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The prospects of modifying the antimicrobial properties of milk

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Abstract

Milk contains a variety of substances, which inhibit the infection of pathogens. This is of benefit to the mother, safeguarding the integrity of the lactating mammary gland, but also of huge importance for protection of the suckling offspring. The antimicrobial substances in milk can be classified into two categories. First, nonspecific, broad-spectrum defense substances, which have evolved over long periods of time, and secondly, substances like antibodies, which are specifically directed against particular pathogens and have developed during the mother's lifetime. Substances in both categories may be targets for biological intervention and manipulation with the goal of improving the antimicrobial properties of milk. These alterations of milk composition have applications in human as well as in animal health. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

The major physiological function of milk is the transport of amino acids, carbohydrates, lipids, and minerals (especially calcium) to mammalian offspring. In addition to its nutritional importance, milk also contains a variety of molecules, which provide protection against pathogen infections or modulate immune responses. Antibodies are the most prominent of these molecules, but other nonspecific, broad-spectrum defense substances possess antimicrobial activity as well. Some of these molecules can be altered to improve the antipathogenic properties of milk. These modifications are of particular interest if no suitable vaccines are

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available and newborn mammals are most severely affected by the infection. This scenario can be the consequence of the low virulence of the pathogen, which does not elicit a major immune response in the adult host [e.g. the human respiratory syncytia virus (RSV)], or the variability of the pathogen, which evades an immune response targeted at variants encountered previously (e.g. influenza virus). Under these circumstances, mothers do not supply protective antibodies to their offspring via placenta and milk and nonspecific defense mechanisms may be too weak to prevent disease. This in turn puts the offspring at an increased risk of infection, as the immune system of newborn mammals is often not sufficiently developed during the early stages of life to cope efficiently with the pathogens. Milk, which has been manipulated to contain large amounts of specific or nonspecific defense substances, may therefore provide a useful tool for disease prevention in these instances.

2. Milk and human health

There is convincing evidence that with regard to infant health, breast-feeding is superior to bottle-feeding (Filteau, 2000; Hanson et al., 2000; Hanson, 1998). Protection against infections during lactation has been demonstrated in cases of acute and prolonged diarrhea, respiratory tract infections, otitis media, urinary tract infection, neonatal septicemia, and necrotizing enterocolitis (Hanson, 1998). Breast-feeding provides the newborn baby with a battery of immunologically active substances (Tables 1, 3, 4, and 5). Antibodies have been demonstrated to efficiently control a variety of pathogen-induced diseases (including malaria, Fried et al., 1998; Kassim et al., 2000), and a wide variety of virus infections (e.g. RSV, Englund, 1994, and rotavirus, Ebina et al., 1992). Lactoferrin, which is present in substantial amounts in human milk, prevents the population of the infant's gut by coliform enterobacteria and acts as an antiinflammatory immune modulator (Nuijens et al., 1996). Other nonspecific defense molecules enhance this protection (Newburg, 1996; Labeta et al., 2000). These substances are of particular importance in the newborn prior to acidification of the stomach, which provides an effective barrier to many bacterial infections of the gastrointestinal tract in later life. In addition, there is growing evidence that the transfer of cytokines, growth factors,

Table 1
Immunologically active components of milk

Specific substances	Nonspecific substances
Antibodies	Lactoferrin/lactoferricin
Antiidiotypes	Casein and casein peptides
Lymphocytes	Oligosaccharides
	Glycolipids
	Glycosaminoglycans
	Mucin
	Growth factors
	Cytokines
	Complement

B-cells, and other lymphocytes from mother to offspring via milk (Zhou et al., 2000) assists the development of the infant's immune system. Babies, who are not breast-fed, are deprived of some of these defense mechanisms. The antibodies in cow's milk will be directed against bovine rather than human pathogens. Bovine lymphocytes even if they would survive the processing of milk to infant formula would be of no benefit to humans. Cow's milk contains very little lactoferrin (Nuijens et al., 1996). In addition, many babies show allergic reactions to cow's milk, with β-lactoglobulin being the major immunogen (Pecquet et al., 2000).

However, even breast-feeding fails to supply adequate protection against a variety of pathogens. This is exemplified by the frequent hospitalization of children (below the age of 6 months) infected with RSV. Although the levels of maternal antibody are inversely correlated with the severity of RSV-induced disease (broncholitis and pneumonia; Lamprecht et al., 1976), most mothers transfer negligible amounts of virus-neutralizing antibodies to their children. This is a consequence of the minor immune reaction that RSV elicits in adults with most infections being subacute. Available vaccines do not lead to a long-lasting immune response. The first RSV vaccine (using formalin-inactivated virus) even resulted in an increased incidence and severity of disease in vaccinated children (Englund, 1999). Therefore, although RSV-specific antibodies in milk are able to control RSV-induced disease, they are usually scarce. In a scenario like this, it is obvious that the manipulation of antimicrobial substances in milk would have beneficial health effects.

3. Potential targets for manipulation

Of the plethora of immunologically active substances in milk, only a few are realistic targets for biological or genetic modification (Table 2), as discussed next.

3.1. Antibodies and antiidiotypes

Antibodies are the most prominent and most specific defense molecules in milk. Depending on the species, immunoglobulin A or G (IgA or IgG) is the predominant isotype in colostrum and milk. They are able to efficiently control a variety of bacterial and viral diseases, which affect newborn mammals.

Table 2
Potential targets for the manipulation of immunologically active milk-components

Immunization of mothers	Immunization of ruminants	Transgenic expression
Antigen	Antigen	Neutralizing antibodies
Neutralizing antibody	Neutralizing antibody	Antiidiotypes
		Nonspecific defense proteins
		Overexpression of IgA transporter
		Insertion of human immunoglobulin gene loci

3.1.1. Immunization of mothers

Milk contains antibodies directed against a variety of pathogens that the mother has encountered during her lifetime. Some pathogens elicit a major immune response and a long-lasting immunity. Other pathogens only lead to a transient immune response. In this case, the antibody content of human milk can be manipulated by immunizing mothers during pregnancy. This can be done as a passive immunization by infusing preparations of pathogen-specific antisera or, more frequently, as active immunization by vaccination. Vaccination in pregnancy leads to a normal immune response and no pregnancy-specific side effects have been reported (Englund et al., 1998; Munoz and Englund, 2000). This approach is particularly useful in cases (like RSV), where vaccination only leads to a short-term immune response and infants can be seriously affected by the infection. Immunization of mothers during pregnancy with RSV antigen leads to the transfer of antibodies to the offspring via placenta and colostrum and helps to prevent RSV-induced disease (Englund, 1994; Englund et al., 1998). A potential drawback to this approach may relate to the finding that maternal antibodies can suppress the infant's own immune response to certain pathogens (Englund et al., 1998). Therefore, careful analysis of the host–pathogen interactions is

Table 3
Nonspecific antiinfective components of milk: lactoferrin and lactoferricin

Milk component	Pathogen	Disease	Mode of action	Evidence	Reference
Lactoferrin	Enterobacteria	Diarrhea	Sequestering of iron and deprivation of pathogenic bacteria	In vitro	Bishop et al. (1976)
	Haemophilus influenzae	Respiratory tract infections	Inactivation of colonization factors	In vitro	Qiu et al. (1998)
	HIV	AIDS	Inhibition of virus–cell fusion via negative charges	In vitro	Harmsen et al. (1995)
	hCMV	Necrosis affecting salivary gland, liver, kidney, lung, and brain	Inhibition of virus–cell fusion via negative charges	In vitro	Harmsen et al. (1995)
	Hepatitis C virus	Hepatitis, liver cancer	Specific interaction of virus surface proteins with LF N-terminus	In vitro, in vivo	Tanaka et al. (1999)
	Candida albicans	Oropharyngeal candidosis	Unknown	In vitro	Kuipers et al. (1999)
	Pneumocystis carinii	Pneumonia	Unknown	In vitro	Cirioni et al. (2000)
Lactoferricin	*C. albicans*	O. candidosis	Unknown	In vitro, in vivo	Bellamy et al. (1993)
	Gram-negative and Gram-positive bacteria	Various	Effect on membrane function	In vitro	Chapple et al. (1998)

LF = lactoferrin.

Table 4
Nonspecific antiinfective components of milk: casein and casein fragments

Milk component	Pathogen	Disease	Mode of action	Evidence	Reference
Isracidin (f1-23 bovine αS1 casein)	*Staphylococcus aureus*, *Streptococcus pyogenes*, *Listeria monocytogenes*	Anaphylactic shock, mastitis	Unknown	In vitro, in vivo	Lahov and Regelson (1996)
Casocidin I (amino acids 150–188 of bovine αS2 casein)	*Escherichia coli*	Enteric disease	Unknown	In vitro	Zucht et al. (1995)
Fragments of bovine αS2-casein (amino acids 183–207 and 164–179)	*E. coli*, *Bacillus cereus*, *S. thermophilus*	Enteric disease	Unknown	In vitro	Recio and Visser (1999)
Human (not bovine) κ-casein	*H. pylori*	Gastric disease, heart disease (?)	Glycosylated κ-casein inhibits adhesion of *H. pylori* to gastric cells	In vitro	Stromqvist et al. (1995)

required before embarking on vaccination during pregnancy. Nevertheless, immunization during pregnancy may help to prevent a wide spectrum of diseases.

Milk can also be used as a medium for the active immunization of the neonate with antiidiotypes. This can be achieved by immunizing mothers with pathogen-neutralizing antibodies. The mother will develop an immune response against the variable region of the neutralizing antibody and transmit secondary antibodies (i.e. antiidiotypes) to the offspring via placenta and milk. The offspring's immune system will respond by generating tertiary antibodies, which recognize the same epitope as the primary neutralizing antibody. The viability of such an approach has been demonstrated in animal experiments (Okamoto et al., 1989). The transfer of antiidiotypes via placenta and milk may also be a mechanism of natural immunization in humans (Hanson et al., 2000).

3.1.2. Immunization of ruminants

Immunization during pregnancy requires a substantial financial input in terms of vaccine development and clinical testing (Englund et al., 1998), as the outcome of the vaccines is not always predictable. As an alternative, the manipulations of antimicrobial properties of milk can be carried out in ruminants. Cattle can be successfully immunized against a battery of human pathogens (Jenkins et al., 1999). Pathogen-specific antibodies can be found in substantial concentrations in bovine colostrum and milk and can be processed to provide a marketable product (Stephan et al., 1990). Cow's milk containing antibodies directed against rotavirus has been successfully used to prevent rotavirus-induced diarrhea in children (Ebina, 1996). In another experiment, disease symptoms caused by enterotoxigenic *E. coli* (ETEC) were efficiently suppressed in adult volunteers by cow's milk containing ETEC-specific

immunoglobulin (Freedman et al., 1998). These examples demonstrate that bovine antibodies in milk are able to control pathogen-induced diseases in humans. Cattle may also be immunized with pathogen-neutralizing antibodies to generate milk containing antiidiotypes, thereby providing a recombinant vaccine.

3.1.3. Transgenesis

Alternatively, ruminants expressing pathogen-neutralizing antibodies can be generated by transgenesis. This has a variety of advantages. Transgenesis circumvents the problem of differences in isotype usage in milk. Cows mainly produce IgG, whereas human milk mainly contains IgA (Larson, 1992). IgG can activate complement via its Fc portion and is very potent against bacteria, whereas IgA (due to its dimeric nature) has a strongly neutralizing effect against viruses. Humanized IgA molecules can theoretically be produced in the milk of transgenic ruminants. The concentration of the recombinant antibody present in milk would

Table 5
Nonspecific antiinfective components of milk: other molecules

Milk component	Pathogen	Disease	Mode of action	Evidence	Reference
Mucin	Rotavirus	Diarrhea (mainly in infants)	Glycosylation-dependent inhibition of virus infection	In vitro, in vivo	Yolken et al. (1992)
Mannosylated glycopeptide	Enterohaemorrhagic *E. coli* (EHEC)	*Haemorrhagic colitis*	Binding of bacteria to cell surface receptor inhibited	In vitro	Ashkenazi et al. (1991)
CD14	*E. coli*	Enteric disease	Binding of bacterial LPS and whole bacteria	In vitro	Labeta et al. (2000)
Complement	General cytotoxicity			In vitro	Ogundele (1999a)
Glycolipid (ganglioside fraction of human milk)	*Vibrio cholerae, E. coli, Campylobacter jejuni*	Diarrhea	Inhibition of toxin action by competition of milk fat globule-bound glycolipids with cell surface receptors	In vitro, in vivo	Laegreid and Kolsto-Otnaess (1987)
Glycosaminoglycan	HIV	AIDS	GAG[a] binds/competes with HIV cell surface receptor CD4	In vitro	Newburg et al. (1995)
Lactadherin	Rotavirus	Diarrhea	Inhibits virus binding	In vivo	Newburg et al. (1998)
Lipids and fatty acids	Enveloped viruses, *S. epidermidis, E. coli*	Various	Free fatty acids impair membrane function	In vitro, in vivo	Isaacs et al. (1990)
	Giardia lamblia trophozoites	Diarrhea	Free fatty acids impair membrane	In vitro	Rohrer et al. (1986)
α-Lactalbumin fragments	Gram-positive bacteria		Unknown	In vitro	Pelligrini et al. (1999)

[a] GAG = Glycosaminoglycan.

exceed the levels of antibodies generated by vaccination by some orders of magnitude. Antibody concentrations of 10 mg/mL have been obtained in transgenic goats (Pollock et al., 1999), while the total immunoglobulin content of ruminant milk is about 1 mg/mL. In addition, the most potent antibodies directed against a given pathogen can be selected in vitro.

However, there are also serious disadvantages of the transgenic approach. Transgenesis will lead to milk containing only one monospecific antibody directed against a single epitope (in contrast to vaccination, which leads to a whole battery of pathogen-specific antibodies). 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 will have to be selected carefully, so that they are directed against essential epitopes, which cannot be mutated without impairing infectivity (Sola et al., 1998). Transgenesis in ruminants is also expensive and time consuming. The market for milk as a nutraceutical containing a recombinant, pathogen-neutralizing antibody may be therefore only profitable enough for some exceptional human pathogens with worldwide significance. The biggest disadvantage of transgenic technology will be the current adverse public perception. However, the transgenic approach may find public approval in the area of animal health where it could partly obviate the need for antibiotics.

The porcine coronavirus transmissible gastroenteritis virus (TGEV) illustrates the possible application of transgene-mediated modification of milk as a means to prevent disease. TGEV causes gastroenteritis and diarrhea in pigs (Enjuanes and van der Zeijst, 1995). Newborn animals are most severely affected by the infections with a mortality of up to 100%. Coronaviruses in general elicit an immune response that is highly strain-specific and often short-lived. Although a variety of routes were investigated to obtain useful vaccines against TGEV, no marketable vaccine has been developed yet. Oral administration of neutralizing antibody has been shown to efficiently prevent TGEV infection (Torres et al., 1995). Milk containing this neutralizing antibody may therefore provide a route to protecting piglets against TGEV infections (Sola et al., 1998; Castilla et al., 1998). To provide a proof of principle, a mouse model for this approach was established (Fig. 1). Transgenic mice, which express 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 challenge with a lethal dose ($7000 \times LD_{50}$) of MHV-JHM. Cross-fostering experiments demonstrated that the milk-borne recombinant antibody was sufficient for this protection (Kolb et al., 2001). Although there are differences in the disease pattern elicited by MHV and TGEV and the immunoglobulin isotypes typically found in the milk of mice and pigs, these experiments demonstrate that modification of milk via a transgenic route can have a very beneficial impact on animal health.

Combinations of transgenesis and vaccination may provide other promising approaches for the generation of milk containing pathogen-specific antibodies. Cattle mainly express antibodies of the IgG isotype, whereas IgA is the predominant isotype in humans (Larson, 1992). In mice, IgG and IgA are present at roughly equal levels (Larson, 1992). By overexpressing the polymeric immunoglobulin transporter in the lactating mammary gland of transgenic mice, the IgA content of murine milk could be increased twofold (De Groot et al., 2000). A similar approach may also be used to generate cattle that are able to secrete increased amounts
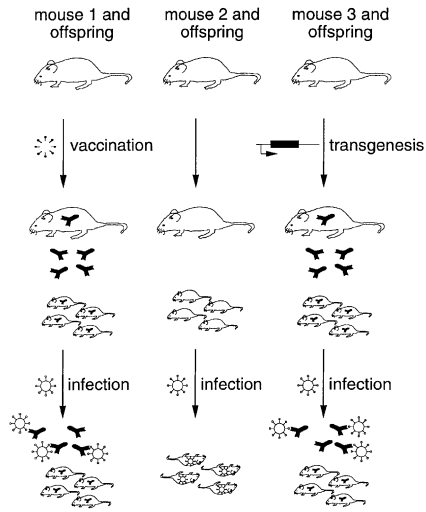
Fig. 1. A transgenic approach to lactogenic immunity: schematic representation of the outcome of a coronavirus infection in naive mice (Mouse 2 and offspring), immunized mice (Mouse 1 and offspring), and transgenic mice expressing a coronavirus-neutralizing antibody (Mouse 3 and offspring). Immunizations against coronaviruses are often short-lived. Therefore, Mouse 1 would only supply virus-neutralizing antibodies to their offspring for a limited time-span. Mouse 3, however, will consistently supply their offspring with large quantities of the recombinant virus-neutralizing antibody throughout every lactation period.

of IgA into milk. Immunization of these transgenic cows would then lead to milk rich in pathogen-specific IgA.

By using homologous recombination, researchers at Abigenix (⟨www.abigenix.com⟩) have replaced the murine immunoglobulin loci by their human counterparts. The mice will therefore respond to immunization by producing human antibodies. Monoclonal antibodies derived from these mice are human monoclonal antibodies and can be used therapeutically with minimal side effects. A similar approach could be taken in ruminants by using nuclear transfer techniques. Ruminants thus modified could be immunized against pathogens of interest and produce milk containing human antibodies.

3.2. Lymphocytes

There is growing evidence that lymphocytes are transferred from mother to offspring via milk. In mouse experiments, B-cells carrying a reporter gene could be transferred to suckling mice via milk (Zhou et al., 2000; Arvola et al., 2000). Breast-feeding mothers transfer lymphocytes to their babies, which induce an immune tolerance of the baby against the mother. One well-documented consequence of this is the fact that breast-feeding mothers are often suitable organ donors for their children in later life (Hanson, 2000). Immunization during pregnancy may also affect the properties of these transferred immune cells. However, too little is known about lymphocyte transfer from mother to offspring to warrant direct manipulations of this process.

3.3. Lactoferrin and lactoferricin

Lactoferrin is a glycosylated milk protein, which is also found in other mucosal secretions and is expressed in neutrophils. It possesses a wide variety of antimicrobial functions (Table 3). Its actions against viruses, bacteria, and fungi can be classified into several different modes of action. (1) By sequestering free iron, lactoferrin can restrict the growth of Gram-positive and Gram-negative bacteria and a variety of fungi (Kuipers et al., 1999; Cirioni et al., 2000; Andersson et al., 2000). 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 (Hwang et al., 1998). Lactoferrin's activity against fungal infection in vitro and in vivo (Kuipers et al., 1999; Bellamy et al., 1993; Wakabayashi et al., 2000) may have the same biochemical basis. (3) Lactoferrin can also bind bacterial lipopolysaccharide (LPS), thereby impairing bacterial cell wall/membrane function (Nuijens et al., 1996; Odell et al., 1996). By binding free LPS or LPS bound to other proteins, it can also act as an antiinflammatory immune modulator (Baveye et al., 1999, 2000). (4) Lactoferrin purified from milk and colostrum is able to block human cytomegalovirus (hCMV) replication as well as inhibit HIV-1-induced cytopathic effects. Negatively charged lactoferrin derivatives obtained by acylation of the amino function of lysine residues even had a fourfold stronger antiviral activity against HIV-1 but lost their anti-hCMV activity. Native and acylated lactoferrin strongly binds to the V3 domain of the HIV envelope protein gp120, suggesting that the shielding of this domain results in the inhibition of virus–cell fusion and virus entry. Addition of positive charges to lactoferrin by amination resulted in an increased anti-hCMV activity and a loss of anti-HIV activity (Swart et al., 1998). (5) Lactoferrin also inhibits the entry of hepatitis virus C (HCV) into susceptible cells by direct interaction with the viral surface glycoproteins (Ikeda et al., 1998). Pilot studies in HCV-infected patients, however, only demonstrated a moderate antiviral activity in vivo (Tanaka et al., 1999). Whereas the antibacterial effects of lactoferrin probably reflect a natural function, the antiviral activities may be fortuitous. However, lactoferrin, α-lactalbumin, and β-lacto-globulin, which can also be derivatized to yield compounds with strong anti-HIV activity (Swart et al., 1996), may provide lead structures for the development of antiviral drugs (Swart et al., 1999).

In addition to its direct effects on pathogens, lactoferrin fulfills a variety of other functions. Lactoferrin binds to a cell surface receptor (McAbee et al., 1998). It is taken up into cells and transported to the nucleus, where it binds to DNA (Nuijens et al., 1996; Kanyshkova et al., 1999). It inhibits growth of tumor cells by modulating the expression and the activity of key G1 regulatory proteins (Damiens et al., 1999) and metastasis of tumors (Iigo et al., 1999). It also influences cellular responses to *Listeria* infections (Moriishi et al., 1999; Valenti et al., 1999). Lactoferrin is also reported to significantly decrease the cell number of *Helicobacter pylori* (a suspected cause of stomach cancer) in the stomach of experimental animals (Wada et al., 1999).

primary structure	name	origin	cleaving agent
	lactoferricin B	bov. lactoferrin	pepsin
	isracidin	bov. αS1-casein	chymosin
	casocidin I (αS2 casein aa 150-188)	bov. αS2 casein	trypsin
	αS2 casein aa 164-179	bov. αS2 casein	pepsin
	αS2 casein aa 183-207	bov. αS2 casein	pepsin
	α-lactalbumin f17-31 and 109-114	bov. α-lactalbumin	trypsin
	α-lactalbumin f61-68 and 75-80	bov. α-lactalbumin	chymotrypsin

Due to the wide variety of applications, lactoferrin has long been a prominent target for recombinant production in a variety of host organisms (Krimpenfort et al., 1991; Salmon et al., 1998; Sun et al., 1999; Chong and Langridge, 2000). Milk containing high amounts of lactoferrin may be used directly as a nutraceutical to prevent or treat bacterial infections of the digestive tract. Lactoferrin purified from milk could serve a variety of other purposes. Transgenic cattle expressing human lactoferrin have been generated (Krimpenfort et al., 1991) and the product is in clinical trials (⟨www.pharming.com/products⟩).

Lactoferricin B and H are derived from lactoferrin by pepsin cleavage in the digestive tract. The 25 amino acid lactoferricin peptide folds into a distorted antiparallel β-sheet, whereas the N-terminal 13 amino acids in the native lactoferrin adopt a α-helical structure (Hwang et al., 1998). The bacteriocidal effect of lactoferricin is far greater than that of native lactoferrin (Bellamy et al., 1992). Two tryptophan residues in the bovine lactoferricin peptide are indispensable for this activity (Strom et al., 2000). Although lactoferricin has been shown to depolarize bacterial membranes (Aguilera et al., 1999; Ulvatne et al., 2001), the precise basis of its antimicrobial action remains unclear. In order to induce the activity of lactoferricin in milk, the pepsin cleavage site may be engineered to be recognized by a protease, which is resident in the lactating mammary gland. This may boost the antibacterial potency of milk and reduce the number of bacteria (especially enterobacteria, which are particularly susceptible to lactoferricin action) in milk. These manipulations, however, would probably alter the processing abilities of milk. In addition, the modified milk may also have an unwanted immunogenic/allergenic effect.

3.4. Casein and casein fragments

Caseins and casein fragments also display antimicrobial activities, which, however, are not very well defined (Table 4). Isracidin is a chymosin cleavage product of bovine αS1 casein encompassing amino acids 1–23 (Fig. 2). It 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 (Lahov and Regelson, 1996). Surprisingly, isracidin is most potent when injected intramuscularly in vivo but only displays a modest antibacterial activity in vitro. This suggests that the effect of isracidin may be indirect. Therefore, it is uncertain whether transgenic overexpression of an isracidin-peptide in the lactating mammary gland would increase the antibacterial potency of milk. The chymosin cleavage sites in αS1 casein are conserved in ruminant proteins but not in other mammals, indicating that the formation of isracidin may not be essential for the well-being of mammals in vivo (Fig. 3). Although isracidin may not augment the

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Fig. 2. Primary structure of bactericidal peptides derived from milk proteins. Charged amino acids are marked (+) or (−); hydrophobic amino acids are designated with (*). Disulfide bridges between cysteine residues are indicated as lines. The distribution of charged and hydrophobic residues in lactoferricin B allows the peptide to fold into antiparallel amphipathic β-sheets (Hwang et al., 1998), which can interact with membranes. Similar structures could be formed by the other casein-derived peptides whose structures have not been identified yet. Bov.: bovine, aa: amino acids.

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      cow   MKLLILTCLVAVALARPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKE
  buffalo   MKLLILTCLVAVALARPKQPIKHQGLPQGVLNENLLRFFVAPFPEVFGKE
     goat   MKLLILTCLVAVALARPKHPINHQGLSPEVLNENLLRFVVAPFPEVFRKE
    sheep   MKLLILTCLVAVALARPKHPIKHQGLSPEVLNENLLRFVVAPFPEVFRKE


            signal peptide              isracidin


      cow   MKLLILTCLVAVALARPKHPIKHQ..GL.......PQEVLNE.NLLRFFVAPFPEVFGKE
      pig   MKLLIFICLAAVALARPKPPLRHQ..EHLQNEPDSREELFKERKFLRFPEVPLLSQFRQE
    mouse   MKLLILTCLVAAAFAMPRLHSRNAVSSQTQQQHSSSEEIFKQPKYLNL.NQEFVNNMNRQ
guinea pig   MKLFIFTCLLAVALA..KHKSEQQSSS....EESVSISQEK.FKDKNM......DTISSE
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Fig. 3. Alignment of the N-terminal regions of ruminant (cow, buffalo, sheep, and goat) and nonruminant (pig, mouse, and guinea pig) αS1 casein proteins. Dark and light background shading indicate the sequences, which correspond to the signal peptide and isracidin, respectively. Whereas there is a high degree of sequence conservation between the ruminant and nonruminant signal peptides, there is no significant sequence conservation between the N-terminal segments of the mature proteins, which correspond to isracidin.

antibacterial properties of milk, transgenic expression may nevertheless be useful, as large-scale production of isracidin was mainly hampered by the lack of suitable production systems (the peptide had to be isolated from milk after chymosin treatment). Production of larger amounts of isracidin in the milk of transgenic animals could be achieved either by expressing it as a peptide or by introduction of a cleavage site for a mammary-resident protease.

Two C-terminal pepsin fragments of bovine αS2 casein have also been shown to possess antibacterial activity (Zucht et al., 1995; Recio and Visser, 1999; Fig. 2). The precise mechanism by which these peptides exert their bacteriocidal effect is unclear. However, the αS2 casein-derived peptides share some similarities with other antibacterial peptides (Boman, 1995) and may be a component of an innate defense system of the mammary gland.

Human κ-casein strongly inhibits binding of *H. pylori* to susceptible intestinal cells (Stromqvist et al., 1995). Glycosylation of κ-casein was found to be crucial for this activity. Casein proteins, in general, may be more difficult to manipulate than whey proteins as caseins also contribute to the generation of casein micelles. Manipulations, which disturb the micelle formation, may therefore impair the animal's ability to lactate. This would influence efficiency of production as well as animal welfare.

3.5. Complement

The cytotoxic activity of milk is inactivated by incubation at 56°C, suggesting that complement is a major cytotoxic factor (Ogundele, 1999a,b). Complement activity, however, needs to be regulated to maintain the integrity of the mammary epithelium of the mother and the epithelium of the digestive tract in the offspring. Lactoferrin, complement protein C1, and the complement regulator CD59, which are expressed in the mammary epithelium, have been implicated in that control (Bjorge et al., 1996). Manipulation of complement proteins in milk without endangering the integrity of the mammary epithelium may be difficult.

3.6. Other milk-borne molecules

Other milk-borne molecules also have antimicrobial potency (Table 5). Milk mucin inhibits rotavirus infections in vitro and in vivo (Yolken et al., 1992), presumably by its sialic acid residues competing with natural virus receptors (Guerrero et al., 2000). Tryptic fragments of bovine α-lactalbumin (Fig. 2) efficiently restrict the growth of Gram-positive bacteria (Pellegrini et al., 1999). Other milk proteins have been shown to have neutralizing effects against specific pathogens (Table 5) but the protective mechanisms are not clear (Labeta et al., 2000; Newburg, 1999). Milk is also rich in oligosaccharides and glycoconjugates (Newburg, 1996). Many of which have been demonstrated to possess antiviral, antibacterial, or antifungal effects (see Table 2). Lipids in milk have also shown antimicrobial effects, which are not well defined at present (Goldman et al., 1996). These molecules may represent components of an innate defense system of the mammary gland. Synthesis of these substances, however, depends on the interplay of a number of enzymes. Manipulation of these pathways would require major efforts and may therefore not be cost-effective.

Various milk proteins, which are chemically modified, are also powerful antagonists of virus infections (Neurath et al., 1996; Berkhout et al., 1997). Although some of these activities are fortuitous, milk may provide a cheap source for the production for these agents. In addition, a wide variety of immunomodulatory substances are found in milk (Goldman, 2000; Table 5). Examples include interleukins, transforming growth factors α and β, or the complement regulatory protein CD59 (Bjorge et al., 1996). Some of the abovementioned molecules (e.g. lactoferrin and isracidin) also possess immune modulatory activity. Little is known about the precise role of these proteins in the mother or in the offspring (Goldman et al., 1996). However, the importance of these factors is exemplified by the fact that TGF-β knockout mice, which die shortly after birth, can be rescued by TGF-β supplied by maternal sources (via placenta and milk; Letterio et al., 1994). As the precise significance of these factors is not established, they are not likely candidates for targeted manipulation.

3.7. Nonmilk proteins and peptides

Other molecules with antimicrobial properties, e.g. porins, can theoretically be expressed in the milk of transgenic animals. However, these molecules may also have a deleterious effect against the mammary epithelium of the dam or the gut epithelium of the offspring. Therefore, these proteins or peptides would have to be expressed as preproteins, which have to be cleaved proteolytically (ideally by a pathogen-specific protease) to yield a functional toxin.

4. Conclusions

There are two routes for the manipulation of immunologically active, protective components of milk: vaccination and transgenesis (Table 2). Vaccination of mothers during pregnancy requires a careful consideration of potential risks and benefits for each

pathogen in question. Vaccination of ruminants can lead to the production of milk highly enriched in antibodies directed against particular pathogens (Ebina, 1996; Freedman et al., 1998). Since antibodies in milk are relatively stable (Schmidt, 1982), formula containing antibodies directed against a variety of pathogens can be generated and used for the prevention and treatment of infectious diseases of the digestive tract. The modified milk will have its major application in infectious diseases, which are acute but not life-threatening and do not lead to hospitalization. However, the economic value of this strategy may be considerable. In addition, the time span between the occurrence of a new pathogen (or a new variant) and the development of a vaccine may be shortened significantly by DNA vaccination. DNA vaccines have been effective in many instances (Turnes et al., 1999) and lead to the production of pathogen-specific antibodies in colostrum and milk (Jenkins et al., 1999). Manipulation of milk by transgenesis will be useful in the veterinary field. Milk containing either a defined, highly neutralizing antibody directed against a pathogen or a high concentration of a nonspecific defense molecule may be applied for the prevention of infectious diseases, when suitable vaccines are unavailable. This approach, however, will be subject to a stringent cost-benefit analysis. The transgenes encoding the antipathogenic molecules will not be integrated into the conventional breeding programs but used for the generation of a herd of animals, which provides modified milk as a product (Whitelaw, 1999).

The modification of the immunological properties of milk by vaccination, transgenesis, or a combination of both may be a promising approach, which leads to products with a clear health benefit in the medical and veterinary field. Different mammalian species employ different strategies to provide immune protection to their offspring. Also, pathogens use different strategies to overcome the host's immune defenses. Therefore, there will not be a single approach for the prevention of infectious diseases via immunologically modified milk, but a variety of approaches tailored to specific classes of pathogens will have to be used to turn milk into an effective disease-preventing nutraceutical.

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