showed a delayed decline in comparison to that observed in animals vaccinated with DNA vectors in saline.

To assess the cellular immune response, peripheral blood mononuclear cells were isolated from all animals at 8 wk postprimary vaccination and stimulated in vitro with purified GFP and Protein A. The cellular immune response (Figure 4) is represented as stimulation index. Four of 10 immunized animals mounted a cellular immune response to Protein A. In contrast, none of the animals showed a detectable cellular response to GFP.

DNA Prime/Protein Boost Regimen for Protein A Intravulvamucosal Vaccination

Six animals from the pcDNA3/Protein A injection study were selected to receive an additional booster injection of purified Protein A (Figure 5). These animals included three previously vaccinated with Protein A DNA and three nonvaccinated controls. No significant difference in response to the protein boost was observed between animals previously vaccinated and nonvaccinated controls. However, each of the DNA vaccinated animals appeared to maintain their elevated Protein A antibody levels from the previous study. Following immunization with purified Protein A, the DNA vaccinated group responded with an increase in antibody levels similar to the nonvaccinated controls. Consequently, although the magnitude of the response to protein A was not different for the two groups, the DNA prime/protein boost group maintained a superior antibody level for each of the recorded time points in comparison to protein injection only.

DISCUSSION

In a comparative study of six different injection methods, our results demonstrated that intradermal injection

Figure 4. Cellular immune response as stimulated by intravulvamucosal injection of cattle with DNA encoding green fluorescent protein (GFP) and Protein A. Peripheral blood mononuclear cells were isolated at 8 wk postprimary vaccination and exposed to protein antigen (1 μ g/ml) in media: either GFP (dotted) or Protein A (solid). Treatment groups were; A) nonvaccinated controls (C), B) vaccinated with a 1:1 mixture of pcDNA3/GFP and pcDNA3/Protein A vectors in Saline (VS), or C) vaccinated with a 1:1 mixture of pcDNA3/GFP and pcDNA3/Protein A vectors in aluminum phosphate adjuvant (VA). Vaccinated animals were injected with the high pressure jet injection device (Ped-o-Jet). These results represent the average of quadruplicate wells. Lymphocyte proliferation in the presence of antigen to proliferation in the absence of antigen. *A value determined to be two S.D. above the mean of the controls; indicates cellular response to antigen.

tion with the high-pressure injector, Ped-o-Jet, stimulates a superior response to intramuscular injection in dairy animals. Needle and syringe injection proved to be ineffective using either intramuscular or intradermal routes, while low-pressure injection elicited an intermediate humoral response. Intradermal DNA-based vaccination has been shown to be superior to intramuscular injection in animals, such as cats (Osorio et al., 1999) and pigs (van Rooij et al., 1998). Our results are in agreement with van Drunen Littel-van den Hurk et al. (1998) who reported that immunization of cattle with 500 μ g of DNA encoding bovine herpesvirus-1 glycoprotein induced a superior response when intradermally injected as compared to intramuscular vaccination. The increased immune response resulting from intradermal

vaccination is considered a result of the antigen presenting functions of dendritic cells in the dermis known as Langerhans cells. These cells can migrate through the dermis and lymph system and are able to process both endogenous or exogenous antigen. Their ability to interact with T lymphocytes through major histocompatibility complex (MHC) class I or MHC class II presentation makes them a powerful effector cell in the immune response. In addition, dendritic cells may be transfected directly and subsequently migrate to distant sites further stimulating immune cells through antigen presentation (LaCava et al., 2000; Larregina et al., 2001). In contrast, an inferior response results from injection of muscle cells perhaps because they express predominantly MHC class I and low levels of costimulatory molecules (Ulmer et al., 1996). Therefore, MHC class II dependent immune stimulation likely does not occur as a result of antigen presentation from the transfected muscle cells but rather depends on interactions between the encoded foreign protein and antigen presenting cells.

In seeking further immune enhancement to DNA-based vaccination, alternative sites have been investigated. Injection of the vulva mucosa in cattle has recently been shown to elicit an even greater response than intradermal injection (Loehr et al., 2000). Animals vaccinated intravulvamucosally developed stronger humoral and cellular immune responses than those receiving intradermal vaccination. The conclusion resulting from this work was that the enhanced immune stimulation caused by injection at this site was due to increased dendritic cell activity in the mucosal tissue. This is a logical supposition since the lamina propria of mucosal membrane consists of loose connective tissue where there are a variety of cells participating in immune defenses. Loehr et al. (2000) also showed that there is an

Figure 5. Humoral immune response stimulated by intravulvamucosal protein boost vaccination with Protein A. Each value is the average $\pm$ the standard error of the mean of the optical densities observed for each animal in each of two treatment groups ($n = 3$). Animals used in the pcDNA3/Protein A vaccination trials as nonvaccinated controls; protein boost only (●), and animals previously vaccinated with pcDNA3/Protein A that received subsequent boosting with protein; DNA prime/protein boost (■), are represented. DNA and protein injections are indicated by a down arrow.

increased presence and deeper penetration of dendritic cells in the mucosa and that they are directly transfected by DNA. The results of our investigation using the vulva mucosal injection site did not show an apparent enhanced response to pcDNA3/GFP compared to intradermal vaccination. However, since there was not a direct comparison of intradermal and intravulvamucosal vaccination within the same experiment, we cannot draw firm conclusions. We did find immune stimulation resulting from intravulvamucosal vaccination with pcDNA3/Protein A, confirming that the vaccination route is effective in dairy cows. Surprisingly, there was little response to the cotransfected pcDNA3/GFP, even though we had previously shown the ability of this plasmid to stimulate the production of antibodies when given intradermally and i.m. If the negligible immune response to GFP was the result of deficient interactions between DNA and immune components at the injection site, the response to Protein A would likely not have occurred. Another unknown consideration is that GFP may not be as effective an immunogen as Protein A.

The humoral immune response stimulated by intravulvamucosal vaccination of pcDNA3/Protein A and pcDNA3/GFP vectors was measured by assessing serum antibodies using an ELISA, while the cellular response was determined by a lymphocyte proliferation assay. The results of these assays showed that animals vaccinated with Protein A DNA elicited a significantly higher antibody response than the nonvaccinated controls. Interestingly, the two individuals producing GFP specific antibody also showed the greatest antibody production against Protein A DNA. This humoral response was not predictive of the cellular response stimulated by vaccination in either GFP or Protein A DNA injection.

When comparing the cellular and humoral responses to intravulvamucosal injection in general, Protein A DNA injection elicited a response in more animals. Six of 10 animals elicited a humoral response to Protein A compared to two of 10 for GFP. Four of 10 individuals responded with cellular immunity to Protein A DNA as opposed to no responders with GFP DNA. The pcDNA3/GFP vector was used in preliminary trials to compare intramuscular and intradermal injection methods, resulting in the stimulation of a humoral response in three of five animals for most treatment groups. Therefore, this plasmid is known to be immunogenic in dairy cows. One possible contributing factor to the differences in the response to the GFP DNA injection may be a variation between batches of the plasmid attributed to contamination by residual lipopolysaccharide, a component of the *E. coli* cell wall known to effect immune stimulation. Although the same pcDNA3/GFP plasmid

was used for all vaccinations, two different preparations were performed, the first for the intradermal and intramuscular trials and the second for the intravulvamucosal trial.

Alternatively, the contrasting responses between the intradermal and intravulvamucosal injections may be a result of the differing concentrations of plasmid DNA injected. While the concentration of pcDNA3/GFP remained the same for both injections, the total plasmid concentration was doubled for intravulvamucosal vaccination with the addition of an equivalent amount of pcDNA3/Protein A. The volume injected was also doubled, and as a result, intravulvamucosal-injected animals received a final dose of 2.0 mg of plasmid DNA, while 0.5 mg of pcDNA3/GFP was injected intradermally. When considering *in vitro* transfection of mammalian cells, an optimal DNA concentration is necessary to achieve maximum transfection efficiency. Exceeding this optimal DNA concentration can result in high levels of DNA precipitate on the cell surface leading to cell death (Gluzman, 1981). In our laboratory, we observed a decrease in protein production corresponding to an increase in DNA concentration (data not shown). In addition, an increase in the dose of plasmid DNA injected may have accentuated a localized cytotoxic response through cellular mechanisms stimulated by the bacterial CpG sequences within the DNA backbone. CpG motifs may serve as a danger signal to the mammalian immune system. Coinjection of CpG motifs has been reported to increase the proliferative response as well as INF- γ production, both indications of cellular immunity (Brtko et al., 2000). However, further consideration is necessary before we can conclude that the increase in DNA concentration is a contributing factor to the differing immune responses observed. In pigs, injection of 1200 μ g of plasmid DNA encoding multiple antigens resulted in high levels of antibody production as well as a cellular immune response that proved to be partially protective against challenge with a pseudorabies virus (van Rooij et al., 1998). In a human trial, volunteers were injected with 2500 μ g of plasmid DNA encoding multiple *P. falciparum* proteins (Doolan and Hoffman, 2001). This work demonstrated that this dose was safe and well tolerated, although the immune response was less than ideal.

Progress in recent years has been made with DNA-based vaccine technology with the introduction of DNA prime/protein boost regimes. The success of this strategy is attributed to effective priming of numerous immune cells followed by a comparatively large dose of purified protein. Upon injection of DNA, small amounts of protein are produced by transfected cells stimulating interaction with antigen presenting cells at the site of injection or at distant sites, such as the spleen or lymph

nodes (LaCava et al., 2000). Once in the lymph organs, antigen presenting cells interact with many lymphocytes and stimulate them to become effector cells in an immune response specific for the antigen encoded by the DNA. Because injection of the purified protein introduces a considerably greater dose of antigen than can be encoded by further injection of DNA, the purified protein in a booster vaccine stimulates a large population of lymphocytes that are primed to respond. Thus, a greater immune response is stimulated by this strategy than with either DNA or protein only. An additional benefit of this strategy is that smaller amounts of DNA are required to elicit a protective response against challenge (Degano, 2000), thereby demonstrating the potential for more cost-effective vaccine production. In the current investigation, we did not observe an enhanced response to the protein A booster injection in animals previously vaccinated with the Protein A gene compared to nonvaccinated controls. However, the previously vaccinated animals did have higher anti-Protein A antibody levels.

CONCLUSIONS

In summary, our results indicate that specific immune responses can be stimulated in dairy cattle using plasmid DNA-based vaccines. Although our findings did not reflect high antibody titers in response to either GFP or Protein A DNA, the data we reported was not dissimilar to other work previously done in cattle (Guidry et al., 1994; Braun et al., 1999; Wagter et al., 2000). A number of other factors including the site and method of injection, the inclusion of an adjuvant, and the concentration of DNA in the vaccine appear to affect the immune response. In addition, genetic variation, health status, or previous antigen-specific exposure may alter the immune response stimulated by immunization. Taken together, these factors may have contributed to the inconsistent immune response we observed to the DNA-encoded antigen. Our work supports that of others who have found that intradermal injection in cattle is superior to intramuscular injection. The intravulvumucosal injection site was found to be readily accessible, well tolerated, and effective for delivery of DNA-based vaccines by high-pressure jet-injection. While the vaccine formulation requires further refinement, this preliminary work suggests that intravulvumucosal vaccination with the pcDNA3/Protein A vector may prove to elicit a protective immune response against challenge with *S. aureus* in future investigations.

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