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Engineering Immunity in the Mammary Gland

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The major physiological function of milk is the transport of amino acids, carbohydrates, lipids, and minerals to mammalian offspring. However, milk is also a rich collection of antimicrobial substances, which provide protection against pathogenic infections. These molecules safeguard the integrity of the lactating mammary gland, but also provide protection for the suckling offspring during a time when its immune system is still immature. The protective substances can be classified into two categories: 1) nonspecific defense substances, which provide innate immunity, and 2) molecules such as antibodies, which provide adaptive immunity and are directed against specific pathogens. The antimicrobial potency of milk has not been a target for farm animal breeding in the past, and present day ruminants provide suboptimal levels of antimicrobial substances in milk. Altered breeding regimes, pharmacological intervention, and transgenesis can be utilized to improve the antimicrobial properties of milk. Such alterations of milk composition have implications for human and animal health.

KEY WORDS: milk; immunology; pathogen; protection; neutralization.

INTRODUCTION

Milk contains a wide variety of antimicrobial substances, which provide innate and adaptive immunity (Table I). Innate immunity in milk is provided by nonspecific, broad-spectrum defense molecules (including lipids, carbohydrates, and proteins), which share the common feature that they suppress the growth of pathogenic microorganisms. Innate immunity-related molecules are also expressed in other mammalian epithelia and can also be found in bacteria, fungi, plants, and lower vertebrates (1). Adaptive or acquired immunity is provided by an immune system, which is only found in higher vertebrates. In contrast to innate immunity, adaptive immunity is directed against specific pathogens and includes the molecular tools to memorize antigen stimuli. Milk contains a variety of molecules which are products or inducers of adaptive immune reactions, most prominently antibodies, which are secreted into milk mainly as IgG or IgA (2). In addition, milk contains a variety of cytokines and growth factors which interact with lymphoid or myeloid cells and may play a critical role in establishing the immune system of the offspring (3). Moreover, a variety of lymphocytes reside in the mammary gland, including neutrophils, macrophages, CD4+ and CD8+ T-cells, and IgA-producing plasma cells. Some of these (especially neutrophils and macrophages) are also found in milk, where they provide (in collaboration with antibodies and components of the complement system) a cell-based defense system against bacterial infections (4). The inflammatory processes orchestrated by these cells, however, may damage the mammary epithelium (4). Some lymphocytes can also be transmitted to the offspring via milk (5,6). The innate and adaptive immune systems overlap, since lymphoid and myeloid cells sometimes secrete and are sometimes induced by "innate immunity" molecules (7).

Molecules and pathways involved in innate and adaptive immune responses are potential targets for biological manipulation. The major aim for

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Abbreviations used: AMP, antimicrobial peptide; ETEC, enterotoxic *E. coli*; Ig, Immunoglobulin; J, Joining chain; LPS, lipopolysaccharide; MHC, major histocompatibility complex; RSV, respiratory syncytial virus; SC, secretory component; TGEV, transmissible gastroenteritis virus; UHT, ultra high temperature.

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Table I. Immunologically Active Components of Milk

Adaptive immunity	Innate immunity
Antibodies	Lactoperoxidase
Anti-idiotypes	Lactoferrin/lactoferricin
Cytokines/growth factors	Casein and casein fragments
Lymphocytes	Mucin
	Lactalbumin fragments
	Lactadherin
	Antimicrobial peptides (e.g. defensins)
	Lipids and glycolipids
	Cytokines/growth factors
	Complement

engineering immunity in the mammary gland is to support the natural protective functions of milk, namely to defend the gland against microbial infections and to guard the newborn offspring against pathogen insult. Modified milk may be used as a food supplement to improve the disease resistance of farm animals or to provide a "nutraceutical" for human consumption. The adaptive immune response can be manipulated by vaccination of ruminants against animal or human pathogens. This approach leads to an enrichment of pathogen-specific antibodies in milk. Alternatively, the concentration of antimicrobial proteins and peptides in milk can be increased by transgenesis. Pharmacological stimulation can be used to enhance the synthesis of endogenous antimicrobial components in the lactating mammary gland. In the long run genes correlated with the antimicrobial properties of milk can be used as genetic markers in breeding programs.

TARGETS FOR MANIPULATION—ADAPTIVE IMMUNITY

The most prominent result of adaptive immune responses with respect to milk is the secretion of antibodies which are specific for pathogens that the mammal has encountered. IgA, IgM, and IgG are found in colostrum and milk of all mammals, albeit in varying concentrations. IgA is the major immunoglobulin in milk of humans and rodents, whereas IgG is the major immunoglobulin in milk of ruminants and pigs (2,8) (Table II).

IgA is synthesized by B-cells, which are resident in the mammary gland (Fig. 1). These lymphocytes home to secretory epithelia after contact with their cognate antigen in the gut-associated lymphoid tissue. This stimulus induces expression of specific surface antigens called addressins (e.g. L-selectin and $\alpha 4\beta 7$ integrin). After release into the circulation, these addressin-expressing lymphocytes bind to cognate cell surface receptors ("homing receptors," e.g. MAdCAM-1) present on the vascular endothelial cells in secretory epithelia (9). By a mechanism involving sequential lymphocyte–endothelium interactions and chemotaxis, the lymphocytes differentiate into IgA-producing cells and migrate into the lamina propria (the connective tissue underlying the epithelial cells) (9,10). The mature, mammary-resident B-cells generate high local concentrations of pathogen-specific IgA, which is subsequently transcytosed into milk. IgA is secreted from B-cells as a dimer, which is covalently linked by a joining chain (Fig. 1) and transported across the epithelial cells via the polymeric Ig receptor (pIgR) (9) (Fig. 1). The receptor–IgA complex is secreted at the apical surface of the epithelial cell after proteolytic cleavage of the pIgR molecule (9). The resulting mature dimeric IgA in milk consists of a total of four heavy chains, four light chains, a joining chain (all synthesized in the B-cell), and the secretory component (i.e. the remainder of the polymeric Ig receptor, provided by the epithelial cell). Targeted inactivation of the genes encoding pIgR and the joining chain have demonstrated that these proteins are essential for the production and secretion of polymeric IgA from secretory epithelia (11,12).

IgG is synthesized by B-cells residing in lymph nodes, collected from serum and transferred across the mammary endothelial and epithelial cells (Fig. 1). The molecules involved in transepithelial transport of IgG into colostrum and milk are still unknown (9).

Table II. Immunoglobulin Transfer in Different Species[a]

Species	Major Ig in colostrum/milk	Absorption by gut epithelium	Gut closure	Transplacental transfer of Ig
Human, rabbit	IgA	Probably none	24 h	Yes
Mouse, rat	IgA/IgG	Moderate, selective	19 days	Yes
Horse, pig, ruminants	IgG	Extensive, selective	48 h	No

[a] Adapted from Ref. 8.

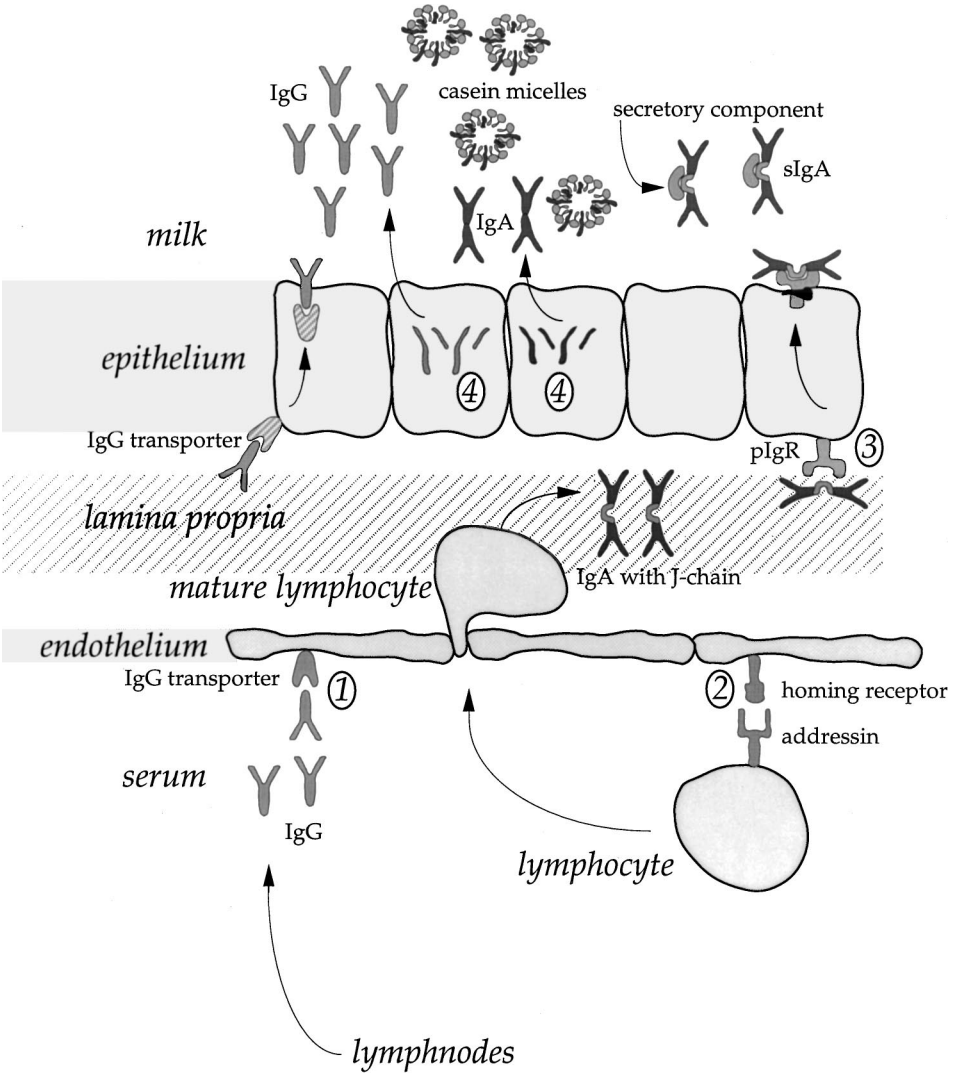

Fig. 1. Potential targets for engineering adaptive immunity in the mammary gland. Schematic representation of the lactating mammary gland including serum, endothelium, lamina propria, epithelium, and milk (as indicated). IgG is synthesized by lymphnode-resident B-cells, collected from serum and transcytosed through the endothelial and epithelial cell layers by a prolactin-sensitive mechanism. This putative transporter system is particularly active prior to parturition and is responsible for the high IgG concentration in ruminant colostrum [1]. Activation of the putative transporter system during later stages of lactation could be used to increase the IgG concentration in milk. After contact with antigen, IgA-expressing B-cells home to secretory epithelia (including the mammary gland) from mucosa-associated lymphoid tissues. In an activated state these cells carry an $\alpha 4\beta 7$-integrin surface marker, which interacts with the MAdCAM-1 receptor on the vascular endothelium of secretory epithelia. After further receptor- and cytokine-mediated maturation steps IgA-producing B-cells migrate into the lamina propria (i.e. the connective tissue underlying the epithelial cell layer) [2]. When the rate-limiting factors (receptors and cytokine molecules) involved in the migration and maturation of IgA-producing B-cells are identified, the expression of the corresponding genes could be stimulated by transgenesis or pharmacological intervention. IgA is transcytosed through the epithelial cell layer via the polymeric Ig receptor (pIgR) [3]. Stimulation of pIgR expression could increase the IgA concentration in ruminant milk. IgG and IgA can be expressed and assembled in and secreted from mammary epithelial cells [4]. Transgenic expression of either antibody isotype could increase the pathogen-specific antimicrobial activity of milk.

Table III. Immunoglobulin Concentrations in Serum, Colostrum, and Milk[a]

Species	Serum	Colostrum	Milk
Human	IgA: 3 mg/mL	IgA: 12 mg/mL	IgA: 1 mg/mL
	IgG: 12 mg/mL	IgG: 0.1 mg/mL	IgG: 0.05 mg/mL
Cow	IgA: 0.4 mg/mL	IgA: 3 mg/mL	IgA: 0.1 mg/mL
	IgG: 25 mg/mL	IgG: ≤200 mg/mL	IgG: 0.7mg/mL

[a] Adapted from Ref. 8 and 13.

However, as the concentration of IgG in colostrum is 10 times higher than in serum such a transport mechanism is likely to exist (13) (Table III). Transepithelial IgG transport in the gut is catalyzed by the FcRn receptor (Fc receptor neonatal), which is a heterodimer consisting of a β2-microglobulin subunit and a MHC class I-like 50 kDa protein (14). While transepithelial transport in the gut is largely abolished in mice carrying a targeted deletion of the β2-microglobulin gene, IgG levels in milk are unaffected (15). This result suggests that (at least in mice) the serum to milk transport of IgG involves a transporter system which does not require β2-microglobulin. The IgG concentration in cow's milk is far lower than in colostrum or serum (Table III), suggesting that the IgG transport is down-regulated at the commencement of lactation. Prolactin has been demonstrated to inhibit IgG transport into milk and to down-regulate the expression of an IgG-binding protein on mammary epithelial cells (13,16,17).

In order to engineer the adaptive immune response in the mammary gland, two objectives can be envisioned. First, the specificity of the response (i.e. the specificity of the antibodies) can be modulated. This can be accomplished by vaccination. Second, the concentration of antibodies in milk can be modulated. This can be done by transgenesis or pharmacological manipulation of gene expression. In a best-case scenario both parameters (qualitative and quantitative) can be regulated to improve the antimicrobial potential of milk.

Vaccination

Generally, immunological protection against infectious diseases in mammals is best obtained by vaccination. However, in humans, as in animals, there are a variety of microbial pathogens against which no suitable vaccine is available. Some pathogens (e.g. influenza virus) mutate quickly, so that vaccinations do not have a lasting effect and have to be repeated at short intervals. Other pathogens (e.g. RSV in humans or TGEV in pigs) do not induce a strong immune response in adult mammals, but cause serious disease in newborns. In both cases levels of maternal antibody, which are inversely correlated with the severity of the virus-induced disease (18,19), are usually scarce. In a scenario like this, milk/colostrum enriched in pathogen-specific antibodies would provide clear health benefits.

Cow's milk can be enriched with antibodies directed against veterinary and human pathogens by conventional vaccination (20). Pathogen-specific antibodies can be found in substantial concentrations in colostrum and can be processed to provide a marketable product (21) (see also www.immucell.com/human.php and www.mucovax.nl). Standard pasteurization and UHT treatments destroy 30 and 90% of immunoglobulin (Ig) activity, respectively, but antibodies can be extracted from milk and then added back to UHT-treated milk (22). Ig activity in milk is also well conserved by freeze-drying (22). Preparations of antibodies from milk and colostrum have been used for some time as food supplements for animals (22), but may also be useful for medical applications or human nutrition, as has been demonstrated in numerous examples (23). Immunologically active components in bovine colostrum (probably bovine IgG) are able to ospsonize bacteria for ingestion by human leukocytes (24). This phenomenon suggests that these components of the bovine and human immune system are sufficiently conserved to interact. Bovine antibodies are therefore able to control pathogen-induced gastrointestinal diseases in humans.

Systemic inoculation has been shown to be the most effective route for the vaccination of cows (23). Adjuvant material has been shown to be a critical factor for eliciting a satisfactory immune response (23). The major limitation of the ruminant vaccination approach is the moderate yield. Most successful experimental approaches were carried out using colostrum (or colostrum-based material), and all of the marketed products are derived from it (23). Immunoglobulin isolation from milk is usually not cost effective. Stimulation of immunoglobulin secretion into milk therefore is an important objective for future research.

Milk can also be used as a medium for the active immunization of the neonate with anti-idiotypes. This was demonstrated in a murine system (25). Lactating mice, which were immunized with an antibody directed against a surface glycoprotein of RSV (4 and 8 days postpartum), secreted corresponding

anti-idiotypes into milk. Offspring suckling immunized mothers responded significantly better to a subsequent immunization with the RSV surface protein (1 week after weaning) than control animals, who were not primed by the milk-borne anti-idiotype (25). This finding demonstrates that the rudimentary immune system of the suckling mice was able to respond to milk-borne antigenic stimulation. The transfer of anti-idiotypes via placenta and milk may also be a natural mechanism of immunization in humans (5).

Transgenic Expression of IgG or IgA

As an alternative to vaccination, ruminants secreting pathogen-neutralizing antibodies into milk can be generated by transgenesis, as mammary epithelial cells are able to express, assemble, and secrete Ig molecules (26). Transgenesis enables the selection of the desired antibody isotype, thereby overcoming species-specific restrictions (Table II). The concentration of the recombinant antibody present in milk is likely to exceed the levels of pathogen-specific immunoglobulin generated by vaccination by some orders of magnitude. Antibody concentrations of 10 mg/mL have been obtained in transgenic goats (26), while the total immunoglobulin content of ruminant milk is about 1 mg/mL (Table II). In addition, the most potent antibodies directed against a given pathogen can be selected in vitro. On the other hand transgenesis will lead to milk which contains only one monospecific antibody directed against a single epitope (in contrast to vaccination, which leads to a collection of pathogen-specific antibodies recognizing numerous epitopes). Pathogens are often able to evade the neutralizing effect of an antibody by mutating the recognized protein domains. Therefore, antibodies expressed in the milk of transgenic animals should be selected carefully so that they are directed against essential epitopes of the pathogen, which cannot be mutated without impairing infectivity (27). In addition, synthesis of recombinant IgG requires the expression of two transgenes to be optimized. IgA consists of four protein chains—heavy chain, light chain, joining chain (J), and secretory component (SC), a fragment of the polymeric Ig receptor—which are derived from two different cell types (Fig. 1). The natural mechanism of IgA production is therefore difficult to mimic by transgenesis. However, IgA molecules, which are synthesized in epithelial cells, tend to dimerize spontaneously even in the absence of J-chain and SC (27). Dimeric IgA possesses a greater neutralization activity than monomeric IgG and elicits no inflammatory responses, as it does not contain a Fc portion. As transgenesis in ruminants is expensive and time consuming, milk containing a recombinant, pathogen-neutralizing antibody may only be commercially viable in a few exceptional instances with major economic significance.

One such example is the fatal infection of piglets with the porcine coronavirus TGEV (transmissible gastroenteritis virus). TGEV causes gastroenteritis and diarrhea in pigs, with significant economic consequences (19). TGEV elicits a highly strain-specific and short-lived immune response in adult animals. Newborn animals, however, are severely affected by the infection, with a mortality of up to 100%. Despite major efforts, no marketable TGEV vaccine has been developed yet. Oral administration of neutralizing antibody has been shown to efficiently prevent TGEV infection (27). Milk containing a neutralizing antibody may therefore provide a route to protect piglets against TGEV infections (27).

To provide a proof of principle, a mouse model for this approach was established. Transgenic mice expressing a highly neutralizing antibody directed against a murine coronavirus (the mouse hepatitis virus: MHV-JHM) in the lactating mammary gland, were generated. Newborn mice suckling the milk of transgenic dams were fully protected against a lethal MHV-JHM challenge (Table IV). Cross-fostering experiments demonstrated that the milk-borne recombinant antibody was sufficient for this protection (35). The passive immune protection was long lasting, as offspring which were nursed by transgenic dams were resistant to a lethal virus challenge up to 6 weeks after weaning (Perlman and Kolb, unpublished). This result demonstrates that viral escape mutants could not overcome the neutralizing effect of the monospecific antibody provided in milk during the course of the infection. Although there are differences in the disease patterns elicited by MHV and TGEV and the immunoglobulin isotypes typically found in the milk of mice and pigs, these experiments demonstrate that the modification of milk via a transgenic route can have a beneficial impact on animal health.

A number of transgene expression systems have been used successfully to produce significant amounts of immunoglobulin in milk. Using an expression system based on the ovine β-lactoglobulin gene (36), up to 6 mg/mL IgA could be produced in the milk of transgenic mice (27). A concentration of 10 mg/mL of IgG in the milk of transgenic goats could be obtained by expressing IgG encoding transgenes under

Table IV. Examples of Transgenic Mice Expressing Recombinant Antimicrobial Proteins in Milk

Protein	Transgene promoter	Origin of gene	Observed effect	Reference
Lactoferrin	Bovine αS1 casein Bovine β casein	Human	Improved gut development in neonates	(28–30)
TAP (antimicrobial peptide)	Murine WAP	Cow	Not published	(31)
Lysozyme	Bovine αS1 casein	Human	Antibacterial activity of milk in vitro, altered processing properties	(32,33)
Lysostaphin	Ovine β-LG	Staph. simulans	Increased resistance to mastitis	(34)
IgG (monoclonal)	Ovine β-LG	Mouse/human	Systemic protection against a lethal virus infection	(35)
IgA (monoclonal)	Ovine β-LG	Mouse/pig	Not determined	(27)

the control of the caprine β-casein promoter (26). Antibodies are assembled from two protein chains, which are usually encoded by two separate transgenes. When transgenic animals are generated by conventional microinjection, the copy number and the integration site of the transgene cannot be preselected. In addition, the process of transgene expression variegation influences the rate of transgene transcription (37). Therefore equimolar expression of both antibody chains is unlikely. Because of the cytotoxicity of the IgG heavy chain the light chain is reproducibly expressed at a higher level than the heavy chain in vitro and in vivo (35). In order to generate equimolar levels of heavy and light chain, thereby maximizing the antibody yield, the transgenes encoding the two antibody chains may be inserted into the two alleles of a highly expressed milk protein gene (Fig. 2) by strategies based on homologous recombination (38).

Combinations of Transgenesis and Vaccination

The expression of transgene-encoded antibodies in the lactating mammary gland may only be commercially viable in a few instances. In contrast, transgenic animals in which the antibody concentration in milk is increased or isotype ratio of milk-borne immunoglobulins is altered would have a much wider range of applications. However, at present the rate-limiting steps involved in immunoglobulin transport into milk are not well defined. Once the proteins involved in the transport of IgG or the homing of IgA secreting lymphocytes are identified, the corresponding gene(s) could be expressed as transgenes (Fig. 2).

The polymeric Ig receptor (pIgR) was suspected as a factor which limits IgA transport into ruminant milk. In order to test this hypothesis transgenic mice expressing a pIgR transgene under the control of the bovine αS1 casein promoter were established (39). The transgene was expressed at high levels resulting in a 270-fold increase in pIgR protein in mammary epithelial cells. However, the IgA content of murine milk was only modestly increased by twofold (39). This suggests that the availability of the pIgR protein is not an important rate-limiting factor for IgA secretion into murine milk. A similar approach in cattle, however, which mainly secrete IgG into milk (IgG:IgA ratio in colostrum up to 60:1 as opposed to 4:1 in rodents (8)), may result in a more substantial increase in IgA secretion into bovine milk.

Cell surface markers (homing receptors) and chemotactic factors involved in the lymphocyte homing process are other candidates which could be rate limiting for IgA secretion into milk. To test this hypothesis and to overcome these limitations, the corresponding genes must be expressed at high levels in transgenic animals. This could be accomplished by expressing a candidate gene under the control of its own promoter. This protocol will increase the copy number of that gene but retain the tissue specificity (i.e. homing receptors would still be expressed in endothelial cells but at a higher level). Alternatively, the target gene can be expressed under the control of a promoter, which can be activated by an exogenous stimulus (like tetracycline or ecdysone) (40). In order to retain tissue specificity the regulator transgene (encoding the tetracycline-regulator protein, rtTA, or the ecdyson receptor) would be controlled by the promoter of the target gene (Fig. 2). The tetracycline system has been demonstrated to enable high levels of transgene expression in the lactating mammary gland (41).

Transgenic technology could also be utilized to generate ruminants which synthesize fully humanized polyclonal antibodies. Two complementary strategies have been used successfully to generate

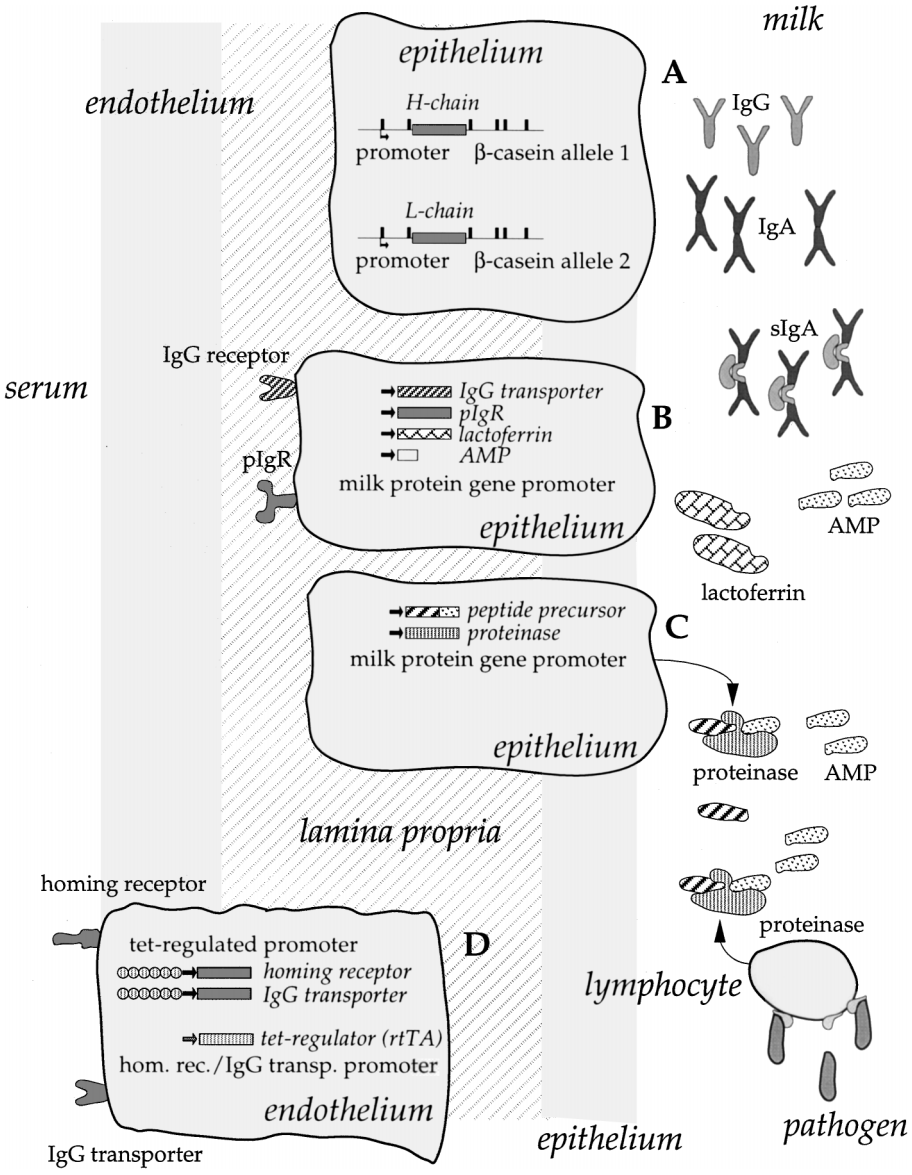

Fig. 2. Transgenic strategies for engineering immunity in the mammary gland. Schematic representation of the lactating mammary gland as in Fig. 1. (A) The heavy and light chain genes of IgG or IgA can be integrated into milk protein gene loci by strategies based on homologous recombination. This approach is expected to lead to high levels of transgene expression and production of equimolar amounts of heavy and light chain protein. (B) A variety of genes involved in adaptive or innate immunity can be expressed in the lactating mammary gland under the control of milk protein gene promoters. (C) Transgenes encoding antimicrobial peptides and proteins can be expressed as inactive precursors (under the control of a milk protein gene promoter), which can be activated by proteolytic cleavage. The required protease can be a milk component (e.g. a component of the complement system or plasmin), which itself is activated by pathogen infection. Alternatively the protease can be transgene-encoded. (D) The transport of antibodies into milk can be enhanced by the expression of transgenes encoding rate-limiting homing receptors or Ig transporter proteins on endothelial and epithelial cells. Conditional gene expression systems can be used to generate high and regulatable levels of transgene expression. The tet-regulatable transcription factor (rtTA) can be expressed under the control of the promoter of the IgG transporter or homing receptor gene. The homing receptor and IgG transporter transgenes in turn are expressed under the control of a tet-responsive promoter. This method ensures that the tissue-specific expression of the target genes is maintained while the level of expression can be regulated exogenously.

transgenic mice synthesizing human antibodies. In both strategies the endogenous murine immunoglobulin gene loci were inactivated by homologous recombination. The corresponding human immunoglobulin loci were subsequently introduced either as yeast artificial chromosomes (42) or as human chromosome fragments (43). In both strains of transgenic mice human immunoglobulin proteins of all isotypes could be detected, and hybridomas synthesizing human monoclonal antibodies for therapeutic purposes could be established (44). In order to produce large amounts of humanized polyclonal antibodies, similar approaches could be pursued in ruminants. Ruminants thus modified could be immunized against antigens of interest and produce milk containing human antibodies. By this route large amounts of polyclonal human antibodies could be produced, which could subsequently be purified and utilized for clinical applications (e.g. intravenous infusion of antiviral antibodies in immuno-compromised patients). Bovine antibodies could not be used for such applications, as they would elicit a major immune response in humans.

POTENTIAL TARGETS—INNATE IMMUNITY

A variety of milk-borne molecules have antimicrobial potency and can be classified as components of the mammary innate immune system (Table I). Proteins and peptides with inherent antimicrobial activity are the most promising targets for biological manipulation. Oligosaccharides, glycoconjugates (45), and lipids (46), many of which possess antimicrobial potency, are synthesized by the interplay of a number of enzymes. Manipulation of these pathways may require major genetic alterations and may therefore not be cost-effective. Transgenes encoding antimicrobial proteins and peptides of mammalian or non-mammalian origin can be expressed in the lactating mammary gland under the control of milk protein gene promoters (Fig. 2; Table IV). Their antimicrobial activity should be as broad as possible to inhibit the growth of many pathogens at the same time, but should be specific enough not to interfere with micro-organisms that are involved in the downstream processing of milk (e.g. in cheese manufacturing). To combat infections of the mammary gland the antimicrobial peptides and proteins could be provided as inactive forms, which are activated by proteolytic cleavage (Fig. 2). The cleavage site could be engineered such that it is recognized by a protease which is preferentially activated upon pathogen infection (e.g. a protease belonging to the complement system or plasmin). To enhance resistance to microbial gut infections, recognition sites for proteases of the gastrointestinal tract (e.g. pepsin) could be introduced, so that the active proteins/peptides are released from their precursors in the GI tract of the milk consumer. The latter strategy appears to be used naturally by a variety of milk components. To date only a small selection of antimicrobial proteins and peptides have been expressed in the milk of transgenic mice (Table IV).

Lactoferrin

Lactoferrin is a glycosylated milk-protein, which is also found in other mucosal secretions and is expressed in neutrophils (7). Lactoferrin owes its antimicrobial activity to a variety of biochemical properties. 1) By sequestering free iron, lactoferrin can restrict the growth of gram-positive and gram-negative bacteria and a variety of fungi (7). The bacteriostatic effects can be counteracted in vitro by addition of excess iron. 2) Lactoferrin and (even more potently) its pepsin-breakdown products lactoferricin B and H (from bovine and human lactoferrin, respectively), which are derived from the N-terminus of the protein, have a bacteriocidal effect by interfering with bacterial membrane function (47). Lactoferrin's activity against fungal infections in vitro and in vivo may have the same biochemical basis. 3) Lactoferrin binds bacterial lipopolysaccharide (LPS), thereby impairing bacterial cell wall/membrane function (7). By binding free LPS or LPS bound to other proteins, it can also act as an antiinflammatory immune modulator (48). 4) Purified lactoferrin neutralizes the infectivity of human cytomegalovirus (hCMV) and human immunodeficiency virus (HIV-1). Noncovalent interactions between charged lactoferrin residues and the viral surface proteins have been implicated in the antiviral effect (49). 5) Lactoferrin also inhibits the entry of hepatitis virus C (HCV) into susceptible cells by direct interaction with the viral surface glycoproteins (49). Antiviral activity of lactoferrin has been reported against herpes simplex virus, respiratory syncytial virus, rotavirus, and poliovirus (49). Lactoferrin influences cellular responses to Listeria infections (50) and decreases the cell number of Helicobacter pylori (a suspected cause of stomach cancer) in the stomach of experimental animals (51). Human milk contains much higher levels of lactoferrin than bovine milk and therefore has a higher antimicrobial potency

(7), which may be one of the reasons that breast-fed infants are significantly healthier than bottle-fed infants (52).

Lactoferrin has long been a prominent target for recombinant production in a variety of host organisms (53). Milk containing high amounts of lactoferrin may be used directly as a "nutraceutical" to prevent or treat bacterial infections of the digestive tract. Recombinant human lactoferrin has been demonstrated to improve the resistance of neonatal rats to bacterial infections (54). This effect appears to be independent of lactoferrin's ability to bind iron, as addition of excess bioavailable iron did not reverse the protective effect (54). Human lactoferrin in the milk of transgenic mice enhanced intestinal growth and maturation in the offspring (28) but had little effect on iron metabolism (55). Lactoferrin purified from milk could serve a variety of other purposes (e.g. as adjuvant in wound healing). Transgenic cattle expressing human lactoferrin have been generated (56), and the product is in clinical trials.

Lactoferricin

Lactoferricin B and H are derived from bovine and human lactoferrin, respectively, by pepsin cleavage in the digestive tract. Lactoferricin B, a 25 amino acid peptide (amino acids 17–47 of bovine lactoferrin), folds into a distorted antiparallel β-sheet, whereas parts of this N-terminal region adopt an α-helical structure in native bovine lactoferrin (57). The bacteriocidal effect of lactoferricin is far greater than that of native lactoferrin (58). Lactoferricin has been shown to depolarize bacterial membranes (59); however, the precise basis of its antimicrobial action remains unclear.

In order to generate lactoferricin in milk the corresponding peptide-coding sequence could be expressed as a transgene under the control of a mammary gland specific promoter (Fig. 2). Alternatively, lactoferrin could be modified such that lactoferricin can be released by the action of a proteolytic enzyme in the mammary gland. The required protease could be co-expressed as a second mammary-specific transgene (Fig. 2). A similar approach has been used successfully to process human protein C to its mature form in milk (60). This method may boost the antibacterial potency of milk and reduce the number of bacteria (especially enterobacteria, which are particularly susceptible to lactoferricin action) in the lactating mammary gland.

Antimicrobial Peptides

Antimicrobial peptides (AMPs) are important members of the innate immune system, especially in nonmammalian species (1). Lactoferricin is a typical antimicrobial peptide in that it folds into an amphipathic helix, contains a high proportion of basic amino acids and interferes with bacterial membrane function without disturbing mammalian cell membranes (1,57). Breakdown products of other milk proteins are also potent AMPs. Two C-terminal pepsin fragments of bovine αS2 casein have been shown to possess antibacterial activity (61,62). Tryptic fragments of bovine α-lactalbumin efficiently restrict the growth of gram-positive bacteria (63). Isracidin, a chymosin cleavage product of bovine αS1 casein encompassing amino acids 1–23, possesses strong antibacterial activity (at concentrations similar to that of penicillin) and was found to be effective against a variety of gram-negative and gram-positive bacteria (64). Surprisingly, isracidin is very potent when injected intramuscularly in vivo but only displays a modest antibacterial activity in vitro. The effect of isracidin may therefore be indirect. Consequently it is uncertain, whether transgenic overexpression of an isracidin-peptide in the lactating mammary gland would increase the antibacterial potency of milk.

The majority of antimicrobial peptides, however, are gene-encoded (1). Mammalian AMPs are secreted by a variety of epithelia including the mammary gland. β-Defensin, for example, is secreted into human milk (65) (Table I). Sequence comparisons of a multitude of antimicrobial peptides have yielded sufficient information to generate synthetic peptides which efficiently inhibit bacterial growth (66). Although the biological relevance of AMPs has been clearly established in nonmammalian species (67), the contribution of these peptides to the host defense in mammals (i.e. in the presence of an adaptive immune system) is not clear. Therefore no firm predictions can be made as to how the presence of AMPs in milk would alter the antimicrobial potency of milk. The targeted inactivation of recently identified mammalian (human and mouse) gene clusters encoding a large number of β-defensin-related peptides (68) will be instrumental in defining the biological role of AMPs in mammals. A β-defensin-related bovine antimicrobial peptide, TAP, could be expressed in the lactating mammary gland of transgenic mice without overt side effects (31). However, the in vivo activity of the peptide was not analyzed by microbial challenge experiments (Table IV).

Lysozyme

Lysozyme is an antibacterial protein present in a variety of mucosal secretions, including milk (32). The human lysozyme gene was expressed in transgenic mice under the control of the bovine αS1 casein promoter. Milk derived from mice secreting human lysozyme displayed enhanced antimicrobial properties in several in vitro assays (32,33) (Table IV). However, pathogen challenge experiments were not done. The presence of lysozyme in milk also altered the processing properties of mouse milk (32).

Lysostaphin

Lysostaphin is a peptidoglycan hydrolase derived from Staphylococcus simulans. When expressed in eukaryotic cells the protein is inactivated by glycosylation at cryptic glycosylation sites. Transgenic mice expressing a modified lysostaphin devoid of these sites under the control of the ovine β-lactoglobulin promoter, displayed an increased resistance to mastitis after Staphylococcus aureus challenge (34) (Table IV).

Other Options

A variety of other proteins could be used to improve the antimicrobial properties of milk. Some of these are natural milk components, like lactoperoxidase, a 78 kDa basic glycoprotein, which is one of the most prominent enzymes in bovine milk and catalyzes the inactivation of a wide range of microorganisms (69). Lactoperoxidase isolated from milk and whey is utilized as a natural biopreservative in food, cosmetics, and related products (70). Transgenic overexpression of lactoperoxidase may therefore improve the antimicrobial properties of milk but also alter the processing properties of milk. Related peroxidase systems are also found in human secretions, such as saliva and tear fluid, and may be expressed in the lactating mammary gland to improve the antimicrobial properties of milk. In addition, a wide variety of other antimicrobial proteins and peptides can be derived from nonmammalian sources.

CONCLUSIONS

Milk contains a variety of antimicrobial agents which are crucial in safeguarding the mammary epithelium and providing passive immune protection to the offspring. The contribution of individual components of innate and adaptive immune systems to the antimicrobial activity of milk is not well defined at present. Tissue-specific inactivation of individual genes related to mammary gland immunity in mice may resolve some of these questions in the future.

Engineering immunity in the mammary gland will endeavor to improve the antimicrobial potency of milk, either by stimulating expression of existing genes or by adding new genes (i.e. transgenes) to the mammalian genome. Modified milk, with improved antimicrobial properties may be used to improve the disease resistance of farm animals or to produce a "nutraceutical" for human consumption. Natural milk components, like lactoperoxidase, lactoferrin, or antimicrobial peptides, have no toxic side effects on mammalian cells and can be overexpressed in the lactating mammary gland of transgenic animals. Transgenic expression of pathogen-specific antibacterial proteins or peptides may increase the antimicrobial potency of milk while retaining its normal processing properties. Stimulation of lymphocyte homing to the mammary gland and immunoglobulin transport in conjunction with vaccination could allow the production of pathogen-specific Ig-rich milk for the prevention and treatment of gastrointestinal infections in animals and man. Which of these options will be realized will be determined not only by technical constraints, but also by commercial viability, legislation, and public acceptance. However, milk with improved antimicrobial properties will have numerous applications in animal and human health.

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