

Mammary expression of new genes to combat mastitis

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ABSTRACT: Continual advances in the ability to produce transgenic animals make it likely that such animals will become important components of animal agriculture. The full benefit of the technology, and justification of its initial cost outlay, will be dependent on the establishment within these animals of new traits not easily achievable by other means. Potential applications include enhanced nutrient digestibility with reduced fecal losses, significantly altered milk composition with superior nutritional properties, and enhanced disease resistance. Our goal is to enhance mastitis resistance of dairy cows by enabling the cells of the mammary gland to secrete additional antibacterial proteins. Proof of concept has been obtained through experimentation with a transgenic mouse model. Three lines of mice were developed that produce varying levels of lysostaphin in their milk. This protein has potent anti-staphylococcal activity and its secretion into milk confers substantial resistance to infection caused by intramammary challenge with *Staphylococcus aureus*, a major mastitis pathogen. Additional antibacterial proteins

are being sought that will complement lysostaphin. A potential benefit of transgenic application of antibacterial proteins is the concomitant sparing in the agricultural use of antibiotics currently used as human therapeutics. Antibacterial proteins, such as lysostaphin, are not typically used as injectable or oral therapeutics because of immune-mediated or digestive destruction of their activity. In contrast, the immune system of transgenic animals will not consider the transgenic protein as being foreign. In addition we are exploring the potential of involution or mastitis responsive promoter elements for use in subsequent transgenic experiments designed to restrict lysostaphin production to these important time points. It is anticipated that genomics will play a role in unveiling candidate genes whose promoter elements will enable desired temporal expression patterns. The transgenic approach to insertion of new genetic material into agriculturally important animals is feasible but requires extensive prior evaluation of the transgene and transgene product in model systems.

Key Words: Mastitis, *Staphylococcus aureus*, Transgenic, Mice

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Introduction

The ability to produce transgenic dairy cows opens the door to countless new strategies aimed at enhancing the efficiency of dairy production. Goals of projects include increasing milk production efficiency, enhancing feedstuff nutrient availability to the cow with reduced fecal losses, increasing milk protein content, and improving animal health through enhanced disease resistance. Advances in the ability to generate dairy cows containing new genes are continuing, and are the basis for continued optimism of this strategy. However, in addition to the substantial cost associated with the generation of founder animals, the cost of the technology in terms of time to implementation will remain enormous (Wall et al., 1997). A 5 to 10-yr time frame between

initial embryo manipulations to generation of transgenic herds of lactating cows is likely. This time does not include the effort involved in the design, assembly, and testing of appropriate gene constructs in model systems that must be performed to ensure success of the technology. Strategies for introgression of transgenes into a dairy cattle breeding population have previously been reviewed and appear economically viable for traits with major effects on net merit (Cundiff et al., 1993). This review will focus on the use of transgenic technology to enhance mastitis resistance. This is currently the most economically important disease of dairy cattle. Effects of mastitis go beyond treatment and prevention costs to include issues of animal welfare, and the impact of agriculture use of antibiotics on the development of antibiotic resistant human pathogens (Smith et al., 2002). In addition, as mastitis is clearly associated with causing apoptosis of mammary epithelial cells there is likely a substantial impact of this disease in preventing the realization of an animal's full genetic potential to produce milk.

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Mastitis

Mastitis is an inflammatory reaction of the mammary gland, usually to a microbial infection, and its prevalence is alarming. Wilson et al. (1997) recently published the results of a retrospective study of milk samples collected from more than 100,000 cows in New York and northern Pennsylvania between 1991 and 1995. They found that IMI were present in 36% of cows enrolled in the Dairy Herd Improvement Association. This disease, in addition to causing distress for the cow, is estimated to cost the producer approximately \$200/cow per year, which corresponds to a US total of \$1.7 billion. The dairy processor also incurs losses from the detrimental changes in milk composition that occur during mammary inflammation. These changes are associated with reduced cheese yields and reductions in the shelf-life of dairy products (Barbano et al., 1991; Auld et al., 1995; Auld et al., 1996; Klei et al., 1998).

Mastitis is characterized by an influx of somatic cells, primarily polymorphonuclear neutrophils (PMN), into the mammary gland and by an increase in milk protease content (Verdi et al., 1987). Clinical infections are diagnosed by red, swollen appearance of the gland and flakes or clots (protein aggregates) in the milk. Subclinical infections, by definition, show no obvious signs of disease. Five bacterial species—*Staphylococcus aureus*, *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis*, and *Escherichia coli*—are responsible for the bulk of bovine mastitis cases. *Staphylococcus aureus*, *S. dysgalactiae*, and *S. agalactiae* exhibit a contagious route of transmission, whereas *S. uberis* and *E. coli* are considered to be environmental agents. The incidence of contagious mastitis has greatly declined over the last 30 yr with implementation of a five-point control plan (Bramley and Dodd, 1984). The plan recommends use of correctly maintained milking equipment, postmilking teat disinfection, both therapeutic and prophylactic use of antibiotics, and culling of persistently infected animals. With this plan, the once-common mastitis pathogens—*S. agalactiae* and *S. dysgalactiae*—have been eliminated from many herds. However, *S. aureus*, which accounts for 15 to 30% of infections, has proven more difficult to control (Sutra and Poutrel, 1994). The cure rate for treatment of *S. aureus* infections with antibiotics is often less than 15%. This is attributed to incomplete penetration of the antibiotics throughout the gland and the potential survival of bacteria within host cells, leading to a recurrence of disease once treatment has ended (Craven and Anderson, 1984; Yancey et al., 1991). Because *S. aureus* mastitis can be induced experimentally with as few as 100 organisms, a few chronic infections within a herd can maintain a persistent bacterial reservoir. Antibiotic treatment of mastitis caused by environmental pathogens is also practiced, but recurrence of infection from environmental reservoirs is a continuing problem.

Susceptibility of the mammary gland to new IMI was markedly increased during early involution and during

the periparturient period (Nickerson 1989; Oliver and Sordillo, 1988). These infections are often associated with clinical mastitis during early lactation and can have a marked detrimental effect on subsequent milk yield and quality. Susceptibility to mastitis can also be quite high during the prepartum period of first-lactation heifers (Nickerson et al., 1995). These infections are associated with a decrease in alveolar epithelial and luminal area and an increase in connective tissue, which could potentially lead to a life-long reduction in milk yield.

Current therapies for mastitis rely heavily on the use of β -lactam antibiotics such as penicillins and cephalosporins. The parent compounds of these orally active agents are naturally produced by *Penicillium* spp. or *Cephalosporium* spp., respectively. They are bicyclic ring structures that contain a 4-membered-lactam ring fused to a five-membered thiazolidine ring (penicillin) or a six-membered dihydrothiazine ring (cephalosporin). A number of enzymatic steps are required for the synthesis of the β -lactam ring structure and although this has been accomplished chemically, these antibiotics are currently produced more economically through biosynthesis or by semisynthesis starting from fermentation intermediates (Andersson et al., 2001). These agents have had an enormously beneficial impact on dairy-animal health and milk production. However, concern that accidental exposure of susceptible consumers may produce drug-induced anaphylaxis has necessitated the imposition of a posttreatment milk discard period and strict industry surveillance of all milk shipments. Further, there is growing concern that the agricultural use of antibiotics contributes to the emergence of antibiotic resistance in human pathogens (Smith et al., 2002).

A transgenic approach to enhance mastitis resistance would enable mammary epithelial cells to produce antibacterial enzymes that, in contrast to β -lactam antibiotics, would be degraded along with other milk proteins during the digestion process and not pose a health risk to the consumer. Furthermore, as antibacterial proteins are not orally active, and are likely to be immunogenic if given by an intravenous route, they are not being widely used in human or veterinary medicine and thus pose a reduced threat for the development of organisms resistant to currently used therapeutics. There are currently a large number of bacterially derived enzymes and products approved by the FDA for use in food production (US FDA, 2001). The dairy products industry makes extensive use of these enzymes for yogurt and cheese manufacture and agents such as nisin and natamycin are used extensively in food manufacture for antibacterial and antifungal purposes, respectively.

Transgenic Approach to Enhance Mastitis Resistance

The use of transgenesis to direct expression of a foreign protein into mouse milk was first reported in 1987

(Gordon et al., 1987). Shortly thereafter, it was proposed that mammary production of trout lysozyme II (Grinde 1989) or bacterial lysostaphin (Bramley and Foster, 1990) would be an effective means to enhance mastitis resistance. Both of these proteins have considerable antistaphylococcal activity. However, the initial applications of the technology were the generation of transgenic mice producing human lysozyme (Maga et al., 1994) or human lactoferrin (Platenburg et al., 1994) in milk.

Milk from various lines of the lysozyme-transgenic mice contained approximately 0.5 mg/ml of human lysozyme (Maga et al., 1995). Bioactivity of the lysozyme, as detected by its ability to degrade *Micrococcus lysodeikticus* cell walls, was retained. Limited, but significant bacteriostatic activity of the milk against *S. aureus*, but not a mastitis-causing clinical isolate of *E. coli*, was also found (Maga et al., 1998). Further, the lysozyme-containing milk also slowed the growth of the cold spoilage organisms *Pseudomonas fragi* and *Lactobacillus viscosus*, indicating the potential of this strategy to also address issues of product shelf life and food safety. Interestingly, the lysozyme also altered the physical and functional properties of the milk. Rennet clotting time was reduced and rennet-induced casein gel strength was increased. Clearly, the potential exists that the production of foreign proteins in milk of dairy cows could positively or negatively affect its antibacterial or functional characteristics. Unfortunately, human lysozyme has very limited potency against *S. aureus*. We find no indication of activity when up to 1 mg/ml aliquots (15 μ l) of human lysozyme in PBS are spotted onto bacterial plates that have just previously been streaked with *S. aureus*. After an overnight incubation, a fully confluent lawn of bacteria develops. In contrast, similarly applied lysostaphin at a concentration of 1 μ g/ml results in a completely clear zone. Both lysostaphin and human lysozyme are ineffective against *E. coli* and *S. uberis* isolates obtained from mastitis milk. Lysozyme from hen egg white also has very limited antistaphylococcal activity but has been shown to substantially enhance the activity of lysostaphin (Cisani et al., 1982).

Transgenic technology has resulted in the production of mice and cows that secrete human lactoferrin into their milk (Platenburg et al., 1994; van Berkel et al., 2002). Lactoferrin is an iron-binding glycoprotein found in most exocrine secretions including tears, saliva, and milk, and there are numerous reports of its antibacterial activity in vitro and in vivo (Nuijens et al., 1996; Nibbering et al., 2001). Although bovine milk normally contains low levels of lactoferrin, the transgenic cows were generated as potential bioreactors to produce large quantities of recombinant hLF (**rhLF**) for applications in human health care. The human form of the protein was chosen to potentially limit immunogenic reactions in humans treated via an intravenous route with human lactoferrin purified from bovine milk. The transgenic cows, which produce approximately 1 g of

rhLF per liter of milk, have now supplied sufficient quantities of rhLF for thorough evaluation (van Berkel et al., 2002). The rhLF from cows was essentially identical to natural hLF except for a different glycosylation pattern. Importantly, both natural hLF and bovine-derived rhLF were equally effective in three different mouse infection models. The models involved infection of mice with *K. pneumoniae* or a multidrug-resistant strain of *S. aureus* followed by intravenous administration of 50 μ g of rhLF or natural hLF. In all cases, a substantial reduction in recovery of viable organisms was observed as compared to control animals. In one model, a substantial dose of *S. aureus* (10^6 cfu) was injected i.m. into the hip muscle with rhLF (50 μ g) given i.v. 24 h later. Up to 99% reduction in recovery of *S. aureus* from the injected muscle was found 24 to 48 h following rhLF injection. The cows that produce the rhLF are reported to have normal milk production, composition, and SCC. The ability of these animals to resist mastitis has not been reported. Presumably, the pharmaceutical company backing the production of these animals has not wanted to jeopardize their lactational capabilities with a deliberate challenge of mastitis causing pathogens. With three fertile transgenic bulls now available it will likely not be long before mastitis challenge studies are conducted on their daughters.

The potency of bovine lactoferrin (**bLF**) against mastitis causing organisms is not very great, and is clearly not sufficient to prevent mastitis. Purified bLF has very limited antistaphylococcal activity even at concentrations of 1 mg/ml, although there is indication that it enables the activity of β -lactam antibiotics, such as penicillin G (Diarra et al., 2002). The mechanism of lactoferrin activity has not been clearly defined but appears to stem primarily through iron sequestration or through direct interaction of its cationic *N*-terminal region with bacterial components. The contribution of bLF to protection of the mammary gland is compromised by its low concentration in milk and the presence of other milk constituents. Thus, milk citrate can effectively compete with lactoferrin for iron binding and the resulting iron-citrate complex can be utilized by bacteria (Schanbacher et al., 1993). Action of the cationic *N*-terminal region of lactoferrin in milk may be inhibited in much the same manner as we observe inhibition of a potent, cationic antimicrobial peptide, tachyplesin, when diluted in milk (unpublished observation). The inhibitory effect may be related to the abundance of anionic, phosphorylated casein molecules binding the cationic peptide, or insertion of the peptide into milk fat globule membranes. Interestingly, bovine lactoferrin, but not hLF has been shown to inhibit the growth of bovine mammary epithelial cells in vitro (Hurley et al., 1994). Thus, overexpression of bovine lactoferrin does not seem to be a candidate for enhancing mastitis resistance, although its gene regulatory region may be suitable to direct expression of new antibacterial proteins (see below).

In addition to the antibacterial proteins previously mentioned, there is a class of compounds—the defensins—that are produced in neutrophils, macrophages, and epithelial cells lining mucosal surfaces [for review see (Kaiser and Diamond, 2000)]. Defensins are relatively small, cationic peptides with an amphiphilic charge distribution that enables them to interact with, and disrupt, bacterial cell membranes. Their antibacterial action appears to result from their ability to form pores in target membranes leading to cell lysis. Analogous antibacterial peptides have been isolated from a diverse array of multicellular organisms including insects, amphibians, and plants and are currently being evaluated as topical antibiotics for human medicine (Zaslloff 2002).

Transgenic mice have been generated that produce small quantities (5 $\mu\text{g}/\text{ml}$) of a mammalian defensin—bovine tracheal antimicrobial peptide (**bTAP**)—in their milk (Yarus et al., 1996). The bTAP purified from milk was bioactive in vitro, but no challenge studies were reported. Transgenic mice that secrete lysostaphin into their milk provide a clear demonstration of the transgenic approach to enhancing mastitis resistance (Kerr et al., 2001). Lysostaphin is a potent peptidoglycan hydrolase naturally secreted by *Staphylococcus simulans*. The lysostaphin gene is contained on a large plasmid and encodes a preproenzyme of 493 amino acids that is processed extracellularly to a 246 amino acid mature form (Recsei et al., 1987). The activity of the enzyme is specific to hydrolysis of the polyglycine interpeptide bridges of the staphylococcal cell wall (Schindler and Schuhardt, 1964). This specificity restricts its antibacterial activity to staphylococcal species, having little effect on other mastitis-causing organisms. However, the enzyme's specificity for pentaglycine peptides also makes it an ideal candidate for use as an antibacterial in milk as it does not appear to degrade milk proteins even after 4 d at 37°C (Kerr, unpublished). Furthermore, we have found that bacterial lysostaphin, at concentrations up to 100 $\mu\text{g}/\text{ml}$, has no effect on yogurt production using bovine milk and a commercial, freeze-dried starter culture.

The potential of the lysostaphin protein for the therapeutic or prophylactic control of staphylococcal mastitis was demonstrated initially in a mouse model (Bramley and Foster, 1990) and subsequently in dairy cattle (Oldham and Daley, 1991). The application of lysostaphin, a prokaryotic protein, to a transgenic animal program first required that it be successfully produced and secreted by eukaryotic cells (Kerr et al., 2001). Transfection studies were conducted with the lysostaphin gene and the COS-7 monkey kidney fibroblast-like cell line (ATCC #CRL1651). These studies revealed that the cells could produce lysostaphin, but that it was secreted in an inactive, glycosylated form. Two potential N-linked glycosylation sites (Asn-Xxx-Ser/Thr) exist within the native lysostaphin enzyme and apparently these had become targets of the eukaryotic glycosylation machinery. We modified these sites by a PCR tech-

nique that generated asparagine to glutamine codon substitutions within the glycosylation motifs. The resulting Gln^{125,232}-lysostaphin variant produced by the COS-7 cells retained approximately 20% of the activity of the native form. Given that lysostaphin exhibits substantial bioactivity at concentrations of less than 1 $\mu\text{g}/\text{ml}$, we decided to pursue the transgenic mouse experiment knowing that production of transgenic proteins in milk at 0.1 to 1.0 mg/ml can be typically obtained.

Three lines of mice were produced that secrete the bioactive variant of lysostaphin into milk (Kerr et al., 2001). Production is under the control of the 5'-regulatory region of the ovine β -lactoglobulin (**BLG**) gene (Whitelaw et al., 1991). The concentration of lysostaphin in the transgenic mouse milk was approximately 100 $\mu\text{g}/\text{ml}$ in two of the lines and 1 mg/ml in the third line. Mice from the transgenic lines, and non-transgenic controls, were challenged on d 10 of lactation with an intramammary infusion of a strain of *S. aureus* (M60) that had been isolated from a case of bovine mastitis. Two glands per mouse were infused with a substantial dose of bacteria (10⁴ cfu/50 μl). Pups were then removed and dams returned to their cages. Twenty-four hours later the mice were euthanized, and infection status of the glands was determined by visible inspection and enumeration of viable *S. aureus* in mammary homogenates. Approximately 80% of the challenged, nontransgenic glands became heavily infected (>10⁸ cfu/gland) and showed visible signs of hemorrhagic inflammation. The remaining, challenged, non-transgenic glands were infected but to a lesser extent. In marked contrast, none of the glands from the transgenic mice were visibly infected. No bacteria survived in mammary glands from the highest expressing line and approximately 50% of glands from the other two lines were also free of infection. The infected transgenic glands in these lines contained less than 10% of the bacterial load observed in the heavily infected controls, and it is possible that these infections would have cleared given additional time. Clearly these transgenic mice will be protected from *S. aureus* mastitis when the oBLG regulatory region is active.

Another transgenic approach to enhance disease resistance involves the production of pathogen-specific antibodies into milk (Castilla et al., 1998; Sola et al., 1998; Kolb et al., 2001). To date, the primary goal of these projects has been to supply antibodies to offspring and thus provide enhanced passive immunity. However, with these projects as proof of concept it is not inconceivable that antibodies to mastitis causing pathogens could enhance protection of the mammary gland. Two of the reports indicate the potential of producing coronavirus neutralizing antibodies into milk under the control of the 5'-regulatory regions of the murine whey acidic protein (WAP) gene (Castilla et al., 1998) or the oBLG gene (Sola et al., 1998). In both instances transgenic mice secreted functional antibody into milk as determined by an in vitro neutralization assay. The production of functional antibodies was made possible

by the coinjection of two constructs encoding light and heavy chain variable regions, respectively, cloned from a specific monoclonal antibody producing hybridoma cell line. The beauty of the technique is that a specific high titer antibody is first produced and characterized by hybridoma technology, and then the appropriate gene is cloned for the production of transgenic animals. A recent report describes the production of an encephalitis-neutralizing antibody using similar technology (Kolb et al., 2001). In this case the antibody was transferred to offspring via the milk and conferred full protection to an experimental challenge with an otherwise lethal dose of the virus. Application of this technology to enhance mastitis resistance awaits generation of the appropriate hybridoma cell line.

One other reported transgenic model that may find application in mastitis resistance is the over-expression of the polymeric immunoglobulin receptor (**pIgR**) gene in mammary epithelial cells (de Groot et al., 2000). This receptor transports immunoglobulin A (IgA) across epithelial layers and into the secretions of various mucosal tissues including the mammary gland. The receptor binds circulating IgA at the basolateral side of the cell then transports it via transcytosis secretory component into the secretion. Secretory IgA is not naturally a major component of bovine milk perhaps due to a deficit of the pIgR. In transgenic mice, expression of 60- up to 270-fold above normal levels of pIgR resulted in only a modest 1.5- to 2-fold higher levels of total IgA in milk. The discrepancy suggests a shortage of circulating IgA available for transport. An interesting finding from this experiment was that milk from the highest pIgR expressing line of mice was significantly altered (de Groot et al., 2001). This milk contained little if any κ -casein and contained substantial quantities of a non milk protein identified as serum amyloid A-1. These mice were unable to support the growth of suckling offspring. The mechanism of the transgene effect on the endogenous proteins is unknown. It could result from over expression of the transmembrane receptor protein or be a function of the integration site within the genome. The lack of effect on other milk proteins suggest that it is in some fashion specific to κ -casein rather than a general effect on transcriptional ability of the mammary gland. Clearly milk from transgenic animals will have to be extensively characterized prior to its approval for human consumption.

Candidate Antibacterial Proteins for Mastitis Resistance

Our goal is to enhance mastitis resistance of dairy cows by enabling the cells of the mammary gland to secrete additional antibacterial proteins. The proteins that we are focusing on are enzymes—such as lysostaphin—that are able to cleave the peptidoglycan of the bacterial cell wall. The peptidoglycan is the structural framework of the cell wall, and its degradation exposes the bacteria to mechanical damage, and os-

motically lysis. A complete description of peptidoglycan hydrolases is beyond the scope of this review. However, two classes of these enzymes that may be particularly relevant are the bacteriophage lysins required to complete the phage lytic cycle and the bacterial autolysins involved in bacterial cell division. As seen with lysostaphin, the transgenic animal strategy opens the door to utilization of these nonmammalian enzymes with the possibility that a combination of enzymes may be effective against all species of mastitis pathogens, and that the combination approach against any particular species may limit the development of resistant bacteria.

The potential for development of resistance to a peptidoglycan hydrolase does exist. However, it is difficult to predict how this will affect the virulence of the pathogen. Resistance to lysostaphin is property of *S. simulans*, its natural producer organism, but most other staphylococcal species are very sensitive to its lytic activity (Cisani et al., 1982). A lysostaphin immunity factor (*lif*) gene has been located within *S. simulans* and is thought to enable the substitution of serine for glycine residues in the cross-bridge of the peptidoglycan (Thumm and Gotz, 1997). The catalytic action of lysostaphin, a glycyl-glycyl endopeptidase, is thus prevented. A second resistance mechanism may be the inability of lysostaphin to bind to the *S. simulans* peptidoglycan. The binding domain of lysostaphin has been identified, and reporter proteins containing this region are able to bind to *S. aureus*, but not to *S. simulans* (Baba and Schneewind, 1996). Further, removal of the binding domain from lysostaphin disables its ability to kill *S. aureus*. A third mechanism of resistance has been identified in lysostaphin-resistant *S. aureus* mutants that developed following low dose administration of lysostaphin for 3 d in a rabbit model of endocarditis (Climo et al., 2001). The mutation resulted in a monoglycine, rather than the usual penta-glycine cross-bridge. Interestingly, these mutants were more susceptible to β -lactam antibiotics, and furthermore, the development of the lysostaphin mutants was largely prevented by coadministration of β -lactam antibiotics. Thus, bacterial resistance to peptidoglycan hydrolases may develop along with some cost to the pathogen's fitness or virulence. It is our strategy to develop additional antistaphylococcal proteins such that a combination will reduce the development of resistant organisms. Proteins such as *S. aureus* autolysins, and bacteriophage lysins (see below) have evolved over time to bind to, and catalyze degradation of peptidoglycan. These evolutionarily selected binding/catalytic sites are likely to be critical to the organism, and not amenable to mutation (Schuch et al., 2002).

Bacteriophage lysis is generally mediated by the production of two proteins, a holin and a lysin (Wang et al., 2000). The holin creates a hole in the cell membrane that enables the lysin to access the peptidoglycan. The lysin then degrades the peptidoglycan resulting in lysis of the bacteria. This phenomenon is known as "lysis from within," and in contrast, external addition of the

lysin to a gram-negative bacteria such as *E. coli* does not result in lysis presumably because the lysin can not access the peptidoglycan through the outer cell membrane. However, the application of phage lysins to treat infections caused by gram-positive bacteria, which lack an outer membrane, is seeing a resurgence. Recently, streptococcal phage lysins have been shown to be very effective in the treatment of experimentally induced nasopharyngeal, streptococcal infections. Mice that had been inoculated with a strain of group A streptococci—and subsequently demonstrated significant oral colonization—were cleared of infection within 2 h of lysin application (Nelson et al., 2001). In a similar study, mice previously colonized with *Streptococcus pneumoniae* were cleared of infection by a single dose of a specific lysin protein (Loeffler et al., 2001). Finally, a third report from this group demonstrates the ability of PlyG lysin, isolated from the γ phage of *Bacillus anthracis*, to kill *B. anthracis* (Schuch et al., 2002). In this study repeated exposure of a susceptible organism to PlyG did not result in the generation of spontaneously resistant mutants suggesting that the lysin targets essential cell-wall molecules. Given that bacteriophage have been described for nearly all bacteria, it is likely that appropriate lysins will be found that can kill mastitis-causing pathogens.

Peptidoglycan degradation is also required for bacterial growth and division. In fact, the peptidoglycan layer is a highly dynamic structure undergoing continual synthesis and degradation. Endogenous hydrolases are referred to as autolysins. The major autolysin of *S. aureus* is encoded by the *Atl* gene (Oshida et al., 1995; Foster 1995). This bifunctional gene product contains both N-acetylglucosaminidase (GL) and N-acetylmuramoyl-L-alanine amidase (AM) activities. The relative bacteriolytic activities of GL and AM were found to be 250-fold and 25-fold less than lysostaphin (Sugai et al., 1997). These enzymes are promising candidates but it remains to be determined if they will be potent enough to confer mastitis resistance.

Candidate Gene Regulatory Regions

The production of novel proteins into milk of transgenic animals has relied on the incorporation of milk protein regulatory regions into the transgene constructs. This ensures production of the protein in lactating mammary epithelium. However, mastitis susceptibility is not confined to the lactating state. In fact, transition periods, either from the dry to lactating state or vice versa, are periods of enhanced susceptibility to mastitis. This has led us to explore genes known to be active in these states as candidate regulatory regions for a transgenic approach to enhance mastitis resistance.

Lactoferrin

The concentration of lactoferrin in normal bovine or murine milk is reported to be between 20 and 200 $\mu\text{g/}$

ml (Neville et al., 1998), approximately one-hundredth that of human milk. However, upon cessation of bovine lactation, the lactoferrin concentration in mammary secretion begins to increase after 2 to 4 d, reaching peak levels 14 to 21 d later that are 100-fold greater than during lactation (Welty et al., 1976). These protein levels are a reflection of mammary gene expression. In both mouse and cattle, lactoferrin mRNA is barely detectable by northern blot analysis of RNA from lactating tissue, but is dramatically induced upon cessation of lactation (Goodman and Schanbacher, 1991; Lee et al., 1996). In the mouse, this induction can easily be detected within 1 d of involution, while the first time point in the bovine report was 3 d. The intense signal on Northern blots of involuting bovine mammary tissue indicates that lactoferrin production is a major function of the tissue.

Perhaps related to its antibacterial properties, lactoferrin concentrations in bovine milk are increased by mastitis (Harmon et al., 1975). Furthermore, in acute experimentally induced infection, lactoferrin concentrations can increase 30-fold within 90 h of inoculation (Harmon et al., 1976). The major source of this appears to be the mammary epithelium and the additional PMNs found in milk from inflamed glands (Harmon and Newbould, 1980). These authors estimated that PMN contributed only about 5% of peak lactoferrin levels in endotoxin-induced inflammation of the bovine mammary gland. Changes in lactoferrin gene expression by mammary epithelial cells in response to mastitis have not been specifically investigated. However, Molenaar et al. (1996) reported some interesting observations in this regard during a survey of abattoir-derived mammary tissue. The glands surveyed were from virgin, pregnant, lactating, and dry cows. Using in situ hybridization, these authors found that lactoferrin gene expression varied inversely with the lactational state of the secretory cells, being generally restricted to developing alveoli in pregnant animals, generally low in actively lactating alveoli, and generally high during involution. Importantly, they also noted that lactoferrin expression was high at any lactational stage in those alveoli that contained somatic cells in their lumen, indicative of mastitis. Thus, a strong body of evidence exists suggesting that inflammation and involution induces mammary expression of lactoferrin. For our goals of strengthening the mammary gland's repertoire of antibacterial proteins it appears that the regulatory region of the lactoferrin gene would provide appropriate tissue and developmental expression and the ability to respond to inflammation.

Genes Induced by Mastitis or Involution

Appropriate regulatory regions may also be found through studies of mammary tissues either responding to infection or undergoing involution. One similarity between these states is a marked increase in epithelial cells undergoing apoptosis. Perhaps gene regulatory re-

gions upregulated within apoptotic cells or within the neighboring surviving cells would be effective candidates for transgene regulation.

It is now well documented that bacterial infection of epithelial cells stimulates apoptosis (for review see (Weinrauch and Zychlinsky, 1999). The relationship of this to lost milk production is difficult to measure but potentially has a substantial negative effect. Experimental challenge of lactating cows with *E. coli* has clearly documented the resulting stimulation of apoptosis (Long et al., 2001). In that study, infected mammary glands were biopsied 24 h postinfection with the resulting tissues processed for RNA, protein, and histological examination. Both mRNA and protein analysis indicated a substantial up-regulation of pro-apoptotic factors—Bax and interlukin-1 β converting enzyme—and a down-regulation of the antiapoptotic factor Bcl-2. Further, induction of a 92-kDa gelatinase—presumably MMP-9—was clearly observed by gelatin zymography. Finally, the number of apoptotic epithelial cells/10 microscopic sections, as determined by TUNEL assay, increased from 1.8 ± 0.5 to 8.8 ± 2.8 cells. Interestingly, an increase in epithelial cell proliferation was also observed that might be a restorative mechanism to maintain alveolar integrity. Whether these cells represent scar tissue formation or do in fact go on to produce milk proteins is unknown. Evidence for induction of apoptosis by other mastitis pathogens has been provided by experimental infection of the bovine mammary gland with *Streptococcus agalactiae* (Sheffield 1997). In this study a fivefold induction of a putative marker of apoptosis—testosterone-repressed prostate mucin-2 (TRPM-2) mRNA was observed. Finally, in vitro studies indicate that *S. aureus* causes apoptosis in a bovine mammary cell line (Bayles et al., 1998).

An increase in apoptotic epithelial cells is also a characteristic of mammary gland involution in the cow (Wilde et al., 1997; Capuco and Akers, 1999), and the mouse model (Jerry et al., 2002). In the mouse model, genes associated with cell cycle progression and arrest are rapidly induced along with pro-apoptotic genes. The activation of the tumor repressor protein, p53, appears to play a central role in modulating the expression of various involution responsive genes. Activation of nuclear factor- κ B (NF- κ B) is one of the most rapid events, clearly visible 24 h postweaning (Brantley et al., 2000). In fact, the activation of NF- κ B can be observed within 30 min in a mammary cell culture model system (Clarkson et al., 2000). In this system, KIM-2 mammary cells can be induced to differentiate in the presence of prolactin and dexamethazone with complete suppression of NF- κ B after 11 d. Abrupt withdrawal of the lactogenic hormones by media replacement induces apoptosis and NF- κ B activity. However, the role of NF- κ B in mediating the apoptosis is not clear. Rather, it appears that only about one third of the cells become apoptotic after 17 h of hormone depletion, and it seems that it is the surviving cells that produce the active NF- κ B. Examining KIM-2 cells that had been stably

transfected with an NF- κ B-responsive green fluorescent protein (GFP) reporter construct further substantiated this finding. Under apoptotic conditions a large number of cells expressed GFP; however, in an analysis of over 1000 cells positive for annexin V (a marker of apoptosis) not one instance of GFP co-localization was observed. Thus, NF- κ B appears to have been selectively activated in surviving rather than dying cells. A gene regulatory region responsive to NF- κ B may be a good candidate to drive mastitis resistance genes.

Microarray-based experiments are now being employed to evaluate changes in murine mammary gene expression that occur during pregnancy, lactation, and involution (Lemkin et al., 2000). Similar experiments are also shedding new light on the response of cultured cells to infection (Rosenberger et al., 2000; Coussens et al., 2002). The application of these high throughput techniques to bovine mammary cells undergoing involution or responding to infection will reveal genes, whose promoter regions may be ideal for directing the expression of antibacterial proteins during these critical periods. We are pursuing antibacterial proteins that function in mammary secretions to kill mastitis causing pathogens. The location and secretory capability of the mammary epithelial cells appear to make them the best cell type to produce these proteins in a localized fashion. However, locating epithelial specific responses with microarray experiments based on mammary tissue samples will be challenging given that the mammary gland also contains numerous other cell types such as fibroblastic, endothelial, adipose, myoepithelial, and infiltrating lymphoid cells. Further, the relative proportions of these cell types changes drastically with the physiological state of the tissue. Techniques such as laser dissection microscopy, or mammary cell culture may be more appropriate sources to identify candidate genes that can then be verified histologically on tissue sections.

Transgenic Technology to Enable Mammary Production of New Proteins

The use of transgenesis to direct mammary gland expression of foreign protein in the mouse was first reported by Gordon et al. (1987). This technology has now progressed to transgenic pigs, sheep, goats, and cattle, powered primarily by pharmaceutical interests seeking to generate animal bioreactors (Wall, 1996). Human clinical trials using livestock-milk-derived human α 1-antitrypsin and antithrombin III are now in progress. However, due primarily to costs and the time frame involved, this technology has yet to be used for strictly dairy purposes. The cost of producing transgenic founder animals which, using standard microinjection techniques, may approach \$60,000 for a sheep or goat and \$300,000 for a cow, with approximately 7 yr required for generation of a herd of production of milk (Wall et al., 1997). Progress in techniques for nuclear transfer from transgenic cells to enucleated embryos

should reduce this cost considerably as the number of recipient animals required will be greatly reduced (Schnieke et al., 1997). Recent advances in production of transgenic ruminants include targeted, nonrandom, insertion of transgenes into the genome (McCreath et al., 2000), and insertions of very large DNA segments containing multiple genes (Kuroiwa et al., 2002). These and other advances will aid in the realization of transgenic, agriculturally important livestock. However, the cost, and the 5 to 10-yr time frame are still daunting.

Conclusion

The amazing advances in agriculture that have already been produced by transgenic plant technologies foretells advances in animal agriculture to be delivered by application of transgenic animal technologies. Lyso-staphin is but one of a host of bacterial proteins that could have transgenic application in the prevention of mastitis. The development of an inflammation- and involution-inducible expression construct may be superior to constitutive expression of antibacterial proteins during lactation, delivering antibacterial proteins only when needed. New technologies often raise public concerns. These concerns must be addressed through education on the scientific basis of transgenic animal technology and through demonstration of the safety of the food produced and the well-being of the animals. The need for new technologies to meet future demands for food production must also be explained. The goal is not only to satisfy future needs, but also to do so with far less use of resources and production of waste than would be required through simple expansion of current agricultural practices. In addition, if the goal of enhanced mastitis resistance can be realized, it will positively affect animal welfare and lessen the need for antibiotics to treat this disease.

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