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Recombinant human lactoferrin treatment for global health issues: iron deficiency and acute diarrhea

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Abstract

Iron deficiency and diarrhea are two of the most significant issues for global health. Iron deficiency anemia is the most common nutritional deficiency in the world, affecting nearly 25% of the world population (UNICEF/WHO 1999). The prevalence of iron deficiency in developing countries is illustrated by comparison with other deficiencies: iron deficiency affects 3.5 billion people, while vitamin A and iodine deficiency affect 0.3 billion people and 0.8 billion people, respectively. The prevalence is highest among young children and women of childbearing age (particularly pregnant women). It is estimated that national productivity levels could be raised as much as 20% by correcting iron deficiency in developing countries. Recombinant human lactoferrin (rhLF), expressed and extracted from rice seed, is being evaluated by Ventria Bioscience for use as a dietary supplement to treat iron deficiency and/or iron deficiency anemia. Diarrhea is also a major world health issue. Sixty percent of children who die under age five die of pneumonia, diarrhea or measles. World Health Organization oral rehydration solution (WHO-ORS) is one of the major medical advances in the past 50 years, saving the lives of 1 to 2 million children annually. Many studies have demonstrated similar efficacy of rice-based ORS. There are studies documenting the reduced frequency of diarrhea in breast-fed children and this health improvement is attributed to the antimicrobial action of the human milk proteins lactoferrin and lysozyme. *In vitro* data document the growth inhibition of the diarrheal associated organisms: rotavirus, ETEC, cholera, salmonella, and shigella by human lactoferrin (hLF) and human lysozyme. Using Ventria's ExpressTec^{TM} system, we have expressed human lactoferrin and human lysozyme in rice. In a rice-based ORS formulation, these proteins have the potential to provide not only the benefits of reduced stool volume and improved weight gain, but also shorten the course of diarrheal episodes via antimicrobial activity against the causative agent.

Introduction

Despite progress made in dealing with global health issues, there are still staggering numbers related to morbidity and mortality in developing countries. In addition to infectious diseases such as respiratory infections, diarrhea, malaria, measles, and HIV/AIDS, there are also malnutrition problems and micronutrient deficiencies. In the report on the State of the World's Children in 1998, it was estimated that over half of the child deaths had an underlying association with malnutrition (1998). The link between nutrition, growth faltering and diarrhea continues to be an area of concern in developing nations, and research has shown a bi-directional interaction between malnutrition and diarrhea (Guerrant, Schorling *et al.* 1992).

Iron deficiency and diarrhea are two significant issues for global health. Iron deficiency is the most common nutritional deficiency in the world. The World Health Organization (WHO) reports that as many as 4–5 billion people may be iron deficient. The prevalence is highest among young children and women of childbearing age (particularly pregnant women). It is estimated that correcting iron deficiency alone would reduce maternal deaths by 20%, decrease child mortality by 23% and increase work capacity by 15% (WHO 2003). The micronutrient initiative, following the United Nations World Summit for Children in

Table 1. Comparison of expression levels of rhLF in transgenic plants

Expression system	rhLF/Total solulble protein (%)	rhLF/Total mass (%)	Reference
Tomato (leaf)	Expressed	Expressed	(Anzai *et al.* 2000)
Tobacco (culture)	Expressed	Expressed	(Mitra & Zhang 1994)
Corn (Grain)	Expressed	0.02%	(Personal communication)
Potato (leaf)	0.1%	–	(Chong & Langridge 2000)
Potato (tuber)	0.1%	–	(Chong & Langridge 2000)
Tobacco (leaf)	0.8%	–	(Salmon *et al.* 1998)
Rice (Grain)	–	0.05%	(Anzai *et al.* 2000)
Ventria rice	**25%**	**0.5%**	**(Nandi *et al.* 2002)**

1990, set a target goal of reducing anemia in pregnant women by 30%. In the decade that followed, iodine and vitamin A deficiencies were significantly reduced, but little progress was made in the reduction of iron deficiency (Gleason 2002). In 2002, the goal of reducing iron deficiency by 30% was set once again and has received a higher priority in the agendas of developing countries.

Morbidity and mortality of children under the age of five due to diarrheal diseases remain a major health challenge in developing nations as well as in lower socio-economic groups in the developed world. It was not until the 1950s that diarrheal diseases were identified as a major cause of morbidity and mortality in children less than five years of age. Through interventions and the development of programs for the control of diarrheal diseases (CDD) the annual mortality in children under five has decreased from 13.6 deaths per 1000 children per year in 1955–1979 to 4.9 in 1992-2000 (Kosek, Bern *et al.* 2003). WHO reported approximately 1.5 million children under the age of five died from diarrheal disease in 2000 (Victora, Bryce *et al.* 2000). However, during this same time period, the morbidity rate has remained remarkably stable. The median number of diarrhea episodes was 3.2 per child per year for children under five years of age. This value did not differ from the median episodes reported in the reviews for 1955–1979 and 1980–1989 (Kosek, Bern *et al.* 2003). Most of the interventions in the CDD programs are expected to have a direct effect on the reduction of mortality and morbidity. These include promotion of optimal breast-feeding practices, improved supplemental feeding and weaning, access to clean water and sanitation facilities, and improved personal and domestic hygiene. In addition, immunization against measles and use of vitamin A may play a role in decreasing mortality (Victora, Bryce *et al.* 2000).

Production of recombinant human lactoferrin in rice grain

Ventria Bioscience has spent more than six years developing and optimizing its proprietary protein expression system, ExpressTec™. ExpressTec™ allows the production of recombinant proteins and peptides in cereal grains, including rice, at large volume with low cost. Recombinant proteins are targeted to protein bodies for safe expression and accumulation through specific promoters and signal peptides. Some of the work related to development and utilization of ExpressTec™ has been published (Huang *et al.* 2001, Yang *et al.* 2001, Huang *et al.* 2002, Huang *et al.* 2002, Humphrey *et al.* 2002, Nandi *et al.* 2002). With ExpressTec™, rhLF is expressed in rice at significantly higher levels than in other transgenic plant systems (Table 1), reaching 0.5% of grain weight.

Kilograms of rhLF have been purified to near homogeneity and compared to native human lactoferrin. The results of the comparisons are summarized in Table 2.

One of the important considerations in the production of recombinant proteins for human consumption is the glycosylation pattern. Use of various available expression systems, *E. coli*, yeast, mammalian cells and plants, results in glycosylation patterns characteristic of the species expressing the protein. Thus, it is predictable that hLF and rhLF will have different glycosylation patterns. SDS-PAGE and MOLDI demonstrated that hLF and rhLF exhibited slightly different molecular weights, though they have identical amino acid composition (unpublished data). The different

Table 2. Comparison of physical, chemical and biochemical properties of native human lactoferrin and recombinant human lactoferrin from rice

Property	Human LF (hLF)	Recombinant hLF (rice-derived)
Molecular mass (MALDI)	80.6 kDa	78.5 kDa
Isoelectric focusing point	8.2–8.7	8.2–8.7
Solubility	>10 mg/ml PBS at RT	>10 mg/ml PBS at RT
Physical appearance	Apo-form: colorless Holo-form: Pink	Apo-form: colorless Holo-form: Pink
Resistance to pepsi/ pancreatic digestion	Resistant to digestion	Resistant to digestion
Iron binding and release capacity	pH dependent release and saturation	pH dependent release and saturation
Receptor binding affinity (CACO-2 cells)	Kd = 0.16 +/− 0.03 μM	Kd = 0.27 +/− 0.04 μM
Immunogenicity	Reactive to rabbit polyclonal Ab against hLF	Cross-reactive to rabbit polyclonal Ab against hLF
Binding to Porins	Binds to porins	Binds to porins
Bacteriostatic effect (EPEC)	Growth reduction of *E. coli* by 5 fold at 37 °C for 12 h	Growth reduction of *E. coli* by 10 fold at 37 °C for 12 h
Peptide mapping by Lys-C digestion	Similar pattern to each other	Similar pattern to each other
N-terminal sequence	GRRRRSVZWCA	Identical (GRRRRSVZWCA)
Amino acid composition	Identical to each other	Identical to each other
Glycosylation	Mammalian pattern	Plant pattern

carbohydrate moieties coupled to the lactoferrin polypeptide could explain this difference. In fact, based on the MOLDI data and primary amino acid sequence information, sugar content constitutes 5.5% and 2.9% of hLF and rhLF mass, respectively. Although glycosylation sometimes plays an important role in the functionality of the molecules, it has been demonstrated that the carbohydrate moieties are not required for the interaction of LF and its receptor (Kawakami & Lonnerdal 1991, Iyer & Lonnerdal 1993). Furthermore, studies in infant rhesus monkeys fed rhLF for four months (unpublished data), indicated that there is no immunological response to the variation in glycosylation.

Lactoferrin-iron: A solution to iron deficiency (ID) and iron deficiency anemia (IDA)

There are three possible ways to prevent and control the development of ID and IDA. They are dietary diversification, food fortification and individual supplementation (Zlotkin 2002). Dietary diversification involves promotion of diet with foods rich in iron (e.g. meat and fish). This approach is not feasible in the developing world due to the high cost of foods rich in bioavailable iron. Food fortification, especially fortified commercially prepared infant food, is an excellent way to increase iron uptake in children. However, it is also cost-prohibitive in the developing world. The final approach is iron supplementation through iron drops/syrup or tablets. Recently, Supplefer Sprinkles were proposed as another way to supplement iron intake (Zlotkin 2002). Due to the poor bio-availability of inorganic iron (1–7%), however, a large quantity of iron must be consumed by IDA patients. Heavy iron loads cause many problems in the gut, including gastrointestinal discomfort, nausea, vomiting, diarrhea, constipation, iron toxicity. The potential risk of increased bacterial infections associated with iron supplementation was recently addressed in a comprehensive review (Gera & Sachdev 2002). Combined data from 28 controlled studies showed iron supplementation had no effect on the incidence of infectious disease. Due to the poor compliance with current treatments, there is a need for an alternative, low-cost treatment for ID and IDA.

Lactoferrin is a naturally existing iron binding protein. Each mole of lactoferrin binds two moles of ferric iron with high affinity. Lactoferrin receptors have been identified in several types of mammalian cells (Suzuki & Lonnerdal 2002, Testa 2002). Recently, a human intestinal lactoferrin receptor was cloned. When CACO-2 cells were transfected with the cloned gene, they were able to take up 3.4 times more iron than non-transfected cells (Suzuki, Shin *et al.* 2001). LF is thought to be internalized through endocytosis (Bi, Liu *et al.* 1996). Iron is then released from hLF-Fe complex in intestinal cells and LF is degraded (Mikogami *et al.* 1994). The released iron is then transported through the basolateral membrane into the circulation by transferrin. This proposed apical-to-basolateral hLF-Fe transport mechanism via a specific receptor in the intestinal cells provides an efficient mechanism for iron uptake (Mikogami *et al.* 1994, Bi *et al.* 1996, Suzuki *et al.* 2001). Studies also confirmed that the binding of hLF to the human LF receptor was greater than bovine lactoferrin (bLF), indicating a specific recognition mechanism (Cox *et al.* 1979, Kawakami & Lonnerdal 1991, Iyer & Lonnerdal 1993). Furthermore, the interaction of LF and receptor is not dependent on the carbohydrate moieties (Kawakami & Lonnerdal 1991, Iyer & Lonnerdal 1993).

The concept of using LF-Fe complex to treat anemia is supported by a study conducted with artificially induced anemia in rats (Kawakami *et al.* 1988). Four groups of rats were given 35 mg of apo-bLF, 35 mg iron-saturated bLF, 250 μg $FeSO_4$ or H_2O orally once a day. Changes in red blood cell density, hematocrit, and hemoglobin values were measured at 14-day intervals for 70 days. A statistically significant increase in these values was demonstrated for the rats fed iron-saturated lactoferrin (50 μg Fe/35 mg lactoferrin/day), while the ferrous sulfate group showed only slight improvement. The results suggest that iron from iron-saturated lactoferrin is absorbed across the intestinal mucosa much more readily than ferrous sulfate.

Ventria Bioscience has proprietary technology for expressing rhLF at large scale in a cost effective way and has demonstrated the capacity to produce kilograms of iron saturated rhLF. In order to prove the concept that ID and IDA can be alleviated and treated by oral consumption of rhLF-iron complex, clinical studies are planned.

Use of recombinant human lactoferrin in management of acute watery diarrhea

The CDD programs in developing countries have contributed to the reduction in mortality and morbidity in children younger than five years of age. The first intervention in the CDD program, and probably the most significant contributor to the reduction in mortality, is not linked to reduction in morbidity. That intervention is the administration of oral rehydration solution and therapy (ORS/ORT) developed in 1970 (Cash *et al.* 1970). Oral rehydration solution (salt/sodium, sugar/carbohydrate and water) is considered one of the most significant medical advances of the 20^{th} century (Farthing 2002). It has played an important role in preventing diarrheal-associated dehydration. It is not, however, anti-diarrheal. One of the early modifications of ORS was in the source and form of the carbohydrate (glucose). In many cultures rice and rice water have been used as a home remedy for diarrhea. A number of clinical and field studies comparing rice-based ORS with WHO glucose-based ORS demonstrated reduced stool output, decreased duration of diarrhea and fewer unscheduled interventions with intravenous fluids (Molla *et al.* 1985, Faruque *et al.* 1997, Zaman *et al.* 2001).

In considering alterations of the ORS formulation that might add anti-diarrheal activity, it is logical to look at the success of breast-feeding, since breast-fed children have a lower incidence of diarrhea, as well as other infections (Victora *et al.* 1987, Lopez de Romana *et al.* 1989, Shah *et al.* 2003). Breast milk contains a number of innate antimicrobial proteins that may play a role in the reduction of diarrhea. *In vitro* data suggest that the milk proteins lactoferrin and lysozyme play an important part in the antimicrobial role of breast milk (Lonnerdal 1985). Lactoferrin and lysozyme individually and in combination have demonstrated antimicrobial activity against a wide spectrum of bacteria, viruses, parasites, and fungi. Etiologic agents associated with diarrhea in children have been identified. The organisms most frequently associated with diarrhea in developing countries are rotavirus (15–25%), enterotoxigenic *Escherichia coli* (EPEC) and enteroadherent *Escherichia coli* (EAEC) (10–20%), shigella (5–15%), salmonella (1–5%), *Campylobacter jejuni* (10–15%) and cryptosporidium (5–15%) (PAHO 1997). A two-year study of children from a peri-urban area of Lima, Peru, found that 60% of the patients had viruses, 34% bacteria and 5% parasites. Rotavirus was the most commonly detected etiologic agent, present in 52% of the cases. In one-third of the rotavirus cases, there was a co-infecting pathogen (Cama *et al.* 1999). Lactoferrin alone, or in combination with lysozyme, has demonstrated *in vitro* bacteriostatic/bactericidal activity against the etiologic agents associated with diarrhea (Sterling *et al.* 1991, van der Strate *et al.* 2001, Huang *et al.* 2002, Nandi *et al.* 2002, Gomez *et al.* 2003).

Lactoferrin also has immunomodulatory activity, up-regulating the anti-inflammatory cytokines and down-regulating the pro-inflammatory cytokines in the intestinal tract (Togawa *et al.* 2002). Studies in animals with oral administration of lactoferrin have demonstrated a protective effect against development of colitis via modulation of the immune system. This is accomplished by lactoferrin-induced increases in the anti-inflammatory cytokines IL-4 and IL-10 and the inhibition of release of the pro-inflammatory cytokines IL-6, TNF-α, and IL-1β. This protective mechanism may result in less damage and more rapid repair of gut mucosal tissue leading to normal permeability and growth. Lactoferrin also protects gut mucosa against the effects of bacterial lipopolysaccharide in mice (Kruzel *et al.* 2000).

The extensive *in vitro* data using lactoferrin and lysozyme, as well as the positive effects of breast-feeding, suggest that an ORS that provides antimicrobial activity along with rehydration would provide another advance in the management of infectious diarrhea. The addition of recombinant human lactoferrin and lysozyme to an ORS may result in a reduced duration of diarrheal disease and faster rate of recovery.

Conclusion

The use of recombinant human lactoferrin extracted from rice is an attractive approach to addressing global health needs through nutritional supplements. Rice is the predominant cereal grain consumed by a large percentage of the world's population and among the first foods recommended for introduction to infants. It has nutritional value and low allergenicity. After extraction, any residual protein or carbohydrate from the rice introduces little risk and may be viewed as nutritionally sound. Ventria Bioscience's ExpressTec™ expression technology provides for the production of high volume, low-cost proteins for oral administration. These proteins, incorporated into supplements for iron deficiency and diarrhea have the potential to address two global heath needs.

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