Alternative Approaches for the Prevention and Treatment of Mastitis

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Abstract

Current methods of mastitis control based on proper milking hygiene, reduced exposure to environmental pathogens, and dry cow antibiotic therapy have reduced occurrences of the disease. However, mastitis continues to be the most costly disease affecting the dairy industry. The incidence of mastitis increases when defense mechanisms of the mammary gland are impaired. If immunomodulators can be used to augment immune functions at critical periods during the production of food animals, then the economic loss caused by mastitis should be reduced. This paper will review some of the recent advances in the area of mammary gland immune regulation.

Introduction

The prevention and treatment of mastitis is a primary concern of the dairy industry. Mastitis is the most devastating disease affecting adult dairy cattle and the associated losses continue to present serious economic burden to the producer. Current mastitis control practices based on proper milking hygiene, reducing exposure to environmental pathogens, and dry cow antibiotic therapy have reduced occurrence of the disease. However, the most recent estimates of the National Mastitis Council suggest that 38% of the cows in the United States have intramammary infections in an average of 1.5 quarters.[1] Clearly, new and innovative approaches to mastitis control are needed.

One means of decreasing the impact of mastitis on the dairy industry is by increasing the cow's natural ability to resist infections. Defense of the mammary gland against mastitis-causing pathogens is mediated by several cellular and soluble protective factors. Once bacteria successfully penetrate the teat end opening, it is the efficiency of these defense mechanisms which determines the resistance of the mammary gland to new intramammary infections. There are certain times in the lactation cycle when mammary gland defenses fail to operate properly and cows become more susceptible to mastitis. Strategies aimed at enhancing immune systems of the mammary gland during these periods of immunosuppression would greatly impact the cow's ability to resist infection. This paper will review the potential future role of immunomodulators for the control of mastitis in dairy cattle.

Cytokines Immunomodulation

Cytokines are naturally-produced proteins that play an important role in essentially all aspects of inflammation and immunity. The term 'cytokine' describes a heterogeneous group proteins produced by a spectrum of both immune and non-immune cells under diverse circumstances. The immunomodulatory capacity of the cytokine network is complex. Individual cytokines can interact with other cytokines in a synergistic, additive, or antagonistic fashion on multiple cell targets.[2] Cytokines are often referred to as hormones because they are usually produced transiently and locally with potent biological activity at extremely low doses. Because of their extreme potency, elevated levels of certain cytokines can be detrimental to the host as well. To date, there are over 20 cytokines which have been identified, purified, and characterized by their regulatory activities.[2] As large quantities of recombinant cytokines become available for research, more information is being generated concerning the potential immunotherapeutic application of cytokines against infectious diseases in food-producing animals.

One of the more exciting potential applications of cytokines is for the control of bovine mastitis. It is well documented that increased incidence of mastitis is associated with impaired host defense mechanisms and cytokines are the major mediators of mammary gland immunity during establishment of infection. Therefore, in devising strategies to counteract these various dysfunctional mammary gland defenses, cytokines are logical candidates to aid in the prevention and treatment of mastitis.

Over the last several years, numerous reports have

shown the immunomodulatory capabilities of recombinant cytokines on important mammary immune cell functions (Table 1). Information also is available which describes the potential clinical application of recombinant cytokines for the control of experimental *S. aureus* and coliform mastitis in dairy cattle (Table 2). Based on availability and species biological cross-reactivity between human cytokines and bovine cells, much of the earlier work with recombinant cytokines in cattle involved the human recombinant forms.

Table 1. Summary of cytokine effects on mammary immune cells.

Cytokine	Observation	Reference
granulocyte colony stimulating factor	increased milk somatic cell counts	3
	increased numbers of milk neutrophils	4
granulocyte-macrophage colony stimulating factor	enhanced neutrophil chemotactic and bactericidal activity	5
	enhanced neutrophil antibody-dependent cytotoxic activity	6
	enhanced neutrophil activity	7
interferon-gamma	enhanced neutrophil phagocytosis and bactericidal activity	8
	enhanced neutrophil bactericidal activity	9
interleukin-1	increased numbers of neutrophils	10
	no change in mononuclear cell proliferation	11
interleukin-2	enhanced mammary mononuclear cell proliferation	12
	enhanced cytotoxic and bactericidal activities of lymphocytes	13
	increased plasma cell numbers	14

Table 2. Summary of the efficacy of cytokines against bovine mastitis.

Cytokine	Mastitis Model	Observation	Reference
granulocyte colony stimulating factor	*S. aureus*	no effect	3
	S. aureus	47% reduction in new infections	4
interferon-gamma	*E. coli*	reduced rate, duration and severity of disease	14
interleukin-2	*S. aureus*	improved antibiotic efficacy	15

Colony Stimulating Factors

Recombinant human granulocyte colony stimulating factor (GCSF) was one of the first cytokines to be tested in an experimental mastitis model. The colony stimulating factors (CSF) are a group of cytokines required for the proliferation and differentiation of a variety of hematopoietic stem cells. These growth factors are distinct glycoproteins that bind to cells by a common receptor and are produced by a variety of cells including fibroblasts, endothelial cells, macrophages, and T cells. Each CSF tends to target a specific lineage to expand and/or activate its function. Originally characterized by its ability to stimulate myeloid colonies in soft agar, GCSF is required for the growth, survival, and differentiation of granulocyte phagocytic cells. The pronounced influence of GCSF on phagocytic cell populations suggest possible clinical applications in the prevention of infectious bacterial diseases, such as mastitis.

Administration of human GCFS to lactating dairy cattle by subcutaneous infection reduced new infection rates by 47% following experimental *S. aureus* challenge when compared with placebo treated controls.[4] It was suggested that the reduction in new *S. aureus* intramammary infection were related to the GCSF-induced recruitment of neutrophils into the mammary gland prior to challenge. There have been no reports on the prevention or treatment of *S. aureus* mastitis using an intramammary infusion of GCSF.

Interleukins

The term 'interleukin' was originally introduced to describe cell-free soluble factors that function as communicator molecules between leukocytes. Although all cytokines share this basic property, many of the well-characterized cytokines are designated as interleukins. Interleukin-2 (IL2) is the most extensively characterized of all the cytokines.

Interleukin-2 was originally described as T-cell growth factor. It is primarily produced by T cells of the helper phenotype and is responsible for clonal expansion of the initial T cell immune response and establishment of immune memory following mitogenic or antigenic stimulation. This cytokine also plays a role in B cell growth and differentiation, enhancing thymocyte proliferation, activation of natural killer cells, and inducing cytotoxic T cell activation.[16]

The diverse functions of IL2 in orchestrating the immune response suggest that this cytokine may be an important factor in augmenting compromised or suppressed immune systems. The possibility of enhancing bovine mammary gland defenses with IL2 to increase resistance to mastitis has received considerable research attention. Both *in vitro* and *in vivo* studies indicate that recombinant bovine IL2 may enhance the antibacterial capacity of mammary gland leukocytes and increase the resistance of the mammary gland to bacterial infections in a nonspecific manner.[10,11,12] Recent studies have shown that intramammary administration of bovine IL2 can enhance cellular and humoral immune response in quarters infected with *S aureus*.[13] Prophylactic administration of IL2 was shown to protect the mammary gland from subsequent intramammary challenge with *S.*

aureus, Therapeutic administration of these cytokines into *S. aureus* infected quarters were less efficacious at eliminating preexisting intrammary infections.[15] However, there is evidence to suggest that combination of homologous cytokines with current antibiotic formulations may improve the overall efficacy of these therapeutic agents.[15]

Interferons

Interferons (IFN) are a group of closely related proteins of three major types. IFN-α and IFN-β are produced by a variety of cell types in response to several inducers including viral infections, bacterial products, and tumor cells. IFN-γ is a T cell-derived cytokine often produced in response to antigen or mitogen stimulation. Over 30 years ago IFNs were discovered and named based on their ability to induce antiviral states *in vitro*.[2] Since then, it has been recognized that IFN exhibits a variety of immunomodulatory properties to many aspects of the immune system. For example, IFN-γ enhances natural killer cell activity, antibody dependent cellular cytotoxicity, and cytotoxic T cell activity. This cytokine also enhances macrophage-mediated cytotoxicity against tumor cells, induces membrane bound Fc receptors for IgG on macrophages, and stimulates the synthesis and release of reactive oxygen species from both macrophages and neutrophils. It has been shown that IFN-γ can also regulate host responses to bacterial toxins. Depending on the dose and the timing of administration relative to challenge, IFN-γ was shown to decrease the morbidity and mortality associated with endotoxemia.[2]

Based on the biological actions of this cytokine, it was suggested that intrammary IFN-γ treatment may enhance bacterial clearance by mammary gland phagocytes and regulate acute inflammatory responses to bacterial toxins during acute coliform mastitis. To test this hypothesis, researchers examined the influence of recombinant bovine IFN-γ treatment on the establishment and severity of experimentally induced *E. coli* mastitis in postpartum dairy cattle.[14] Dairy cows treated intramammarily with IFN-γ 24 hours before *E. coli* challenge had fewer infected quarters, infections of shorter duration, and lower clinical scores compared to placebo-treatment animals. All INF-γ treated cows survived the experimental *E. coli* challenge, whereas there was a 42% mortality rate in the placebo-treated group, attributed to coliform mastitis within 3 days of the experimental challenge.

These experiments clearly indicate the ability of recombinant cytokines to modify the outcome of mastitis during instances when the immune system has been compromised. Recombinant cytokines are capable of modifying the outcome of mastitis through a combined effect of recuitment of effector cells to the mammary gland, enhanced bacterial clearance by phagocytic cell populations, and regulation of acute inflammatory reactions. However, research into the role of cytokines in bovine mastitis is in the beginning stages. The challenge that confronts researchers now is to gain a better understanding of the complex interaction between the pathogenesis of bacteria, host responses needed to eliminate pathogens from the mammary gland, and mechanisms by which cytokines can modulate these responses. Further developments in these areas are necessary before experimental findings can be transferred to field conditions. However, information generated thus far concerning our ability to effectively manipulate and regulate mammary immune functions suggest great potential for the future therapeutic application of cytokines for the control of bovine mastitis.

Mastitis Vaccines

Vaccination programs are designed to potentiate the host's immune system toward a unique, specific antigen. For mastitis vaccines, this can be accomplished by eliciting a prompt recruitment of neutrophils to the site of infection and stimulating the production of specific antibody. The interaction between mammary gland neutrophils and bacteria during the initial stages of colonization is critical to the outcome of infection. Adequate numbers of functional competent neutrophils are necessary for the elimination of bacteria during the pathogenesis of disease. Immunization can enhance neutrophil recruitement through the release of inflammatory mediators by localized antigen-specific lymphoid populations. Specific antibodies are required for the opsonization of bacteria and the promotion of phagocytosis by mammary gland neutrophil populations. In addition to serving as opsonins, antibodies may neutralize bacterial toxins, interfere with bacterial adherence mechanisms, and induce cell lysis of the invading pathogen. Both neutrophil numbers and antibody concentration are low in the healthy, non-infected mammary gland. Immunization protocols that are capable of potentiating these essential bactericidal components should contribute to the effect control of mastitis.

Mastitis vaccines are expected to eliminate chronic infection, prevent the establishment of new infections, and reduce the frequency and severity of clinical disease. Vaccines currently available do not appear to eliminate chronic mastitis or consistently reduce the incidence of new infections. However, several recently available vaccines have been shown to effectively reduced the incidence of clinical mastitis.

Staphylococcus aureus Mastitis Vaccines

Numerous attempts have been made to ameliorate or prevent *S. aureus* mastitis through vaccination pro-

grams. Many of the earlier studies used systemically injected bacterins derived from *in vitro* grown cultures.[17,18] Although serum antibody titers increased following immunization, adequate antibody concentrations in milk were only achieved after inflammation to the challenge organisms had occurred. Increased milk antibody titers were effective in lessening the severity of disease, but had no effect in preventing new intramammary infections.

As more information became available concerning important *S. aureus* pathogenic mechanisms, different antigenic formulations were developed. The cell walls of *S. aureus* contain and produce a number of factors that are known to interfere with the ability of neutrophils to phagocytize and kill bacteria. Protein A is a cell wall component of *S. aureus* that binds immunoglobulin (Ig) by the non-specific Fc receptor instead of by the antigen-specific Fab terminal. It was suggested that a vaccine against protein A may improve the opsonic activity of specific antibody by allowing the binding of Ig to the bacterial surface. Attempts to vaccinate dairy cattle with a *S. aureus* protein A vaccine was found to improve the spontaneous cure rate, but had no effect on the rate of new infections.[19]

Many *S. aureus* are able to produce an extracellular polysaccharide psuedocapsule that is known to have antiphagocytic properties. Investigators have recently developed a *S. aureus* vaccine composed of bacteria that were cultured *in vivo* to promote the expression of important capsular antigens.[20] It was shown that immunization of animals with this vaccine resulted in improved protection from experimental challenge with *S. aureus.* These findings suggest that altered *in vitro* growth conditions may interfere with the expression of important virulence factors that are normally expressed *in vivo.*

There also is evidence to suggest that staphylococcal toxins are important factors that can damage host tissues and promote bacterial growth. Recent studies have shown that heifers vaccinated with and *S. aureus* vaccine formulated to stimulate anti-staphylococcal pseudocapsule antibodies and well as both α-toxin and β-toxin had reduced new infection rates and infections of shorter duration compared to an unvaccinated group.[21] To date, the efficacy of these newer vaccine formulations when used under field has not been evaluated.

Coliform Mastitis Vaccines

Considerable progress has been made over the last several years in the development of an effective mastitis vaccine against coliform mastitis. Initial studies showed that cattle with low pre-existing serum titers against common gram-negative core antigens were more susceptible to clinical coliform mastitis than cows with higher tiers.[22] From this observation, cows were then immunized with an R-mutant *E. coli* which resulted in a dramatic reduction in the incidence of clinical coliform mastitis.[23] This bacterial stain is unique from the standpoint of lacking enzymes required for normal bacterial cell wall synthesis. As a consequence, the R-mutant have nearly complete LPS assembly, but no O or somatic side chains. The exposed inner cell wall structure are highly uniform and vaccines containing killed R-mutant bacteria should provide broad-spectrum immunity against a wide variety of gram-negative bacteria. To test this theory, a heat-killed *E. coli* J-5 mutant vaccine was developed and tested in several field trials. Although the vaccine has little impact on the prevalence of coliform infections, immunization with gram-negative core antigens decreased the incidence and severity of clinical disease.[24] The practical application of coliform mastitis vaccines will be as a supplement to traditional methods of mastitis control based on good management and nutritional practices.

Nutritional Supplementation

The nutritional status of an animal is directly related to overall health, and proper nutrition has long been associated with the ability to fight disease. Although studying the effects of specific nutrients is complicated by their diversified functions and complex interaction with other nutrients, researchers have been able to better define the role of several micronutrients in the process of infection and immunity. Because a variety of these substances and their deficiencies have been shown to have profound effects on the immune systems of many animals, adequate nutrition has received increasing attention as an essential element in the prevention and control of mastitis. Most of the available information on micronutrients and their immunomodulatory properties with regard to bovine mastitis focuses on selenium, vitamin E, vitamin A, β-carotene, copper and zinc. A summary of their primary roles in mammary defense is outlined in Table 3.

Selenium

Selenium (Se) is probably the best characterized micronutrient with regard to immunoregulatory effect. Selenium is a vital component of the antioxidant enzyme gluthathione peroxidase (GSH-Px). This enzyme is essential to the protection of cells and body tissues from auto-oxidative damage as a result of oxygen radical production by certain leukocytes during phagocytosis and killing.[25] Deficiencies in Se result in compromised neutrophil function which are a primary effector cell in the initial elimination of infections.[26,27] Since Se-deficient soil is widespread across much of North America, Se deficiency often results in animals whose primary source of nutrition is derived from plants grown in these areas.

Table 3. Summary of micronutrient effects on mammary gland immunity.

Micronutrient	Observation	Reference
Selenium	↓ efficiency in neutrophil function	25, 27, 28
	Improves bactericidal capabilities of neutrophils	26, 27
	↓ severity and duration of mastitis	26
Vitamin E	↑ neutrophil bactericidal activity	27, 31
	↓ incidence of clinical mastitis	28, 31
	In combination with Se, ↓ prevalence of IMI at calving	31
Vitamin A	↓ SCC counts	25, 32 (rev)
	Moderates glucocorticoid levels	32 (rev)
β-carotene	↑ bactericidal function of phagocytes	34
	↑ mitogen-induced proliferation of lymphocytes	25, 32 (rev)
Copper	Cu deficiency ↓ neutrophil killing capability	36
	Cu deficiency ↑ susceptibility to bactericidal infection	37 (rev)
Zinc	Zn deficiency ↓ leukocyte function	37 (rev)
	Zn deficiency ↑ susceptibility to bacterial infection	25, 37 (rev)

Based on the role of Se in important immune cell function and protection, deficiencies in this micronutrient can have serious consequences on mammary gland health. Many studies have documented the benefits of dietary Se supplementation for the control of bovine mastitis. Neutrophil killing of *S. aureus*, *Candida albicans* and *E. coli* is greatly enhanced in Se-supplemented versus Se-deficient dairy cattle.[26,27,28] Erskine, *et al.*[26] have also shown that Se-supplemented cows experience clinical infections of reduced severity and duration compared to non-supplemented cows. The same study revealed lower peak bacterial numbers in cows provided with dietary selenium when infection did occur. This observation is most likely due, in part, to the more rapid influx of neutrophils into the mammary gland upon bacterial infusion in Se-supplemented cows. These beneficial effects of Se can be attributed to the decreased damage to cells by oxygen radicals and peroxidases with an increased efficiency of the enzymes involved in intracellular killing mechanisms. Regardless of the means, overwhelming evidence for the protective role of Se against bovine mastitis clearly warrants inclusion of dietary Se supplementation in mastitis control protocols.

Vitamin E

Similar to selenium in its biological properties, vitamin E is an important component of all cell membranes which provides stability and prevents the debilitating peroxidation of membrane lipids. Vitamin E also plays a regulatory role in the biosynthesis of various inflammatory mediators.[25] It is necessary for the integrity of integument and wound healing and has shown immunostimulatory effects, both cellular and humoral.[29,30] This essential antioxidant is found in high quantities in fresh, green foodstuffs; however, its concentration decreases with age of plants, length of storage, and is often destroyed in silages. Therefore, deficiencies are common in unpastured animals and during seasons when pasturing is not possible.

Because of its positive role in immunity and the widespread potential for deficiency in farm animals, vitamin E supplementation could provide great benefit to the control of bovine mastitis. Indeed, Hogan *et al.*[27] reported an increased intracellular kill of both *S. aureus* and *E. coli* when cows' diets were supplemented with this micronutrient. Smith *et al.*[31] were able to demonstrate a reduction in the incidence of clinical mastitis by 37% when supplements of 1g/cow/day were provided. Additionally, a synergistic effect of Se and vitamin E has been observed, reducing the prevalence of clinical mastitis, new intramammary infections at calving, and somatic cell counts, as well as the severity and duration of clinical mastitis, to a greater degree than the supplementation of either micronutrient alone.[27,28] The necessity of nutritional supplementation, especially during the dry period before deficiencies are most likely to develop, should not be overlooked in the attempt to bolster an animal's resistance to mastitic infections.

Vitamin A and Beta-carotene

Vitamin A and its precursor, β-carotene, have long been known for their effect on vision, normal cell growth, and epithelial cells and therefore mucosal surface integrity and stability. Vitamin A deficiency has been linked to an increased glucocorticoid response to stress, which has an immunosuppressive effect.[32] β-carotene can act independently as an oxygen radical scavenger and is incorporated into cell membranes as such. Both vitamin A and β-carotene have been shown to have stimulatory effects on immune cell populations and have been correlated with a generally increased resistance to disease.

Deficiencies in both these nutrients have been related with severity of mastitis, and both decrease at a time when cows become increasingly susceptible to new intramammary infections.[32] The role of vitamin A in epithelium health may be due to an affect on mammary gland defense mechanisms. Several researchers have reported a negative correlation between levels of vitamin A and β-carotene with somatic cell counts in lactating dairy cows. Supplementation with both of these nutrients improved the status of clinical mastitis over the provision of vitamin A alone, indicating the protec-

tive role of β-carotene independent from its function as precursor to vitamin A.[33] The work of Daniel *et al.*[34] seems to support this idea, as the *in vitro* ability of phagocytic blood and milk leukocytes to kill *S. aureus* was enhanced in the presence of β-carotene but not various forms of vitamin A. Nevertheless, the importance of adequate dietary intake of both these micronutrients is still apparent, and mastitis control programs should ensure that proper levels are maintained in all animals.

Copper

Little information on the role of copper in disease is available, however its importance in normal biological function is recognized. Copper is required for the synthesis of hemoglobin and is an essential element in the antioxidant Cu-dependent enzyme superoxide dismutase. It is also present in the serum protein ceruloplasmin, recognized as an acute phase protein in cattle. The latter two proteins are important to immune function partly in terms of their protection of cells from oxidative products released as a result of phagocytosis and killing by leukocytes. Ceruloplasmin has also been indicated as a possible modulator of extracellular lysosomal enzyme activity as a result of inflammation.[35]

Deficiencies in copper have been shown to result in lowered bactericidal activity of blood leukoctyes[36] and increased susceptibility to bacterial infection.[25,37] As with vitamin A and beta carotene, Cu levels fall to their lowest levels at a time when cows are most vulnerable to mastitis. Studies at the University of Kentucky[37] showed that dietary copper supplementation resulted in approximately a 2 fold reduction in percent of infected quarters at calving compared to untreated controls. The amount of quarters infected by major mastitis pathogens was reduced more than four fold. Somatic cell counts also tended to be lower in the supplemented group. Additionally, Suttle and Jones[38] report a decrease in proliferation of lymphocytes to mitogens in hypocupremic animals which was restored by supplementation with Cu. These results indicate that Cu as well may have regulatory role in the immunity of the mammary gland.

Zinc

Another micronutrient essential to various biochemical pathways, zinc has also been linked to proper immune function. Zinc is essential for the integrity of skin, the first physiologic barrier to infection. It is also a component of the antioxidant zinc-dependent superoxide dismutase. Zinc is likely to have a stabilizing, antioxidative role in cellular membranes and therefore protects them from damage.[25] Zinc deficiency can result in the atrophy of thymus and other lymphoid tissue and irregular profiles of serum antibodies and immune cells.[25,27] Deficiencies in zinc also set up a predisposition for secondary infections, which can be reversed by supplementation.[25,37]

The problems associated with zinc insufficiencies can be exacerbated by high calcium diets, a condition common to cows in early stages of lactation. Therefore, an even greater concern is created for the proper dietary intake of zinc by dairy cows as a means to maintain mammary immunity. However, every few studies have been carried out which elucidate the specific relation of zinc to bovine mastitis. Nevertheless, the interaction of zinc with immune cells and overall health indicates it could certainly pass a role in prevention or control of the disease.

Diet plays an important role in the ability of dairy cows to resist disease. Not only gross malnutrition, but merely suboptimal levels of any one micronutrient is sufficient to adversely effect mammary gland immunity. Key to ensuring adequate levels of these important micronutrients is direct testing of animals at least at the herd level to delineate patterns in overall nutrient deficits. Providing dietary supplements of the deficient vitamins or minerals in accordance with accepted doses is one practical means to enhance a cow's inherent defense against invading mastitis pathogens.

Summary

The development of immunomodulatory strategies for the control of bovine mastitis are active areas of research. The advent of recombinant DNA technology has allowed the production of large quantities of animal cytokines. An increased understanding of the role of cytokines in host defense will lead to the most appropriate use proteins under field conditions. Mastitis vaccine technologies have improved considerably over the years. To date, mastitis vaccines are not widely used in mastitis control programs. However, the recent development of the R-mutant vaccines for gram-negative mastitis should prove to be beneficial for the control of coliform mastitis on well managed dairy operations. Although the role of nutrition in mammary resistance to infection has been best defined for antioxidants, supplementation with other micronutrient appear to hold promise as well. If immunomodulators can be used to augment immune function at critical periods during the production of food animals, then the economic loss caused by mastitis should be reduced. Enhancing the host's natural ability to resist mastitis without introducing undesirable residues into the food chain will be fully compatible with current public concerns and demands.

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