Persistency of Adenoviral-Mediated Lysostaphin Expression in Goat Mammary Glands

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ABSTRACT

Gene therapy has great potential to enable synthesis of protein molecules in targeted cells of an animal. One application may be the production of antibacterial enzymes by the mammary gland as a means of preventing or treating mastitis. We have previously demonstrated that goat mammary cells are capable of producing lysostaphin, an antistaphylococcal enzyme, after being transduced in vivo with a recombinant adenoviral vector containing a modified lysostaphin gene (*Ad-lys*). The current study examined duration of expression, and antibody response to lysostaphin and the adenoviral vector. Following intramammary infusion into nonlactating goats ($n = 4$), recovery of transducible adenoviral vector in mammary secretions persisted for 11 d. Transducible vector was not detected in serum, saliva, urine, or feces. Peak lysostaphin concentrations ($< 20 \mu\text{g/mL}$) in mammary secretions of infused udders were detected approximately 1 wk postinfusion, and generally returned to undetectable levels after an additional 1 to 2 wk. The poor persistency of expression was likely due to the very potent immune response to both the adenovirus and the expressed lysostaphin. Serum IgG antibodies recognizing the adenoviral vector developed within 7 d of the infusion, and titers rose dramatically to greater than $1:1 \times 10^5$. Similar titers of serum IgG antibodies to lysostaphin developed in 3 goats, with more moderate titers in the fourth goat. The antibody response to lysostaphin was delayed by approximately 4 d in comparison to the response to the adenovirus. Serum IgG antibody profiles were reflected in mammary secretions. No IgA antibodies to adenovirus or lysostaphin were detected in sera or mammary secretion. We demonstrate that while the lysostaphin gene can be introduced to the mammary gland using an adenoviral-mediated gene transfer technique, the strong

immune response that it provokes makes the approach unsuitable for combating mastitis.

(**Key words:** adenovirus, lysostaphin, mastitis, antibody)

Abbreviation key: *Ad-lacZ* = adenoviral vector containing the *E. coli lacZ* gene, *Ad-lys* = adenoviral vector containing a modified lysostaphin gene, **BAV-3** = bovine adenovirus type 3, **BHV-1** = bovine herpesvirus type 1, **HD-Ad** = helper-dependent adenoviral vector, **FIX** = factor IX, **gD** = glycoprotein D, **PBS-T** = PBS-0.1% Tween 20, **PBS-T-G** = PBS-0.1% Tween 20-0.1% gelatin.

INTRODUCTION

Intramammary infections are of great detrimental burden to the dairy industry. While there are numerous mastitis-causing pathogens, *Staphylococcus aureus* is one of the most frequently isolated bacterial pathogens and often results in a chronic state of infection (Nordhaug et al., 1994). Current strategies to protect against *S. aureus* mastitis rely on preventative hygiene, prophylactic and therapeutic use of antibiotics, and ultimately on removal of infected animals from the herd. New strategies are being explored with the goal of providing the cells of the mammary gland with a new gene that will enable them to produce the antistaphylococcal enzyme, lysostaphin (Kerr et al., 2001; Fan et al., 2002). Lysostaphin has a potent staphylolytic activity and has been shown to be an effective therapy in experimentally induced murine and bovine mastitis (Bramley and Foster, 1990; Oldham and Daley, 1991; Ulmer et al., 1993). In a previous study (Fan et al., 2002), we demonstrated that a recombinant, replication-deficient human adenoviral vector containing a modified lysostaphin gene (Kerr et al., 2001) could transduce bovine mammary epithelial cells in vitro and goat teat canal epithelium in vivo. Bioactive lysostaphin was detected in mammary secretions; however, as part of the experimental design, the animals were euthanized after 2 d for evaluation of the mammary tissue. The objective of the current study was to measure the persistency of intact viral

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particles in mammary secretions, the duration of lysostaphin expression, and to determine whether an antibody response was stimulated by the procedures.

Adenoviral-mediated gene transfer has been used experimentally to deliver a variety of exogenous genes to various target cells in vivo as a prelude to correcting hereditary deficiencies of functional proteins such as cystic fibrosis transmembrane conductance regulator (Engelhardt et al., 1993), ornithine transcarbamylase (Ye et al., 1996), human factor IX (**FIX**) (Dai et al., 1995), human factor VIII (Connelly et al., 1996), and dystrophin (Clemens et al., 1996). Poor persistence of transgene expression and marked host immune responses against both the adenovirus and/or the transgene products have been observed in most adenoviral-mediated gene transfer experiments (Xiang et al., 1996; Yang et al., 1996). The host immune response diminishes the persistence of transgene expression, but a number of reports indicate that expression of the foreign protein may last a few months (Connelly et al., 1996; Ye et al., 1996). This length of time, for mammary expression of lysostaphin, may be suitable for protection of cows against staphylococcal infection during the mastitis susceptible nonlactating period between lactations, or as a means to protect animals prior to their first lactation.

MATERIALS AND METHODS

Transduction of Goat Mammary Gland Cells in Vivo

Replication-deficient adenoviruses containing either the *E. coli lacZ* gene (**Ad-lacZ**) or a modified version of the *S. simulans* lysostaphin gene (**Ad-lys**) were previously described (Fan et al., 2002). The vectors were constructed from the pAvS6a shuttle plasmid (Smith et al., 1993) and an E3 deletion mutant of human adenovirus type 5 (Ad-dl327). The resulting E1-, E3-deleted, replication-deficient adenoviruses were propagated in the human adenovirus type 5 transformed 293 packaging cell line (CRL 1753, American Type Culture Collection, Manassas, VA) and purified by density gradient ultracentrifugation (Rosenfeld et al., 1992). Four nonlactating, multiparous, Toggenburg goats were purchased locally and housed individually in metabolism crates for a 2-d acclimation period and for 14 d following intramammary infusion. After 14 d the crates allowed for total collection of urine and feces that were subsequently autoclaved before disposal or incinerated. The goats were then group housed in an 18-m² pen with straw bedding that was subsequently disposed of in typical agricultural practice. At all times the animals had free access to feed and water. During the trial, for all mammary infusions and sample collections, teats were routinely prepiped with alcohol and postdipped

with iodine to prevent mammary gland infection. Housing and care was conducted in accordance with the Office of Animal Care Management, following approval by the University of Vermont IACUC.

On d 0, after collecting preinfusion samples (blood, urine, feces, saliva, and mammary gland secretions), the right teat of four goats received 5 mL of 2×10^{10} pfu/mL Ad-lys in infusion diluent (10 mM Tris pH7.4, 1 mM MgCl₂, with 10% glycerol). Left teats received 5 mL of 2×10^{10} pfu/mL Ad-lacZ (three goats) or 5 mL of infusion diluent (Archer et al., 1994) as a negative control. Blood, urine, feces, saliva, and mammary gland secretions were collected on d 2, 4, 7, 11, 14, 21, 28, 35, and 42 post-infusion. On d 42 the goats were euthanized. All mammary secretion samples were examined for bacteriological contamination and SCC by the University of Vermont's Quality Milk Research Laboratory.

Detection of Transducible Adenovirus in Goat Secretions

The presence of transducible Ad-lacZ particles in test samples was monitored by β -galactosidase expression in replication permissive 293 cells exposed to the samples. Sample transduction medium was prepared by adding 50 μ L of sample (either mammary gland secretion, serum, urine, or dissolved feces) to 450 μ L of medium containing 2% fetal bovine serum (GIBCO, Grand Island, NY). Saliva samples collected on cotton-tipped applicator swabs were immersed into 500 μ L of transduction medium. A series of standards containing known quantities of transducible adenoviral particles was used to monitor transduction efficiency and to estimate the concentration of transducible adenovirus in the samples. Confluent monolayers of 293 cells cultured in 6-well dishes were incubated with 500 μ L of either standard or sample transduction medium (Fan et al., 2002). After exposure for 1.5 h at 37°C in a 5% CO₂ atmosphere, complete medium (10% FBS) was added and the cells were incubated for 24 h to allow production of β -galactosidase. Detection of the β -galactosidase was then performed (Fan et al., 2002).

Analysis of Lysostaphin in Mammary Gland Secretions

Immunological lysostaphin concentrations in mammary secretions were determined by ELISA and bioactive concentrations determined by staphylocytic assay (Fan et al., 2002). Recombinant lysostaphin (Sigma) was used as standard.

Detection of Antibodies in Sera and Mammary Gland Secretions

Antibodies to adenovirus and lysostaphin in sera and mammary gland secretions were detected by indirect ELISA. A dilution series of serum samples were prepared in PBS containing 0.1% Tween 20 and 0.1% gelatin (**PBS-T-G**). Mammary secretion samples were analyzed for total protein (Bio-Rad, Richmond, CA) then initially diluted in PBS-T-G to a protein concentration of 25 $\mu\text{g/mL}$. A subsequent dilution series was then prepared in PBS-T-G for analysis. The diluted serum and secretion samples were stored at 4°C for 12 h. High binding EIA/RIA plates (Corning) were coated with recombinant lysostaphin (1 $\mu\text{g/mL}$) or Ad-*lacZ* (5 μg of protein/mL) in 0.05 M sodium carbonate buffer (pH 9.6) at 4°C for 12 h. These plates were washed three times with PBS containing 0.1% Tween 20 (**PBS-T**) and blocked with PBS-T-G at room temperature for 1 h. The plates were washed and incubated for 2 h with 100 μL of diluted serum or mammary secretion. After incubation the plates were washed with PBS-T and incubated for 2 h at room temperature with anti-goat IgG or IgA, peroxidase conjugate (Sigma) diluted in PBS-T at dilution factors of 10,000 (for serum samples) or 20,000 (for mammary secretions). Plates were then washed and bound enzyme was detected by color development (OD_{405}) following addition of 3, 3', 5, 5' tetramethylbenzidine dihydrochloride (Sigma, St. Louis, MO). Preinfusion values were considered as background and were subtracted from values obtained from postinfusion samples. Antibody titer was defined as the highest dilution of the sample that gave OD_{405} values twice that of the background.

RESULTS

The goat teats appeared normal during the experiment period with the exception of moderate swelling on d 2 in two of the four goats. The swelling in one goat disappeared by d 4, and in the other by d 14. A mild coagulase-negative staphylococcal infection was detected in the left teat of one goat preinfusion, which persisted until the end of experiment. No bacterial infection was detected in other glands prior to or during the experiment.

Presence of Viable Ad-*lacZ* Virus in Secretion Samples

At no time following intramammary vector infusion was Ad-*lacZ* detectable in the saliva, serum, urine, or feces. However, Ad-*lacZ* was detectable in mammary secretions from all Ad-*lacZ* treated glands until d 11. An approximation of the Ad-*lacZ* particle concentrations in

Figure 1. Lysostaphin concentrations in mammary secretions obtained from glands of four goats following intramammary infusion of adenovirus (10^{11} pfu/gland) containing the lysostaphin gene (goat A, $\blacklozenge$; goat B, $\blacklozenge$; goat C, $\triangle$; goat D, $\times$).

secretions was determined by comparing the numbers of *LacZ* -expressing 293 cells resulting from exposure to dilutions of mammary secretions with the numbers of *LacZ* -expressing cells resulting from exposure to known quantities of virus. Ad-*lacZ* particle concentrations in the mammary secretions were approximately 10^9 , 10^7 , and 10^5 pfu/mL on d 2, 4, and 7, respectively, with no particles detectable on d 11.

Lysostaphin in Mammary Gland Secretions

Lysostaphin was not detectable in non Ad-*lys* treated mammary gland secretions. Lysostaphin expression in Ad-*lys* treated glands increased gradually after infusion and peaked at d 7 (3 goats) or 11, then declined dramatically (Figure 1). The lysostaphin concentrations returned to basal levels in 3 goats after 14 d and by d 21 in the fourth goat. Examination of the ELISA used to quantitate lysostaphin indicated that detection of lysostaphin added to preinfusion secretion (10 $\mu\text{g/mL}$) was 92%, while detection of lysostaphin added to postinfusion secretion ranged from 28 to 90%. The assay interference was likely due in part to antibodies to lysostaphin that were present in the postinfusion secretions (see below). Consequently, lysostaphin concentrations are underestimated by ELISA in postinfusion secretion. The highest lysostaphin bioactivity, observed as a zone of lysis on a lawn of *S. aureus*, was 0.1 $\mu\text{g/mL}$ (data not shown). Some reduction in bioactivity was expected as the modified lysostaphin produced by mammalian cells has previously been shown to have approximately 20% of the activity of the recombinant, bacterially produced form (Kerr et

Figure 2. Serum anti-lysostaphin IgG in 1:1000 dilute samples obtained from four goats following intramammary infusion of adenovirus (10^{11} pfu/gland) containing the lysostaphin gene (symbols for each animal as in Figure 1).

al., 2001; Fan et al., 2002). The substantial reduction bioactivity may be related to the presence of neutralizing antibodies in the secretion samples.

Antibody Response to Lysostaphin and to Adenovirus

Serum IgG reactive to lysostaphin was observed in all animals 11 d after the infusion of Ad-lys, and in 3 animals the levels remained elevated for the entire 42-d duration of the experiment (Figure 2). Serum antibody titers to lysostaphin reached 1:50,000 or greater in 3 of the 4 goats (Table 1). A more moderate titer of 1:1600 developed in the fourth goat. Anti-lysostaphin titers in mammary secretions were similar. Of note is that the animal with the lowest anti-lysostaphin response achieved the greatest lysostaphin concentrations in mammary secretions. Also, the titers in both glands were similar even though the Ad-lys was only infused into the right-hand gland.

Figure 3. Serum anti-adenovirus IgG in 1:5000 dilute samples obtained from four goats following intramammary infusion of adenovirus. Adenovirus containing either the lysostaphin gene or the *lacZ* gene was infused into each gland with (10^{11} pfu/gland), except for goat D, which only received adenovirus containing the lysostaphin gene in one gland and diluent in the other (goat A, $\blacklozenge$; goat B, $\diamond$; goat C, $\triangle$; goat D, $\times$).

The serum antibody response to adenovirus was also substantial, but developed even more rapidly than the anti-lysostaphin response and was consistent in all 4 animals (Figure 3). Within 7 d of the infusion, a marked anti-adenovirus response was detected in all animals. In comparison, the anti-lysostaphin response required 14 to 21 d to fully develop. Serum IgG titers to adenovirus of 1:800,000 were consistently detected (Table 1). Similar titers were also detected in mammary secretions. Antibodies of the IgA isotype against either adenovirus or lysostaphin were not found in goat sera or mammary secretions.

DISCUSSION

We have previously reported transduction of cells lining the teat cistern in Ad-*lacZ* infused goat mammary glands, and the presence of lysostaphin in mammary

Table 1. Maximum antibody titers in goat sera and mammary secretion samples obtained over a 42-d period following intramammary infusion of adenovirus. The right gland in all goats was infused with adenovirus containing the lysostaphin gene (10^{11} pfu/gland). The left gland in goats A, B, C was infused with adenovirus containing the *lac-Z* gene (10^{11} pfu/gland), while the left gland of goat D was infused with diluent. Antibody titers represent the largest dilution of sample resulting in assay OD₄₀₅ values of twice of the background.

Goat	Serum antibody titers		Left mammary gland secretion antibody titers		Right mammary gland secretion antibody titers	
	Anti-adenovirus	Anti-lysostaphin	Anti-adenovirus	Anti-lysostaphin	Anti-adenovirus	Anti-lysostaphin
A	800,000	100,000	800,000	100,000	800,000	800,000
B	800,000	100,000	800,000	100,000	800,000	800,000
C	800,000	1600	800,000	6400	800,000	6400
D	800,000	50,000	800,000	3200	800,000	400,000

secretions of Ad-lys infused glands (Fan et al., 2002). In that study, the animals were infused with 1 mL of solution containing 1.5×10^{10} pfu of Ad-lys, and were euthanized 48 h postinfusion for tissue collection and examination. We have now examined an extended time course of lysostaphin production following transduction with the same recombinant adenovirus but administered at a higher dose of 5 mL of 2×10^{10} pfu/mL. The higher dose had minimal effect on the average concentration of lysostaphin in mammary secretions obtained 48 h postinfusion that was approximately 1 $\mu\text{g/mL}$ in both studies. Further studies would be required to determine whether the number of epithelial cells transduced differed in the two studies. As adenoviral DNA generally does not integrate into the host cell chromosome, we expected only transient expression of the lysostaphin gene. However, a number of reports indicate that expression can persist for 1 to 2 mo (Connelly et al., 1995, 1996; Chen et al., 1997), which may be ideal for short-term protection of the mammary gland from *S. aureus* infection.

In the current study we have found that although lysostaphin concentrations in mammary secretion increase until approximately 7 d posttransduction, the persistency of lysostaphin production was relatively short-lived. Lysostaphin concentrations approached nondetectable levels 21 d postinfusion. The peak concentrations observed were on average less than 10 $\mu\text{g/mL}$, and only limited bioactivity could be detected. The native form of lysostaphin has very potent staphylocytolytic activity that can easily be detected at a concentration of 0.01 $\mu\text{g/mL}$. The lysostaphin encoded by the adenovirus has been modified by 2 amino acid substitutions to enable production of an active form by mammalian cells (Kerr et al., 2001). The modified form has approximately 20% of the activity of the native form. Our inability to detect more bioactivity in the secretions was likely due to neutralizing antibodies in the secretions. It seems unlikely that the moderate level of lysostaphin produced in the current study would be of benefit in preventing *S. aureus* mastitis.

An aggressive immune response to both lysostaphin, and the adenoviral vector undoubtedly contributed to the short-lived ability of the transduced cells to produce lysostaphin. This was reflected by the apparent inverse relationship between the profiles of lysostaphin concentrations in mammary secretions and the serum titers of anti-lysostaphin IgG. For 3 of the goats, lysostaphin concentrations in mammary secretions reached peak at d 7, while serum antibodies to lysostaphin were still undetectable. Between d 7 and 11, the antibody levels climbed, while lysostaphin concentration in secretions dropped dramatically. A similar response, albeit delayed, was observed in the fourth goat. Of note is the

finding that the goat whose mammary secretions obtained the highest concentration of lysostaphin was also the goat with the lowest lysostaphin antibody titers. The potent immune response to lysostaphin was unexpected in consideration of a previous study in cattle that showed minimal antibody response in animals given up to 27 intramammary infusions of recombinant lysostaphin over a 9-mo period (Daley and Oldham, 1992). These authors concluded that the intramammary administration of recombinant lysostaphin to the bovine was relatively nonimmunogenic. However, in the present study, lysostaphin was synthesized within the transduced cells of the animals as opposed to exogenous delivery of protein. In agreement with the present study, a substantial anti-human FIX response was observed in monkeys following intravenous administration of an adenoviral vector encoding FIX, whereas a much lower response was produced by administering the purified protein (Lozier et al., 1999). Endogenous synthesis of foreign proteins is known to stimulate both humoral and cell-mediated immune responses, and has been demonstrated in numerous studies exploiting this finding in the development of DNA-based vaccines (Donnelly et al., 1997).

Poor persistence of transgene expression and substantial host immune responses are related. The importance of the immune response in limiting transgene expression has been clearly demonstrated in mice injected with adenoviral vectors containing the canine FIX gene. In one study, Dai et al. (1995) injected mouse legs with adenoviral vectors containing the FIX gene and analyzed FIX production in mouse plasma by ELISA. They found that FIX concentrations peaked at 0.1 $\mu\text{g/mL}$ 5 to 6 d postinfusion and retreated rapidly to basal levels after 10 d. In comparison, similar treatment of nude immune-deficient mice resulted in peak FIX levels that were approximately 10-fold higher and persisted for more than 300 d. Different viral vectors are now being developed for use in human gene therapy. One goal is to further reduce the quantity of viral protein produced and thus limit the immune response. In one type of vector, helper-dependent adenoviral vector (**HD-Ad**), almost all of the adenoviral genome has been deleted, resulting in complete elimination of viral protein coding sequences (Morsy et al., 1998). This vector retains high transduction efficiency yet also elicits a diminished host immune response. More recently, Kim et al. (2001) reported that HD-Ad mediated transfer of an apolipoprotein E gene into apolipoprotein E-deficient mice resulted in lifetime (2.5 yr) correction of the hypercholesterolemia associated with the genetic defect. The toxicity associated with the treatment was negligible. Perhaps the HD-Ad system will also be appropriate for cattle.

Our findings are in agreement with other research groups that have been exploring the vaccine potential of replication-deficient human adenovirus that has been reported to stimulate potent immune responses in mice (Xiang et al., 1996), rats (Mittal et al., 1996), cats (Gonin et al., 1995), chickens (Adam et al., 1995), monkeys (Sullivan et al., 1997), and calves (Gogev et al., 2002). The recent experiments in calves demonstrated the development of a very potent neutralizing antibody response to the antigen (bovine herpes virus-1 glycoprotein) encoded by the recombinant vector. Interestingly, the antibody response to the vector was relatively poor, even after a second immunization given 21 d after the first. In contrast, in our present study with mature goats, the immune response to the adenovirus itself was more rapid, more potent, and more consistent than the response to lysostaphin. Antibody titers soared between 4 and 7 d postinfusion. The magnitude and speed of response suggests that the goats had been preexposed to the antigen and had an existing pool of memory B cells. The doses of vector were similar between the 2 studies, although methodological differences in vector preparation might have affected potency. Other potential differences between the studies that may have contributed to the different responses are the species difference (bovine vs. goat), immunological history of the animals, and/or the site of vector administration, intranasal in the calves, intramammary in the goats. The mammary gland is a very active site of immune surveillance as evidenced by the extensive draining lymph nodes. A further difference is the persistence of shedding of transducible vector. In the calve study vector was only detected in nasal swabs in 3 of 15 inoculated animals, and then only for 1 or 2 d. In contrast, the goat mammary secretion contained considerable vector (10^5 pfu/mL) 7 d postinfusion. Perhaps the thick consistency of the mammary secretion provided a depot-like effect allowing for extended time course of transduction of the epithelial cells.

CONCLUSION

Our results indicate that an adenoviral-mediated gene therapy strategy to combat mastitis is not currently feasible due to low expression persistency and potent immune responses to both the vector and the to encoded therapeutic protein. New vectors are being pursued by numerous groups for use in human gene therapy and might be of use in mastitis prevention. However, development of a strategy to avoid an immune response to the therapeutic protein itself will be a challenge. In fact, with the appropriate encoded antigen, the very potent immune response that develops after intramammary infusion of a recombinant adenoviral

vector may form the basis for future vaccine development.

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