Recombinant human milk proteins—an opportunity and a challenge \(^1,^2\)

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**ABSTRACT** Several human milk proteins have physiologic functions in infants. These proteins are involved in defense against infectious agents and in the optimization of nutrient uptake from milk. Therefore, interest in producing recombinant human milk proteins to use in infant formula has been growing. Microorganisms and transgenic animals can now be used for the production of bioactive proteins. However, the benefits of each protein must be evaluated in cells, animal models, and infants before claims can be made that adding them to formula improves the health or nutrition of infants. Once benefits are shown, proper manufacturing conditions must be developed for introducing the protein or proteins into formula. Processing conditions must be evaluated to ensure that biologic activity is maintained. Dry blending, aseptic processing, sterile filtration, and other techniques will likely be necessary for introducing proteins that require specific tertiary structure for activity. The importance of posttranslational modifications must also be considered: some proteins may require proper glycosylation or phosphorylation for physiologic activity. *Am J Clin Nutr* 1996;63:622S-6S.

**KEY WORDS** Milk, human milk, milk proteins, transgenic organisms, recombinant proteins, bioactive proteins

**INTRODUCTION**

Human milk provides infants with several benefits and there is consensus that breast-feeding is the optimal mode of feeding infants (1). Several studies showed that breast-fed infants have a lower incidence of infections (2-6) and also that the infections that do occur may be of shorter duration than those in formula-fed infants (7). Epidemiologic studies showed an association between feeding breast milk in early life and mental scores (IQ) in later life (8); in addition, an association between early introduction of cow milk and the development of insulin-dependent diabetes mellitus was observed (9), although this disease is multifactorial in origin and numerous other etiologies have also been suggested. Thus, direct cause-and-effect relations have not yet been shown.

It is also well known that nutrients are well absorbed from human milk and that they are provided in balanced quantities. Thus, in many clinical studies in which infant formulas are evaluated, a breast-fed control group is used and all indexes are evaluated against this standard. In addition, most nutrient requirements for infant formulas are based on the intakes of breast-fed infants, with an additional safety factor added to compensate for possible lower absorption from formula. With an increased knowledge of infant nutrition and of the qualities of breast milk, the composition of infant formulas has regularly been modified and significant improvements have been made. Current infant formulas are different from those of just a decade ago. Changes in composition have increased the nutritional quality of formulas, but still, some of the properties of human milk are difficult to achieve with components from cow milk or plant proteins (soy). Recent advances in molecular biology and genetic engineering have made it possible to add human milk components or to modify the components of infant formula to make them similar to those of human milk. Because many of the physiologic advantages of human milk are provided by proteins (10), one of the major areas in which progress has been made is in the production of recombinant human milk proteins.

**PHYSIOLOGIC FUNCTIONS OF HUMAN MILK PROTEINS**

The lower incidence of bacterial and viral infections in breast-fed infants is likely due in part to the immunoglobulins in human milk. It was shown that maternal immunity can be transferred to breast-fed infants by specific secretory immunoglobulin A (sIgA) antibodies (11). Human milk is exceptionally high in sIgA during early lactation, and all through lactation this is one of the major proteins in human milk (10-12). Other immunoglobulins, such as IgG, IgM, and IgA are also present in human milk, but at considerably lower concentrations. It is less likely that these immunoglobulins will provide substantial activity in breast-fed infants; the amounts provided are low and they are likely to be digested. It was shown, however, that sIgA survives digestion because of a stable molecular arrangement, and therefore remains intact in the gastrointestinal tract in breast-fed infants (13). The possibility of producing antigen-specific sIgA by genetic engineering may prove more difficult than the production of other human milk proteins, but some attempts have been made to produce specific human antibodies by in vitro methods. It may be possible to produce specific antibodies against some of the more common pathogens; however, at this time it appears unlikely that epitope-specific antibodies can be made against the whole panorama of pathogens that the mother has been exposed to.

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Antibacterial activity is also provided by lysozyme in human milk. The concentrations of lysozyme are high in human milk (eg, 3000 times higher than in tears, another site of lysozyme antibacterial activity); this enzyme can degrade the specific linkage between N-acetylglucosamine and N-acetylmuramic acid in the cell walls of mainly gram-positive but also some gram-negative bacteria (10, 14). Definitive evidence for such an effect in the gastrointestinal tract of breast-fed infants is lacking, but it is likely that lysozyme can have an effect on the bacteriologic quality of the milk.

Another component of human milk that is believed to have bacteriostatic or bactericidal activity is lactoferrin (15), a major protein in human milk (1-2 g/L). It can bind two ferric ions, but usually occurs in a highly unsaturated form (16). Thus, highly unsaturated lactoferrin can bind iron with a high affinity and can successfully compete with iron-requiring bacteria. This was shown to occur in vitro for several pathogens, such as Vibrio cholerae, Streptococcus mutans, and Escherichia coli (17, 18), but there is little evidence to date for such an effect in vivo. More recently, it was found that a particular fragment of human or bovine lactoferrin can have a bactericidal effect in vitro (19). Whether such a peptide is formed in the infant in vivo is not yet known.

Several human milk proteins were shown to have glycans that are analogues of the receptors used by bacteria in the gastrointestinal tract of infants (20). Human colostrum slgA (21) and 8-casein (22) were shown to inhibit the adherence of Helicobacter pylori to human gastric mucosa. Whether it is only the glycan part of these glycoproteins that is needed to inhibit the attachment of bacteria or whether there is some synergism between the glycan and the rest of the protein is not yet known. Molecular biology techniques should easily resolve some of these questions.

Lactoferrin may facilitate the uptake of iron in breast-fed infants. The presence of a lactoferrin receptor in the brush border membrane of the small intestine of human infants supports such a hypothesis (23). Clinical studies in which infants were given formula fortified with bovine lactoferrin did not show increased iron absorption (24-26). However, the human receptor does not appear to recognize bovine lactoferrin, which may explain these results. Studies of human lactoferrin in infant rhesus monkeys (27) and in human infants given breast milk and breast milk from which the lactoferrin was removed (28) have not provided strong support for a direct enhancing effect of lactoferrin on iron absorption. Lactoferrin may deliver the iron effectively to the mucosal cell, but there may be other mechanisms that determine the fate of the newly absorbed iron after that. Lactoferrin was also shown to have a positive effect on crypt cell growth in vitro, but this effect appears to occur independently of iron saturation (29).

Caseins are believed to be easily digested and to be a readily accessible source of calcium, phosphate, and amino acids. However, some caseins, particularly 8-caseins, are digested to only a limited extent (10). The resulting peptides may have several physiologic activities. For example, the aminoterminal, highly phosphorylated casein phosphopeptide was shown to increase calcium solubility and facilitate calcium absorption in vitro (30,31) and in animal experiments (32). Caseomorphins have opiate-like activity and may have an important role in newborns, not only neurologically in the gut mucosa but also, possibly, on sleeping patterns (10). These compounds and their activity require further evaluation.

Bile salt-stimulated lipase (BSSL) was shown to facilitate the digestion and absorption of lipids in infants (10, 33). This enzyme can hydrolyze triacylglycerols completely to yield fatty acids and glycerol. This is important in premature infants who have poor lipase activity; thus, BSSL may play a role in early life to ensure lipid digestion.

Many enzymes have been detected or analyzed in human milk (34). It should be noted, however, that the mere presence of these enzymes does not necessarily mean they have a function in breast-fed infants. Enzymes are part of the mammary gland and they participate in its metabolic activities and are also transferred from the bloodstream. Similarly, growth factors and cytokines are present in human milk at physiologically relevant concentrations (35, 36) but it is not known whether they act on the gland or on the infant, even if some animal studies favor a role of growth factors in neonatal development (35).

**RECOMBINANT HUMAN MILK PROTEINS**

Several human milk-protein genes have been cloned and sequenced, most of them at the level of complementary DNA (cDNA), but in a few cases the entire gene has been characterized. One of the first milk proteins to be cloned from a mammary gland library was &-lactalbumin (37). Human lysozyme was also cloned, but from a placental cDNA library (38). However, this protein is expressed in several tissues, and milk lysozyme was shown to be identical to that in other tissues. Lactoferrin was cloned by two groups; there are minor differences in the reported nucleotide sequences (39, 40). In addition, lactoferrin was cloned from a neutrophil cDNA library, and in this case enough of the 5'-flanking region was obtained to describe the promoter region (41). BSSL was also cloned and sequenced by two research groups, with some minor inconsistencies in the sequence (42, 43).

Caseins are among the major milk proteins in virtually all species. Human 8-casein cDNA was first cloned and sequenced (44) and subsequently the entire 8-casein gene was isolated and sequenced (45). This gene contains eight exons and seven introns and the entire gene is &-10.5 kb. Human &-casein cDNA was also cloned and sequenced (46).

Human milk also contains growth factors, such as epidermal growth factor and insulin-like growth factor, and cytokines (35, 36). Most of these were cloned from cells and tissues other than those of the mammary gland and are commercially available. Many clinical trials evaluated the functions of these recombinant proteins and peptides, but to date there have been no attempts to use them as components of infant diets, even if there are known physiologic functions for them. These compounds are potent biomolecules; in some situations (eg, in prematurity), giving them parenterally would make it possible to more precisely control their dosage, and might be preferable.

**EXPRESSION SYSTEMS FOR PRODUCING RECOMBINANT HUMAN MILK PROTEINS**

Depending on the need for posttranslational modification of the protein in question or the regulatory requirements of bodies
such as the US Food and Drug Administration (FDA) to ensure a safe product, different expression systems for producing recombinant proteins can be used. Bacterial expression of recombinant proteins was part of the advent of genetic engineering and is still used for large-scale production of proteins. This approach was used for human β-casein (47). The expression vector can be chosen to make the protein available in the supernate (secreted) or in the bacterium (intracellular). However, proteins will not be phosphorylated or glycosylated in bacteria. If such modifications are needed for physiologic activity, yeast or fungi can be used. For example, Saccharomyces cerevisiae was used for producing human lactoferrin (48) and human β-casein (47) and Aspergillus nidulans and A. oryzae were used to produce human lactoferrin (49, 50). Another possibility is to use animal cells in culture. For example, human lactoferrin was expressed in baby hamster kidney cells (51).

The patterns of glycosylation and phosphorylation in recombinant proteins may be quite different from those of the native proteins. Little is known about how this will affect the activity of milk proteins. Tissue-specific expression of human milk proteins in transgenic animals should result in recombinant proteins with glycans and phosphorylation patterns more like those of human milk proteins, but they will still not be identical. At first, transgenic mice are likely to be used. To date, human lactoferrin (52) and human lysozyme (53) have been expressed in transgenic mice with reasonable levels of expression in the milk. Subsequently, other species such as sheep, swine, and cattle will be used. Human lactoferrin was recently produced in transgenic cows (54). It is not yet known whether the minor differences in posttranslational modification will affect the biologic activity of this protein; this will have to be determined for each protein expressed in transgenic animals.

EVALUATION OF THE PHYSIOLOGIC ACTIVITY OF RECOMBINANT HUMAN MILK PROTEINS

A recombinant milk protein that has enzymatic activity can be evaluated initially in vitro. For example, various substrates (triacylglycerol and monacylglycerol) can be incubated with BSSL. The activity of recombinant BSSL can then be assessed and compared with that of the native enzyme. Substrate binding and specificity of the enzyme can be evaluated. The activity of the entire enzyme as well as of deglycosylated and truncated versions of BSSL (deletions of various regions) can also be assessed (55). As another example, the capacity of recombinant lactoferrin to bind iron can also be evaluated in vitro.

At a level of higher complexity, cells can be used to assess the capacity of recombinant proteins to interact with specific sites. For example, lactoferrin is known to bind to specific receptors on monocytes (56) or enterocytes (57); binding studies can then confirm whether the recombinant form interacts in the same manner. From a slightly different perspective, human κ-casein was shown to prevent the adhesion of H. pylori to gastric mucosal cells (22). Thus, recombinant κ-casein can be evaluated for its capacity to prevent adhesion.

Animal models can subsequently be used to evaluate the activity of a recombinant protein in vivo. The choice of animal model is important for obtaining data that are relevant to human infants. In many cases, rodent (rat and mouse) models are inadequate because the metabolism or gastrointestinal function, or both, of rodents is different from that of infants. For example, rat small intestine has receptors for transferrin, a major iron-binding protein in rat milk, but no receptors for lactoferrin (58). Thus, the data obtained for recombinant human lactoferrin in rats are not likely to be valid for human infants. On the other hand, proteins like β-casein that facilitate calcium uptake by keeping calcium soluble and delivering it to the mucosal cells could be evaluated in a rat pup model because there may be no specific receptor mechanisms involved (10).

Nonhuman primates may be a better alternative for preclinical trials to assess the activity of recombinant proteins. Infant rhesus monkeys were shown to have postnatal development similar to that of human infants with regard to gastrointestinal function and nutrient requirements. The composition of rhesus monkey milk is also similar to that of human milk (59). Thus, proteins that have physiologic activity in infant monkeys may have the same function in human infants. For example, it was shown that rhesus monkey milk has a high concentration of lactoferrin, that its structure is similar to that of human lactoferrin, and that the lactoferrins show immunologic cross-reactivity (60). Therefore, infant rhesus monkeys can be used to evaluate the function of recombinant human lactoferrin, and monkey tissues can be used to more specifically evaluate interactions with the lactoferrin receptor (61). Because radioisotopes can be used in infant monkeys, iron absorption can be studied with a high degree of precision (27).

The final tests of recombinant milk proteins must be performed in human infants. Several hurdles will have to be overcome when this stage is approached. Ethics boards are likely to approve clinical protocols because the recombinant proteins are not likely to have any adverse affects; in the worst-case scenario, the recombinant proteins may be ineffective. It will be necessary to ensure that any impurities have no negative side effects, but many of the expression systems described above have been used to produce food components (eg, rennin) that are on the FDA’s GRAS (generally regarded as safe) list. Approval by the FDA and subsequent acceptance by the consumer are important issues to resolve, as discussed in another article in this supplement (62).

ADDING BIOACTIVE RECOMBINANT HUMAN MILK PROTEINS TO INFANT FORMULA

The biological activity of a recombinant human milk protein will also need be evaluated as a component of infant formula. The possibility that several components of formula may interact with the human protein cannot be immediately excluded. Differences in concentrations of several major nutrients such as protein, calcium, and phosphate may cause protein aggregation or possibly precipitation, which may lower or inhibit the protein’s activity. The presence of vegetable oils and emulsifiers may also, at least in theory, interfere with the bioactivity of the protein. Thus, the experiments mentioned above should also be performed with the recombinant protein present in the formula.

Heat treatment of infant formula is likely to have a substantial effect on the activity of proteins. Regular “in-can” sterilization of liquid formula involves temperatures >100 °C for several minutes, which will denature and inactivate most proteins. An alternative is spray-drying, which is used for
powdered formula. In this process, 90 °C is reached for a few seconds. More recently, some infant formulas were prepared by UHT (ultra-hightemperature) treatment; i.e., treatment at ≈130 °C for 3–8 s. Few studies to date addressed the issue of heat treatment of recombinant proteins, but some studies explored the effect of temperature on milk proteins. For example, Abe et al (63) studied the effect of pH and heat treatment of bovine lactoferrin on its iron-binding capacity. They found that at pH 4 and treatment at 90 °C for 5 min, bovine lactoferrin retained its iron-binding capacity, antigenic properties, and bacteriostatic activity. The effect of heat treatment of bovine lactoferrin on its ability to interact with monocytes was also investigated (56). The authors found that heat treatment of apo- or hololactoferrin for 20 s at 72 °C or 8 s at 137 °C had no effect on thymidine incorporation (proliferation) but that treatment at 85 °C for 20 min inhibited the effect of hololactoferrin. Formula manufacturers will likely develop new technologies that will make it possible to incorporate bioactive proteins into their products. Such methods may include dry-blending into powdered products and aseptic packaging.

Other important considerations when bioactive components are added to formula are the greater ionic strength and the stability (shelf life) of the product. In some cases, evaluating all of these factors will require that the active component be isolated before the effects of the treatment or conditions are evaluated in detail.

ADDITIONAL CONSIDERATIONS

Clear demonstrations of the benefits of adding bioactive proteins to infant formula will be important for the formula industry. Investments in genetic engineering and other aspects of novel bioactive foods must be substantial to overcome technical hurdles and address regulatory and consumer concerns. Consumers are sensitive to any increase in cost and without strong arguments for an improved product it is unlikely that the market will tolerate a significant increase in the price of the product. Consumers who purchase special formulas, such as those for premature infants or infants with inborn errors of metabolism, are likely to tolerate a larger increase in cost, but many of these products are given away at hospitals.

Although, from the scientist’s perspective, adding a component to formula that is “identical” to a component of human milk should be perceived as beneficial by consumers, such modifications should be considered with great care. Some consumers and activist groups may react negatively toward any addition that is considered to be genetic engineering or biotechnology. As scientists, we should be aware that although some proteins may be considered to be identical to human milk proteins, proteins with different glycosylation or phosphorylation patterns may be used, and that, in the future, truncated versions of the protein or variants in which certain amino acids are substituted through site-directed mutagenesis may also be used. Thus, although consumers may be prepared to accept “the real thing,” they may be hesitant to accept products produced in a way that they believe can be characterized as tampering with nature. Only with extensive information and education are these hurdles likely to be overcome.

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