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December 16, 2004

Robert L. Martin, Ph.D.
U. S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
CFSAN/OO/OFAS/DBGNR
HFS-255
Harvey W. Wiley Federal Building
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Dear Dr. Martin,

We wish to notify you that Ventria Bioscience has determined that recombinant human lactoferrin produced in rice is "generally recognized as safe" (GRAS) for use as an ingredient in functional foods and beverages and medical foods. Accordingly, recombinant human lactoferrin is exempt from the premarket approval requirements of the Federal Food, Drug and Cosmetic Act.

Enclosed please find an original, two copies and one electronic copy on disk of the document used by Ventria Bioscience to make its GRAS determination. As directed by the agency, the information is formatted in accordance with proposal 21 CFR 170.36(c) (62 Federal Register 18937 (April 17, 1997)).

The data and information that serve as the basis for this GRAS notification will be sent to the FDA upon request or are available for the FDA's review and copying during business hours at the office of Delia Bethell, Ventria Bioscience, 4110 N. Freeway Blvd., Sacramento, CA 95834, telephone: 916-921-6148 ex 21, fax: 916-921-5611, email: dbethell@ventria.com.

Sincerely,

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GENERALLY RECOGNIZED AS SAFE (GRAS) NOTIFICATION FOR RECOMBINANT HUMAN LACTOFERRIN PRODUCED IN RICE

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December 16, 2004

Contains No Confidential Business Information

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I. GRAS EXEMPTION CLAIM

A. Name and Address of Notifier

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B. Common or Usual Name of GRAS Substance

Recombinant Human Lactoferrin from Rice is the common or trade name for the iron-binding glycoprotein isolated from transgenic rice. The published literature may also refer to lactoferrin as red milk protein (Masson and Heremans 1968), lactosiderophilin (Montreuil and Mullet 1960), ekkrinosiderophilin (Schade 1977), or lactotransferrin (Legrand, Mazurier et al. 1991).

C. Conditions of Use

The nutrient properties of lactoferrin have many potential physiological benefits. Ventria Bioscience intends to add recombinant human lactoferrin to functional foods and drinks at levels not to exceed 1000 mg/serving and to medical foods at levels not to exceed 1 mg/mL.

D. Basis for GRAS Determination

The GRAS determination for recombinant human lactoferrin produced and purified from rice grain is based on scientific procedures.

Ventria Bioscience is providing this notification that the intake of recombinant human lactoferrin from rice from its intended use is safe and also Generally Recognized as Safe (GRAS) under the Federal Food Drug and Cosmetic Act. This document establishes the substantial equivalence

of recombinant human lactoferrin to human lactoferrin. It also demonstrates that the safety of human lactoferrin is generally recognized. Information on lactoferrin was reviewed with respect to background information and biological studies *in vitro* and *in vivo* in animals and humans. The sources of information included studies identified through searches of online bibliographic systems, such as PubMed from the National Library of Medicine.

Determination of the GRAS status of recombinant human lactoferrin from rice as a food ingredient has been made by a panel of experts qualified by scientific training to evaluate food safety and selected for their expertise in issues related to allergic and immunologic issues.

E. Statement of Availability of Data and Information

The data and information that serve as the basis for this GRAS notification will be sent to the FDA upon request or are available for the FDA's review and copying at reasonable times at the office of

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II. DESCRIPTION OF SUBSTANCE

A. Physical and Chemical Composition

Human lactoferrin is present in human biological fluids and mucous secretions (milk, tears, saliva, synovial fluid, genital and nasal secretions) as well as in the granules of circulating neutrophils. Lactoferrin is an 80 kD glycoprotein member of the transferrin family of iron binding proteins. Human lactoferrin is a single chain polypeptide of 692 amino acids organized into two globular lobes, representing its N-terminal and C-terminal halves (the N-lobe and C-lobe) (Baker, Anderson et al. 1991). Each lobe is itself folded into two domains (N-lobe: N1 and N2; C-lobe: C1 and C2) that enclose the iron binding site. This two-lobe, four-domain structure is presented in Figure 1. Lactoferrin undergoes conformational changes as iron is bound (closed form) or released (open form).

Figure 1. Human lactoferrin with two bound iron molecules and two glycan chains

The protein sequence for human breast milk lactoferrin was initially determined by amino acid sequencing (Metz-Boutigue, Jolles et al. 1984) followed by a deduced amino acid sequence of cloned human neutrophil lactoferrin obtained from a myeloid cDNA library (Rado, Wei et al. 1987). Although gene polymorphisms occur in human lactoferrin, it is generally accepted that there is only a single functional human lactoferrin gene located on chromosome 3 and mapping to 3q21-q23 (http://www.ncbi.nlm.nih.gov/ entrez/query.fcgi?db=gene&cmd=retrieve&dopt= Graphics&list_uids=4057) (Teng, Pentecost et al. 1987; Teng 2002).

Human lactoferrin (hLF) has 3 potential glycosylation sites. However, only two sites (Asn^{137} and Asn^{478}) are normally glycosylated (Shimazaki 2000). Crystallographic studies with hLF and recombinant hLF expressed in Aspergillus indicate that carbohydrate moieties do not play a role in polypeptide conformation (Sun, Baker et al. 1999). With a human kidney-derived cell line that constitutively expresses recombinant hLF, it was shown that both glycosylated and non-glycosylated rhLF can be completely saturated with iron (van Berkel, Geerts et al. 1995).

Human lactoferrin from milk and other secretions differs from human neutrophil lactoferrin in the type of glycosylation pattern. The neutrophil lactoferrin glycan does not contain a fucose on the core, making it more similar to the glycan pattern of human serum transferrin (Derisbourg, Wieruszeski et al. 1990; Spik, Coddeville et al. 1994). This difference in glycan structure between human milk and neutrophil lactoferrin has not resulted in any reported differences in function, rate of clearance, or immunogenicity of lactoferrin (Moguilevsky, Retegui et al. 1984).

Lactoferrin is a member of the iron binding protein family characterized by transferrin, the common iron binding protein found in blood. Lactoferrin's iron affinity is 3×10^{25} compared to serum transferrin at 10^{24}. The protein has two binding sites for iron and when fully saturated has 1.4 mg Fe^{3+} per gram of lactoferrin.

Recombinant human lactoferrin is identical to human lactoferrin in amino acid sequence, structure and iron binding capacity. The proteins differ in the structure of the N-glycans on the molecules. The number and location of glycosylation sites are the same on human lactoferrin and recombinant human lactoferrin. The structures of the N-glycans differ as plant glycans differ from mammalian glycans.

- The absence of the terminal sialic acid found in many mammalian glycosylation patterns and no galactose at the non-reduced end
- The presence of a ß(1,2)-xylose on the core manose
- The presence of an a(1,3)-fucose on the proximal N-acetyl glucosamine.

The details of the structural difference in glycosylation is discussed in Section V, page 37. The safety of the glycosylation difference is presented in Section V, page 56.

Lactoferrin is ubiquitous in the human body. The concentration of hLF is highest in breast milk (1 - 3 mg/mL) and tear secretions (0.5 – 4.2 mg/mL) (Lönnerdal 1985; Ohashi, Ishida et al. 2003). Table 1 provides published levels of lactoferrin measured in human exocrine secretions, neutrophils and serum.

Table 1. Lactoferrin levels in human exocrine secretions

Fluid	Lactoferrin Concentration	Reference
Breast milk	1 -3 mg/mL	(Lönnerdal 1985)
Tears	0.5 – 4.2 mg/mL	(Ohashi, Ishida et al. 2003)
Genital secretions	0.5 – 1.0 mg/mL	(Laibe, Bard et al. 2003)
Nasal secretions	200 µg /mL	(Pupek, Mikulewicz et al. 2003)
Lung secretions	11.8 µg/mL	(Thompson, Bohling et al. 1990)
Amniotic fluid	2 – 37 µg/mL	(Niemela, Kulomaa et al. 1989)
Saliva	3.4 – 11 µg/mL	(Lin, Johnson et al. 2001) (Laibe, Bard et al. 2003)
Serum	<1.0 µg/mL	(Caccavo, Sebastiani et al. 1999)
Synovial fluid	0.45 – 100 µg/mL	(Caccavo, Sebastiani et al. 1999)
Neutrophils	0.57 µg/10^6 cells	(Deicher, Exner et al. 2000)

B. Molecular Biology of Production

Recombinant human lactoferrin was produced through genetic modification of *Oryza sativa, Japonica,* Taipei 309. This parent rice variety (TP309) is a commercially cultivated Japonica variety that has ancestral origins in China. This variety is not grown commercially in the United States. The strain of Taipei 309 used by Ventria Bioscience was provided by a collaborator at the University of California in 1993. The variety has been maintained in the greenhouse to produce material for plant transformation on an as-needed basis.

Ventria Bioscience has produced transgenic rice that expresses human milk lactoferrin in the endosperm of the seed. Ventria used a seed-specific promoter and signal peptide sequence to direct the lactoferrin molecule to the protein bodies in the seed endosperm cell. While the gene for the lactoferrin and selectable markers are present in every transgenic plant cell, lactoferrin expression is limited to the seed endosperm and does not occur in other parts of the rice plant. The promoters, signal peptide sequences, and selectable markers used in the transformation process were either isolated from the rice genome or are those used in other genetically modified grains currently in the food supply. As described below, Ventria Bioscience has used a selection technology which does not express the antibiotic resistance proteins in the plant or grain.

A two-vector system was used in the transformation process and DNA from these vectors was introduced into plant cells via particle bombardment. One vector contains the chimeric target gene and the other contains the chimeric selectable marker gene. The frequency with which both vectors are integrated into the same cell in the plant transformation process is approximately 90%. The

details of the transformation process and characterization of the resulting lactoferrin protein have been published (Nandi, Suzuki et al. 2002; Suzuki, Kelleher et al. 2003).

The human milk lactoferrin gene sequence was based on the DNA sequence from GenBank (GenBank accession number U07643). The production of the recombinant human lactoferrin in the grain was accomplished through the use of a seed-specific promoter and a signal peptide directing the resulting lactoferrin to the protein body of the seed endosperm cell. The Gt1 promoter was derived from the rice glutelin gene family (Okita, Hwang et al. 1989) and controls the specificity and level of expression of the lactoferrin gene in rice endosperm. The Gt1 promoter was isolated from the rice genome and used in chimeric gene construction. The corresponding Gt1 signal peptide was also derived from this rice glutelin gene. The signal peptide is used to guide the lactoferrin through the endoplasmic reticulum and target it to the protein bodies of the endosperm cells in maturing rice grain. It is cleaved from the lactoferrin as the protein is translocated into the endoplasmic reticulum.

Transformed rice calli were selected by the expression of hygromycin B phosphotransferase (hpt), an enzyme that deactivates the antibiotic hygromycin B (Waldron, Murphy et al. 1985). Hygromycin B phosphotransferase (hpt) has been used in transformation of crop plants intended for human consumption. It has been the subject of an FDA consultation on the use of antibiotic resistant marker genes (FDA 1998). Although the hpt protein is present in the calli of the cultured non-differentiated plant cells after transformation, it is not present in tissues of the mature plant including the grain. The selectable marker gene (hpt) is under tight control of the 940 bp rice ß-glucanase 9 (Gns9) gene promoter that is specific for expression in rice callus tissue. Therefore, hygromycin phosphotransferase is expressed and detected in rice calli, but not in the rice plant (Huang, Wu et al. 2001).

During chimeric gene construction, the gene encoding kanamycin phosphotransferase (npt) was utilized as a selectable marker for plasmid maintenance in *E. coli*. The npt gene is under the control of a bacterial promoter. This promoter and gene pair were used in the propagation of the plasmid in bacteria prior to plant transformation by bombardment. The npt gene sequences are not active in plants. The npt promoter and gene pair have been used in the transformation of crop plants intended for human consumption and the safety addressed in the FDA consultation on antibiotic resistant markers (FDA 1998).

In summary, of the three open reading frames for the new genetic material introduced into Taipei 309 rice, only one is functional in the mature rice plant, the human lactoferrin gene. The hygromycin B phosphotransferase gene is under control of a promoter that is not functional in the vegetative portions of the rice plant or the grain and the kanamycin phosphotransferase gene is under control of a bacterial promoter and is not functional in plants.

The correct functioning of the Gt1 promoter and signal peptide control sequences has been documented through the use of Northern blot analysis of RNA expression, Western blot analysis and ELISA measurement for levels of protein expression. Figures 2 and 3 present Northern blot and ELISA analysis of the rice seed (grain) during the maturation process, respectively. Figure 2 illustrates that when total seed mRNA is probed for the lactoferrin sequence, lactoferrin mRNA is present during the seed maturation process. mRNAs from the antibiotic resistance genes and the vector backbone sequences are not detected during seed development. Figure 3 demonstrates the increasing accumulation of lactoferrin protein in the rice grain during the maturation process.

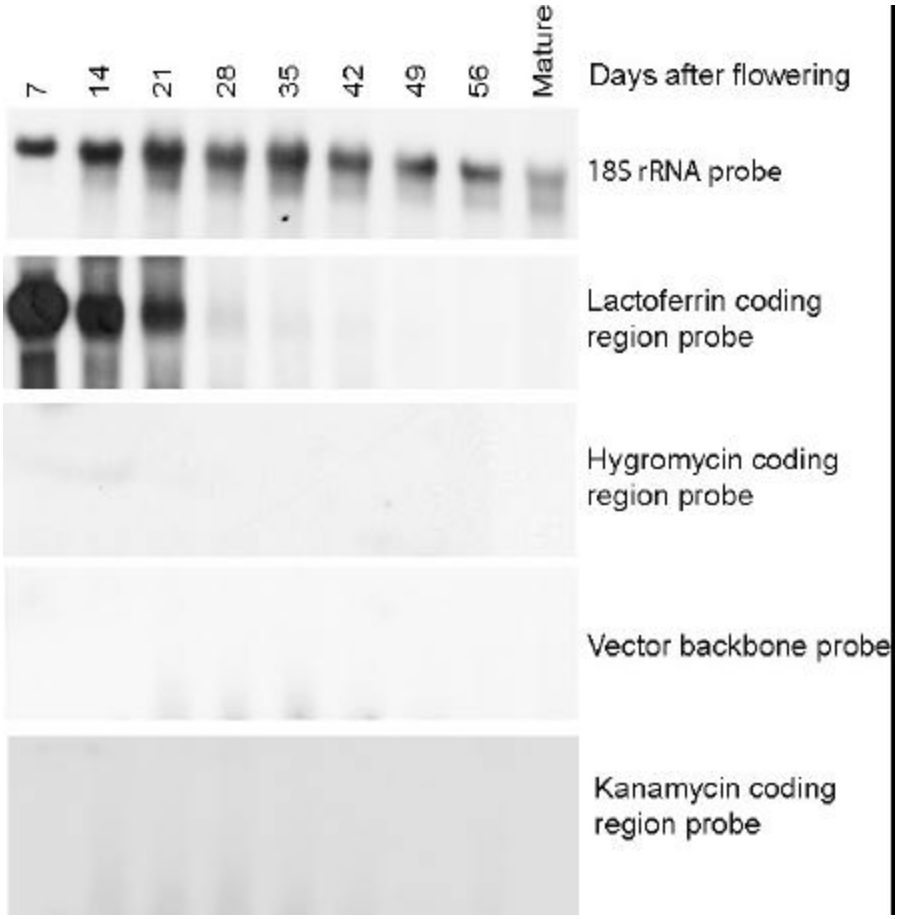

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Figure 2. Northern blot analysis of maturing seed in LF164 rice

The lactoferrin gene expression profile in grain of LF164 was analyzed by Northern blotting. Total RNAs were isolated from developing panicles of LF164 at 7, 14, 21, 28, 35, 42 49 and 56 (matured) days after flowering. The RNAs were blotted onto a nylon membrane after separation in 1 % agarose gel and probed

with lactoferrin, hygromycin, vector backbone DNA sequences and the kanamycin resistance gene. The 18S rRNA was used as internal control.

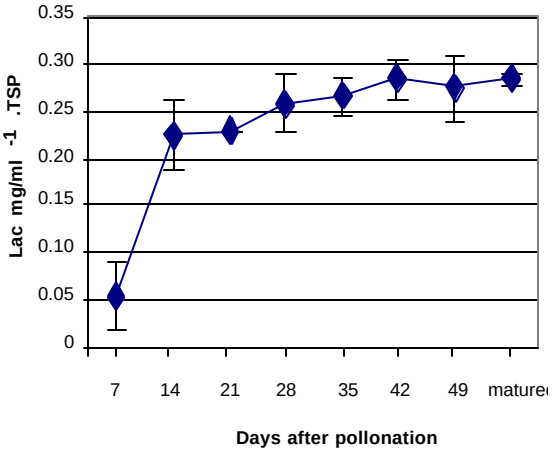


Figure 3. Lactoferrin protein in the developing grain
Ten seeds from rice plants corresponding to the developmental stage shown were dehusked, pooled and ground with a mortar and pestle. Soluble protein was extracted with 0.35 M NaCl in PBS at room temperature for 2 h and clear supernatant was obtained by centrifugation at 14,000 rpm at 4°C for 10 min. Lactoferrin and total protein content were determined by ELISA and Bradford assays, respectively.

Figures 4 and 5 illustrate the tissue specificity of the lactoferrin protein by Northern and Western blot analyses, respectively. In Figure 4 plant tissues were analyzed for lactoferrin and antibiotic resistance protein RNAs and in Figure 5 the plant tissues were analyzed for the presence of lactoferrin protein by Western blot (Nandi, Suzuki et al. 2002).

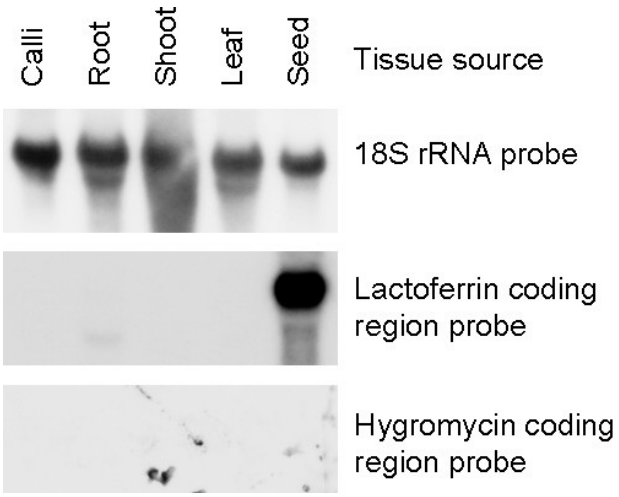


Figure 4. Northern blot analysis of various LF164 plant tissues
Tissue-specific expression of the lactoferrin gene in line LF164 was determined by Northern analysis. Total RNA was extracted from roots, shoots, leaves, and seed of mature plants as well as calli derived from LF164 mature embryo. The RNAs were blotted onto nylon membrane after separation by 1% agarose gel and probed with lactoferrin and hygromycin genes. The 18S rRNA was used as an internal control. The faint band seen in the lane with root tissue is residual 18S rRNA after stripping the blot.

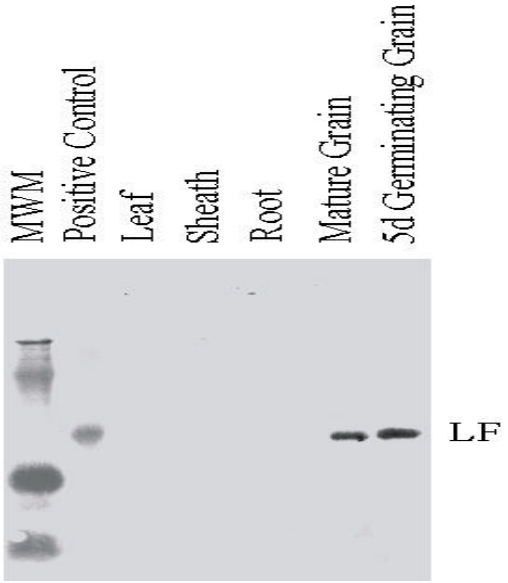


Figure 5. Tissue specificity of rhLF protein expression
Total soluble protein was extracted from roots, shoots, leaves and mature grain with 0.35 M NaCl in PBS at room temperature for 2 h. Proteins were separated by PAGE and transferred to nitrocellulose

membrane. Lactoferrin expression was determined by Western blot analysis using polyclonal antiserum against human lactoferrin.

In addition to the Northern blots, extracts from transformed and non-transformed control rice have been compared through the use of 2-D gel analysis (Kendricks Laboratories, Madison, WI). The data in Figure 6 compare the LF164 transformed rice extract with an identical extract from the parent Taipei 309 rice variety using 2-D gel electrophoresis. The gels were loaded with 200 µg of rice extract in 50 µL and stained with Coomassie Blue. Approximate levels of detection are 1 µg of a protein specie as a reasonably dark spot and 0.2 µg protein specie as a visually discernable spot. The 2-D gels were compared by HBC, Human Brain Comparison. There were no proteins in the LF164 rice, other than lactoferrin, that were not also found in the Taipei 309 rice. Additionally, only one protein was identified that may have been present in slightly greater amounts in the LF164 rice. An additional difference observed was the reduced amount of natural rice proteins in the transformed rice, probably due to their replacement in the endosperm by recombinant lactoferrin. These data support our determination that there are no unintended proteins produced in the transgenic rice due to any other introduced open reading frames.

A. Extract from LF 164 **B. Extract from Taipei 309**

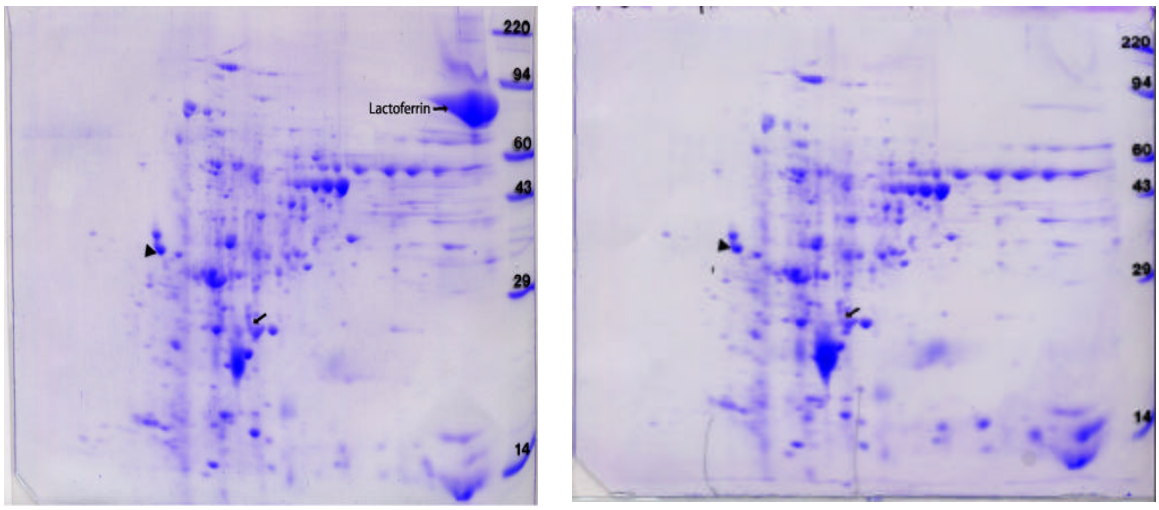


Figure 6. 2-D gel comparision of soluble protein extracts from LF164 and the parent Taipei 309 rice variety
Rice flour from LF164 and TP309 was extracted with phosphate-buffered saline (PBS) and 0.35M NaCl and then analyzed by 2-D gels stained with Coomassie. An internal standard, tropomyocin (MW 33,000, pI 5.2) was included and is marked on the gel with an arrow head (?). HBC was done to compare the gels and the only protein identified in the LF164 and not present in the parent rice was lactoferrin (labeled in gel A). Protein spot judged by HBC to be greater in the LF164 rice is marked by an arrow (→).

The newly introduced genetic locus meets the single locus model of Mendelian inheritance in the R_1 generation based on Chi square analysis. During the screening of R_1 grain, it was observed that all rice grains positive for lactoferrin expression were opaque-pinkish in color as compared to the negative segregating rice grains. This pinkish color is due to the high expression of lactoferrin and the iron bound to the lactoferrin. Lactoferrin without iron, apo form, is a white powder, but as iron is bound, the color changes to a pinkish-brown. The lactoferrin present in the rice grain is about 50% saturated with iron, producing the pinkish color. Grain appearance (color) was used to identify homozygous lines from the R_2 generation. A transgenic plant was considered homozygous for lactoferrin expression if all grains from the plant were opaque-pinkish. Homozygous lines were further confirmed by ELISA for lactoferrin expression.

At each generation, the expression of rhLF in the transgenic rice grain was confirmed by ELISA and Western blot analysis using rabbit anti-lactoferrin antiserum. The level of lactoferrin expression has been monitored over ten succeeding generations (R_2-R_{10}) and is stable and reproducible.

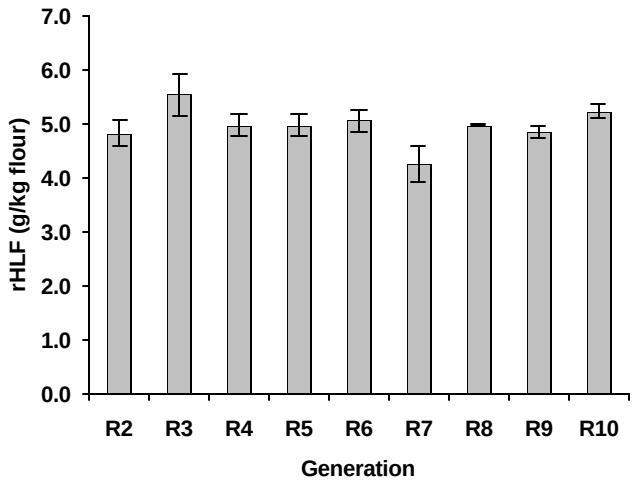


Figure 7. Stable expression of rhLF in transgenic rice grain.
Rice grain from generations R_2 through R_{10} was analyzed. One gram of brown rice flour was extracted with 40 mL of extraction buffer and clarified by centrifugation to obtain total soluble protein. The extract was analyzed via ELISA.

Conclusion

Recombinant human lactoferrin is produced through standard methods of genetic modification and plant transformation. The rice grain in which the protein accumulates is stable and well suited as a production system. There is no evidence of any new material being expressed in the transformed rice grain other than the human lactoferrin. The genetic elements used in the transformation process are currently found in the food supply, either in native rice or genetically modified foods designated as safe for food or feed by the FDA.

C. Manufacturing Process

The production of recombinant human proteins in rice grain has several advantages.

- Rice grain proteins can accumulate to 9-19% of grain weight (Huang, Wu et al. 2001)
- The endosperm proteins are synthesized during grain maturation and stored in protein bodies for use in the germination and seedling growth of the next plant generation.
- Grain can be stored for years without loss of functionality, and therefore the downstream processing can be conducted independent of growing seasons.
- Specific promoters for expression of recombinant proteins are available from the genes of the major rice storage proteins, the glutelin, *Gt-1*, *Gt-2* and *Gt-3* (Okita, Hwang et al. 1989)
- Rice is a self-pollinating crop, which significantly reduces the possibility of gene flow in the environment
- There are standard extraction methods available in the food industry for the extraction of rice protein fractions.
- Any material, other than the target protein, remaining after purification is from rice and thus considered to have low allergenicity. The plant matrix can actually serve as an excipient or inert carrier (Peterson and Arntzen 2004).

1. General Description of the Production Process

To date Ventria Bioscience has purified 8.7 kg of recombinant human lactoferrin from rice flour. The protocols developed at pilot scale for 10 kg of rice flour have been scaled to 180 kg of rice flour in a linear fashion. Current efforts are underway to scale to 2000 kg of rice flour.

Recombinant human lactoferrin is produced in the LF164 rice variety derived from *Oryza sativa, Japonica*, Taipei 309. The LF164 rice is grown under USDA permit conditions with physical separation from commercial rice crops. The rice is planted, harvested, stored and transported using equipment and facilities dedicated to the production of Ventria Bioscience's rice. The equipment and storage facilities are not used in the production or storage of commercial rice for food use. Following harvest remaining straw and residual materials are plowed under and taken through flood and plow cycles.

The rice is dehusked and milled to 100 mesh flour using standard food industry procedures. Hulls and the solid material remaining after the extraction described below are destroyed by land application in the fields. Recombinant human lactoferrin is extracted from ground rice flour using 0.02 M phosphate buffer with 0.3 M sodium chloride, pH 6.5. After 1.5 – 3.5 hours of extraction, the solid rice

flour is separated from the liquid phase by centrifugation. The liquid phase, containing the soluble protein, is filtered and concentrated. At this point the product is a protein concentrate that is >10% lactoferrin protein. The concentrate is further purified to an isolate using ion exchange chromatography. The concentrate is loaded on a column of SP Sepharose Big Bead Media, the column is washed and the bound lactoferrin eluted with 0.8 M sodium chloride. The isolate is diafiltered (30kD) to remove excess salt and concentrated prior to lyophilization. Details of the extraction and purification method development have been published (Nandi, Yalda et al. 2005). Prior to drying the lactoferrin isolate, it can be saturated with iron or have the iron removed, resulting in three different forms based on the level of iron saturation. The isolate forms of lactoferrin are all >85% lactoferrin protein. Residual materials are moisture, salt, rice proteins and carbohydrates.
The recombinant human lactoferrin can be produced in three forms and these forms can be incorporated into different products.

- Holo-lactoferrin, iron saturated
- Apo-lactoferrin, iron depleted
- “Asis” lactoferrin, as extracted, partially iron saturated

Following completion of the initial extraction and ion exchange procedures, the lactoferrin is in the form designated “asis” lactoferrin by Ventria. The iron saturation of the “asis” lactoferrin is dependent on the background agronomic variation which results in different levels of iron in commercial rice, but is generally in the range of 50-60% or 0.7 – 0.8 mg iron/gram lactoferrin. This is the starting material for the production of holo or apo lactoferrin.

Holo-lactoferrin is produced through saturation of the lactoferrin with iron in the presence of ferric nitrate and bicarbonate. When lactoferrin is fully saturated with iron, it has 1.4 mg of ferric iron per gram of lactoferrin protein. Any residual unbound iron is removed during the diafiltration process prior to drying.

When the iron is removed from the lactoferrin, the form is designated as apo-lactoferrin. Although the designation is for lactoferrin without any iron, the apo form of lactoferrin generally is about 10% saturated with approximately 0.14 mg iron/gram lactoferrin. The iron is removed from the lactoferrin by using low pH in the presence of urea. Unbound, released iron is removed during diafiltration. After removal of iron, part of the quality control process is the resaturation of the lactoferrin with iron. This assures that the desaturation process has not damaged the lactoferrin protein.

The three forms of lactoferrin demonstrate different activity profiles in *in vitro* studies. The apo-lactoferrin is able to sequester more iron and thus has better bacteriostatic activity. The holo-lactoferrin has shown greater activity in supporting cell growth and tissue repair. The asis-lactoferrin has activity in all

areas. The different forms can be used based on the nutritional support role of the functional or medical foods.

All chemicals and processing aids are of food grade or higher quality. The chemicals would include but not be limited to food grade sodium chloride, sodium phosphate, and demineralized water. Processing aids would include but not be limited to SP Sepharose (or other cation exchange resin with an inert solid support), regenerated cellulose and/or polyvinylidene fluoride membranes. Figure 8 gives a schematic of the purification process. Safety and information sheets for the processing chemicals are located in Appendix B.

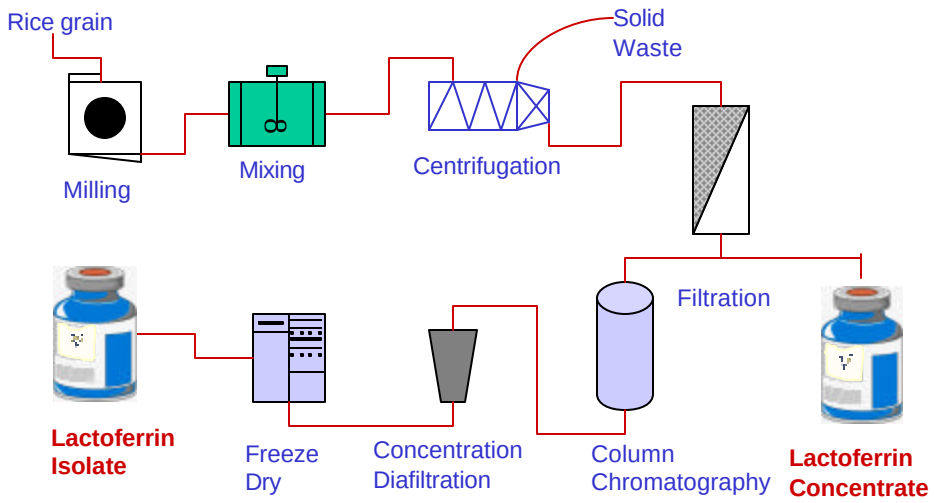


Figure 8. Purification of recombinant human lactoferrin

The purification process has been consistent and reproducible. During the recent production of a 2 kg lot of recombinant human lactoferrin, five batches were purified and blended to produce the lot. Table 2 gives the yield and purity of the five batches prior to blending. Each batch was purified from 180 kg of brown rice flour.

Table 2. Batch results from recombinant human lactoferrin purification

Batch No.	Yield weight (gm)	LF as % of Protein
30404	404.6	97.9
30704	388.8	94.5
30904	311.1	93.9
31404	411.9	95.6
31704	373.2	95.4

Rice is a production crop species that is well understood and characterized as to the presence of toxins and anti-nutrients. There are no toxins and only three antinutrient factors in rice, all concentrated in the bran fraction. They are phytic acid, haemagglutinin-lectin and trypsin inhibitor. Ventria Bioscience has sponsored analysis of these factors and shown that there is no increase in the levels of these factors in the transformed rice grain. Phytic acid and lectin are removed during the extraction process based on solubility and size for phytic acid. Phytic acid is extracted using an acid-based extraction (Chen 2004). It is also has a molecular weight of 923 and would be removed during the diafiltration to remove excess salt. The lectins of rice are extracted with an alkaline based buffer, so they remain in the residual solids (Tabary, Font et al. 1987). The trypsin inhibitor is in the albumin family of rice proteins and is extracted with water or saline. This protein has a molecular weight of 16kD and is removed during the 30kD diafiltration step (Matsuda, Sugiyama et al. 1988).

2. Finished Product Specifications

Specifications for the different forms lactoferrin are in Table 3. The sodium concentration is variable depending on the final product specifications. For example, an oral rehydration solution would be specified to contain sodium, whereas other forms might be sodium free.

Table 3. Manufacturing specifications for recombinant human lactoferrin

Specification	Holo-lactoferrin Isolate	Apo-lactoferrin Isolate	Asis-lactoferrin Isolate	Asis-lactoferrin Concentrate
Appearance	Pink powder	Off white powder	Pink powder	Pink powder
Assay of lactoferrin	> 85% (w/w)	> 85% (w/w)	> 85% (w/w)	> 10%
Total protein	> 90%	> 90%	> 90%	> 50%
Moisture	< 10%	< 10%	< 10%	< 10%
Solubility (1%)	A_{600} <2.5	A_{600} <2.5	A_{600} <2.5	na
pH (1% in H_2O)	5.0 – 7.0	5.0 – 7.0	5.0 – 7.0	5.0 – 7.0
Bound Iron	= 1.2 mg/g LF	< 0.2 mg/g LF	0.5 – 1.0 mg/g LF	0.5 – 1.0 mg/g LF
Total Iron	< 2 mg/g	< 0.21 mg/g	TBD*	TBD*
Iron Re-uptake	na	> 1.0 mg/g	na	na
S. aureus	Neg in 1 g	Neg in 1 g	Neg in 1 g	Neg in 1 g
Pseudomonas spp	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g
E. coli	Neg in 1 g	Neg in 1 g	Neg in 1 g	Neg in 1 g
Salmonella	Neg in 25 g	Neg in 25 g	Neg in 25 g	Neg in 25 g
Total Plate Count	< 500 cfu/g	< 500 cfu/g	< 500 cfu/g	< 500 cfu/g
Yeast	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Mold	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g	< 10 cfu/g
Sodium	Product specific	Product specific	Product specific	Product specific
Heavy metals (as Pb)	< 10 ppm	< 10 ppm	< 10 ppm	< 10 ppm
Lead	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm	< 0.5 ppm
Urea	na	= 1% (w/w)	na	na

* Iron will vary in the lactoferrin based on background agronomic conditions.

Conclusion

Recombinant human lactoferrin has been purified from transgenic rice using standard food industry procedures and current good manufacturing practices. The process is reproducible and scalable.

III. USE AND CONSUMER EXPOSURE

A. Consumer Safety

1. Use of lactoferrin in animals and humans

An extensive library of literature is available to support the safety of lactoferrin from human, bovine and recombinant human origin. Human and bovine lactoferrin have been consumed by humans through consumption of breast milk in infancy and cow's milk throughout life. Infants who are breast-fed consume human lactoferrin, a natural constituent of human milk. Human milk contains from 5.8 ± 4.3 g/L in colostrums to 3.3 ± 0.8 g/L in mature milk (Montagne, Cuilliere et al. 2001).

Bovine lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found in milk. DMV International, a major manufacturer of bLF has determined that bLF is "Generally Recognized As Safe" (GRAS, GRN 00077) for use as an ingredient in sports drinks and functional foods. bLF is currently marketed in dietary supplement form with dose recommendations ranging from 10-1200 mg/day.

Human lactoferrin has not been able to compete with bLF in the market due to the difficulty and expense of producing large quantities. However, several clinical experiments had been conducted to evaluate the turnover rate in man as well as its use some disease states. The result showed that up to 15 g of rhLF could be safely administrated within a 24-h period. In reviewing the clinical studies, there are no comments on the efficacy of the lactoferrin in the studies as the focus of this notice is on exposure level and the safety of lactoferrin.

2. Animal Studies

Bovine lactoferrin (bLF) was administered orally to groups of 12 male and 12 female rats at dose levels of 200, 600 and 2000 mg/kg/day once daily for 13 weeks and toxicity on repeated administration was examined. Throughout the administration period, there were no deaths caused by administration of the test compound, nor were there any adverse effects noted in the general condition of the animals. The study findings concerning body weight and food consumption, ophthalmology, urinalysis including water consumption, haematology, blood chemistry, necropsy, organ weights and histopathology revealed that there were no apparent changes due to administration of LF. Therefore, the level of LF at which no adverse effect was observed was considered to be 2000 mg/kg/day for

both sexes (Yamauchi, Toida et al. 2000) Ventria Bioscience has also sponsored two 28-day feeding trials in rats with recombinant human lactoferrin the holo and apo forms. In one study the maximum dose of 1000 mg/kg/day of holo-rhLF resulted in a No Observed Adverse Effect Level (NOAEL). In the second study the maximum dose of apo-rhLF was 1800 mg/kg/day. No adverse events were reported in that study.

Data has been published to support safety of rice derived recombinant human lactoferrin. The recombinant protein was used in combination with a second recombinant human protein produced in rice, lysozyme. The study included two trials using 660 3-day old broiler chicks (Humphrey, Huang et al. 2002). Chicks were fed corn-soy-rice diets containing conventional or recombinant rice extract at levels from 0% to 5% lactoferrin. There were no adverse events and feed efficiency based on grams body weight gained/gram feed consumed was significantly higher for chicks receiving lactoferrin containing rice extract as compared to commercial rice extract supplemented in the feed.

Table 4. Data on feed efficiency of LF164 rice fed to broiler chicks

Dietary Treatment	Feed Efficiency (gm wt gain/gm feed)
Experiment 1	
Commercial rice	0.79
5% LF + 10% LZ	0.84 ($p<0.05$)
Experiment 2	
Commercial rice	0.72
5% LF + 10% LZ	0.77 ($p<0.05$)

4. Infant Formula Supplementation Studies

Numerous studies have been done in infants to test the use of lactoferrin addition to infant formula. Inclusion levels have generally been at the 1 mg/mL, the level close to that found in breast milk. Various endpoints of iron balance (McMillan, Oski et al. 1977; Fairweather-Tait, Balmer et al. 1987; Schulz-Lell, Dorner et al. 1991; Chierici, Sawatzki et al. 1992) and gastrointestinal fecal flora (Balmer, Scott et al. 1989; Roberts, Chierici et al. 1992) have been measured in the infant formula studies. No safety or allergenicity issues have been reported in any of the trials.

5. Adult Dosing of Lactoferrin

The turnover of ^{125}I-labelled human milk-derived lactoferrin was measured in ten adults and simultaneous organ radioactivity counting was performed. Of the administered ^{125}I label 99% was recovered in the urine, as free iodine, within the first 24 h, suggesting a rapid catabolism. Organ radioactivity counting showed

that lactoferrin was rapidly taken up by the liver and spleen (Bennett and Kokocinski 1979). These results suggest the metabolism in humans is identical to that described from animal models.

Bovine lactoferrin has been dosed in studies looking at the effects of lactoferrin. There were no material related incidents in any of these studies. Studies include

- the immune system (10 subjects, 2 g/day for 4 weeks) (Yamauchi, Wakabayashi et al. 1998)
- saliva lactoferrin supplementation (6 subjects, 500 mg/2 hours) (Kuipers, Heegsma et al. 2002)
- oral Candidiasis in HIV and xerostomia (21 subjects, mouth wash with 0.6mg/mL)(Kirstila, Lenander-Lumikari et al. 1996; Masci 2000)
- tinea pedis (14 subjects, 600 mg/day, 8 weeks and 12 subjects, 2000 mg/day, 8 weeks) (Yamauchi, Hiruma et al. 2000)
- digestion studies (12 subjects, 4.5 g, 3 days of apo or holo) (Troost, Steijns et al. 2001).

In Japan, four studies have been published on the treatment of chronic hepatitis C patients with bLF. In the first pilot study eleven patients with chronic hepatitis C received an 8-week course of bovine lactoferrin. Seven patients received 1.8 g/day and 4 received 3.6 g/day (Tanaka, Ikeda et al. 1999). A second study enrolled twenty-five patients in a blinded study comparing 0.4 (n = 10) and 3.6 (n = 15) g/day of bLF. The patients received oral bLF for six months (Iwasa, Kaito et al. 2002). The third study involved dosing for a year at 600 mg/day bLF. Sixty-three patients were randomly enrolled, 36 received bLF and 27 were control (Ishii, Takamura et al. 2003). The final study was a randomized trial treating chronic hepatitis C patients with recombinant type 1 interferon with or without lactoferrin. Ten of twenty-one subjects received subcutaneous interferon and 9 grams (3 g three times a day) oral bLF for 26 weeks (Hirashima, Orito et al. 2004). There were no adverse or allergic reactions observed in any of the 78 patients dosed with oral bLF for up to a year.

Both bovine lactoferrin and recombinant human lactoferrin have been used to treat *H. pylori* infections. Recombinant human lactoferrin from yeast was used in three studies. Two open-label studies (low and high dose) were performed to assess the safety and efficacy of orally administrated rhLF to cure or suppress *H. pylori* infection in adult subjects. The rhLF was given orally five times throughout a 24-h period. Six subjects received 250 mg per dose (1.25 g/day) and six subjects 1 g per dose (5 g/day). No significant adverse effects were observed during this acute dose study. (Opekun, El-Zaimaity et al. 1999) A similar study dosed six subjects with 5 grams a day for 5 days and three subjects with 5 grams a day for 14 days. No significant adverse effects were reported although one patient complained of flatulence and dysmenorrhoea and one of abdominal cramps. No changes were observed in routine laboratory values. (Guttner, Windsor et al. 2003). A more extensive study combining one week of triple antibiotic therapy with bovine lactoferrin versus triple therapy alone for one week

or 10 days dosed with 100 mg bLF BID (200 mg/day) for seven days. The adverse events were similar across all three groups suggesting the bLF was safe and free from side-effects. (Di Mario, Aragona et al. 2003; Di Mario, Aragona et al. 2003)

Human lactoferrin was used for the prevention of enterogenic infection in patients with myelogenous leukemia undergoing therapy resulting in neutropenia. Five patients received 800 mg of human lactoferrin purified from breast milk for 10 days. The lactoferrin was not associated with any side-effects or adverse events (Trumpler, Straub et al. 1989). Human recombinant lactoferrin was used in two studies in humans, one single dose study in women with ileostomies and one multi-dose study in healthy volunteers taking indomethacin. The single dose study used a single 5 gram dose of lactoferrin in 8 subjects. In the multi-dose study, healthy volunteers (15) consumed three 5 gram doses of recombinant human lactoferrin in a 24 hour period. No side effects from the lactoferrin ingestion were observed (Troost, Saris et al. 2003).

rhLF has also been used in patients with failed chemotherapy for solid tumors. Thirty-three patients received 1.5, 4.5 or 9 grams/day of rhLF from Aspergillus for 14 days or 28 days with a 14 day gap in the middle (Hayes, Varadhachary et al. 2003). A total of 210 individuals have been treated with rhLF with no lactoferrin-related serious adverse events or laboratory abnormality greater than grade 2 (Anderson 2004).

Ventria has recently completed a limited safety study in adult women. Four women took 4.8 grams of holo-lactoferrin daily for 42 days. Subjects were asked about known allergies or reactions to rice or rice products during the enrollment screening, but no testing for allergies was performed. Serum was collected on a weekly basis from the women. General serology chemistries and hematological markers were measured at time zero and week 6. There were no changes noted in any markers. Serum lactoferrin levels were measured at weekly intervals and did not change over the six weeks, Table 5.

Table 5. Serum Lactoferrin Values

	Week 0 µg/mL	Week 1-6 ± SD µg/mL
P-004	0.34	0.32 ± 0.2
P-005	0.45	0.49 ± 0.2
P-007	0.99	0.96 ± 0.4
P-008	0.58	0.48 ± 0.1

B. Consumer Exposure

1. Background exposure to lactoferrin in saliva

Lactoferrin is a natural constituent of the human body, found in exocrine secretions including saliva and breast milk, and in neutrophils and plasma. Human milk and neutrophil lactoferrins are identical except for their glycosylation; the milk glycans contain fucose, while the neutrophil glycans do not (Taylor, J et al. 2004).

Humans produce about 1.5 liters of saliva each day. Under basal conditions, the salivary glands produce saliva at the rate of approximately 0.5 mL/min, with a much slower flow rate during sleep. After stimulation, flow increases 10-fold over the basal rate (Marino and Gorelick 2003).

Several researchers have measured lactoferrin levels in both unstimulated and stimulated saliva. Tanida (Tanida, Okamoto et al. 2003) reported data on unstimulted saliva from thirty-five healthy individuals. The mean unstimulated flow rate of the saliva was 0.46 ± 0.11mL/min. This unstimulated flow rate would produce more than 600 mL of saliva each day. Lactoferrin was measured at 33 ± 14 µg/mL saliva. Assuming 600 mL saliva production/day, this would result in an exposure to lactoferrin in unstimulated saliva of 19.8 mg per day. The concentration of lactoferrin is less in stimulated saliva due to the increased flow rate. Lin et al. (Lin, Johnson et al. 2001) reported stimulated saliva flow rates from 34 healthy control subjects. Flow rates ranged from 1.5 to 2.4 mL/min with a mean of 2.0 mL/min. This corresponds to a mean stimulated saliva production of about 2.8 L per day. The stimulated lactoferrin levels ranged from 3.4 to 7.1 µg/mL saliva. Assuming a mean production of 2.8 L stimulated saliva/day, mean lactoferrin production from stimulated saliva is 14.7 mg/day. The combination of unstimulated and stimulated saliva measured in these studies is comparable to that stated in the standard Medical Physiology text book (Marino and Gorelick 2003). The data from this research support an estimated mean exposure to lactoferrin from saliva of 15-20 mg/day. In addition to saliva, there is additional daily exposure from bronchial, gastrointestinal, tear, nasal, and other exocrine secretions

Although bovine and native lactoferrin share some amino acid sequence homology, the absence of immunological cross-reactivity (monospecific IgE sera of milk allergic patients positive for bovine lactoferrin do not cross-react with rhLF; see discussion in Section V, page 50) indicates that this homology has no biological significance with respect to consumer safety. Therefore, background exposures to bovine lactoferrin from dairy products and GRAS-Notified uses are not considered here.

2. EDI of rhLF from uses proposed by Ventria

Ventria proposes 2 categories of uses for recombinant hLF enrichment:

1. General population: 1000- mg/serving in yogurts, and meal replacement and performance beverages and bars, including granola bars and "Ensure"-type drinks
2. Medical foods (under supervision of a physician only): 1 mg/mL in oral rehydration solution (ORS)

a. General population uses

Exponent examined the CSFII food codes included in the EDI analysis of GRN 000042. There is nearly complete correspondence between the foods included in that analysis and the foods of interest to Ventria. The only exception is frozen popsicles (containing yogurt), which appear not to have been included in GRN 000042. The contribution of these foods to the EDI for added lactoferrin is negligible. This is consistent with the CSFII data indicating that yogurt consumers tend to consume only one form of yogurt on any given day.

Exponent estimated the EDI associated with adding rhLF to the proposed foods at 1000 mg/serving, using food consumption data from the 1994-96-98 CSFII and their FARE™ software. This analysis included all the food codes from GRN 000042 and the yogurt-containing popsicles discussed above. The comprehensive list of codes included in the analysis is included in Appendix A. Results of the analyses are in Table 6 (Total rhLF intake from all enriched foods) and Table 7 (rhLF intake by food category). "Per user" values are based on persons who reported consuming the foods of interest during the survey. Intake of rhLF by the highly exposed consumer (represented by the 90th percentile per user values) ranges from 1.4 g/day (children) to about 2 g/day (teens and adults).

Table 6. Average daily intake of lactoferrin from proposed uses, mg/day (1000 mg/serving)

Population Subgroup	Per capita		**Per user**	
	Mean	90th pctl intake	**Mean intake**	**90th pctl intake**
Children, 1-12 yr	192	667	**723**	**1446**
Teens, 13-19 yr	210	650	**1131**	**2043**
Adults, 20+ yr	176	545	**1034**	**2000**

Table 7. Category contribution: Average daily Intake of lactoferrin from proposed uses, mg/day (enrichment at 1000 mg/serving)

Food Group	Children, 1-12 yr		Teens, 13-19 yr		Adults, 20+ yr	
	Per Capita	**Per User**	Per Capita	**Per User**	Per Capita	**Per User**
Dairy Containing Popsicles*	13	**607**	9	**576**	9	**752**
Dry Meal Replacements*	2	**778**	6	**1029**	13	**1035**
Fluid Meal Replacements*	29	**859**	86	**1722**	38	**1399**
Solid Meal Replacements*	37	**607**	34	**628**	27	**800**
Sports Beverages*	NA	**NA**	NA	**NA**	0	**23**
Yogurts Including Frozen	49	**475**	49	**902**	80	**852**

*Population size insufficient for reliable estimate of the 90 th percentiles, per USDA guidance.
NA: No CSFII survey respondents in this age grouping

b. Medical food uses

The World Health Organization (WHO) has issued guidance for administration of ORS in the first 4 hours to children with some dehydration (http://www.who.int/child-adolescenthealth/publications/referral care/chap4/ chap41.htm). Using these recommended dosages, an oral rehydration solution containing 1 mg/mL rhLF (the proposed enrichment level) would correspond to the lactoferrin intakes in Table 8.

Table 8. Estimated rhLF intake from ORS enriched at 1 mg/mL

Weight	Age	Amount of ORS in first 4 hours	rhLF intake
<5 kg	<4 months	200-400 mL	200-400 mg
5-<8 kg	4-<12 months	400-600 mL	400-600 mg
8-<11 kg	12 months to <2 years	600-800 mL	600-800 mg
11-<16 kg	2-<5 years	800-1200 mL	800-1200 mg
16-50 kg	5-15 years	1200-2200 mL	1200-2200 mg

Conclusion

Recombinant human lactoferrin from rice has been shown to be safe and free from adverse reactions in studies with oral delivery of lactoferrin for up to a year. Lactoferrin is well tolerated and not related to any serious adverse events or

allergic reactions. The dietary exposure to rhLF associated with the proposed uses at the specified levels is safe.

IV. INTENDED EFFECTS

A. INTRODUCTION

Since the discovery of lactoferrin by Sorensen more than 60 years ago, studies have been performed *in vitro*, in experimental animals, and in humans to analyze the functions of lactoferrin (Sorensen and Sorensen 1939). It is increasingly clear that lactoferrin has multiple functions depending on its cellular or extra-cellular environment (for reviews, see (Brock 1980; Levay and Viljoen 1995; Brock 2002; Valenti, Berlutti et al. 2004)). Lactoferrin is a member of the iron binding protein family characterized by transferrin, the common iron binding protein found in blood. Lactoferrin receptors have been found in human cells (for review, see (Testa 2002)), including monocytes-macrophages, brush border membrane cells of the small intestine, parenchymal liver and breast epithelial cells.

The use of lactoferrin in functional foods and beverages and medical foods can lend nutritional support to issues associated with conditions of gastrointestinal disease and stress. Lactoferrin can have effects on pathogenic, as well as beneficial flora in the intestine. It also can play a role in modulating the immune response and inflammation. A summary of the *in vitro* and *in vivo* studies on the various functions of lactoferrin reported in the literature are below:

- Antimicrobial
- Gastrointestinal flora
- Anti-inflammatory/immune modulation

B. ANTIMICROBIAL STUDIES

Lactoferrin has been shown to exhibit antibacterial, antiviral, antifungal and antiparasitic activities. Although most of the studies were conducted *in vitro* (for review, see (Naidu 2000; Shimazaki 2000)), there are *in vivo* studies as well (Kirstila, Lenander-Lumikari et al. 1996; Opekun, El-Zaimaity et al. 1999; Tanaka, Ikeda et al. 1999; Masci 2000; Yamauchi, Hiruma et al. 2000; Iwasa, Kaito et al. 2002; Troost, Saris et al. 2002).

The antimicrobial spectrum of lactoferrin includes stasis, cidal, cationic, phagocytic, and colonization/decolonization effects on susceptible microorganisms. Investigators have hypothesized that lactoferrin could impede iron utilization and cause bacteriostasis as a result of its iron-sequestering properties (Lonnerdal and Iyer 1995). However, the fact that some strains are resistant or unaffected by lactoferrin implies that bacteriostasis is not simply the result of iron sequestration (Bhimani, Vendrov et al. 1999). The selective

interaction of lactoferrin with the bacterial membrane may also play an essential role in lactoferrin's antimicrobial action (Alugupalli, Kalfas et al. 1995; Naidu and Arnold 1995). A key event in this process is the binding of lactoferrin to specific outer membrane pore-forming proteins (porins) (Naidu, Andersson et al. 1992; Naidu and Arnold 1994; Naidu and Bidlack 1998). Both human and bovine lactoferrin have been shown to bind to these proteins (Kishore, Erdei et al. 1991). The magnitude of the lactoferrin-microbe interaction is directly related to the bacterial susceptibility to lactoferrin (Naidu, Andersson et al. 1991). Certain strains show resistance to lactoferrin and do not demonstrate lactoferrin binding (Naidu, Svensson et al. 1993). Although most bacteria express porins, the resistance of these bacteria was attributed to the shielding of the porin accessibility by the O-antigentic carbohydrate chains of lipopolysaccharide (LPS) (Tigyi, Kishore et al. 1992; Naidu, Svensson et al. 1993; Erdei, Forsgren et al. 1994).

Lactoferrin has been shown to actively impair Shigella virulence. Epidemiologic studies in Bangladesh have demonstrated that breast-fed infants have reduced incidence and severity of Shigella infection (Ahmed, Clemens et al. 1992). *In vitro* studies suggest that lactoferrin acts at the surface of the bacteria to cause the invasion antigens to be released and become more susceptible to a protease (Gomez, Ochoa et al. 2003). These same studies demonstrated that the loss of invasion antigens was not associated with iron saturation or the N-terminal cationic peptide of lactoferrin.

Lactoferrin is effective against *E.coli*. The *in vitro* activity has been measured by Ventria Bioscience as part of the comparison of the recombinant proteins produced in rice to native proteins from breast milk (Huang, Nandi et al. 2002; Huang, Wu et al. 2002; Suzuki, Kelleher et al. 2003). Other investigators also have evaluated lactoferrin against *E.coli* (Hughey and Johnson 1987; Naidu, Erdei et al. 1991).

The N-terminal peptide of human lactoferrin, lactoferricin H, has also been shown to have bactericidal activity against a number of gram-positive and gram-negative organisms. It is of note that most species of *Bifidobacterium*, the major organism in the intestine of healthy breast-fed infants, is minimally susceptible to lactoferricin (Bellamy, Takase et al. 1992).

Lactoferrin has been shown to have *in vitro* antiviral effects against rotavirus infection of HT-29 cells, an enterocyte-like cell line. The antiviral mechanism of action appears to be two-fold. The lactoferrin binds directly to the virus particle and prevents it from binding to the target cell. It also has an inhibitory effect on viral antigen synthesis and viral yield when cells are exposed to lactoferrin after the attachment step (Superti, Ammendolia et al. 1997; van der Strate, Beljaars et al. 2001). This second mechanism requires uptake of lactoferrin by specific cell receptors. Another significant observation was that the activity of bovine lactoferrin against rotavirus was increased following desialylation (Superti,

Siciliano et al. 2001). It is of note that recombinant human lactoferrin produced in rice has no sialic acid. Although not as commonly detected, adenovirus can also be associated with diarrhea. Lactoferrin is able to inhibit adenovirus infection of cells by binding to the glycosaminoglycan receptors and blocking viral attachment to the cell membrane (Arnold, Di Biase et al. 2002; Di Biase, Pietrantoni et al. 2003).

Most recently, hLF has been shown to block biofilm development by the opportunistic pathogen *Pseudomonas aeruginosa* (Singh, Parsek et al. 2002). The activity of lactoferrin against biofilm was at concentrations below that required to inhibit growth of the Pseudomonas and was attributed to its iron binding capabilities.

Soukka (Soukka, Tenovuo et al. 1992) also tested the ability of human lactoferrin, purified from colostrums, to inhibit fungal growth. They investigated lactoferrin from 25 – 100 µg/mL and found dose dependent inhibitory activity. In addition, they studied the effect of pH, time, temperature, and iron saturation on the fungicidal activity of lactoferrin. The results indicated lactoferrin was more effective at pH 7.0 than 5.5. At pH 7.0 there was no time dependence after 25 minutes, and although partially saturated lactoferrin showed candidacidal activity, full saturation with iron resulted in loss of activity. At 0°C the killing effect of lactoferrin was not significant, but at all other temperatures (21, 37 and 42°C) there was equivalent inhibition. However, growth inhibition could be blocked by phosphate and bicarbonate ions, suggesting lactoferrin might not be active *in vivo*.

Arnold (Arnold, Brewer et al. 1980) studied the sensitivity of a number of microorganisms, including *Candida*, to lactoferrin. In these studies, purified human apo-lactoferrin was added to culture medium at concentrations of 4.2 µM to 83 µM. Fungal growth was significantly inhibited at the lowest concentration and completely inhibited at the higher levels. Iron saturated lactoferrin at 83 µM showed no ability to inhibit growth.

Human milk and lactoferrin also have been studied for anti-parasitic activity. Both have shown cidal activity against *Giardia lamblia* trophozoites. In the case of cryptosporidiosis, breast-feeding has been an effective deterrent. This could be both an antimicrobial role and an anti-inflammatory role through cytokine modulation (Sterling, Gilman et al. 1991; Kirkpatrick, Daniels et al. 2002).

C. GASTROINTESTINAL FLORA STUDIES

Lactoferrin may have a role in modulating the microflora of the gastrointestinal track. The levels of bifidobacteria in the gastrointestinal microflora has been implicated as an important aspect in human health, from newborn infants to geriatrics (Balmer, Scott et al. 1989; Balmer, Scott et al. 1989; Balmer and

Wharton 1989; Bennett, Gorbach et al. 1996). Bifidobacteria colonize the human large intestine and are one of the first bacterial groups to become established in the infant intestine (Poupard, Husain et al. 1973; Mitsuoka 1982; Mitsuoka 1984; Hoover 1993). Studies on the health of breast-fed infants have attributed the predominance of bifidobacteria with reduced morbidity and mortality (Yoshioka, Iseki et al. 1983; Ogawa, Ben et al. 1992; Roberts, Chierici et al. 1992). Bifidobacteria have also been used to restore normal intestinal flora following antibiotic therapy (Miller-Catchpole 1989; Wang and Gibson 1993; Gibson, Beatty et al. 1995; Gibson and Roberfroid 1995; Ballongue 1998).

In vitro and *in vivo* studies have been done to demonstrate the ability of lactoferrin to promote the growth of lactoferrin. Human lactoferrin and bovine lactoferrin have been used to support the growth of various species bifidobacterium as well as lactobacillus (Petschow and Talbott 1991; Petschow, Talbott et al. 1999; Griffiths, Duffy et al. 2003; Kim, Ohashi et al. 2004).

Two groups have studied the effect of lactoferrin in human infants. In both studies the effects were minimal. In one study the levels were from 0.1 to 1 mg/mL, below the levels found in early breast milk (Roberts, Chierici et al. 1992), although the high dose group did demonstrate some enhancement of bifidobacteria at three months. The second study used 2.8 mg/mL, but saw little effect (Balmer, Scott et al. 1989).

D. ANTI-INFLAMMATORY STUDIES

Lactoferrin may have a positive role in protection of the intestinal mucosa. Studies in animals with oral administration of lactoferrin have demonstrated a protective effect against development of colitis via modulation of the immune system (Togawa, Nagase et al. 2002). 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-a, and IL-1ß. When the animals received lactoferrin either as a preventative or treatment, the pro-inflammatory cytokines were inhibited and IL-4 and IL-10, the anti-inflammatory cytokines, were increased. When lactoferrin was given to control animals, there was no difference in cytokine levels between lactoferrin controls and saline controls. Recombinant human lactoferrin produced in yeast has also demonstrated a protective effect on the intestine in humans taking lactoferrin and indomethacin (Troost, Saris et al. 2003). This protective mechanism may result in less damage and more rapid repair of gut mucosal tissue injury, leading to more rapid restoration of normal permeability and growth. Lactoferrin also protected gut mucosa against the effects of bacterial lipopolysaccharide in mice (Kruzel, Harari et al. 2000).

Lactoferrin has also been implicated as an anti-inflammatory/anti-oxidant agent. This function is facilitated through the sequestration of free iron at inflammatory

sites (Gutteridge 1989; Morris, Earl et al. 1995) or direct binding to lipopolysaccharides (LPS) (Baveye, Elass et al. 1999). Lactoferrin has also been shown to decrease cutaneous inflammatory reaction by inhibiting the migration of epidermal Langerhans cells in mice and in human volunteers (Cumberbatch, Dearman et al. 2000; Griffiths, Cumberbatch et al. 2001; Kimber, Cumberbatch et al. 2002).

Lactoferrin has been shown to have a modulatory effect during episodes of inflammation both *in vitro* and *in vivo* (Kruzel, Harari et al. 1998). This mechanism of action appears to be through the balancing of the cytokine response during inflammation.

V. SAFETY ASSESSMENT

A. Substantial equivalence of recombinant human lactoferrin to native human lactoferrin

1. Biochemical Equivalence

Recombinant human lactoferrin has been purified from LF164 rice and compared to native human lactoferrin from breast milk. Lactoferrin found in breast milk, saliva, tears and other secretions is produced by the same gene sequence. The results of the physical, chemical and biochemical comparisons are summarized in Table 9 and have been published (Nandi, Suzuki et al. 2002; Suzuki, Kelleher et al. 2003); the data from each comparison follows. The data support substantial equivalence between human lactoferrin and recombinant human lactoferrin from rice. Data are provided from Ventria and the literature that support the safety of the plant glycosylation pattern in the rhLF.

Table 9. 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)
Amino acid composition	Based on DNA sequence	Homologous
N-terminal sequence	GRRRRSVZWCA	GRRRRSVZWCA
Glycosylation	Mammalian pattern[a]	Plant pattern[b]
SDS-PAGE/Western	~80 kDa	Slightly more mobile
Molecular mass (MALDI)	80.6 kDa	78.5 kDa
Peptide mapping by Lys-C trypsin		Good homology Mascot score 63
Isoelectric focusing point	> pI 8.2	> pI 8.2
Resistance to pepsin digestion	Digested in 0.5 minutes	Digested in 0.5 minutes
Physical appearance	Apo-form: colorless Holo-form: Pink	Apo-form: colorless Holo-form: Pink
Solubility	>10 mg/mL PBS at RT	>10 mg/mL PBS at RT

[a] Terminal sialic acid residues
[b] a(1,3)-fucose and ß(1,2)-xylose sugar residues on the core

a. Amino Acid Sequence and Composition

The amino acid sequence, based on the nucleic acid sequence, is identical for human and recombinant human lactoferrin. Amino acid composition analysis was done by ion exchange chromatography on three hydrolyzed samples of purified recombinant human lactoferrin. The theoretical amino acid composition values for native human lactoferrin were calculated based on the nucleic acid sequence. Analysis was done according to the manufacturer's instructions on an Hitachi L8800. Asn and Gln are deamidated to Asp and Glu during hydrolysis and thus are combined and reported as Asx and Glx, respectively. Recovery of Cys is typically incomplete unless oxidized procedures are performed. Tryptophan is destroyed upon hydrolysis and not recovered therefore data is not available for this residue. Table 10 provides the theoretical amino acid composition for native human lactoferrin, the measured average amino acid composition for three lots of recombinant human lactoferrin and the percent of theoretical. The measured amino acid composition of recombinant human lactoferrin was also compared to the theoretical value for human lactoferrin based on the nucleic acid sequence (not shown). Amino acid composition of recombinant human lactoferrin is identical within the acceptable analytical variation to native human lactoferrin.

Table 10. Amino acid composition of recombinant human lactoferrin compared to the theoretical value in human lactoferrin

Amino Acid	Theoretical	Avg n = 3	% Theoretical
Asx	70	69	99
Thr	31	28	90
Ser	50	45	90
Glx	70	72	103
Pro	35	37	106
Gly	54	53	98
Ala	63	62	98
Val	48	44	92
Met	5	4	80
Ile	16	15	94
Leu	58	58	100
Tyr	21	21	100
Phe	30	30	100
His	9	12	133
Lys	45	45	100
Arg	45	45	100

b. N-terminal sequence

N-terminal sequencing of native human lactoferrin and recombinant human lactoferrin gave identical results. In expressing rhLF in rice grain, a signal peptide obtained from rice storage protein glutelin 1 was fused with mature peptide of human lactoferrin to direct the rhLF into the endoplasmic reticulum and the protein bodies. This data indicates that signal peptide is properly removed (Nandi, Suzuki et al. 2002).

c. Glycosylation

The use of 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. Crystallographic studies with hLF and recombinant hLF (rhLF) expressed in Aspergillus indicate that carbohydrate moieties do not play a role in polypeptide conformation (Sun, Baker et al. 1999). Although glycosylation can play an important role in the functionality of molecules, it has been demonstrated that the carbohydrate moieties are not required for the interaction of LF and its receptor (Kawakami and Lonnerdal 1991; Iyer and Lonnerdal 1993). Using a human kidney-derived cell line that constitutively expresses rhLF, it was shown that both glycosylated and unglycosylated rhLF can be completely saturated with iron (van Berkel, Geerts et al. 1995). Human lactoferrin contains three potential N-glycosylation sites. Human lactoferrin has complex type N-glycans at Asn^{138} and Asn^{479}, while the third site, Asn^{624} is generally unglycosylated (Spik, Strecker et al. 1982; Van Berkel, van Veen et al. 1996).

Glycans have been studied and characterized in lactoferrins from humans, mouse, goat, cow, pig, and others. To date there is no evidence of O-glycosidic glycan linkages in any of the transferrin family proteins (Spik, Coddeville et al. 1994). The molecular mass of the recombinant lactoferrin with the N-glycan structures identified below is not compatible with additional glycans.

Structure of N-linked glycans of hLF and rhLF were analyzed by a combination of reverse-phase and size-fractionation HPLC, two dimensional sugar chain mapping, exoglycosidase digestion and mass spectrometry. Sugar chains were released from glycoproteins by hydrazinolysis, N-acetylated and the oligosaccharides fractionated. The oligosaccharides were pyridylaminated with 2-amino-pyridine and fractionated by HPLC. The molecular mass of PA-oligosaccharides was determined by MALDI-mass spectroscopy (Nandi, Yalda et al. 2005).

Analysis indicates a carbohydrate content equivalent to 6.4% and 2.9% of hLF and rhLF mass, respectively. There are three variations between the plant and

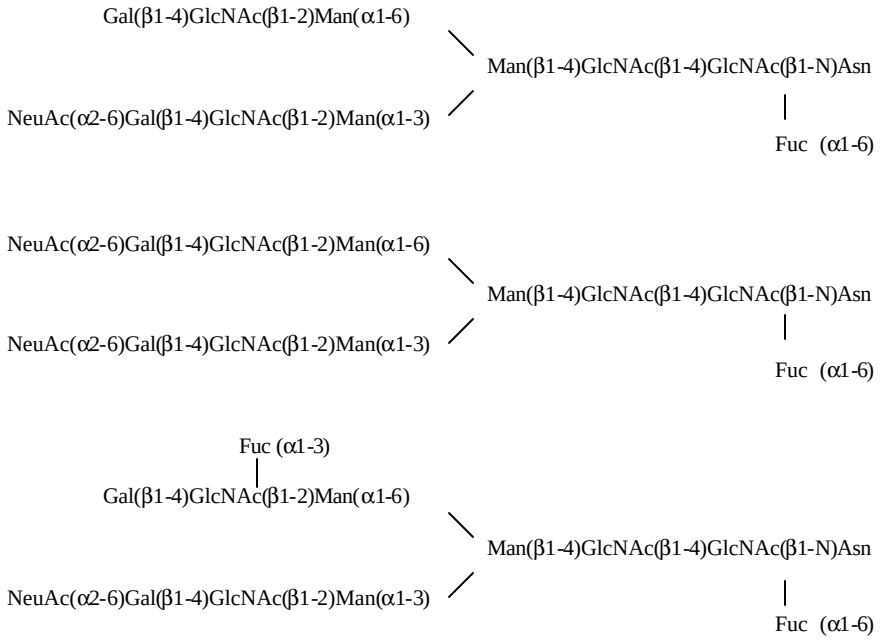
mammalian glycosylation pattern. The variations are listed below and summarized in Table 11.

- The absence of the terminal sialic acid found in many mammalian glycosylation patterns and no galactose at the non-reduced end
- The presence of a ß(1,2)-xylose on the core manose
- The presence of an a(1,3)-fucose on the proximal N-acetyl glucosamine.

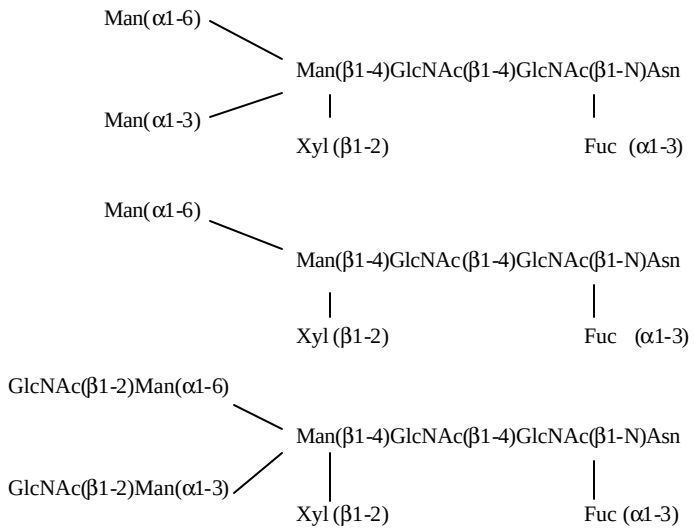
Table 11. Comparison of glycosylation patterns of human milk and LF164 recombinant human lactoferrin

Source	Sugar content (% w/w)	Fucosylation on the core a (1,3)	a (1,6)	Xylosylation on the core b (1,2)	Sialic acid and Galactosylation
Human milk LF (Spik, Coddeville et al. 1994)	6.4	No	Yes	No	Yes
rHLF (Nandi, Yalda et al. 2005)	2.9	Yes	No	Yes	No

The major structures of human milk lactoferrin glycans are below. They differ from lactoferrin from polymorphonuclear leukocytes by the fucosylation of the N-acetylglucosamine residues.



The three major structures of recombinant human lactoferrin glycans are shown below. These three configurations represent 57.2, 14.5 and 11.0% of the N-glycans, respectively, for a total of 82.7% of the glycan content.



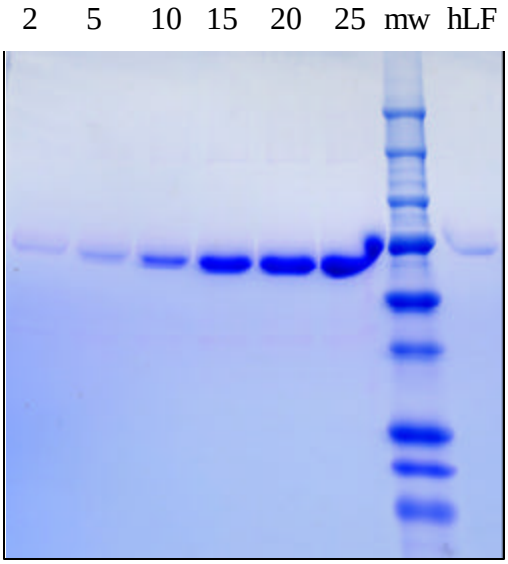
There are two areas to address when assessing the significance of plant glycosylation patterns in human proteins. One is the presence of cross-reactive carbohydrate determinants (CCDs). In the early 1980s the first reports of crossreactive IgE antibodies in pollen and venom allergic subjects directed against carbohydrate structures were published (Aalberse, Koshte et al. 1981). These CCD structures were identified as two typical non-mammalian substitutions found at N-glycosylation sites of plants: an a(1,3)-linked fucose on the proximal N-acetyl glucosamine and a ß(1,2)-linked xylose on the core mannose (van Ree and Aalberse 1999). Ventria's recombinant human lactoferrin has both of these structures. The analysis of the glycosylation demonstrated that 100% of the recombinant lactoferrin carries the ß(1,2)-linked xylose and 92.1% has the a(1,3)-linked fucose. The clinical relevance of these structures will be discussed in this section on page 56.

The presence of the complex glycan representing the Lewis^a antigen found on mammalian red blood cells is another possible source of development of cross-reacting antibodies. Foods such as kiwi, strawberry, hazelnut and walnut have a major glycan component with the Le^a glycan. Ventria's recombinant human lactoferrin may contain Le^a glycan, however, it is at such a low level that initial analysis was not able to confirm the presence of Le^a glycan. This is compared to some foods with Le^a levels as high as 50% of glycan content (Wilson, Zeleny et al. 2001). Investigations have been done to determine whether the Le^a epitope from plants was involved in IgE binding. No involvement of Le^a (or Le^x) was found in the IgE recognition of plant N-glycans (van Ree, Cabanes-Macheteau et al. 2000). Therefore, the presence of even low levels of this glycan structure in recombinant human lactoferrin does not constitute a safety issue.

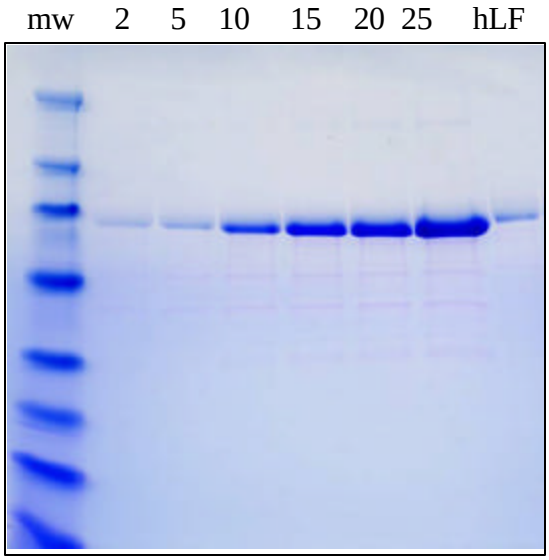
d. SDS-PAGE/Western

SDS-PAGE of purified recombinant lactoferrin compared to human lactoferrin from milk illustrates a small difference in mobility and thus molecular weight. This difference is attributed to the difference in glycosylation as discussed above. The data below compare reduced and non-reduced gels stained with Coomassie Blue (9A) or silver stain (9B). The third pair (9C) is Western blots using rabbit anti-lactoferrin antibody. In all cases the far right lane is human lactoferrin from milk.

A. Coomassie Blue stain of 4-20% gel. Recombinant human lactoferrin at 2-25µg/lane, human lactoferrin at 5µg/lane.



Non-reduced gel



Reduced gel

C. Silver stain, 4-20% Novex gel. Recombinant human lactoferrin at 0.4 – 5µg/lane, human lactoferrin at 0.1µg/lane.

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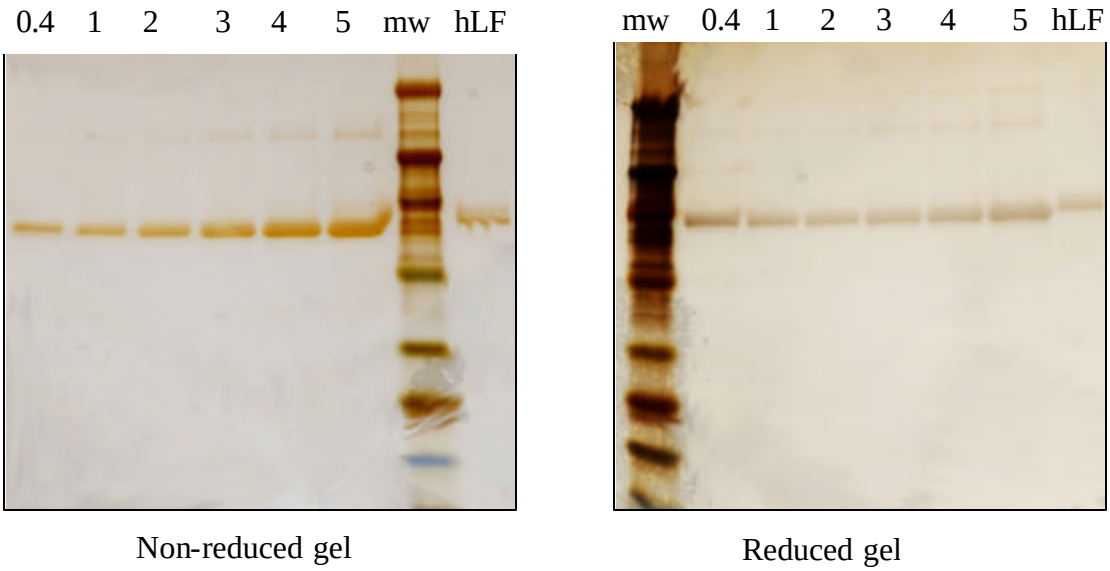


C. Western blot, 4-20% Novex gel, transferred overnight. Recombinant human lactoferrin at 0.4 – 5µg/lane, human lactoferrin at 0.1µg/lane.

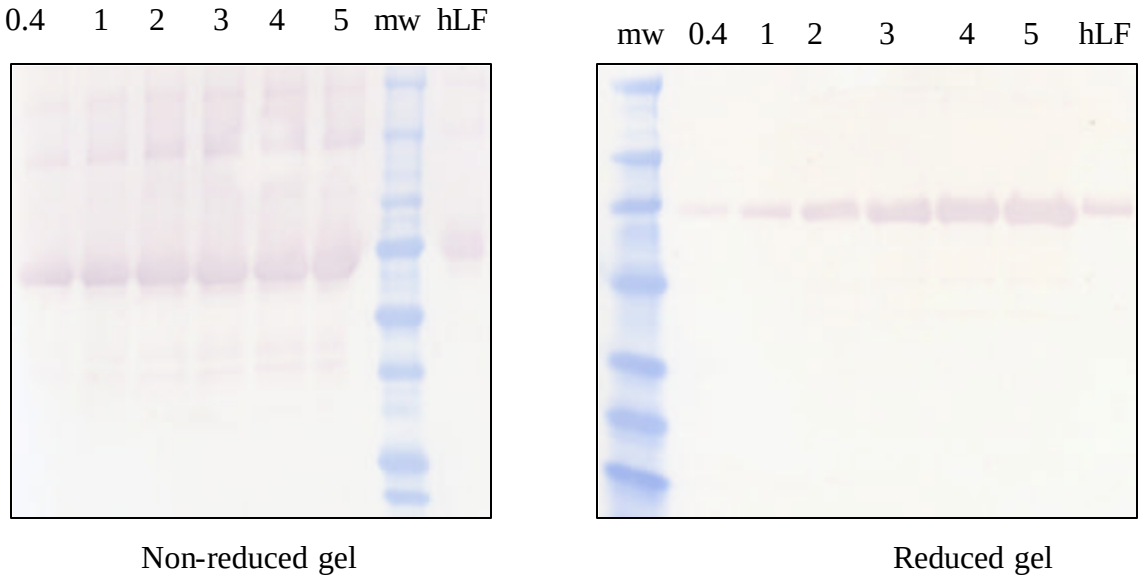
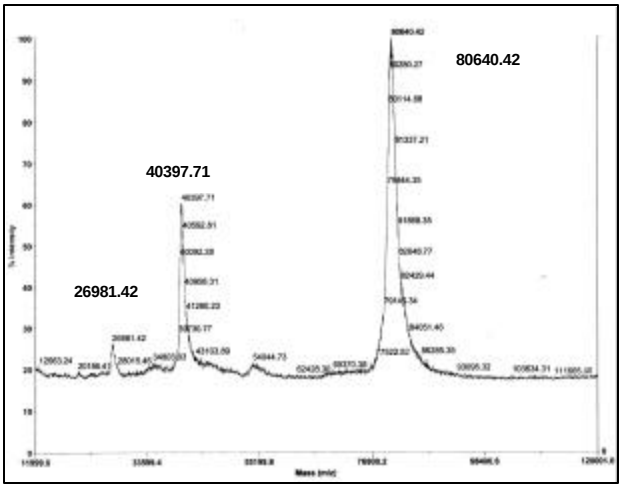


Figure 9. SDS-PAGE of recombinant human lactoferrin and human lactoferrin

e. Molecular Mass (MALDI)

The molecular mass was determined by MALDI (matrix-assisted laser desorption/ionization) mass spectroscopy. The molecular weight is 80.6 kD for human lactoferrin and 78.5 kD for recombinant human lactoferrin. The different carbohydrate moieties coupled to the lactoferrin polypeptide can explain this difference. The peaks at approximately 26 and 39 kD are artifacts caused by the multiple charged ions (+2,+3) of the molecule.

Human lactoferrin from milk – 80.6 kDa



rHuman lactoferrin – 78.5 kDa

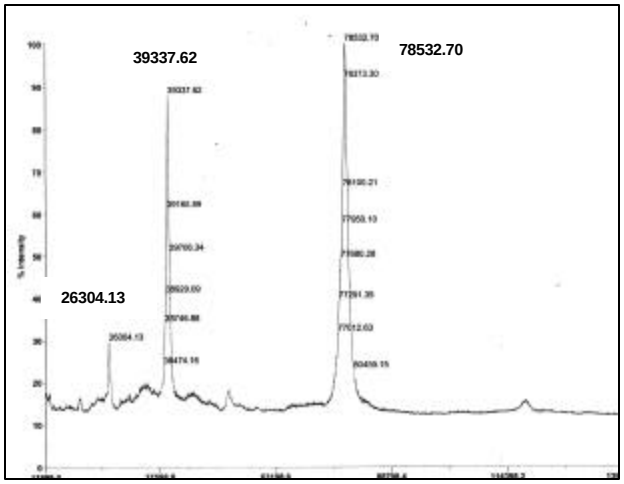


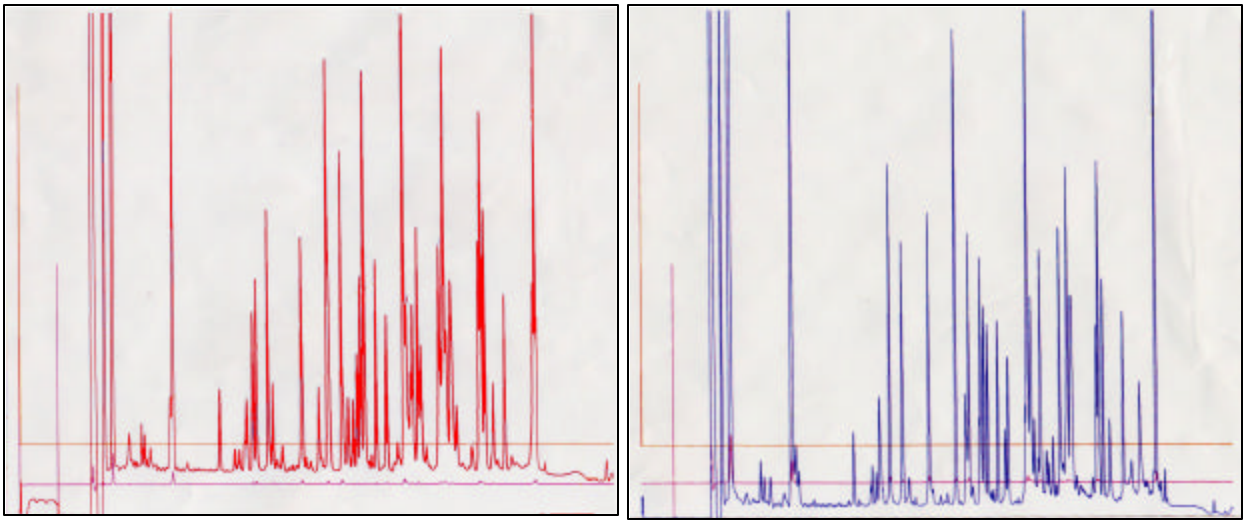
Figure 10. MALDI data on lactoferrin

f. Peptide mapping

The data in Figure 11 were created by a single enzymatic cleavage using Lys-C digestion. Additional data done on another preparation of recombinant human lactoferrin using a single enzyme cleavage with trypsin was analyzed with Mascot software. That analysis resulted in probability matching scores with human lactoferrin of 63. These matches are considered significantly close for single enzyme cleavage (Papayannopoulos 1995; Shevchenko, Wilm et al. 1996).

Figure 11. HPLC of Lys-C degraded human lactoferrin and recombinant lactoferrin

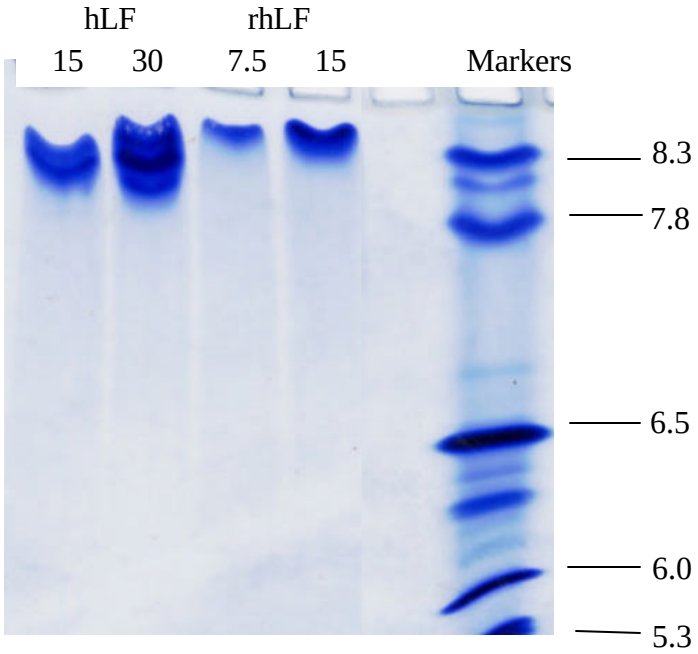
Human lactoferrin Recombinant human lactoferrin



g. Isoelectric focusing

Isoelectric focusing was performed using a Novex IEF gel, pH 3 – 10. The human lactoferrin and recombinant human lactoferrin are basic proteins with a pI greater than 8.2 (Nandi, Suzuki et al. 2002).

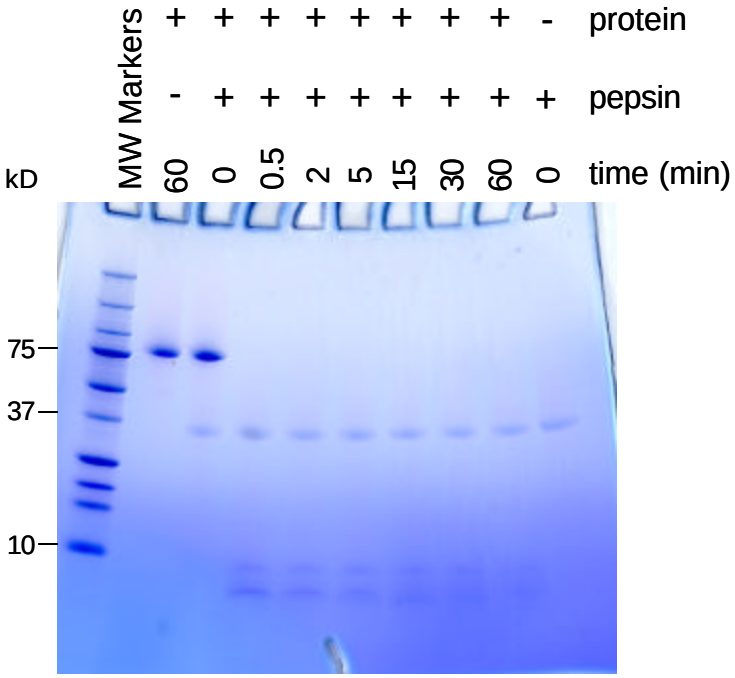
Figure 12. Isoelectric focusing gel with human lactoferrin and recombinant human lactoferrin



h. Resistance to pepsin digestion

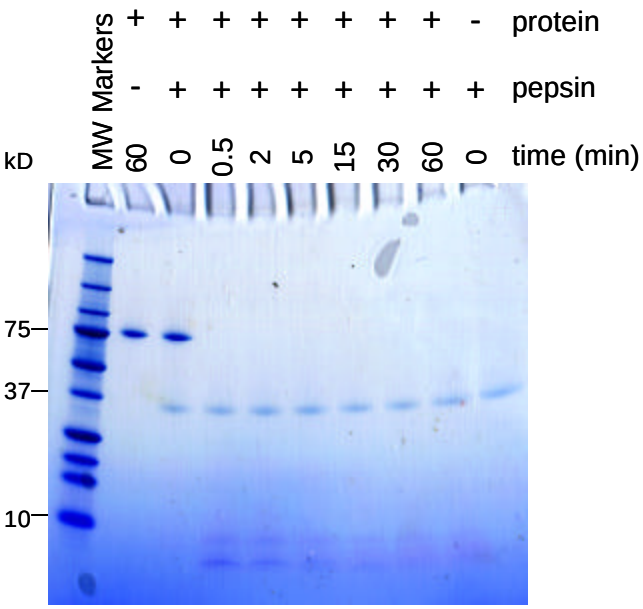
Ventria recombinant lactoferrin preparations, iron saturated (holo-rhLF) and iron unsaturated (apo-rhLF), were compared with native human lactoferrin from breast milk for resistance to digestion. The studies were done using the digestion protocol evaluated by nine different laboratories working with the ILSI Health and Environmental Sciences Institute (Thomas, Alalbers et al. 2004). Lactoferrin samples were digested in simulated gastric fluid, pH 1.2. Samples were removed for analysis at time 0, 0.5, 2, 5, 15, 30, and 60 minutes and neutralized with 0.2 M sodium carbonate, pH 11. Digests were run on 10-20% Tris/Tricine gels from Invitrogen under reducing conditions. In 0.5 minutes of incubation all forms and sources of lactoferrin were digested when analyzed according to the standard protocol. Figure 13 A and B show the complete time course of digestion for native human lactoferrin and the apo and holo forms of recombinant human lactoferrin. Figure 13 C illustrates human and recombinant human lactoferrin on the same gel.

A.

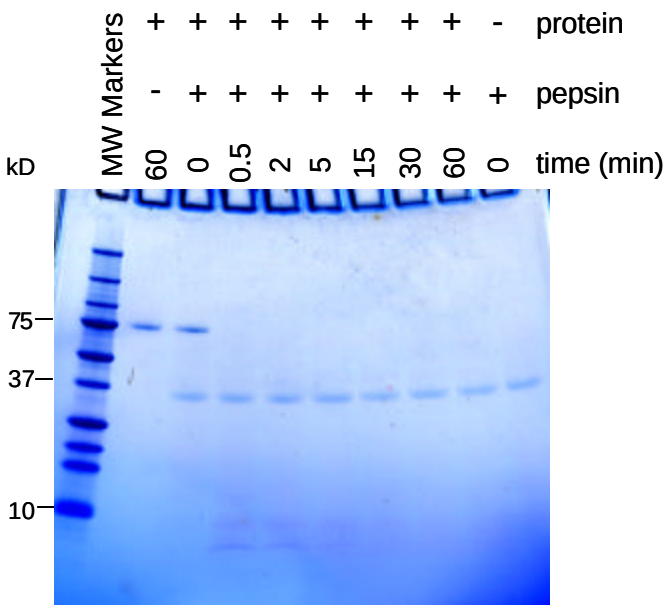


Human lactoferrin from breast milk

B.



Recombinant human apo-LF



Recombinant human holo-LF

C.

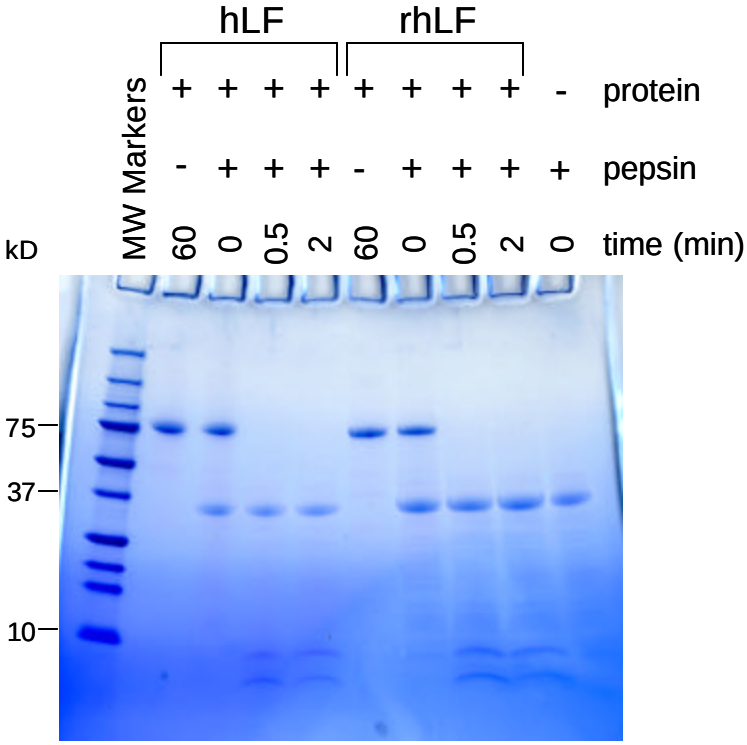


Figure 13. Digestion of human lactoferrin from breast milk and recombinant human lactoferrin using the ILSI protocol.

In addition a theoretical digestion was performed using the computer program on ExPASy Proteomics tools, (http://kr.expasy.org/tools/peptidecutter/), Peptide Cutter. This program predicts the potential enzyme cleavage sites in a given protein sequence. The program was run using pepsin digestion of the rhLF sequence at pH 1.3 and pH >2. Cleavage at pH 1.3 resulted in 170 peptides and amino acids, with 93% smaller than 12 amino acids. From epitope mapping studies the theoretical peptide length for binding is 8 - 12 amino acids for a T-cell epitope and longer for B-cell epitopes (Rothbard and Gefter 1991; Metcalfe, Astwood et al. 1996). The largest fragment was 36 amino acids near the N-terminus of the molecule. It is of note that in the gels, there is evidence of a possible fragment near the dye front at less than 10,000 molecular weight. This fragment may correspond to the computer predicted 36 amino acid fragment. This 36 amino acid fragment had no matches in the allergenonline.com database. Cleavage with pepsin at pH > 2 resulted in 129 peptides and amino acids, with the longest fragment being 39 amino acids that included the 36 amino acid peptide resulting from the pH 1.3 cleavage. Results from the computer generated digestion and the actual digestion indicate there is no pepsin-resistance for native or recombinant human lactoferrin.

i. Solubility

Both native and recombinant lactoferrin are soluble at >10 mg/mL in phosphate buffered saline at room temperature.

2. Activity Equivalence

In addition to comparing the physical and chemical properties of human lactoferrin from milk to recombinant human lactoferrin from rice, activity comparisons were done. Table 12 summarizes the studies and the results. The data from the individual studies follow the table.

Table 12. Activity of recombinant human lactoferrin compared with human lactoferrin

Property	Human LF (hLF)	Recombinant hLF (rice-derived)
Iron binding and release capacity	pH dependent release and saturation	Kinetics identical to native human
Receptor binding affinity (CACO-2 cells)	Kd = 1.6×10^{-7} M ± 0.3×10^{-7} M	Kd = 2.7×10^{-7} M ± 0.4×10^{-7} M
Bacteriostatic effect (EPEC)	Growth reduction of *E. coli* at 37°C for 12 h	Growth reduction of *E. coli* at 37°C for 12 h
Binding to Porins	Binds to porins	Binds to porins

a. Iron binding and release capacity

Lactoferrin is an iron binding glycoprotein. Native human lactoferrin begins to release iron as the pH is lowered. The release of iron from both native and recombinant human lactoferrin from rice begins at pH 4.0 and continues to desaturation at pH 1-2. Both forms of lactoferrin have 1.4 mg of ferric iron per gram of lactoferrin when completely saturated (Nandi, Suzuki et al. 2002).

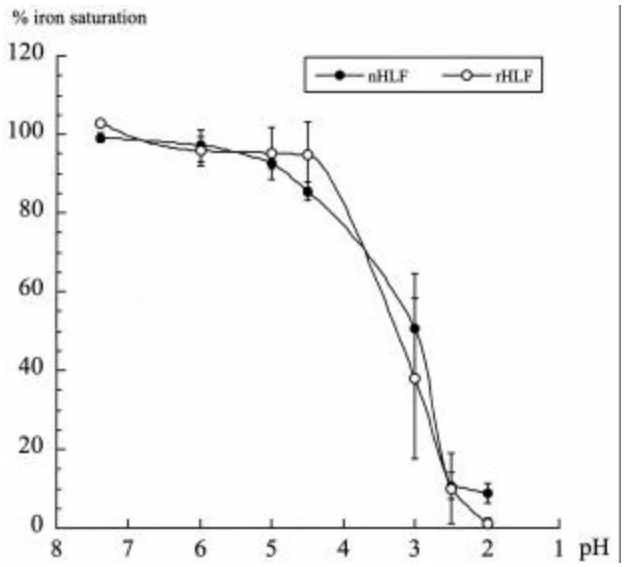


Figure 14. pH dependent iron release from human lactoferrin and recombinant human lactoferrin

b. Receptor binding

Published data of receptor binding using ^{125}I-labeled human lactoferrin and recombinant human lactoferrin was saturable and indicated a non-significant difference in binding between native and recombinant (Nandi, Suzuki et al. 2002). The Kd for human lactoferrin and recombinant lactoferrin was $1.6 \times 10^{-7} \pm 0.3 \times 10^{-7}$ M and $2.7 \times 10^{-7} \pm 0.4 \times 10^{-7}$ M, respectively (Nandi, Suzuki et al. 2002).

Additional human cell lines were tested for binding of hLF and rhLF labeled with ^{59}Fe. Human fetal intestinal cells (HFS cells) and human leukemia T cells (Jurkat cells) were tested. Although there were differences in Kd for the cell lines, there was no significant difference between hLF and rhLF for each cell line. The differences between cell lines were expected considering their different physiological activities. The Kd for hLF and rhLF was $1.63 \times 10^{-6} \pm 0.45 \times 10^{-6}$ M and $1.17\ 10^{-6} \pm 0.30 \times 10^{-6}$ M in the HFS cells. Values for the Jurkat cells were $1.4 \times 10^{-6} \pm 0.60 \times 10^{-6}$ M and $0.56 \times 10^{-6} \pm 0.19 \times 10^{-6}$ M for hLF and rhLF,

respectively. Although there was a minor difference in the Jurkat binding, receptor binding is usually not considered biologically different unless there is an order of magnitude difference. This data indicates there is no functional difference in hLF and rhLF with respect to receptor binding.

c. Bacteriostatic activity

Lactoferrin has been characterized as having bacteriostatic characteristics. One of the mechanisms of action is the ability of lactoferrin to bind and sequester iron required for the metabolic activity of the microorganisms. Although there are other lactoferrin-mediated antimicrobial activities, this mechanism is influenced by the level of iron saturation of the lactoferrin. Human milk lactoferrin and recombinant human apo-lactoferrin have low iron content. When these forms are compared, there is equivalent inhibition of bacterial growth. The more saturated forms of lactoferrin, holo and Ventria's "asis" have 100 and 50% saturation, respectively, and in some *in vitro* conditions are less effective in inhibiting bacterial growth.

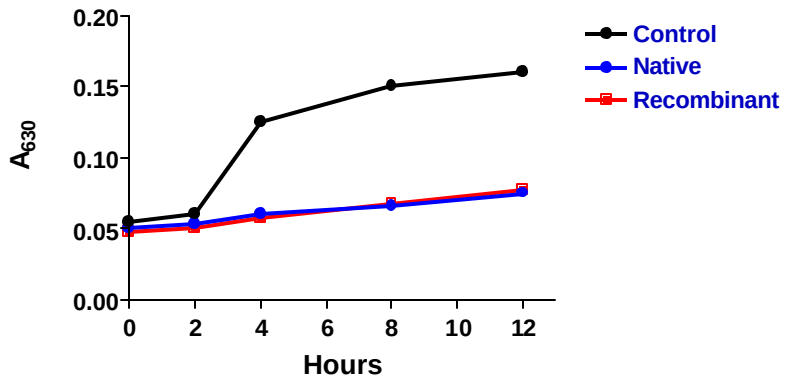
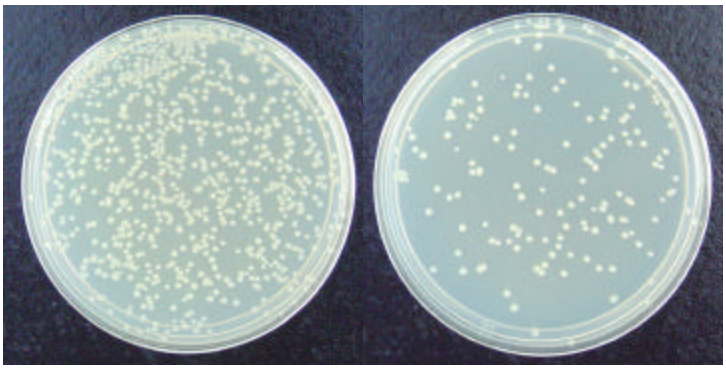


Figure 15. Lactoferrin inhibition of *E. coli* growth

The antimicrobial effect of rhLF was also tested against a laboratory strain of *E. coli*, DH5a (Figure 16). *E coli* was diluted to a concentration of 10^5 CFU (colony forming units) with or without rhLF. After incubation at 37°C for 120 min, the mixture was plated and counted after 24 hours. Colony forming units in cultures treated with rhLF were reduced to 10% of the CFU without rhLF.



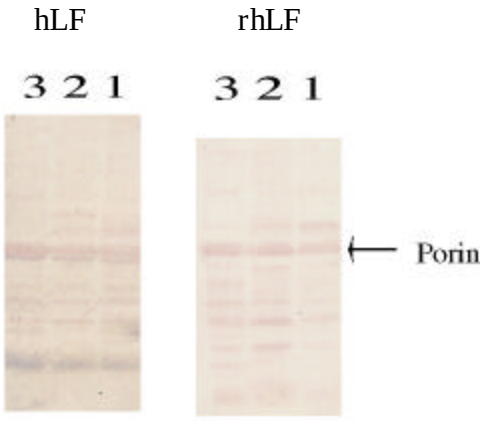
Buffer only Buffer + 1 mg/mL rhLF

Figure 16. Bactericidal effect of rhLF.

d. Porin Binding

Another mechanism of action in the bacteriostatic/bactericidal activity of lactoferrin is through binding to a class of well-conserved outer membrane proteins called porins (Naidu, Svensson et al. 1993). Membrane preparations were made from three strains of *E. coli* and the proteins separated by SDS-PAGE. Following transfer, the blots were reacted with human lactoferrin and recombinant lactoferrin. The porin binding pattern is identical.

Figure 17. Porin binding



1. JF 568 outer membrane fraction
2. JF 701 outer membrane fraction
3. JF 703 outer membrane fraction

3. Safety Equivalence

Immunogenicity and allergenicity are important factors for consideration when determining the safety of recombinant proteins from plants. The goal is to

demonstrate that the recombinant human lactoferrin produced in rice is substantially equivalent to, and thus as safe as, human lactoferrin found in breast milk, saliva and other secretions.

a. Gene Source

The gene for recombinant human lactoferrin is synthetically produced based on the DNA sequence of lactoferrin from human breast milk. Therefore the gene is not from an allergenic source.

b. Sequence similarity, toxin and allergen database search

Lactoferrin is present at significant concentrations in the milk and other biological fluids of most mammals – including humans - and some reptiles. Human colostrum contains up to 7 mg/mL of lactoferrin (Lönnerdal 1985). Due to its ubiquitous presence in human secretions, it is highly unlikely that this molecule is either an allergen or toxin. Recombinant human lactoferrin and human lactoferrin have 100% sequence homology. Human lactoferrin is not listed as a toxin in the SWISS-PROT and TrEMBL databases. Nor does it share any amino acid sequence homology with known toxins, as determined by FASTA search against known toxins listed in the SWISS-PROT and TrEMBL databases. The criteria for the FASTA search are described by Gendel (Gendel 1998). The wordsize used initially was set to two, and the regions of amino acid sequence identity greater than 6 were scored; none were found.

A search against a database of known allergens, identified in the SWISS-PROT and TrEMBL databases, also using techniques described by Gendel, and the Food Allergy Research and Resource Program (FARRP) allergen database, www.allergenonline.com did not identify human lactoferrin as an allergen (Pearson and Lipman 1988).

In searching for sequence similarity, there is 70% homology with bovine lactoferrin. There is little clinical information indicating bovine lactoferrin is a milk allergen. The major allergens in cow's milk allergy are casein, ß-lactoglobulin and a-lactalbumin (Wal 1998; Wal 2001). In investigations where IgE antibodies to lactoferrin have been demonstrated in children with cow's milk allergy, antibodies to one of the major cow's milk allergens has been demonstrated as well. In one study, one milk-allergic infant out of 92 was reported as mono-sensitized to bovine lactoferrin (Wal 1998). We were able to obtain samples of sera from two patients reported as mono-sensitized to bovine lactoferrin. Neither serum reacted with recombinant human lactoferrin. Thus even the rare sensitization by bovine lactoferrin is not relevant to the use of recombinant human lactoferrin.

There have been studies in the Brown Norway rat indicating that bovine lactoferrin can induce an IgE response after intraperitoneal injection (Atkinson and Miller 1994; Miller, Meredith et al. 1999). The Brown Norway rat model is not considered appropriate for the prediction of human responses to the ingestion of lactoferrin or other proteins. The model does not discriminate between allergenic and non-allergenic proteins and following intraperitoneal injection, no protein has had a negative response. That negates the predictive value of the studies. In a recent study with ovalbumin, oral dosing in neonate and young Brown Norway rats actually reduced the IgE response (Madsen and Pilegaard 2003).

In searching www.allergenonline.com database for other sequence similarities, there was a 52% amino acid sequence homology between human lactoferrin and chicken egg ovotransferrin (conalbumin), which is a known allergen. This site uses the FASTA comparison routine of Pearson and Lipman to compare protein sequences in an allergen. Using this data base, historical data indicate cross-reactivity is not likely for proteins with less than 50% homology over the entire protein sequence. Chicken egg ovotransferrin (conalbumin) was the only protein with an identity greater than 50% (52.6%). As there is no immunological cross-reactivity between chicken egg ovotranserrin and human lactoferrin or recombinant human lactoferrin, it is not a safety issue.

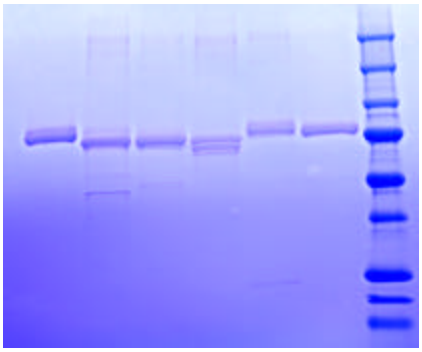
Although there were some matches in the 35-36% range, none of these proteins had 8 identical contiguous amino acids, making cross-reactivity with them highly unlikely (Metcalfe, Astwood et al. 1996).

In vitro cross reactivity studies were done using Ventria Bioscience's recombinant human lactoferrin in Western blots. Lactoferrin is a member of the iron binding glycoprotein family of proteins. Cross reactivity studies compared reactivity of an antibody to human lactoferrin produced in rabbits against recombinant human lactoferrin from rice, human lactoferrin from milk, bovine lactoferrin, human transferrin, bovine transferrin and chicken egg ovotransferrin. In addition, antibodies against bovine lactoferrin and transferrin, human transferrin and chicken ovotransferrin were tested for activity against human lactoferrin and recombinant human lactoferrin. Results indicate there is no cross reactivity among any of the combinations with related proteins. Cross reactivity was present between human and recombinant human lactoferrin antigens and antibodies, as expected. The cross reactivity data are presented in Figure 18.

Figure 18. Western blots for cross reactivity of proteins and antibodies

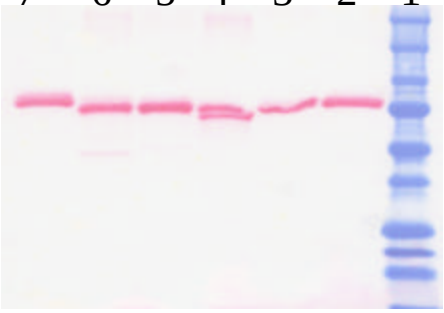
Lane 1. Molecular weight markers
Lane 2. Recombinant human lactoferrin
Lane 3. Bovine lactoferrin
Lane 4. Bovine transferrin
Lane 5. Human transferrin
Lane 6. Ovotransferrin
Lane 7. Human lactoferrin

7 6 5 4 3 2 1



Coomassie Stained Gel

7 6 5 4 3 2 1



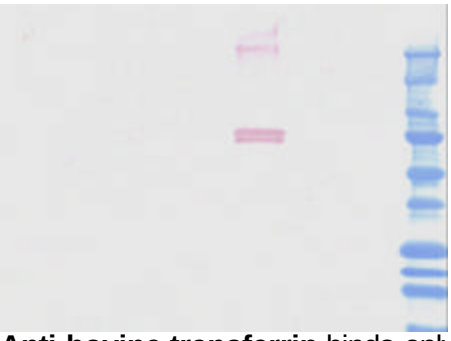
Western blot stained with Ponceau S

Western Blots

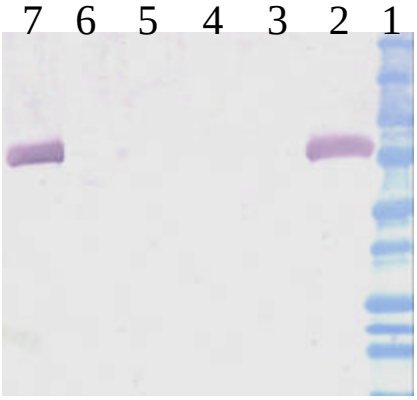
7 6 5 4 3 2 1

Anti-human transferrin binds only human transferrin

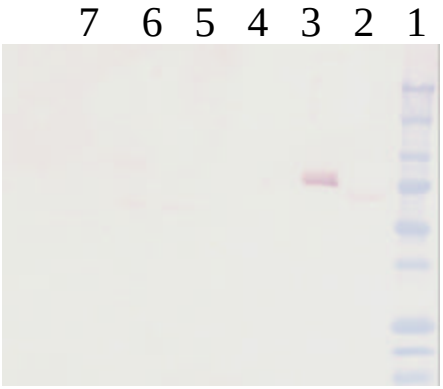
7 6 5 4 3 2 1



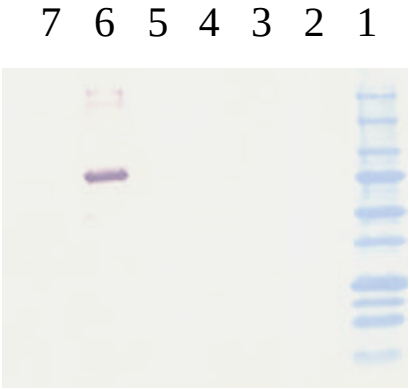
Anti-bovine transferrin binds only bovine transferrin



Anti-human lactoferrin binds human and recombinant human lactoferrin



Anti-bovine lactoferrin binds only bovine lactoferrin



Anti-chicken ovotransferrin binds only chicken egg ovotransferrin

c. <u>Digestibility</u>

There is no difference in the susceptibility of hLF and rhLF to digestion with pepsin at pH 1.2. The gels are shown on page 44.

4. Allergenicity

It is unlikely that human lactoferrin is an allergen. It is a protein component of breast milk, and as such is one of the earliest proteins infants are exposed to in their diet. Second, it has never been identified as an allergen despite a variety of experiments that have tested lactoferrin administered topically and orally (Yamauchi, Wakabayashi et al. 1998; Griffiths, Cumberbatch et al. 2001; Zimecki and Wlaszczyk 2001). There is one report in the literature of lactoferrin antibodies in the serum of breastfed children from Gambia (Brock, Lamont et al. 1998). No additional investigation was performed on these children and there

may have been associated gastrointestinal infection associated with the development of the antibodies. In addition, despite the presence of antibodies, there was no reported clinical allergic reaction.

The allergic potential of recombinant human lactoferrin from rice has been addressed by Dr. Ronald van Ree, a member of the expert panel. The following is his assessment of the allergic potential of the material and data on a histamine release assays performed in his laboratory using Ventria's recombinant human lactoferrin from rice.

In assessing the allergic potential of recombinant human lactoferrin from rice, it is important to investigate the occurrence of IgE binding structures in the product. If these structures are present, it must be determined whether they can induce mediator release in a clinically relevant manner. There are three potential allergic structures:

- Human lactoferrin as an auto-allergen
- Residual rice proteins being known allergens.
- Plant glycans on the recombinant human lactoferrin

a. <u>Human lactoferrin as an auto-allergen</u>

The likelihood of human lactoferrin being an auto-allergen is small, but it can not be excluded completely. Auto-allergens are sometimes also referred to as IgE-dependent histamine-releasing factors (HRF)(Kleine Budde, de Heer et al. 2002; Kleine Budde and Aalberse 2003). IgE-binding to human proteins on the basis of cross-reactivity with a non-self sensitizing allergen has been reported. An example is human profilin that was shown to be a cross-reactive (auto-) allergen in extremely rare patients sensitized to birch pollen profilin (Valenta, Duchene et al. 1991). IgE recognition of recombinant human lactoferrin on the basis of sensitization to e.g. bovine lactoferrin is unlikely, since the sera from reported mono-sensitized milk-allergic patients did not react with recombinant human lactoferrin. Even if such a patient were identified, we can argue that this is not a property that is a result of its expression in rice. The potential role of a human protein as an auto-allergen holds for any human protein, including many blood-derived products like e.g. albumin and immunoglobulin that are administered to patients on a regular basis. In summary, although relevant to be aware of, this possible allergenic property of a recombinant human lactoferrin product can never be a reason to come to a negative verdict.

b. <u>Residual rice proteins being known allergens</u>

The products with recombinant human lactoferrin from rice contain at least 10% residual rice protein, but possibly up to 50%. There is a chance that this contamination includes rice-derived allergens. Cereal grains have been reported

to be a cause of food allergies, however rice is not considered as a major food allergen. *In vitro* studies on the IgE-binding reaction to rice seed proteins have suggested that the major allergenic protein in rice is a 14-16 kD protein. More recent studies have suggested the allergenic protein is a salt soluble albumin belonging to the a-amylase/trypsin inhibitor family (Izumi, Sugiyama et al. 1999), however, the subjects with these antibodies have not been tested by food challenge and it is questionable if this is a true allergen or perhaps a grass pollen allergy. In Europe and the USA, rice is not considered an issue for allergy. In Japan, IgE-mediated rice allergy is reported (Besler, Tanabe et al. 2001), however, there is no indication that these individuals have an allergic response following the ingestion of rice. As discussed in the section on production, it is unlikely that the 15kD proteins identified as rice allergens would be present in the final product. In addition, the product will be labeled as recombinant human lactoferrin from rice.

Although rice is considered to have low allergenicity, since there are reported instances of rice-allergy, Ventria had flour and extracts from the transformed LF164 and parental Taipei 309 rice lines tested for the presence of rice allergens. Testing was performed to confirm that the transformation event did not result in an increase in the allergenicity of the rice. Reactive sera that had at least two of the following three criteria were used: a positive history of rice allergy or food allergy compatible with rice reactivity, a positive skin test to rice extract, or a positive IgE antibody response (>3% binding) to rice by Radio Allergosorbent Test (RAST). Fifteen sera were combined for Pool A, eight sera were pooled for Pool B and eight were individual sera.

Testing was done to demonstrate that rice reactive sera do not exhibit increased IgE antibody binding to the transgenic rice as compared to the wild-type rice. No significant differences were observed by RAST or RAST inhibition or by qualitative analysis of IgE binding through immunoblotting of pooled sera. Additionally, concentrate and isolate preparations of recombinant human lactoferrin were tested with regard to residual rice allergen activity. The concentrate and isolate preparations displayed rice-allergen activity. Generally, there was less IgE antibody reactivity to the isolate than the concentrate with the exception of serum S-6.

Table 13. IgE antibody activity of serum pools/sera from rice reactive subjects as measured by RAST

Sample	Lactoferrin Concentrate	Lactoferrin Isolate	Taipei 309 Flour	Shrimp Extract
Pool A	21.7%	14.1%	22.3%	4.2%
Pool B	16.9%	11.2%	18.5%	0.0%
S-1	29.5%	29.7%	31.9%	0.0%
S-2	25.5%	12.3%	33.8%	0.0%
S-3	27.8%	26.0%	31.1%	0.0%
S-4	21.6%	21.6%	22.1%	0.0%
S-5	24.9%	18.2%	28.5%	0.0%
S-6	26.8%	29.9%	27.5%	0.0%
S-7	22.7%	22.6%	18.8%	0.0%
S-8	22.1%	16.6%	23.7%	0.0%
Shrimp Pool	NA	NA	N/A	26%
Mean	24.0%	20.2%	25.8%	
Std	0.04	0.07	0.05	

Based on the amount of protein and the estimation of biological activity by calculation of the inhibitory dose 50, there is clearly less IgE antibody binding in the isolated preparation. As the sera were not characterized for pollen allergies, this binding may be due to CCD reactivity as described below.

Table 14. 50% inhibitory dose (ID_{50})

Sample	50% Inhibitory Dose (ID_{50})	ID_{50} Dose per 100g Flour
Taipei 309 Flour	252.3 µg	713.4
Lactoferrin Rice Flour	> 1000 µg	< 313.6
Lactoferrin Concentrate	> 1000 µg	< 360.0
Lactoferrin Isolate	> 1000 µg	< 47.2

c. <u>Plant glycans on the recombinant human lactoferrin</u>

The chance that expression of human lactoferrin in rice would result in the introduction of recognition by serum IgE antibodies of pollen allergic subjects was extremely high since two out of three putative N-glycosylation sites are glycosylated in the native protein. Indeed it was confirmed that recombinant lactoferrin from rice was substituted at two positions with plant N-glycans, 100% of the glycoprotein displaying the typical ß(1,2)-linked xylose and 92.1% the core a(1,3)-linked fucose. Both have been shown to be involved in IgE recognition in serum of pollen allergic subjects (van Ree, Cabanes-Macheteau et al. 2000). The most abundant complex N-glycans on rice recombinant lactoferrin are identical to the structures identified on pineapple stem bromelain (14.5% of the glycosylated lactoferrin) and on horseradish peroxidase (57.2%). Both molecules have been

used in several studies as typical examples of glycoproteins carrying IgE-binding glycans (van Ree, Cabanes-Macheteau et al. 2000; Mari 2002). In addition, N-glycans from bromelain have been isolated and conjugated to bovine serum albumin to produce neoglycopeptides. These were used to study the biological activity of IgE antibodies against plant N-glycans (Fotisch, Altmann et al. 1999).

As could be expected, three serum samples of pollen allergic subjects (pf41, pf192 and 2399CV) with reported IgE reactivity to plant N-glycans (van Ree, Cabanes-Macheteau et al. 2000; Mari 2002) demonstrated significant IgE-binding to holo- and apo-lactoferrin isolates from rice but negligible binding to the natural human protein purified from breast milk (Table 15). It is unlikely that this IgE binding is caused by residual rice protein in the lactoferrin isolates because IgE reactivity to rice extracts and lactoferrin isolates was very similar in strength. The observations therefore demonstrate that replacing mammalian for plant N-glycans introduced IgE-recognition.

Table 15. RAST results with plant N-glycan allergic sera, IU/mL

Serum	hLF	holo-rhLF	apo-rhLF	Control Rice	LF-164 Rice
pf 41	0.7	14.3	15.9	20.1	17.8
pf 192	0.1	16.3	17.8	16.3	17.8
2399 CV	0.9	39.0	96.8	105.4	84.9

The key issue for allergenicity assessment is not IgE binding but biological activity of IgE antibodies. Unfortunately, this notion has not really crystallized in the present guidelines for allergenicity assessment. There is much emphasis on IgE binding and very little attention to biological activity, i.e. clinical relevance. This is a dangerous pitfall for evaluation of a novel food or a plant-derived pharma product. It is easy to find some serum samples from food allergic patients that will have serum IgE antibodies recognizing the novel food or plant-derived recombinant protein. First of all, we have to realize that a reported history of food allergy can not always be confirmed by a blinded challenge. But even if serum samples are used from patients with blinded challenge-confirmed food allergy, not all IgE antibodies of those patients need to be involved in induction of symptoms. This issue is particularly relevant for IgE antibodies against plant N-glycans. To illustrate this, let's take the example of a patient with birch pollen rhinitis and fruit and tree nut allergy. This is a very common combination which is explained by cross-reactive IgE antibodies. If we dissect the cross-reactive IgE response of such patients, it is clear that there are 3 to 4 different sources for cross-reactivity. The first one is Bet v 1, the major allergen of birch pollen. The second one is profilin (Bet v 2) and the third one is Bet v 6. Last but not least, there are cross-reactive IgE antibodies against plant N-glycans. In many highly sensitized birch pollen allergic patients with clinical fruit allergy (apple, peach, etc.), these different cross-reactive antibodies go together (Calkhoven, Aalbers et al. 1987; Bauer, Ebner et al. 1996). If such serum samples were used for

assessing the allergenicity of a recombinant glycoprotein from plants, it would certainly come out as an allergen. If we select subjects with sensitization to only Bet v 2 and N-glycans, we rarely, if ever, see clinical food allergy (van der Veen, van Ree et al. 1997; Wensing, Akkerdaas et al. In press). In general, we can say that IgE antibodies against carbohydrate moieties have poor biological activity. Carbohydrates are not generally considered as allergens. There are cross reacting IgE antibodies in the category of the cross-reacting carbohydrate determinant (CCD). Although the *in vitro* reactivity of these IgE cross reacting antibodies can be very strong, they have poor biologic, *in vivo*, activity (van der Veen, van Ree et al. 1997; Aalberse, Akkerdaas et al. 2001).

It may not be wise, however, to completely ignore the reports that claim clinical relevance, or at least biological activity. Most of these reports base these claims on the use of neoglycopeptides that were shown to have the capacity to induce *in vitro* histamine release from basophils (Fotisch, Altmann et al. 1999). In some cases, glycosylated and deglycosylated versions of allergens were compared (Bublin, Radauer et al. 2003; Foetisch, Westphal et al. 2003).

There are some weaknesses in these studies of biological activity. It is known that for efficient mediator release, there are two requirements. The affinity of the interaction between IgE and the allergen should be above a certain (so far unknown) threshold, and the interaction should be multivalent (van Ree 1999). One epitope does not work, two work, but 4-6 epitopes is most likely “ideal”. A weakness of some of these studies is that very high concentrations are needed to achieve biological activity. This points towards low affinity, and it is doubtful whether this has any clinical relevance. A weak point is also that it is difficult to exclude that other (unidentified) allergens are overlooked. The argument that the IgE response of a patient towards a food can be completely inhibited by the neoglycopeptides only stands if the food extract used to perform inhibition assays has all the relevant allergens present. There are several reasons to argue that this can not easily be guaranteed. Some allergens are labile during extraction (Bjorksten, Halmepuro et al. 1980), some get lost during defatting steps, etc. In some studies the clinical background of the patients analysed by histamine release is not mentioned at all (Batanero, Crespo et al. 1999). It is not difficult to guess why this was left out. They simply do not have food allergy. The strongest argument in favor of a lack of biological activity of anti-carbohydrate IgE is that these antibodies are commonly present in pollen allergic patients, but that most of these patients do not have food allergy, let alone for all plant foods, despite the fact that every plant food is loaded with IgE-binding glycoproteins (van der Veen, van Ree et al. 1997). To nevertheless confirm that lactoferrin substituted with plant-like N-glycans can indeed only induce mediator release at very high concentration, compared to established major inhalant and food allergens, histamine release experiments were performed.

d. Histamine Release Assays

To investigate the biological activity of recombinant human lactoferrin from rice, the so-called stripped-basophil protocol was used. In this approach human basophils of a non-allergic donor are semi-purified, stripped from their IgE antibodies by lactic acid treatment and subsequently re-sensitized with serum IgE antibodies. For re-sensitization serum samples were used with moderate (pf41 and pf192) and strong (2399 CV) IgE reactivity to plant N-glycans. In addition, three serum samples (s992, s1249 and s2316) with IgE antibodies to rice were used to evaluate whether residual rice components could possibly induce histamine release (Table 16). Two of these rice-positive serum samples (s992 and s2316) were negative for pollen-specific IgE, making it highly unlikely that they had IgE antibodies against plant N-glycans because pollen is established as the primary source for anti-CCD sensitization. For all three rice-reactive serum samples it is however unclear whether they really had rice allergy. It is more likely that they were rice-reactive on the basis of a different primary sensitizer like peanut (or other legumes) in case of s992 and s2316 or pollen in case of s1249, and that they did not have true rice allergy. It is almost impossible to find challenge-confirmed rice allergic patients, so this is the closest we could get to such controls. For each patient, we also selected an allergen (extract) that was likely to be involved in clinical allergy and therefore expected to have strong biological activity.

Table 16. RAST results with putative rice allergic sera, IU/mL

Serum	hLF	holo-rhLF	apo-rhLF	Control Rice	LF-164 Rice
s992	0.1	0.1	0.1	10.9	2.4
s1249	0.1	1.6	2.7	22.3	16.1
s2316	0.5	0.7	0.6	2.9	2.7

The two serum samples with moderate anti-CCD IgE responses (pf41 and pf192) demonstrated no histamine release with apo-lactoferrin (Figure 19). Holo-lactoferrin gave significant release in case of pf41, not with pf192. This discrepancy between holo- and apo-lactoferrin was also observed with s2316 which did not show significant IgE binding to any lactoferrin preparation. It is unlikely that differences in the presence of residual rice protein are at the basis of these observations, because holo- and apo-lactoferrin both originate from the same source of “asis” –lactoferrin. The main difference is that apo-lactoferrin was depleted of its iron by denaturation with urea. Whether the denaturation of the lactoferrin isolate or the presence or absence of iron results in conformational changes that influence the biological activity is unclear. For the sake of determining the biological activity of plant N-glycans on recombinant lactoferrin, this issue is however not really relevant. The overall conclusion is that moderate anti-CCD IgE titres in combination with recombinant lactoferrin carrying two plant

N-glycans, does not result in histamine release. Even at 1 mg/mL no release was observed.

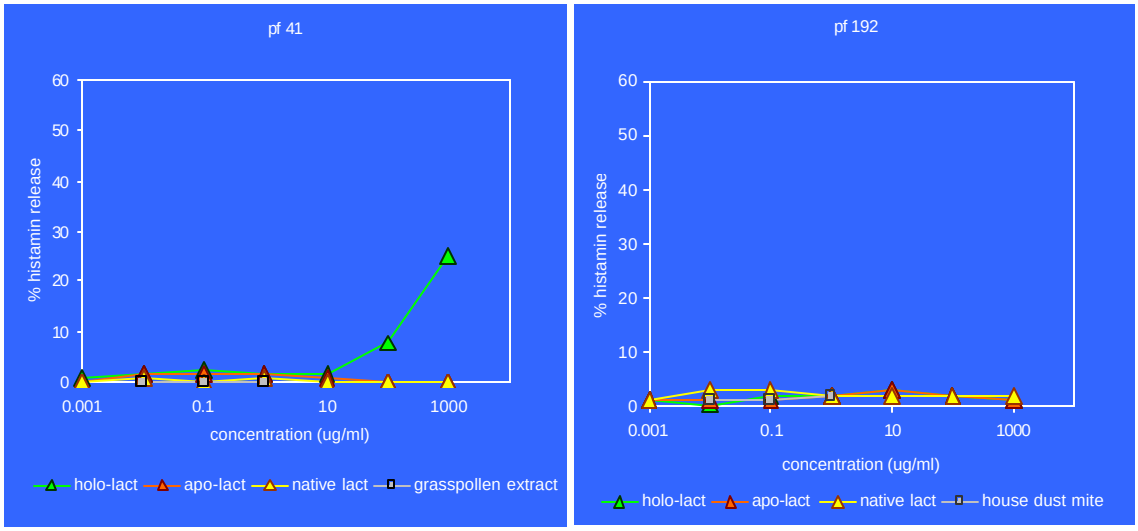


Figure 19. Histamine release by serum samples with moderate anti-CCD IgE responses

When the same experiment was performed with the serum with high anti-CCD IgE titres, significant histamine release was observed at concentrations of 1 µg/mL and higher (Figure 20). The control recombinant grass pollen allergen Phl p 5 demonstrated histamine release at concentrations down to the pg/mL range. In addition, the maximum release observed with rPhl p 5 was approximately twice as high as with lactoferrin. These experiments confirm that IgE antibodies against CCD can be biologically active only at allergen concentrations that are several (2-4) orders of magnitude higher than those needed in case of established clinically relevant allergens such as Phl p 5 (2399 CV), Bet v 1 (s1249), Fel d 1 (s2316) and Ara h 2 (s992) (Figure 21). Furthermore, IgE titers that usually are sufficient to induce histamine release (10-15 IU/mL) are insufficient in case of anti-CCD specificity. Together with the observation that the patients in this study with anti-CCD IgE do not show clinical plant food allergies, these experiments confirm earlier claims that IgE antibodies against plant N-glycans are of poor biological activity and consequently of no or limited clinical relevance. Thus the recombinant lactoferrin from rice can be regarded as safe for pollen allergic patients with anti-CCD IgE.

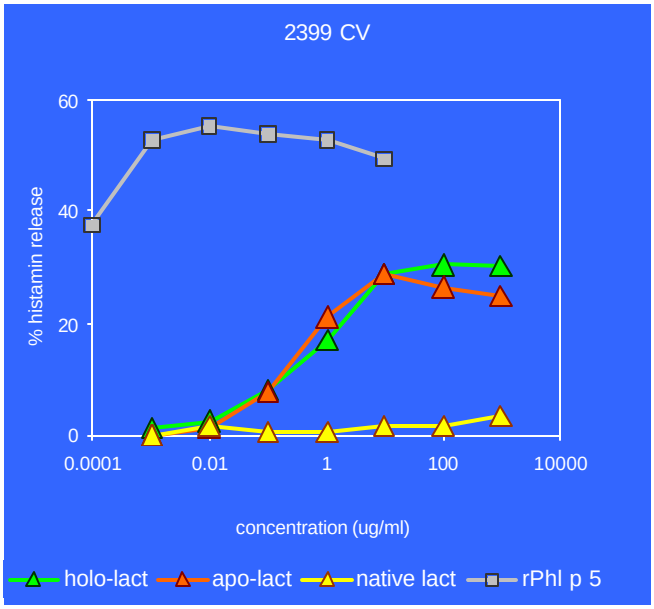


Figure 20. Histamine release by a serum sample with high anti-CCD IgE response

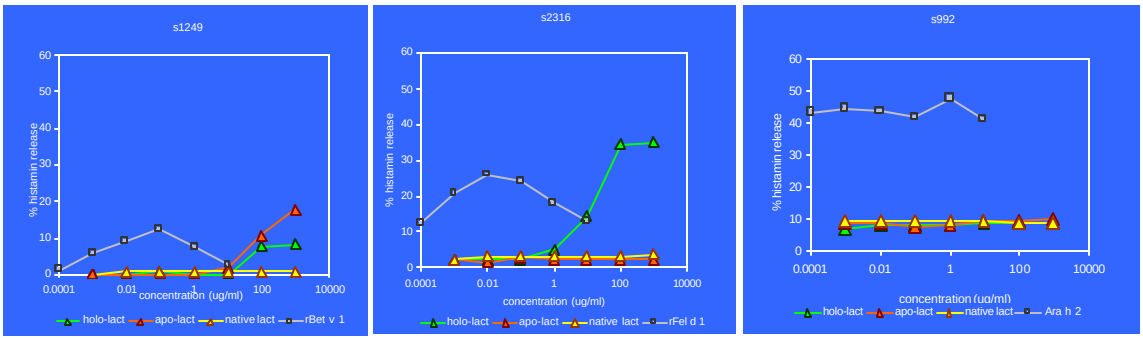


Figure 21. Histamine release by serum samples IgE binding to rice

With respect to the rice-positive controls, s1249 demonstrated low biological activity to rice extract and to both recombinant lactoferrin isolates. Since this patient was also pollen-reactive, this biological activity is probably due to anti-CCD IgE. As mentioned above, s2316 selectively reacted to holo-lactoferrin. The explanation of this is unclear. S992 did not react to rice at all. The control allergen Ara h 2 gave strong release at low allergen concentrations, making it most likely that the rice reactivity is a clinically irrelevant cross-reactivity as a result of primary sensitization to peanut. Rice extract only induced mediator release at very high concentrations. Together these data suggest that IgE reactivity to rice is most likely of limited clinical relevance. Any residual rice protein will not likely trigger an allergic response in these patients.

5. Autoimmune disease and lactoferrin

The final area of concern to be addressed is the presence of lactoferrin antibodies in association with autoimmune diseases. Antilactoferrin autoantibodies are often present in patients with autoimmune diseases, forming a component of anti-neutrophil cytoplasmic antibodies (ANCA; for a review see (Schulz and Tozman 1995)). They have been detected in a variety of diseases, including autoimmune liver disease (Ohana, Okazaki et al. 1998), inflammatory bowel disease (IBD; (Roozendaal, Pogany et al. 1999)), Wegeners granulomatosis ((van der Woude, Rasmussen et al. 1985)), rheumatoid arthritis (Locht, Skogh et al. 2000)), systemic lupus erythaematosus (SLE; (Galeazzi, Morozzi et al. 1998)), and autoimmune pancreatitis ((Okazaki, Uchida et al. 2000)). Consideration therefore needs to be given to whether there is any possibility that ingestion of rhLF by persons suffering from these conditions could exacerbate their disease.

Specifically, one needs to consider

- whether ingestion of rhLF in the form and dose proposed here can stimulate production of circulating anti-hLF autoantibodes
- whether such antibodies have any pathological significance.

Healthy adults constantly ingest small amounts of human lactoferrin from saliva, and this clearly does no harm, but ingestion of a larger bolus resulting from a lactoferrin-fortified food might prove more immunostimulatory. Administration of bovine lactoferrin to mice can induce a systemic immune response, including production of specific IgG anti-lactoferrin antibodies and sensitzation of spleen cells (Debbabi, Dubarry et al. 1998; Sfeir, Dubarry et al. 2004). Likewise oral administration of human milk proteins containing 40% lactoferrin to mice resulted in production of IgG, IgM and IgA anti-hLF antibodies, and spleen cell sensitization (Yuki, Fujihashi et al. 1998). Although these studies clearly show that oral administration of lactoferrin can give rise to a systemic immune response, their relevance to autoimmune disease is questionable as lactoferrin would be acting as a foreign antigen in these circumstances (human/bovine proteins into mice). The only evidence that ingestion of human lactoferrin might induce production of anti-hLF autoantibodies comes from a report of IgG anti-hLF antibodies in African breast-fed infants (Brock, Lamont et al. 1998). However, these infants were likely to be suffering from intestinal infections and the results are probably not representative of ingestion of lactoferrin.

A recent small safety study at Ventria dosed adult women with 4.8 grams of holo-lactoferrin a day for six weeks. Sera were analyzed at time zero and at week six for the presence of IgG and IgE antibodies. Sera were tested by ELISA for IgG and IgE antibodies. There was no evidence in this limited sample of antibody to rhLF; the data is shown below, page 67.

It has also been suggested that increased levels of anti-bovine lactoferrin antibodies found in individuals with autoimmune disease or cow's milk allergy might cross-react with human lactoferrin (Brock, Lamont et al. 1998), but cross-absorption studies revealed no evidence of common epitopes (JH Brock, unpublished data). Thus there is no evidence that ingestion of human lactoferrin by healthy adults can induce an autoimmune response.

It could be hypothesized that individuals with autoimmune disease might be more predisposed to respond to oral administration of a bolus of rhLF, especially if there was concurrent bowel inflammation which could result in leakage of lactoferrin from the gut to the circulation. The question is whether such antibodies, if produced, might have any pathological effect.

The vast majority of studies reporting anti-hLF autoantibodies have either not investigated their pathological significance, or concluded that they played no role in the disease process. Nevertheless, the theoretical possibility exists that they could in some way interfere with the normal function of lactoferrin, or intervene in the disease process. Anti-LF antibodies raised in rabbits prevented binding of iron by apo-hLF but did not cause release of bound iron by holo-LF (Audrain, Gourbil et al. 1996; Brock, Lamont et al. 1998). However, in both studies autoantibodies to hLF from patients had no such effect, suggesting that they were of relatively low affinity. They were also found to have no effect on the antimicrobial activity of neutrophils (Caradonna, Amati et al. 2000). Anti-lactoferrin antibodies have nevertheless been found to induce activation of neutrophils, but again the work was carried out using hyperimmmune rabbit antisera, and although it was stated that human autoantibodies gave similar results the actual data were not presented in the paper (Peen, Sundqvist et al. 1996).

There is one report implicating an immune response to lactoferrin in autoimmune disease (Uchida, Okazaki et al. 2002). Neonatally-thymectomised mice immunized systemically with bovine lactoferrin or carbonic anhydrase developed immune-mediated pancreatitis. Although anti-lactoferrin autoantibodies are present in patients with autoimmune pancreatitis, the relevance of this model is questionable, as systemic immunization of neonatally-thymectomised mice is very different from ingestion of lactoferrin by adult humans, and indeed the authors do not claim that the model represents the autoimmune version of the disease. It was claimed that an extract of murine pancreas reacted with sera from animals immunized with bLF and bCAII, but no data were shown and it was not clear whether LF or CAII was responsible for the cross-reaction. It seems unlikely that LF cross-reactivity was responsible as antisera to bovine lactoferrin do not cross-react with lactoferrins from unrelated species (JH Brock, unpublished data).

Since exposure to lactoferrin is likely to occur as a result of ingestion of a bolus of rhLF in a supplemented food, potentially harmful effects in persons with

ongoing inflammatory bowel disease need to be especially well scrutinized. Although the presence of anti-lactoferrin autoantibodies was associated with inflammation of the colon (Roozendaal, Pogany et al. 1999), there was no evidence for any pathological involvement and the authors concluded that their presence was probably an epiphenomenon. Thus even if ingestion of rhLF were to increase the levels of anti-LF autoantibodies in individuals with IBD there is no evidence that this would exacerbate the disease.

Finally, the possibility exists that lactoferrin itself might traverse the intestine and exacerbate autoimmune disease. The only evidence for this is from a study of collagen-induced arthritis in mice, a model of rheumatoid arthritis, which was found to be exacerbated in transgenic mice expressing human lactoferrin (Guillen, McInnes et al. 2002). Again, it is questionable whether oral ingestion of a bolus of human lactoferrin would mimic the chronic hyperexposure to the protein that exists in transgenic mice and have any significant effect on a chronic disease such as rheumatoid arthritis.

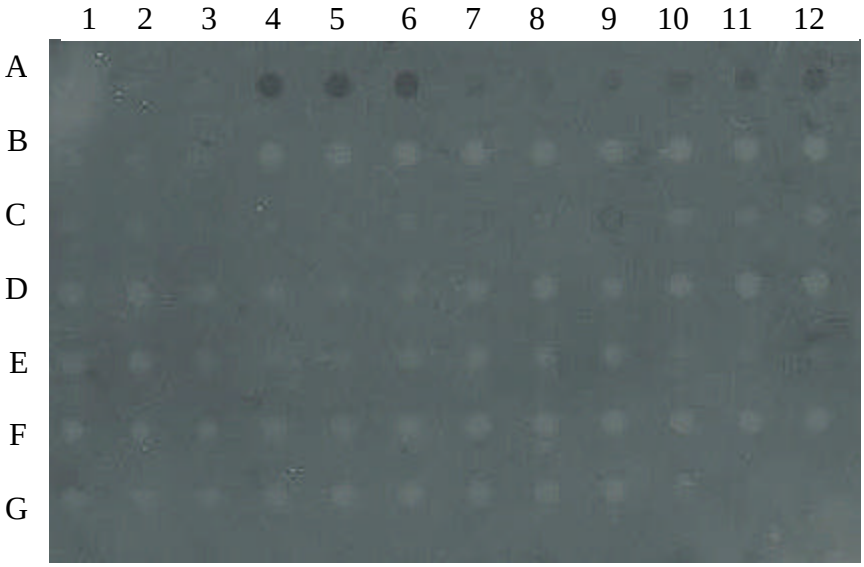
In conclusion, while there is evidence that orally-ingested lactoferrin can induce a systemic immune response, and that antibodies to lactoferrin could theoretically be involved in disease progression, there is no evidence that the former can give rise to the latter. In addition the large clinical literature on anti-lactoferrin autoantibodies as a component of ANCA provides no evidence that anti-lactoferrin autoantibodies play a pathological role, but rather that they are an epiphenomenon in autoimmune disease. It thus seems highly unlikely that ingestion of human lactoferrin as a food supplement, even by individuals with an ongoing autoimmune disease, would have any adverse effect.

6. Studies in animals and humans with oral delivery of recombinant human lactoferrin produced in rice

Ventria Bioscience's recombinant human lactoferrin has been fed to animals and humans with no reports or indication of IgE allergic reactions. The rhesus monkey animal model is often chosen to test the safety of products prior to human trials. In collaboration with a team at UC Davis, infant rhesus monkeys were fed with a modified infant formula containing 1 mg/mL of rhLF and 0.1 mg/mL rhLZ from day one after birth to four months. The ingestion of recombinant human lactoferrin by infant rhesus monkeys for 4 months did not stimulate the production of IgG antibodies to lactoferrin. Animals were fed a whey-based formula containing recombinant human lactoferrin from an extract of the LF164 rice flour. The formula also included recombinant human lysozyme. There were no infant deaths and no adverse effects were observed at a dosage ranging from 0.5 to 0.8 g/kg body weight for rhLF and 0.05 to 0.08 g/kg body weight for rhLZ. Sera from the monkeys were tested for the presence of antibodies to lactoferrin; none were detected. Serum from both control and experimental groups were collected at birth, 1 month, 2 months and 3 months of

age. The potential of immuno-reaction against rhLF was tested by assaying serum for the presence of IgG antibodies against LF using a Bio-Dot Microfiltration apparatus. Results are shown in Figure 22.

Figure 22. Testing of rhesus monkey sera for presence of anti-lactoferrin antibodies



Briefly, 50 µg serum obtained from experimental and control monkeys was added to each well on a nitrocellulose membrane. As a positive control, 200 µL of rabbit anti-hLF antibody at dilutions of 25 K, 100 K and 1 million, corresponding to 30 ng, 15 ng and 1.5 ng protein, was loaded onto the membrane. As a negative control, 50 µg bovine serum albumin was used. After the membrane was blotted with 3% gelatin and washed with Tris buffered saline, 0.1% Tween, it was incubated with 15 µg/mL hLF for 1 h at room temperature. The membrane was then washed and incubated with horseradish peroxidase-conjugated rabbit anti-hLF at 20,000 dilution. The membrane was washed again and color developed with a chemiluminescent system. Results show that no antibody was detected in the sera collected from monkeys, control or experimental groups, indicating that monkeys did not exhibit an immuno-reaction against rhLF. The only color development is seen in the positive control wells, A4 to A12.

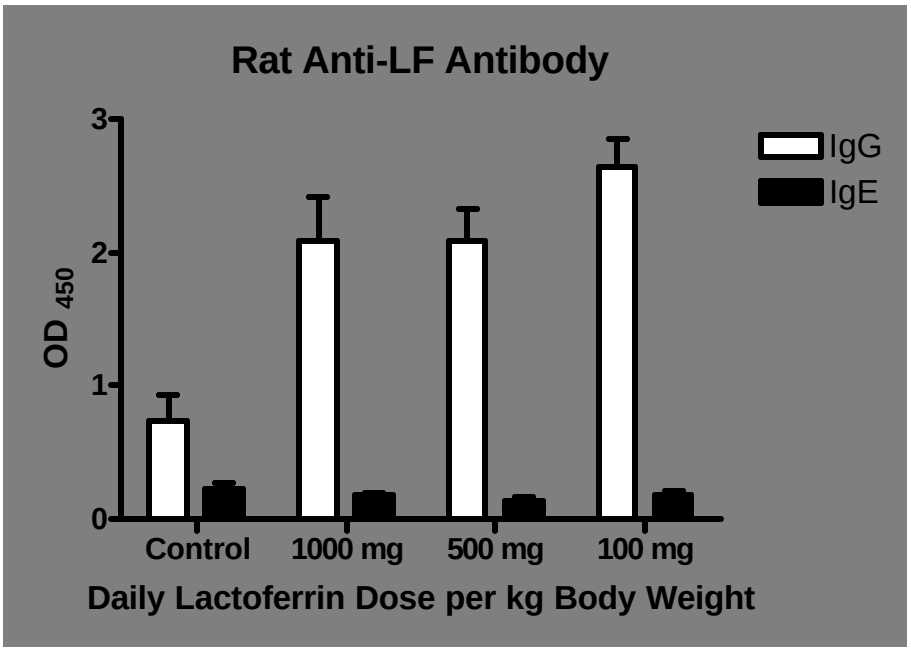
Table 17. Samples and results from dot blot testing of infant rhesus monkey sera for antibodies to lactoferrin

Wells	Sample			Result
A 1-3	Bovine serum albumin			-
A 4-6	Rabbit-anti-human lactoferrin Ab, dilution factor = 25,000			+
A 7-9	Rabbit-anti-human lactoferrin Ab, dilution factor = 100,000			+
A 10-12	Rabbit-anti-human lactoferrin Ab, dilution factor = 1,000,000			+
	MMU #	**Diet**	**Age**	-
B 1-3	33439	Control	Birth	-
B 4-6	33439	Control	1 month	-
B 7-9	33439	Control	2 months	-
B 10-12	33439	Control	3 months	-
C 1-3	33458	Test	Birth	-
C 4-6	33458	Test	1 month	-
C 7-9	33458	Test	2 months	-
C 10-12	33458	Test	3 months	-
D 1-3	33422	Control	2 months	-
D 4-6	33430	Control	2 months	-
D 7-9	33444	Test	2 months	-
D 10-12	33445	Test	2 months	-
E 1-3	33448	Test	2 months	-
E 4-6	33452	Test	2 months	-
E 7-9	33456	Test	2 months	-
E 10-12	Bovine serum albumin			-
F 1-3	33433	Control	3 months	-
F 4-6	33436	Control	3 months	-
F 7-9	33444	Test	3 months	-
F 10-12	33445	Test	3 months	-
G 1-3	33448	Test	3 months	-
G 4-6	33452	Test	3 months	-
G 7-9	33456	Test	3 months	-
G 10-12	Bovine serum albumin			-

In preparation for human studies with purified iron saturated recombinant human lactoferrin, Ventria Bioscience sponsored a 28-day repeat oral dose study in rats. Three groups of animals received recombinant human lactoferrin at dose levels of 1000, 500 and 100 mg/kg body weight and a control group received saline vehicle. At the termination of the study, serum samples from all animals were sent to Ventria Bioscience for analysis of antibody presence. ELISA plates were coated with lactoferrin, serum was added followed by anti-IgG or anti-IgE conjugate antibody. ELISA were run on all sera and low levels of IgG antibody to lactoferrin were detected in all groups, including control animal, indicating a possible matrix effect in the assay due to the undiluted serum. Selected sera from each group were titered. One animal had a titer of 1:128, however, the remainder had antibody titers in the range of 1:4 to 1:16, levels not considered significant. Sera were tested at a 1:4 dilution for the presence of IgE antibodies. No animals demonstrated the presence of IgE antibodies to lactoferrin. No animals exhibited any symptoms of allergic reaction during the feeding phase; the histopathology and serum chemistry indicated no evidence of allergic response. Rats do not have lactoferrin in their milk, the role of lactoferrin being replaced by transferrin (Masson and Heremans 1971). Based on the lack of normal exposure to lactoferrin, it is not unexpected that a low level IgG response

to human lactoferrin would develop during repeated oral dosing. The lack of IgE antibody development indicates there is no allergic response.

Figure 23. IgG and IgE antibodies to lactoferrin in rat sera



Ventria has recently completed a limited safety study in adult women. Five women took 4.8 grams of holo-lactoferrin daily for 42 days. Subjects were asked about known allergies or reactions to rice or rice products during the enrollment screening, but no testing for allergies was performed. Serum was collected on a weekly basis from the women. At completion of the study, these sera were analyzed for the presence of IgG and IgE antibodies to lactoferrin. Sera were screened by ELISA for IgG and IgE antibodies to lactoferrin. No signal difference was seen between day 0 and day 42 samples by ELISA. Data are summarized in Table 18. ELISA values are absorbance at OD_{450}.

Table 18. IgG and IgE in sera from human adults taking rhLF.

Subject	IgG - ELISA		IgE - ELISA	
	Day 0	Day 42	Day 0	Day 42
004	0.242	0.284	0.024	0.020
005	0.085	0.097	0.023	0.017
007	0.082	0.083	0.017	0.016
008	nd	nd	0.016	0.021

Conclusion

The literature and the experimental data provided by Ventria Bioscience support the conclusion that there is minimal risk of allergenic or immunogenic responses in individuals consuming recombinant human lactoferrin produced in rice.

Recombinant human lactoferrin is substantially equivalent to and therefore as safe as human lactoferrin.

- The proteins are biochemically equivalent
- The gene source in non-allergenic
- The proteins are not allergens
- Risk is remote of allergic sensitization to lactoferrin
- Recombinant lactoferrin from rice can be regarded as safe for pollen allergic patients with anti-CCD IgE.
- Residual rice protein will not likely trigger an allergic response
- It is highly unlikely that ingestion of human lactoferrin as a food supplement, even by individuals with an ongoing autoimmune disease, would have any adverse effect.

7. Expert Panel Consensus Statement

In conclusion, there is no indication that adverse effects would result from consumption of foods containing recombinant human lactoferrin from rice.

- The amino acid sequence and structure of the protein are identical to human lactoferrin, which has been studied extensively.
- There is no evidence of immunologic cross reactivity with other iron binding, transferrin-like proteins from humans or other species, some of which may be related to allergic sensitization.
- There is no difference in the resistance to pepsin digestion of recombinant human lactoferrin and human lactoferrin
- Based on histamine release, there is no evidence that the biological IgE binding by sera with pre-existing IgE antibodies to plant N-glycan CCD structures, would result in a clinically relevant reaction in pollen allergic individuals.
- There is no evidence that residual rice proteins would trigger an allergic response in pollen allergic individuals.
- For the rare individual with true rice allergy, the products will be labeled as recombinant human lactoferrin from rice
- The clinical literature on autoantibodies to lactoferrin provides no evidence that these antibodies play a pathological role and it is highly unlikely that ingestion of recombinant human lactoferrin, even by individuals with autoimmune disease, would have any adverse effect.
- Using the data generated for this application and other generally available and accepted scientific data, information, methods, and principles, there is reasonable certainty that recombinant human lactoferrin from rice will be safe under the intended conditions of use in functional and medical foods.

Based on a critical evaluation of the currently available pertinent data and information summarized in this report, the Expert Panel concludes that the use of recombinant human lactoferrin from rice, meeting the proposed specifications and produced by current good manufacturing practice is generally recognized as safe, based on scientific procedures, for use as an ingredient in functional foods in amounts not to exceed 1000 mg/serving and in medical foods in amounts not to exceed 1 mg/mL as specified by Ventria Bioscience.

Jeremy Brock, Ph.D.
Robert Bush, M.D.
Bo Lonnerdal, Ph.D.
Lloyd Mayer, M.D.
Stephen L. Taylor, Ph.D.
Ronald van Ree, Ph.D.

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Jeremy H. Brock, Ph.D.

Signature: _____

Date: 14/12/04

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Robert K. Bush, MD
Department of Medicine, Allergy & Immunology
University of Wisconsin – Madison

Signature: _____

Date: 12/13/04

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Bo Lonnerdal, Ph.D.

Signature: _____

Date: 12/15/04

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Lloyd F. Mayer M.D.
Professor & Director, Center for Immunology

Signature: _____

Date: 12/16/04

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Steve Taylor, PhD
Professor and Head

Signature: _____

Date: 16 Dec 2004

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Ronald van Ree, Ph.D.

Signature: _____

Date: 12-16-2004

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APPENDIX A

CSFII Food Codes Included in EDI Analysis for Ventria Proposed Uses

LIST OF FOODS INCLUDED IN ANALYSES

POPSICLES (DAIRY CONTAINING ONLY)

13120050	ICE CREAM BAR OR STICK, NOT CHOC- OR CAKE-COVERED
13120100	ICE CREAM BAR/STICK, CHOCOLATE COVERED
13120130	ICE CREAM BAR/STICK,RICH ICE CREAM,CHOC COVER,W/NUT
13120140	ICE CREAM BAR/STICK, CHOC ICE CREAM, CHOC COVER
13120400	ICE CREAM BAR/STICK W/ FRUIT
13161630	LIGHT ICE CREAM,BAR/STICK, W/ LOW-CAL SWEETENER, CHOC COAT
13235000	PUDDING, POPS, CHOCOLATE
13235100	PUDDING, POPS, NOT CHOCOLATE
91611050	ICE POP FILLED W/ ICE CREAM, ALL FLAVOR VARIETIES

MEAL REPLACEMENTS (DRY, FLUID AND SOLID)

11830800	INSTANT BREAKFAST POWDER, NOT RECONSTITUTED
11830810	INSTANT BFAST,PWDR,SWT W/ LO CAL SWT,NOT RECONSTUT
11830850	HIGH CALORIE MILK BEVERAGE, POWDER, NOT RECONST
11830900	PROTEIN SUPPLEMENT, MILK BASED, DRY POWDER
11830940	MEAL REPLACEMENT,PROTEIN,MILK BASED,FRUIT JUICE MIX
11830950	NUTRIENT SUPP,MILK-BASED,POWDERED,NOT RECONSTITUTED
11830960	PROTEIN SUPP, MILK BASE, SODIUM CONTROLLED, POWDER
11830970	MEAL REPLACEMENT, PROTEIN TYPE, MILK-BASE, POWDER
11830980	PROTEIN SUPP, MILK-BASE, POWDER (INCL SUSTACAL)
11830990	NUTRIENT SUPP, MILK-BASE, POWDER (INCL SUSTAGEN)
11831500	NUTRIENT SUPPLEMENT,MILK-BASE,HIGH PROT,NOT RECONST
11832000	MEAL REPLACEMENT,MILK-&SOY-BASE,POWDER,NOT RECONST
11832500	MEAL REPLACEMENT,PROTEIN TYPE,MILK-BASE,W/SUGAR&ART
11835000	MEAL REPLACEMENT, CAMBRIDGE, POWDER, NOT RECONST
11835100	MEAL REPLACEMENT, POSITRIM DRINK MIX, DRY POWDER
11835150	DYNATRIM, MEAL REPLACEMENT, POWDER
11835200	LOSE-IT (NANCI), MEAL REPLACEMENT, POWDER
41430000	PROTEIN POWDER, NFS
41430010	PROTEIN SUPPLEMENT, POWDERED
41430310	PROTEIN DIET POWDER W/ SOY & CASEIN
41440000	TEXTURED VEGETABLE PROTEIN, DRY
11611000	INSTANT BREAKFAST, FLUID, CANNED
11612000	INSTANT BREAKFAST, POWDER, MILK ADDED
11613000	INSTANT BFAST,PWDR,SWT W/ LO CAL SWT, MILK ADDED
11621000	DIET BEVERAGE, LIQUID, CANNED
11623000	MEAL SUPPLEMENT / REPLACEMENT,PREPARED,RTD
11631000	HIGH CALORIE BEV, CANNED OR POWDERED, RECONSTITUTED
11641000	MEAL REPLACEMENT, MILK BASED, HIGH PROTEIN, LIQUID

MEAL REPLACEMENTS (DRY, FLUID AND SOLID) CONT'D	
11651010	MEAL REPLACEMENT, CAMBRIDGE, RECONST, ALL FLAVORS
41430200	MEAL REPLACE / SUPP, SOY-MILK-BASE,POWD,WATER ADDED
41440010	MEAL REPLACEMENT/SUPPLEMENT, LIQUID, HI PROTEIN
41440020	ENSURE W/ FIBER, LIQUID
41440050	ENSURE PLUS LIQUID NUTRITION
41440100	MEAL REPLACEMENT,LIQUID,SOY-BASE (ISOCAL, OSMOLITE)
92553000	FRUIT-FLAVORED THIRST QUENCHER BEVERAGE, LOW CAL
92560000	FRUIT-FLAVORED THIRST QUENCHER BEVERAGE
41435110	HIGH PROTEIN BAR, CANDY-LIKE, SOY & MILK BASE
53540000	BREAKFAST BAR, NFS
53540100	BREAKFAST BAR, CAKE-LIKE
53540200	BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, LOWFAT
53540250	BREAKFAST BAR, CEREAL CRUST W/ FRUIT FILLING, FAT FREE
53540500	BREAKFAST BAR, DATE, W/ YOGURT COATING
53541100	BREAKFAST BAR, DIET MEAL TYPE
53541200	MEAL REPLACEMENT BAR (INCL SLIM FAST BAR)
53542100	GRANOLA BAR W/ OATS, SUGAR, RAISINS, COCONUT
53542200	GRANOLA BAR, OATS, FRUIT, NUTS, LOWFAT
53542210	GRANOLA BAR, NONFAT
53543100	GRANOLA BAR W/ PEANUTS, OATS, SUGAR, WHEAT GERM
53544100	GRANOLA BAR, W/ NOUGAT
53544200	GRANOLA BAR, CHOCOLATE-COATED
53544210	GRANOLA BAR, W/ COCONUT, CHOCOLATE-COATED
53544220	GRANOLA BAR W/ NUTS, CHOCOLATE-COATED
53544250	GRANOLA BAR, COATED W/ NONCHOCOLATE COATING
53544300	GRANOLA BAR, HIGH FIBER, YOGURT COATING, NOT CHOC
53544400	GRANOLA BARS, W/ RICE CEREAL
53544450	POWERBAR (FORTIFIED HIGH ENERGY BAR)

YOGURTS, INCLUDING FROZEN	
11410000	YOGURT, NS AS TO TYPE OF MILK/FLAVOR
11411010	YOGURT, PLAIN, NS AS TO TYPE OF MILK
11411100	YOGURT, PLAIN, WHOLE MILK
11411200	YOGURT, PLAIN, LOWFAT MILK
11411300	YOGURT, PLAIN, NONFAT MILK
11420000	YOGURT, VANILLA, LEMON, COFFEE, NS AS TO MILK TYPE
11421000	YOGURT, VANILLA, LEMON, COFFEE, WHOLE MILK
11422000	YOGURT, VANILLA, LEMON, COFFEE, LOWFAT MILK
11423000	YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK
11424000	YOGURT, VANILLA, LEMON, COFFEE, NONFAT MILK, LOW CAL SWEET
11425000	YOGURT, CHOCOLATE, NS AS TO TYPE OF MILK
11426000	YOGURT, CHOCOLATE, WHOLE MILK
11427000	YOGURT, CHOCOLATE, NONFAT MILK
11430000	YOGURT, FRUIT VARIETY, NS AS TO MILK TYPE
11431000	YOGURT, FRUIT VARIETY, WHOLE MILK
11432000	YOGURT, FRUIT VARIETY, LOWFAT MILK
11433000	YOGURT, FRUIT VARIETY, NONFAT MILK
11433500	YOGURT, FRUITED, NONFAT MILK, LOW CAL SWEETENER

YOGURTS, INCLUDING FROZEN-CONT'D

11444000	YOGURT, FRUIT & NUTS, NS AS TO TYPE OF MILK
11445000	YOGURT, FRUIT & NUTS, LOWFAT MILK
11459990	YOGURT, FROZEN, NS AS TO FLAVOR, NS TO TYPE OF MILK
11460000	YOGURT, FROZEN, NOT CHOCOLATE, TYPE OF MILK NS
11460100	YOGURT, FROZEN, CHOCOLATE, TYPE OF MILK NS
11460150	YOGURT, FROZEN, NS AS TO FLAVOR, LOWFAT MILK
11460160	YOGURT, FROZEN, CHOCOLATE, LOWFAT MILK
11460170	YOGURT, FROZEN, NOT CHOCOLATE, LOWFAT MILK
11460190	YOGURT, FROZEN, NS AS TO FLAVOR, NONFAT MILK
11460200	YOGURT, FROZEN, CHOCOLATE, NONFAT MILK
11460250	YOGURT,FROZEN,NOT CHOCOLATE,W/ SORBET/SORBET-COATED
11460300	YOGURT, FROZEN, NOT CHOCOLATE, NONFAT MILK
11460400	YOGURT,FRZ,CHOCOLATE,NONFAT MILK,W/ LOW-CAL SWEET
11460410	YOGURT,FRZ,NOT CHOC,NONFAT MILK,W/ LOW-CAL SWEET
11460420	YOGURT, FROZEN, NS AS TO FLAVOR, WHOLE MILK
11460430	YOGURT, FROZEN, CHOCOLATE, WHOLE MILK
11460440	YOGURT, FROZEN, NOT CHOCOLATE, WHOLE MILK
11461000	YOGURT, FROZEN, CHOCOLATE-COATED
11461100	YOGURT, FROZEN, CAROB-COATED
11461200	YOGURT, FROZEN, SANDWICH
11461250	YOGURT, FROZEN, CONE, CHOCOLATE
11461260	YOGURT, FROZEN, CONE, NOT CHOCOLATE
11461270	YOGURT, FROZEN, CONE, NOT CHOCOLATE, LOWFAT MILK
11461280	YOGURT, FROZ, CONE, CHOCOLATE, LOWFAT MILK

SPORTS BEVERAGES

92900300	FRUIT-FLAV THIRST QUENCH BEV, DRY CONC (GATORADE)

APPENDIX B

Processing Chemicals and Materials

Q & SP Sepharose® Big Beads

BioProcess™ Media

Data File

Ion Exchange Media

Q and SP Sepharose Big Beads are strong ion exchangers designed for industrial applications. The large particle size (100-300 µm) and excellent physical stability of the base matrix ensure maintained speed even with viscous samples. Q and SP Sepharose Big Beads are therefore the ultimate ion exchange media for initial purifications when high viscosity precludes the use of ion exchangers with smaller bead size, such as Sepharose Fast Flow ion exchangers. The unique flow characteristics are also invaluable when adsorption needs to be done quickly, e.g. in order to minimize proteolytic breakdown.

- Easy to scale-up
- High flow rates
- High chemical resistance for effective cleaning-in-place (CIP)
- Easy maintenance

Media characteristics

The ion exchange groups are coupled to the highly cross-linked agarose matrix through chemically stable ether bonds. The strong ion exchange groups maintain full protein binding capacity over the whole operating pH range. Q Sepharose Big Beads and SP Sepharose Big Beads have the same selectivities as the corresponding Sepharose Fast Flow and Sepharose High Performance ion exchangers.

General maintenance

Sepharose Big Beads ion exchange media are easy to pack and handle. The very high flow rates that can be used save valuable time in equilibration and during regeneration. Even with viscosities as high as 2.5 times water a high flow rate (500 cm/h) is maintained in industrial column operation. Packed columns can be cleaned- and sanitized-in-place to minimize production losses. The media can also be autoclaved.

Column packing

Q and SP Sepharose Big Beads are easy to pack in small and large scale columns. Narrow peaks with high symmetry are reproducible whether you pack the ion exchanger bed with a constant pressure of between 1 to 3 bar, or let the slurry sediment and then compress it with the adaptor. Suction packing can easily be performed as well.

Fig. 1. Q and SP Sepharose Big Beads ion exchange media.

Table 2. Characteristics of Q and SP Sepharose Big Beads.

Property	Q	SP
Ion exchange type	Strong anion	Strong cation
Ionic capacity	180–250 pmole/ml gel	
Exclusion limit	4×10^6 daltons (globular proteins)	
Matrix	Macroporous, cross-linked agarose, 6%	
Bead form	Spherical, 100–300 µm	
Flow rate	1200–1800 cm/h*	
Working temperature	4–40°C	
pH stability	2–l 2 (working range) 2–14 (cleaning-in-place)	4–12 (working range) 3–14 (cleaning-in-place)
Chemical stability	All commonly used aqueous buffers – 1 M NaOH – 70% ethanol – Organic solvents	
The following should be avoided	– Oxidizing agents – Long exposure (1 week, 20°C) to pH <4	

* 2.5 cm bed height, 1 bar, 20°C distilled water in Amersham Biosciences XK 50 column.

18-1104-91

Amersham Biosciences

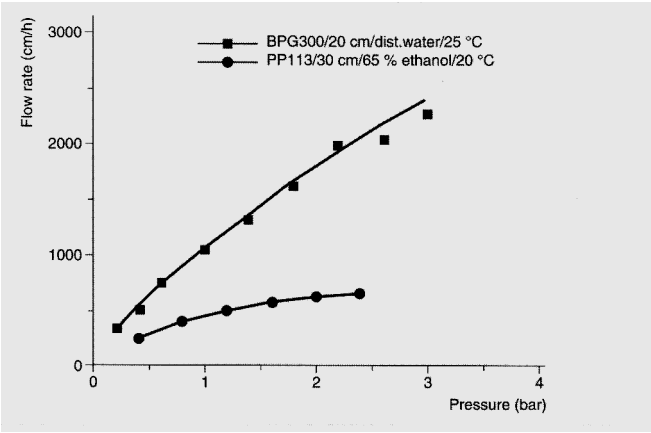

Fig. 2. The excellent flow characteristics of Sepharose Big Beads allow high flow rates even with high viscosity feed.
- Bed height 20 cm, diameter 30 cm in distilled water at 25°C.
- Bed height 30 cm, diameter 11 cm in 65% ethanol at 20°C. i.e. viscosity 2.5 times water.

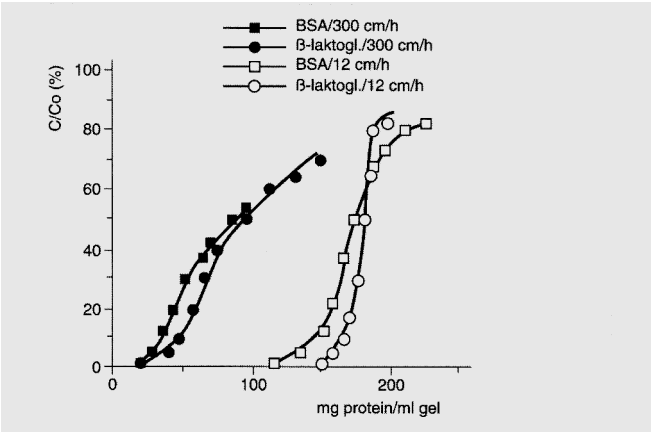

Fig. 3. Typical binding capacities of Sepharose Big Beads media. The above shows the binding capacity of SP Sepharose Big Beads measured with frontal analysis in acetate pH 5 Bovine serum albumin (BSA) and formate pH 4.1 (β-lacto-globulin) at linear flow rates of 12 (correspondmg to total available capacity) and 300 cm/h.

Cleaning, sanitization, regeneration and storage

Due to the high chemical resistance of these media, severe conditions can be used to clean and sanitize the column.

Cleaning-in-place

- **Ionically bound proteins:**
  Wash with filtered 2M NaCl at approximately 100 cm/h. Contact time: l0–15 min.
- **Hydrophobically bound proteins or lipoproteins:**
  Wash with 1 M NaOH at 40 cm/h.
  Contact time: l–2 h.
- **Lipids and very hydrophobic proteins:**
  Wash with 70% ethanol at 40 cm/h, reversed flow, or with saw-tooth gradient 0–30–0% isopropanol. Contact time: l–2 h.

Sanitization

A reduction of microbial contamination in the ion exchanger bed is obtained by washing the column with 0.5–l M NaOH, allowing a contact time of 30–60 min.

Regeneration

Regeneration is performed by passing one bed volume of 1 M NaCl through the column. After regeneration, equilibrate the column with five column volumes of buffer.

Storage

Q and SP Sepharose Big Beads can be stored either at neutral pH in buffer containing 20% ethanol or in 0.01 M NaOH.

Operation

Equipment

Any standard chromatographic system from Amersham Biosciences can be used. Make sure the capacity of the pump is high enough to handle the very high flow rates used during column packing.

Process optimixation

Normal optimization procedures for choosing buffer, ionic strength, pH, gradient shape and elution volume should be followed. The use of a higher bed height can give a better result due to the increased residence time.

Ordering information

Product	Pack size	Code No.
SP Sepharose Big Beads	1 l	17-0657-03
	10 l	17-0657-05
Q Sepharose Big Beads	1 l	17-0989-03
	10 l	17-0989-05

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Amersham Biosciences

Pest Control
Fumigation

P & K PEST & CONTROL

Dennis Bosma

Phone 712-324-4656
1134 10th Street
Sheldon, IA 51201

Pest Control
Fumigation

P & K PEST CONTROL REPORT

CUSTOMER: Sioux Biochemical

INFESTATION INVENTORY:

TYPE: Crickets

LOCATION: Front door + loading room

TREATMENT: Spot spray

	Yes	No
A. Location free of dead rodents	✓	
B. Location free of dead insects	✓	
C. Exterior of premisses inspected / serviced	✓	
D. Interior of premises inspected / serviced	✓	
E. Rodent bait stations inspected / serviced	✓	
F. Storage area clean, free of spillage	✓	
G. Storage lft. or more from walls	✓	
H. Doors rodent proof, windows screened	✓	
I. Building free of holes, harborages, etc.	✓	
J. Lot clean, free of harborages / burrows	✓	

_____ No infestation found. Preventive treatment rendered.
✓ Infestation found. Treatment rendered.

RECOMMENDATIONS:

APPROVED CHEMICALS USED:

Non-residual:

	Concentrate	Qty.	Areas Trtd.
A. **Pyrenone Food Plant** Fogging Insecticide (Fairfield Am. Corp.)			
B. **Pyrenone Industrial** Spray E.C. (Fairfield Am. Corp.)			
C. **PT 555** (Whitmiru)			
D. **BP 100** (Microgen)			
E. **OTHER:**			

Residual:

	Concentrate	Qty.	Areas Trtd.
A. **Dursban LO** (Dow Chemical Co.)			
B. **Dusban 4E Emulsifiable** Concentrate (Dow Chemical Co.)			
C. **OTHER**			

(Used only in inedible areas with no open windows or open vents) (to be used after approval by inspector)

Rodenticides:

	Qty.	Areas Treated
A. **Talon G Rodenticide** Pellets / Weatherblok bait (I.C.I. Americas Inc.)		
B. **P.C.Q Rat and Mouse Bait** (Bell Laboratories, Inc)		
C. **Final Rat and Mouse Bait** (Bell Laboratories, Inc.)		
D. **OTHER**		

Trap / Station Inventory:

No. in place	10
No. added	2
TOTAL:	12
No. replaced	1

Discussed & Accepted: _____

P & K Representative: _____

Date: 8.21.03

MANGOLD ENVIRONMENTAL TESTING, INC.
2004 EXPANSION BLVD
712-732-7786
STORM LAKE IA 50588
FAX 712-732-3925

03/12/2003

FRED CORBIN
SIOUX CENTER WATER DEPT
335 1st AVENUE NW
SIOUX CENTER, IA 51250

Account Number:	C283
Order Number:	2003030025
Sample Number:	8
Lab ID Number:	267447

Sampling Information

Date	03/10/2003 10:50:
Collector	Kruid Harlan
Matrix Type	Water Nitrate Routine
Location	921 9th St SE
RE	

Receiving Information

Date Received	03/11/2003 09:55:
Received By	SYS

Sample Notes

10.5 degrees

Analyte	Results	Test Units	Analyst	Date of Analysis	Method Code
Nitrate (as NO3)	14.6	mg/L	DLF	03/12/2003	SM 2540E
Nitrate-Nitrogen (as N)	3.30	mg/L	MG	03/11/2003	EPA 300.0

Maximum Contaminant Limit for your Nitrate-Nitrogen (as N) is 10.0 mg/L.
Any Nitrate-Nitrogen (as N) result exceeding this value is UNSATISFACTORY.

Prepared and Submitted by:
MANGOLD ENVIRONMENTAL TESTING, INC.

Brent L. Mangold, Manager

CERTIFICATE OF ANALYSIS

Date: 03/26/03

PRODUCT: SODIUM PHOSPHATE, DIBASIC, ANHYDROUS FCC

CATALOG NO: S1402 ** CUSTOMER P.O.: 3306
LOT NO: PT2441

DESCRIPTION	LIMIT MIN.	LIMIT MAX.	RESULT
ASSAY (DRIED BASIS)	98.0 %		100.33 %
LOSS ON DRYING	–	5.0 %	3.06 %
ARSENIC (As)	–	3 mg/kg	0.3 mg/kg
FLUORIDE	–	0.005 %	< 0.005 %
HEAVY METALS (as Pb)	–	10 mg/kg	< 10 mg/kg
INSOLUBLE SUBSTANCES	–	0.2 %	< 0.2 %
IDENTIFICATION	TO PASS TEST		PASSES TEST

APPROVED BY:

encl 1023752

CHRIS YERPAK
QUALITY CONTROL CHEMIST
New Brunswick, N.J. Plant

SPECTRUM®
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East Coast Plant: 755 Jersey Ave. New Brunswick, NJ 08901 • (732) 214-1300

ATTN: Randy

MALLINCKRODT

Mallinckrodt Inc.
P.O. Box 5439
St. Louis, MO 63147

Technical Services:
Telephone (314) 539-1421
Facsimile (314) 539-1128

CERTIFICATE OF ANALYSIS

CUSTOMER PRODUCT: 566570

MSCC ORDER NO.: 402453
CUSTOMER ORDER NO. 097890
SHIPPED TO: BRENNTAG GREAT LAKES L

Item: SODIUM PHOSPHATE DIBASIC USP POWDER (DRIED)
Code: 7920
Lot#: D11313

Specification 23 JUN 2000
Released 16 SEP 2002
Date of Manufacture 9 SEP 2002

COMMENTS:
Retest every 2 years

TESTS	LIMITS	RESULTS
Identification (Sodium) (USP)	dense precipitate	dense precipitate
Identification (Phosphate) (USP)	yellow precipitate	yellow precipitate
Loss on Drying (USP)	5.0% max	0.0%
Insoluble Substances (USP)	0.38% max	0.00%
Chloride (USP)	0.05% max	<0.05%
Sulfate (USP)	0.19% max	<0.19%
Arsenic (USP)	15 ppm max	<15ppm
Heavy Metals (USP)	0.0019% max	<0.0010%
Assay (USP)	98.0-100.5%	99.8%

It is certified that the above is a true copy of the analysis.

Department of Quality Assurance

CARGILL FOODS
Salt Products

Certificate of Analysis

Identification:

Product	TopFlo
Lot	H2193
Date of Manufacture	8/7/2003

Shipped to:
Customer: Johnson Feed
Order # 500153
OrderDate: 8/6/2003 ShipDate: 8/7/2003
PO # Todd e-mail
Trailer # 33 Code #

Comments:

Chemical Analysis:

NaCl %	99.92
Ca & Mg as Ca ppm	254

Additives:

YPS ppm	10.0
TCP %	.00
KI %	.000
Zeolex %	.000
PG %	.000

Physical:

surface moisture (%)	.01
bulk density (#/cu ft)	79
pH (20% solution)	8.50
water insolubles (ppm)	12

Retained on USS Mesh

USS 8	
USS 10	
USS 12	
USS 14	
USS 16	
USS 20	
USS 30	.6
USS 40	23.0
USS 50	61.3
USS 60	12.1
USS 70	2.7
USS 80	
USS 100	
USS 140	
USS 150	
USS 200	
USS 325	
Pan	.3

This salt product is a white, crystaline solid with a characteristic saline taste. Its low water activity and astringent nature inhibit pathogen growth. Periodic testing for microbiological activity confirms this assertion.

This ingredient is tested regularly by the manufacturer and meets all USDA and FDA requirements as specified in the Food Chemical Codex at time of shipment from the manufacturer.

All statements and data above are based on our laboratory results and we believe same to be reliable.

Manufacturer:

Hutchinson Evap. Plant
609 East Avenue G
Hutchinson KS 67501-7574

Quality Assurance (620) 669-2171
Fax (620) 663-8006

Customer Service (800) 282-6777

Submitted by:

QA Chemist

4023 ProdNo: 679375 LotNo:

JUN. 16. 2003 8:41AM ROWELL CHEMICAL NO. 776 P. 2/2

JUN 3 0 2003

REAGENT CHEMICAL

REAGENT CHEMICAL & RESEARCH, INC.
20 SOUTHRIDGE DRIVE
GLENDALE, MO

Product = 679375
Lot # = OM073821127

CERTIFICATE OF ANALYSIS
HYDROCHLORIC ACID

CUSTOMER NAME & ADDRESS	DATE:	CONTROL NUMBER:
ROWELL CHEMICAL	05-29-03	102610
SHIP TO:	LOT NUMBER:	QUANTITY
UNIVAR	RCRX 351053003	190,163 #
OMAHA, NE	RCR I/C	CUSTOMER ORDER #:
	RCRX 1351	OM46739 REL 307802
ATTN: JOHN WARD/ROWELL		PS45225
		PRIORITY: 22185/22188/45225

THIS IS TO CERTIFY THAT TYPICAL SAMPLES OF HYDROCHLORIC ACID HAVE BEEN WITHDRAWN USING STATISTICAL QUALITY CONTROL SAMPLING PROCEDURES AND UPON ANALYSIS IS FOUND TO CONTAIN THE FOLLOWING:

IDENTIFICATION	SAMPLE
STRENGTH IN BAUME'	20.73 Be'
% HCL CONTENT	32.80 %
SPECIFIC GRAVITY	1.1665 @ 60°F
COLOR (A.P.H.A)	10
EXTRACTABLE ORGANIC COMPOUNDS	0.4 PPM
IRON (Fe)	0.4 PPM

Teresa Lee
QUALITY ASSURANCE
(800) 972-3047

Quality First
To Do It Right - The First Time and Every Time

FORM #221174
RVSD 05/03

2302
TOUY

Vulcan CHEMICALS
A DIVISION OF
VULCAN MATERIALS COMPANY

P.O. Box 385015
Birmingham, Alabama 35238-5015
(205) 298-3000

CERTIFICATE OF ANALYSIS

Product = 705849
Lot # = OM0738?1266

ATTENTION : UNIVAR USA INC
KAY WILSON
TRACK 782 ZONE 02
OMAHA NE

50% CAUSTIC, DIAPHRAGM

ORDER NUMBER: 3034890
DATE SHIPPED: JUL 07 03
DESTINATION: OMAHA NE
SOURCE TANK: C17
SEAL NBRS: 133085 86 87

CUSTOMER ORDER NO.: OM468125
TANK CAR NO.: GATX 49533
SHIPPED FROM : WICHITA KS
THIRD PARTY P.O.:

COMPONENT	ANALYSIS
NaOH, Wt. %	50.12
Na2CO3, Wt. %	0.031
Total Alkalinity, as Na2O, Wt. %	26.85
NaCl, Wt. %	1.00
Iron, as Fe, ppm	2.6

Denotes Storage Tank Analysis (**)

MEETS FOOD CHEMICALS CODEX SPECIFICATION

JESSICA REOADES
SUPPLIER'S REPRESENTATIVE

DISTRIBUTION: FAX
FAX NUMBER: 14027333152 KAY WILSON

REMARKS:

FORM NUMBER: 211

PLEASE USE REVERSE SIDE FOR DETAILING DEDUCTIONS OR PAYMENT DISCREPANCIES

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {012K1221} RESULTS
Product Name	Iron(III) nitrate nonahydrate	
Product Number	F1143	
CAS Number	7782618	
Formula	$FeN_3O_9 \cdot 9H_2O$	
Formula Weight	404.0	
ACS TESTS		
ASSAY	98.0 TO 101.0%	99.9%
INSOLUBLE MATTER	NMT 0.005%	PASS
CHLORIDE (CL)	NMT 5 PPM	PASS
SULFATE (SO4)	NMT 0.01%	PASS
		ALL DATA SUPPLIER'S INFORMATION
	DOCUMENT # F1143/01/15/02/2	MEETS REQUIREMENTS OF ACS 9TH ED.
QC ACCEPTANCE DATE		JANUARY 2002

Lori Schulz, Manager
Analytical Services

SIGMA

Certificate of Analysis

TEST	LOT {101K1979} RESULTS
Product Name	Nitrilotriacetic acid disodium salt
Product Number	N0128
CAS Number	15467206
Formula	$C_6H_7NNa_2O_6$
Formula Weight	235.1
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 500 MG PLUS 5.0 ML OF WATER
ICP-ATOMIC EMISSION	19.5% SODIUM
PURITY BY TITRATION	100.3%
QC ACCEPTANCE DATE	NOVEMBER 2001

Lori Schulz, Manager
Analytical Services

SIGMA

Certificate of Analysis

TEST	SPECIFICATION	LOT {049H0407} RESULTS
Product Name	Sodium bicarbonate	
Product Number	S8875	
CAS Number	144558	
Formula	$CHNaO_3$	
Formula Weight	84.01	
APPEARANCE	WHITE POWDER	CONFORMS
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 100 MG/ML IN WATER	CONFORMS
PURITY BY HCL TITRATION	NLT 99.5%	100.2% *
		* SUPPLIER TEST RESULT
SHELF LIFE SOP QC-12-006	3 YEARS	AUGUST 2004
QC ACCEPTANCE DATE		AUGUST 2002

Lori Schulz, Manager
Analytical Services

Univar USA Inc
3002 F Street
Omaha, NE 68107
Ph: (402) 733-3266
Fax: (402) 733-3152
Website: www.univarusa.com

UNIVAR

Product = 678442
Lot # = OM073821113

Certificate of Analysis

Acetic Acid Glacial, FCC

Date 07/01/2003 Product Code # 678442

Univar Lot # OM073821113

	Results	Limits
Assay	101 %	≥99.5 %
Readily Oxidizable	Pass	Pass
Appearance	Pass	Clear liquid free of matter
Solidification Point	16.5°C	≥ 15.6°C
Specific Gravity @ 20°C	1.0455	1.030 to 1.055
Identification (IR)	Pass	Pass

Signed:
Jean Busboom-Piercy
Omaha QC

Date: July 1, 2003

ATTN. Randy

CHURCH & DWIGHT CO., INC.
OLD FORT, OHIO
CHEMICAL ANALYSIS REPORT

DATE:

AMMONIUM BICARBONATE FOOD GRADE - TREATED

BAGS, PACKED IN BAGS OF 50 POUNDS EACH

CONSIGNEE: NCI Tab Chemicals

ORDER #: 349808

SHIPPED BY: CHURCH & DWIGHT CO., INC.
2501 EAST COUNTY ROAD 34
OLD FORT, OHIO 44861

CUSTOMER SERVICE #: 1-800-631-5591

LOT NUMBER: GT 014.1

MANUFACTURE DATE: 01-14-2001

CHEMICAL ANALYSIS

PERCENT AMMONIUM BICARBONATE	99.4 %
PPM ARSENIC	LESS THAN 3 PPM
PPM CHLORIDE	LESS THAN 30 PPM
PPM HEAVY METALS - AS Pb	LESS THAN 5 PPM
PPM SULFUR COMPOUNDS	LESS THAN 70 PPM
PERCENT NONVOLATILES AS MAG CARB.	LESS THAN 0. 55%
IRON	LESS THAN 3 PPM

QUALITY CONTROL LABORATORY

FLORIST. From: TAB. Chemicals 10-02-01

6359

BRENNTAG
PC:03624
10/02/01

Niacet Corporation

TAB CHEMICAL COMPANY
4801 S AUSTIN AVE
FAX: 773-586-9882
CHICAGO, IL 60638
Attn: DENISE

Purchase Order No.: A150662
Date: 01/24/01

Certificate of Analysis for SODIUM ACETATE,ANHYDROUS, FCC

Lot Number: 03624
Date of Analysis: 12/28/00

PURITY (DRY BASIS)	- 99.95 %
pH OF 10% SOLUTION	- 8.41
POTASSIUM COMPOUNDS	- PASS
WATER	- NIL %
HALIDES (AS NaCl)	- <0.05 %
HEAVY METALS (AS Pb)	- < 5 PPM
IRON	- 3.0 PPM
ARSENIC (AS As)	- < 1 PPM
ALKALINITY (AS NaOH)	- 0.0 %
LOSS ON DRYING	- NIL %
APPEARANCE	- FINE WHITE CRYSTAL
SULFATE	- < 25 PPM

S.J. D'Angelo
Manager Quality Assurance

400 47TH STREET, NIAGARA FALLS, NY 14304 • (716) 285-1474 • (800) 828-1207 • FAX (716) 285-1497

TECHNICAL INFORMATION

Oakite Sanitizer 1

Quaternary ammonium germicide disinfectant for all purpose sanitizing, control of mold and algae.

EPA Reg. No. 1020-1

PRIMARY APPLICATION

Oakite Sanitizer 1 is a liquid germicide disinfectant for sanitizing and deodorizing food processing and pharmaceutical plants. A quaternary ammonium compound, it combines fast killing action with excellent residual bacteriostatic protection. Oakite Sanitizer 1 is effective on a wide range of bacteria, fungi, mold, and other disease causing pathogens such as staphylococcus, Streptococcus and escherichiacoli. Sanitizer 1 does not require a potable water rinse when used at concentrations up to 200 ppm.

Oakite Sanitizer 1 contains ingredients acceptable to the FDA and is authorized by the USDA for use in federally inspected meat and poultry plants.

CHEMICAL CHARACTERISTICS

chemical composition	alkyl dimethyl benzyl ammonium chloride
physical form	concentrate: light yellow as used: clear solution
specific gravity (approx.)	0.987 at 20°C (68°F)
bulk density	987 g/ℓ (8.2 lb/gal)
viscosity	4 cps, Brookfield Spindle 1, 60 rpm at 27°C (80°F)
flash point	none
foaming tendency	moderate when circulated; high when sprayed
recommended diluent	water
maximum solubility	completely miscible in cold or hot water
behavior in hard water	unaffected
rinsability	good
biodegradable surfactants	yes
phosphate-free	yes
normal working concentrations	1 fl. oz/8 gals of water
normal working temperatures	21° to 71° (70° to 160°F)
pH at working concentrations	7-9 depending on alkalinity of water
effect of prolonged boiling	some loss of sanitizing properties
effect of working solutions on metals	rate of metal loss from 24-hour immersion in a 200 ppm solution of Oakite Sanitizer 1 at 38°C (100°F), projected for one year, is as follows:

metal (alloy)	mm/yr	in/yr
stainless steel (316)	0.00	0.000
steel (1010)	0.07	0.003
aluminum (2024)	0.06	0.002
aluminum (3003)	0.07	0.003
brass	0.01	0.000
copper	0.00	0.000

Oakite.

APPLICATION PROCEDURE

Directions: Thoroughly clean and rinse all surfaces before applying this solution for sanitizing or germicidal treatment. Prepare a fresh solution daily or when use solution becomes visibly dirty.

General Disinfection: For walls, floors, equipment, other hard nonporous inanimate surfaces. After proper cleaning, apply Oakite Sanitizer 1 by mop, brush or by flooding areas to be treated. Treated surfaces must remain wet for 10 minutes. Prepare solution by adding 1 fluid ounce (about 2 tablespoons) of Oakite Sanitizer 1 to 4 gallons of water. May be used in cold, warm and hot water. Disinfection more rapid with warm or hot water.

Dairy Farms, Milk Plants: For sanitizing milking machines and rubber parts, pails, strainers, separators, sanitary piping, coolers, pasteurizing equipment, etc. After proper cleaning of equipment, sanitize with solution of Oakite Sanitizer 1. Apply by circulating, flooding or spraying 1/4 fluid oz. to 2 gallons of water which approximates 200 ppm (1 to 5000).

Note: Do not use with soap or anionic detergents.

Sanitizing, Mold Control: For general sanitizing in bakeries, food and bottling plants a solution containing 1/2 fluid oz. to 4 gallons of water is applied by spraying or wiping previously cleaned surfaces until they are moist. Solution must have at least one minute contact time. Residual solution will control mold and yeast growth on hard, smooth surfaces such as metal or tile, as well as on painted walls and ceilings. Application should be repeated every 7 days or as necessary depending on the severity of the mold problem.

Algae: To control algae in previously cleaned circulating water systems, apply an initial slug addition of Oakite Sanitizer 1 at the rate of 13-26 fluid oz/per 1000 gal of water in the system (20-40 ppm active) ingredients. Subsequent slug additions of 4-8 oz of Oakite Sanitizer 1 per 1000 gal of water (6-12 ppm) should be applied every 2 to 5 days or as needed depending on the volume of bleed-off and the severity of the algae problem. Additions should be introduced gradually into the sump of the water cooling tower.

Sanitizing food eggs: To sanitize previously cleaned food grade eggs in shell egg and egg product processing plants, spray with 1-1/4 oz Oakite Sanitizer 1 in 10 gallons of water (200 ppm active). The solution should be warmer than the eggs, but not to exceed 130°F. Wet eggs thoroughly and allow to drain. Eggs sanitized with this product shall be subjected to a potable water rinse only if they are to be immediately broken for use in the manufacture of egg products. Eggs should be reasonably dry before casing or breaking. The solution should not be reused for sanitizing eggs.

Restaurants, Luncheon Counters and Taverns: For sanitizing glasses, dishes, utensils. Destroys many disease-producing bacteria. After proper cleaning, apply solution of Oakite Sanitizer 1 (1/2-fluid ounce per 4 gallons of water). Allow to drain thoroughly before reuse.

This product fulfills the criteria of Appendix F of the Grade "A" Pasteurized Milk Ordinance 1965 Recommendations of the U.S. Public Health Service in waters up to 550 ppm of hardness calculated as $CaCO_3$ when tested by the A.O.A.C. Germicidal and Detergent Sanitizers-Official Method.

SOLUTION CONTROL: To determine concentration of active ingredient (quat.), use Procedure 93.

NOTES ON USE

(See Material Safety Data Sheet)

Safety and Handling Precautions: Direct contact causes eye damage and skin irritation. Avoid contact with skin, eyes and clothing. When handling, wear safety goggles or face shield and rubber gloves. Harmful or fatal if swallowed. Avoid contamination of food.

First Aid In Case of Contact: Immediately flush eyes or skin with plenty of water for at least 15 minutes. For **eyes**, get medical attention; for **skin**, remove and wash clothing before reuse. **If swallowed**, promptly drink a large quantity of water. Avoid alcohol. Call a physician immediately.

Note to Physician: Probable mucosal damage may contraindicate the use of gastric lavage. Measures against circulatory shock, respiratory depression and convulsion may be needed.

KEEP OUT OF REACT OF CHILDREN

DISPOSAL

Dispose of according to all federal, state and local regulations.

SHIPMENT

May be shipped by any common carrier. Freight classification is "Cleaning Compound, NOI, Liquid". Product Code No. 5080.

STORAGE

Suitable for general indoor storage. Keep container closed when not in use. Keep from freezing.

effect of high temperature storage ..... slight pressure may build up
effect of low temperature storage ..... will freeze at 0°C (32°F); contents may be restored by thawing
effect of aging ..... none

Oakite Products, Inc. warrants that the product or products described herein will conform with its published specifications. The facts supplied by Oakite and information related to them are intended for use by buyers having necessary industrial skill and knowledge. Buyers should and make sufficient verification and testing to determine the suitability of the Oakite materials for their own particular purpose. Since buyer's conditions of use of products are beyond Oakite's control, Oakite does not warrant any recommendations and information for the use of such products. OAKITE DISCLAIMS ALL OTHER WARRANTIES INCLUDING THE IMPLIED WARRANTY OF MERCHANTABILITY AND FITNESS FOR ANY PARTICULAR PURPOSE IN CONNECTION WITH THE USE OF ITS PRODUCTS.

Oakite

Oakite Products, Inc. P.O. Box 602, 50 Valley Road, Berkeley Heights, NJ 07922
Oakite Canada Limited 115 East Drive, Brampton, Ontario L6T1B7
Tel. (800) 526-4173 (908) 464-6900 FAX (908) 464-4658
Distributors and Licensees Worldwide

F13300R7897
Printed in the USA

CERTIFICATE OF ANALYSIS

Date: 02/09/04

PRODUCT: UREA USP, JP

CATALOG NO: UR107 **
LOT NO: TM0016

CAS NUMBER: 57-13-6
FORMULA WEIGHT: 60.06
CHEMICAL FORMULA: CH_4N_2O

CUSTOMER P.O.: 3017

DESCRIPTION	LIMIT MIN.	LIMIT MAX.	RESULT
ASSAY [$(NH_2)_2CO$]	99.0	100.5 %	99.9 %
MELTING RANGE	132	135 C	132.0 - 134.0 C
RESIDUE ON IGNITION	–	0.1 %	0.09 %
ALCOHOL-INSOLUBLE MATTER	–	0.04 %	0.09 %
CHLORIDE (Cl)	–	0.007 %	< 0.007 %
SULFATE	–	0.010 %	< 0.010 %
HEAVY METALS	–	20 ppm	< 20 ppm
IDENTIFICATION	– TO PASS TEST		PASSES TEST
EXPIRATION DATE			06/30/2006

APPROVED BY:

CHRIS TERPAK
QUALITY CONTROL CHEMIST
New Brunswick, N.J. Plant

encl 1135308

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New Brunswick, NJ 08901
• (732) 214-1300

Submission End

VENTRIA BIOSCIENCE

April 19, 2005

RECEIVED
APR 20 2005
OFFICE OF
FOOD ADDITIVE SAFETY

Robert I. Merker, Ph.D.
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
HFS-255
Harvey W. Wiley Federal Building
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: GRN 000162

Dear Dr. Merker:

In response to our discussions April 19, 2005, regarding GRN 000162, Ventria Bioscience is providing the following additional and correction information. Enclosed are an original, two copies and one electronic copy of the information.

1. A replacement has been provided for page 22 of 90. In paragraph three, four reference citations have been added. All the citations are already included in the Reference section of GRN 162. The details of the studies described in the references are found in the notice as indicated below:

Bennett:	Page 23 of 90, Item 5.
Opekum:	Page 24 of 90, last paragraph
Troost, 2002:	Page 25 of 90, 1st full paragraph
Troost, 2003:	Page 25 of 90, 1st full paragraph

In compiling the reference locations, it was noted that the Troost, 2002, reference was inadvertently omitted on page 25. The reference is cited in the Reference section. A replacement for page 25 of 90 is included with the reference added.

2. A replacement for page 33 of 90 has been provided with the correction in the first full paragraph replacing "lactoferrin" with "bifidobacteria."

3. A replacement for page 43 of 90 with the units provided on Figure 12.

4. A supplement to GRN 162 discussing the interaction of lactoferrin with *Helicobacter pylori*.

If you have any additional questions, please do not hesitate to contact me.

Sincerely,

Delia R. Bethell, Ph.D.
Vice President for Clinical Development

BEST ORIGINAL COPY

cc: Scott Deeter
President & CEO

4110 N. Freeway Blvd. Sacramento, CA 95834 Tel: (916) 921-6148 Fax: (916) 921-5611 http://www.ventriabio.com

April 19, 2005

Robert I. Merker, Ph.D.
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
HFS-255
Harvey W. Wiley Federal Building
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Delia R. Bethell, Ph.D.
Vice President for Clinical Development

cc: Scott Deeter
President & CEO

III. USE AND CONSUMER EXPOSURE

A. Consumer Safety

1. Use of lactoferrin in animals and humans

An extensive library of literature is available to support the safety of lactoferrin from human, bovine and recombinant human origin. Human and bovine lactoferrin have been consumed by humans through consumption of breast milk in infancy and cow's milk throughout life. Infants who are breast-fed consume human lactoferrin, a natural constituent of human milk. Human milk contains from 5.8 ± 4.3 g/L in colostrums to 3.3 ± 0.8 g/L in mature milk (Montagne, Cuilliere et al. 2001).

Bovine lactoferrin has been consumed by humans for thousands of years as a naturally occurring protein found in milk. DMV International, a major manufacturer of bLF has determined that bLF is "Generally Recognized As Safe" (GRAS, GRN 00077) for use as an ingredient in sports drinks and functional foods. bLF is currently marketed in dietary supplement form with dose recommendations ranging from 10-1200 mg/day.

Human lactoferrin has not been able to compete with bLF in the market due to the difficulty and expense of producing large quantities. However, several clinical experiments had been conducted to evaluate the turnover rate in man as well as its use some disease states (Bennett and Kokocinski 1979; Opekun, El-Zaimaity et al. 1999; Troost, Saris et al. 2002; Troost, Saris et al. 2003). The result showed that up to 15 g of rhLF could be safely administrated within a 24-h period. In reviewing the clinical studies, there are no comments on the efficacy of the lactoferrin in the studies as the focus of this notice is on exposure level and the safety of lactoferrin.

2. Animal Studies

Bovine lactoferrin (bLF) was administered orally to groups of 12 male and 12 female rats at dose levels of 200, 600 and 2000 mg/kg/day once daily for 13 weeks and toxicity on repeated administration was examined. Throughout the administration period, there were no deaths caused by administration of the test compound, nor were there any adverse effects noted in the general condition of the animals. The study findings concerning body weight and food consumption, ophthalmology, urinalysis including water consumption, haematology, blood chemistry, necropsy, organ weights and histopathology revealed that there were no apparent changes due to administration of LF. Therefore, the level of LF at which no adverse effect was observed was considered to be 2000 mg/kg/day for

or 10 days dosed with 100 mg bLF BID (200 mg/day) for seven days. The adverse events were similar across all three groups suggesting the bLF was safe and free from side-effects. (Di Mario, Aragona et al. 2003; Di Mario, Aragona et al. 2003)

Human lactoferrin was used for the prevention of enterogenic infection in patients with myelogenous leukemia undergoing therapy resulting in neutropenia. Five patients received 800 mg of human lactoferrin purified from breast milk for 10 days. The lactoferrin was not associated with any side-effects or adverse events (Trumpler, Straub et al. 1989). Human recombinant lactoferrin was used in two studies in humans, one single dose study in women with ileostomies and one multi-dose study in healthy volunteers taking indomethacin. The single dose study used a single 5 gram dose of lactoferrin in 8 subjects (Troost, Saris et al. 2002). In the multi-dose study, healthy volunteers (15) consumed three 5 gram doses of recombinant human lactoferrin in a 24 hour period. No side effects from the lactoferrin ingestion were observed (Troost, Saris et al. 2003).

rhLF has also been used in patients with failed chemotherapy for solid tumors. Thirty-three patients received 1.5, 4.5 or 9 grams/day of rhLF from Aspergillus for 14 days or 28 days with a 14 day gap in the middle (Hayes, Varadhachary et al. 2003). A total of 210 individuals have been treated with rhLF with no lactoferrin-related serious adverse events or laboratory abnormality greater than grade 2 (Anderson 2004).

Ventria has recently completed a limited safety study in adult women. Four women took 4.8 grams of holo-lactoferrin daily for 42 days. Subjects were asked about known allergies or reactions to rice or rice products during the enrollment screening, but no testing for allergies was performed. Serum was collected on a weekly basis from the women. General serology chemistries and hematological markers were measured at time zero and week 6. There were no changes noted in any markers. Serum lactoferrin levels were measured at weekly intervals and did not change over the six weeks, Table 5.

Table 5. Serum Lactoferrin Values

	Week 0 µg/mL	Week 1-6 ± SD µg/mL
P-004	0.34	0.32 ± 0.2
P-005	0.45	0.49 ± 0.2
P-007	0.99	0.96 ± 0.4
P-008	0.58	0.48 ± 0.1

Wharton 1989; Bennett, Gorbach et al. 1996). Bifidobacteria colonize the human large intestine and are one of the first bacterial groups to become established in the infant intestine (Poupard, Husain et al. 1973; Mitsuoka 1982; Mitsuoka 1984; Hoover 1993). Studies on the health of breast-fed infants have attributed the predominance of bifidobacteria with reduced morbidity and mortality (Yoshioka, Iseki et al. 1983; Ogawa, Ben et al. 1992; Roberts, Chierici et al. 1992). Bifidobacteria have also been used to restore normal intestinal flora following antibiotic therapy (Miller-Catchpole 1989; Wang and Gibson 1993; Gibson, Beatty et al. 1995; Gibson and Roberfroid 1995; Ballongue 1998).

In vitro and *in vivo* studies have been done to demonstrate the ability of lactoferrin to promote the growth of bifidobacteria. Human lactoferrin and bovine lactoferrin have been used to support the growth of various species of bifidobacterium as well as lactobacillus (Petschow and Talbott 1991; Petschow, Talbott et al. 1999; Griffiths, Duffy et al. 2003; Kim, Ohashi et al. 2004).

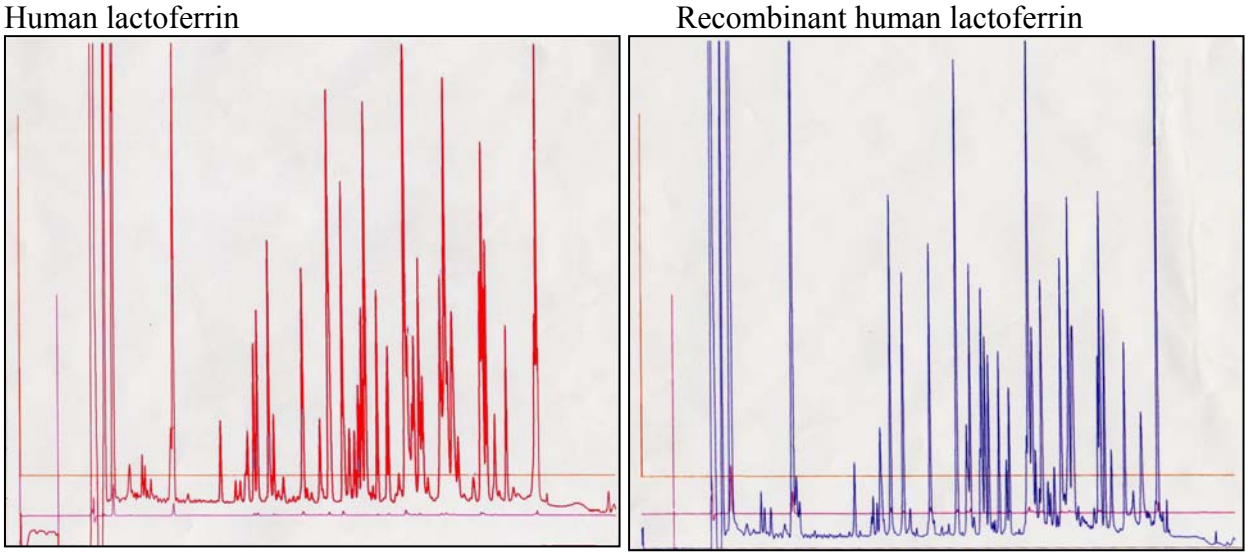
Two groups have studied the effect of lactoferrin in human infants. In both studies the effects were minimal. In one study the levels were from 0.1 to 1 mg/mL, below the levels found in early breast milk (Roberts, Chierici et al. 1992), although the high dose group did demonstrate some enhancement of bifidobacteria at three months. The second study used 2.8 mg/mL, but saw little effect (Balmer, Scott et al. 1989).

D. ANTI-INFLAMMATORY STUDIES

Lactoferrin may have a positive role in protection of the intestinal mucosa. Studies in animals with oral administration of lactoferrin have demonstrated a protective effect against development of colitis via modulation of the immune system (Togawa, Nagase et al. 2002). 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. When the animals received lactoferrin either as a preventative or treatment, the pro-inflammatory cytokines were inhibited and IL-4 and IL-10, the anti-inflammatory cytokines, were increased. When lactoferrin was given to control animals, there was no difference in cytokine levels between lactoferrin controls and saline controls. Recombinant human lactoferrin produced in yeast has also demonstrated a protective effect on the intestine in humans taking lactoferrin and indomethacin (Troost, Saris et al. 2003). This protective mechanism may result in less damage and more rapid repair of gut mucosal tissue injury, leading to more rapid restoration of normal permeability and growth. Lactoferrin also protected gut mucosa against the effects of bacterial lipopolysaccharide in mice (Kruzel, Harari et al. 2000).

Lactoferrin has also been implicated as an anti-inflammatory/anti-oxidant agent. This function is facilitated through the sequestration of free iron at inflammatory

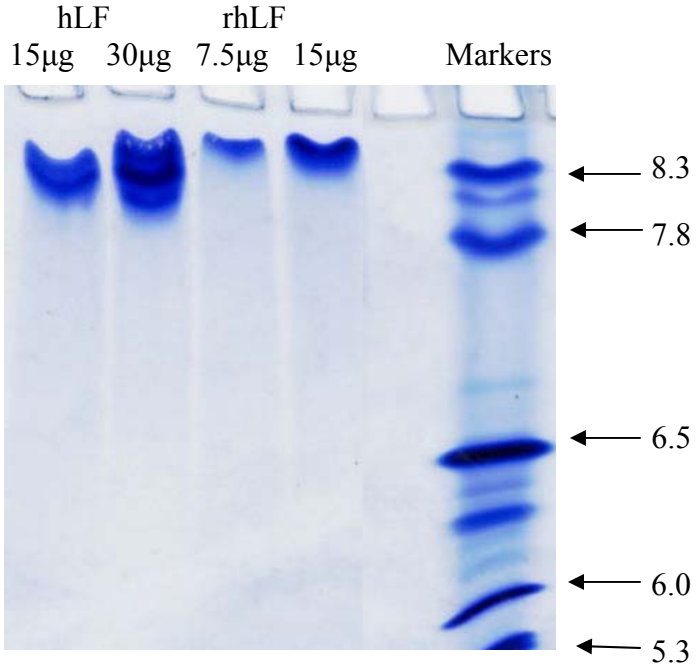
Figure 11. HPLC of Lys-C degraded human lactoferrin and recombinant lactoferrin



g. Isoelectric focusing

Isoelectric focusing was performed using a Novex IEF gel, pH 3 – 10. The human lactoferrin and recombinant human lactoferrin are basic proteins with a pI greater than 8.2 (Nandi, Suzuki et al. 2002).

Figure 12. Isoelectric focusing gel with human lactoferrin and recombinant human lactoferrin



Lactoferrin and *Helicobacter pylori*

Lactoferrin has antibiotic, anti-inflammatory and immune-modulating properties that may be active against *Helicobacter pylori* (Dial and Lichtenberger 2002). However, lactoferrin is also an iron binding and sequestering protein and there have been reports suggesting it can support the growth of *Helicobacter pylori* by providing an iron source (Dhaenens, Szczebara et al. 1997).

Dhaenens et al. were able to isolate a lactoferrin binding protein when *H. pylori* was grown *in vitro* in an iron-starved medium. Using the purified binding protein, they were able to demonstrate it's specificity for human lactoferrin. In binding inhibition studies there was only 35% inhibition by bovine lactoferrin and none by human, bovine or equine transferrins. However, they were only able to use lactoferrin to support the growth of *H. pylori* under very strict iron-free conditions and the extrapolation of this data to the *in vivo* environment is unclear. One factor is that when *H. pylori* is grown under iron-free conditions, it also expresses other outer membrane iron binding and transport proteins which are specific for heme-uptake (Worst, Otto et al. 1995). The iron bound to lactoferrin is non-heme iron, thus *H. pylori* has multiple mechanisms for iron acquisition and the mechanism used *in vivo* has not been resolved (Conneely 2001).

In *in vitro* culture systems used for the study of potential therapeutic agents against *H. pylori*, both bovine and human lactoferrin have demonstrated significant bacteriostatic and bactericidal activity against the bacteria (Miehlke, Reddy et al. 1996; Dial and Lichtenberger 2002). The minimal inhibitory concentration was in the range of 1 – 3 mg/mL. In the Miehlke studies, recombinant human lactoferrin at a concentration of 3 mg/mL was able to inhibit growth of *H. pylori* incubated on an iron-rich blood agar medium and greatly reduce the growth at 0.75 mg/mL.

The effect of lactoferrin in *in vivo* animal models of *H. pylori* and in actual infected patients has also been tested. In a mouse model infected with *H. felis*, oral human lactoferrin at 200 mg/kg/day was optimal for reducing the disease indicators of stomach size (50%), surface hydrophobicity (89%) and bacterial presence (75%) (Dial and Lichtenberger 2002).

There have been a number of studies on the ability of lactoferrin to eradicate *H. pylori* in human infection. In an open label, one week study using standard triple therapy and bovine lactoferrin at 200 mg b.i.d., the patients treated with the bovine lactoferrin had a significantly increased rate of *H. pylori* eradication as indicated by both protocol analysis and intent to treat analysis (Di Mario, Aragona et al. 2003). In two studies using human lactoferrin to treat asymptomatic *H. pylori* infection, the lactoferrin had no effect on disease status. One study involved a one day treatment of 1.25 grams (6 subjects) or 5 grams (6 subjects) of human lactoferrin (Opekun, El-Zaimaity et al. 1999). None of the subjects cleared their infection as determined by the ^{13}C-urea breath test, but there were

also no significant adverse events or changes in serum ferritin or iron. In a second study, nine healthy subjects with a positive ^{13}C-urea breath test were treated for 5 or 14 days with 5 grams of human lactoferrin per day (Guttner, Windsor et al. 2003). Although five patients had an initial decrease in the urea breath test and three showed a further decrease after treatment stopped, none were cured and the decreases were not significant. Although there was no beneficial effect noted, there was also no adverse effect or worsening of the disease status.

Studies in animals with human lactoferrin and the human study with bovine lactoferrin suggest it may have a more therapeutic role when combined with the standard triple therapy (Dial and Lichtenberger 2002; Di Mario, Aragona et al. 2003). Lactoferrin is thought to have a defensive role and as part of the host inflammatory response to *H. pylori*, neutrophils migrate to the stomach where they can release lactoferrin stored in their secondary granules. Elevated levels of lactoferrin are detected in the gastric mucosa and gastric juice of *H.pylori* positive patients (Choe, Oh et al. 2003).

In contrast to the data from Dhaenens suggesting support of *H. pylori* infection by lactoferrin through iron delivery, there are a number of possible mechanisms through which lactoferrin may suppress *Helicobacter*.

- Lactoferrin has been shown *in vitro* to possess direct antimicrobial activity against *H. pylori* (Miehlke, Reddy et al. 1996; Dial and Lichtenberger 2002).
- Lactoferrin may interfere with the adhesion of *Helicobacter* to the gastric surface (Wada, Aiba et al. 1999).
- Lactoferrin may interfere with the immune response (Shimizu, Ikehara et al. 2000).
- Lactoferrin may preserve the function of gastric mucosal tissue (Shimizu, Ikehara et al. 2000; Dial and Lichtenberger 2002).

Based on the studies described, it is unlikely that oral consumption of lactoferrin would have a deleterious effect on individuals infected with *Helicobacter pylori*.

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Shimizu, N., Y. Ikehara, et al. (2000). Effects of lactoferrin administration in *Helicobacter pylori* infections animal model. Lactoferrin: structure, function and applications. K. Shimazaki, H. Tsuda, M. Tomita, H. Kuwata and J.-P. Perraudin. Amsterdam, The Netherlands, Elsevier Science Publishers**:** 209-215.

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AM

Felix Jr, Fred B*

From: Merker, Robert I

Sent: Thursday, May 05, 2005 3:15 PM

To: CFSAN Document Control Center

Subject: FW: Revised Statement on Lactoferrin and H.pylori

Please add this to the record for GRN 162. Thanks.
-----Original Message-----
From: delia bethell [mailto:dbethell@ventria.com]
Sent: Thursday, May 05, 2005 3:09 PM
To: Merker, Robert I
Subject: Revised Statement on Lactoferrin and H.pylori

Dear Dr. Merker,

Attached please find a revised statement on lactoferrin and *H.pylori* for inclusion with the supplement to GRN 000162 submitted April 19, 2005. This statement is labeled as Rev 03 in the footer and replaces the statement labeled Rev 02 in the footer.

Thank you for your assistance. If you have any questions, please don't hesitate to contact me.

Best regards,
Delia Bethell

Delia R. Bethell, Ph.D.
Vice President for Clinical Development
Ventria Bioscience
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Lactoferrin and *Helicobacter pylori*

Lactoferrin has antibiotic, anti-inflammatory and immune-modulating properties that may be active against *Helicobacter pylori* (Dial and Lichtenberger 2002). However, lactoferrin is also an iron binding and sequestering protein and there have been reports suggesting it can support the growth of *Helicobacter pylori* by providing an iron source (Dhaenens, Szczebara et al. 1997).

Dhaenens et al. were able to isolate a lactoferrin binding protein when *H. pylori* was grown *in vitro* in an iron-starved medium. Using the purified binding protein, they were able to demonstrate it's specificity for human lactoferrin. In binding inhibition studies there was only 35% inhibition by bovine lactoferrin and none by human, bovine or equine transferrins. However, they were only able to use lactoferrin to support the growth of *H. pylori* under very strict iron-free conditions and the extrapolation of this data to the *in vivo* environment is unclear. One factor is that when *H. pylori* is grown under iron-free conditions, it also expresses other outer membrane iron binding and transport proteins which are specific for heme-uptake (Worst, Otto et al. 1995). The iron bound to lactoferrin is non-heme iron, thus *H. pylori* has multiple mechanisms for iron acquisition and the mechanism used *in vivo* has not been resolved (Conneely 2001).

In *in vitro* culture systems used for the study of potential therapeutic agents against *H. pylori*, both bovine and human lactoferrin have demonstrated significant bacteriostatic and bactericidal activity against the bacteria (Miehlke, Reddy et al. 1996; Dial and Lichtenberger 2002). The minimal inhibitory concentration was in the range of 1 – 3 mg/mL. In the Miehlke studies, recombinant human lactoferrin at a concentration of 3 mg/mL was able to inhibit growth of *H. pylori* incubated on an iron-rich blood agar medium and greatly reduce the growth at 0.75 mg/mL.

The effect of lactoferrin in *in vivo* animal models of *H. pylori* and in actual infected patients has also been tested. In a mouse model infected with *H. felis*, oral human lactoferrin at 200 mg/kg/day was optimal for reducing the disease indicators of stomach size (50%), surface hydrophobicity (89%) and bacterial presence (75%) (Dial and Lichtenberger 2002).

There have been a number of studies on the ability of lactoferrin to eradicate *H. pylori* in human infection. In an open label, one week study using standard triple therapy and bovine lactoferrin at 200 mg b.i.d., the patients treated with the bovine lactoferrin had a significantly increased rate of *H. pylori* eradication as indicated by both protocol analysis and intent to treat analysis (Di Mario, Aragona et al. 2003). In two studies using human lactoferrin to treat asymptomatic *H. pylori* infection, the lactoferrin had no effect on disease status. One study involved a one day treatment of 1.25 grams (6 subjects) or 5 grams (6 subjects) of human lactoferrin (Opekun, El-Zaimaity et al. 1999). None of the subjects cleared their infection as determined by the ^{13}C-urea breath test, but there were

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Although there are data suggesting support of *H. pylori* infection by lactoferrin through iron delivery, there are also data that indicate lactoferrin may suppress *Helicobacter*. It is not clear how the ingestion of lactoferrin would affect the bacteria in infected individuals. The standard of care for *H. pylori* infected patients is treatment with antibiotics. The ingestion of lactoferrin by these individuals should not present a safety problem, as they are under commonly used antibiotic therapy and therefore not a separate subpopulation.

References

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Worst, D. J., B. R. Otto, et al. (1995). "Iron-repressible outer membrane proteins of *Helicobacter pylori* involved in heme uptake." Infect Immunity **63**: 4161-4165.

Merker, Robert I

From: delia bethell [dbethell@ventria.com]
Sent: Tuesday, May 09, 2006 7:17 PM
To: Merker, Robert I
Cc: Diane B. McColl
Subject: Response to questions from March 15, 2006
Attachments: White Paper 050906.pdf

Dear Dr. Merker,

Attached is a white paper with the response of the Lactoferrin GRAS panel to the questions from FDA/CFSAN from March 15, 2006. The paper was prepared by the members below and reviewed by the complete panel.
Jeremy Brock, Ph.D., Immunologist and Lactoferrin Researcher
Lloyd Mayer, M.D., Gastroenterologist and Mucosal Immunologist
Ronald van Ree, Ph.D., Immunopathology

These three panel members and Steve Taylor, Ph.D., panel chairman, will be attending the meeting on May 31, 2006. In addition Diane McColl and myself will be attending. We would like to begin the meeting with a review of the responses to CFSAN's questions and then provide the opportunity for the CFSAN group to ask the panel questions.

If you have any questions, please don't hesitate to contact me.

Regards,
Delia Bethell

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VENTRIA BIOSCIENCE

May 9, 2006

Robert Merker, Ph.D. (HFS-255)
U. S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
OO/OFAS/DBGNR
Harvey W. Wiley Federal Building
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Response to FDA questions on GRN No. 000162

Dear Dr. Merker:

We look forward to the meeting on May 31, 2006, between the CFSAN team, Ventria Bioscience and members of the Ventria lactoferrin GRAS Panel. In preparation for the meeting, we have prepared a written response to the questions provided on March 15, 2006. At the meeting, the panel members will address the issues associated with their field of expertise and then be available to answer questions.

Regards,

Delia Bethell, Ph.D.
Vice President for Clinical Development

4110 N. Freeway Blvd. Sacramento, CA 95834 Tel: (916) 921-6148 Fax: (916) 921-5611 http://www.ventria.com

Response to concerns of FDA/CFSAN concerning GRN 000162 stated in the correspondence of March 15, 2006.

FDA/CFSAN asked "whether the consumption of large amounts of recombinant human lactoferrin would result in the possibility of a breakdown of tolerance (an autoimmune Th1 response, non-IgE based). Part of this concern rests on whether the antigenic epitopes resulting from plant-based glycosylation could lead to removal of active suppression of existing lactoferrin-reactive T and B cells."

Oral Tolerance and Immunogenicity of Lactoferrin

Antigens elicit qualitatively distinct immune responses based on their portal of entry. Introduction of antigens systemically, whether by injection or injury, leads to local infiltration of inflammatory cells and specific immunoglobulin production. By contrast, antigens introduced at mucosal surfaces, including that of the gastrointestinal tract, elicit active inhibition of the immune response to those antigens systemically (Mayer and Shao 2004). From studies in both mouse and man it is clear that by altering the route of antigen administration, one can change the nature of the immune response. Oral tolerance is the active non-response to an antigen administered via the oral route. While many factors are involved in the development of oral tolerance, the dose of the antigen is a critical factor in the development of tolerance and the mechanism of that tolerance.

Humans consume approximately 30 kg of food proteins during the year and the mucosal immune system is in constant contact with dietary antigens and commensal bacteria. A damaging immune response against these beneficial and/or harmless antigens must be prevented to maintain the integrity of the gut and allow for controlled nutrient absorption (Faria and Weiner 2005). Complete digestion of protein and carbohydrate antigens renders the majority of food antigens soluble. Soluble antigens lead to systemic tolerance, whereas particulate antigens generally prime immune responses. This is of importance in the studies on lactoferrin in autoimmune disease discussed below where lactoferrin is presented on apoptotic neutrophils.

Studies by many groups have shown that systemic tolerance can be induced by two different mechanisms related to administration of two widely separated doses of antigen (Mayer and Shao 2004). The first type of tolerance is associated with low doses of antigen. The low doses of oral antigen tend to elicit tolerance mediated by induction of regulatory T cells that mediate active suppression of the immune response. In contrast, the second mechanism is associated with high doses of antigen which induce anergy or deletion of any antigen reactive clones via mechanisms such as T cell crosslinking (Faria and Weiner 2005). The levels required for low and high dose tolerance vary for different antigens but they generally range from 0.1 – 5 mg/day for low dose tolerance and greater than 25 mg as a single dose for high dose tolerance. The doses of lactoferrin used in the initial Ventria studies range from 196- 378 mg/day, far in excess of what would be considered minimal doses required for high dose or deletional tolerance.

It is well established that it is difficult to elicit protective immune responses by oral antigen administration. This fact is borne out by the paucity of orally effective vaccines. Oral poliovirus is one exception to the rule largely because viral coat glycoproteins bind to specialized epithelial cells called M cells. Microfold cells are specialized epithelial cells found in Peyer's patches that take up particulate antigens. Soluble proteins and glycoproteins are not taken up by M cells and are therefore more likely to elicit a suppressed or tolerant response. Thus it is fully expected that soluble lactoferrin will fail to "immunize" when given orally.

As alluded to earlier, continuous oral antigen administration of an antigen results in deletional tolerance. Previous studies using OVA and MBP as tolerogens have demonstrated that tolerance can be lost if the antigen is removed from the environment. In mice, tolerance induced orally can be maintained for roughly 3 months following initial exposure. Maintenance of the tolerant state requires persistence of the antigen either intrinsically or extrinsically. Continuous exposure to lactoferrin from natural epithelial secretions will maintain a state of tolerance. Thus, in sum, soluble oral lactoferrin is likely to elicit a tolerance response when given orally. At the doses employed, a deletional response would be anticipated.

There is no question that lactoferrin is immunogenic in heterologous species – indeed it is generally recognized to be a very effective antigen. However, there is also a large body of literature showing that patients with autoimmune disease have circulating autoantibodies to human lactoferrin, as a component of ANCA (Anti-Neutrophil Cytoplasmic Antibodies). The antigenic stimulus is probably not the low levels of circulating endogenous soluble lactoferrin, but surface-bound lactoferrin on apoptotic neutrophils at sites of inflammation, creating a particulate antigen (Rauova, Gilburd et al. 2002). It is difficult to see how ingestion of soluble human lactoferrin by adults could mimic this scenario. The IgG isotype predominates, though in some cases IgA lactoferrin autoantibodies have been reported (Chikazawa, Nishiya et al. 2000; Caccavo, Rigon et al. 2005). However, there is no evidence that lactoferrin in the GI tract is involved in their development. It must be emphasized that in adults there is no transmission of lactoferrin across the gut mucosa; work suggesting otherwise invariably cites studies in rodents, calves, or pre-term human infants, in which such transmission can occur. Thus, unless lactoferrin were to be consumed by individuals with an intestinal pathology causing gut leakage, it is highly improbable that ingestion of lactoferrin could exacerbate a systemic autoimmune response. Even in conditions such as IBD, food allergy and celiac disease, which all have defects in permeability, foods (other than gluten or the offending allergen) do not make these conditions worse.

Lactoferrin ANCA are present in some inflammatory conditions of the gastrointestinal tract, including Crohn's disease and ulcerative colitis (Locht, Skogh et al. 1999; Sugi, Saitoh et al. 1999; Taddei, Audrain et al. 1999; Caradonna, Amati et al. 2000). However, there is no evidence that mucosal lactoferrin contributes to their generation, and neutrophils infiltrating the inflamed gastrointestinal mucosa are the likely source of the antigen (Locht, Skogh et al. 1999).

Although cell-mediated immune responses to lactoferrin have been detected in heterologous systems, including oral administration (Debbabi, Dubarry et al. 1998; Yuki, Fujihashi et al. 1998; Sfeir, Dubarry et al. 2004), there appears to be no evidence for a cell-mediated autoimmune response to lactoferrin in humans.

To conclude, it is clear that human lactoferrin is an autoantigen giving rise to circulating anti-lactoferrin autoantibodies in a variety of diseases. There is, however, no evidence that lactoferrin in the GI tract is the antigenic source, even when IgA isotypes are present. Mucosal antibody and cell-mediated responses to oral lactoferrin have been reported only in systems where lactoferrin is acting as a foreign antigen. Oral delivery of lactoferrin is more likely to be associated with the development and maintenance of tolerance.

Immunomodulatory Action of Lactoferrin

There is also a large body of literature testifying that lactoferrin has immunomodulatory effects, including the promotion of a Th1 type response (see review by (Ward, Paz et al. 2005). The vast majority of studies involved the use of bovine or human lactoferrin in rodents, where lactoferrin is itself acting as an antigen. However, there is some evidence for a non-specific adjuvant effect mediated through the mannose residues on lactoferrin glycans (Zimecki, Kocieba et al. 2002), though bovine lactoferrin was more active than human. In addition, in transgenic mice constitutively expressing human lactoferrin, the immune response to experimental arthritis was skewed towards a Th1 response (Guillen, McInnes et al. 2002).

The immunomodulatory effects of lactoferrin have generally been regarded as beneficial rather than harmful. However, the Th1 bias seen in human-lactoferrin transgenic mice resulted in a more severe response in the collagen-induced arthritis model of rheumatoid arthritis (Guillen, McInnes et al. 2002). In these animals, human lactoferrin may be considered as a 'self' protein. Given that lactoferrin is not transferred across the gut mucosa in adult humans (confirmed in the study of (Hayes, Falchook et al. 2005) it is highly unlikely that oral (as opposed to systemic) lactoferrin would affect joint inflammation. Moreover, in an earlier study it was reported that local administration of lactoferrin into the inflamed joints ameliorated the condition (Guillen, McInnes et al. 2000).

IL18, up-regulated by oral lactoferrin, has also been shown to have a pro-inflammatory effect in the mouse arthritis model (Gracie, Forsey et al. 1999). *In vitro* inflammatory cytokine release by synovial membrane cells required 100 ng/mL of IL18 (Gracie, Forsey et al. 1999) In the *in vivo* work, mice received 100 ng of rIL18 intraperitoneally to induce inflammation. In contrast, serum levels in both humans (Hayes, Falchook et al. 2005) and mice (Iigo, Shimamura et al. 2004) are on the order of a few hundred picograms per milliliter.

Oral lactoferrin is known to stimulate both mucosal and systemic IL18 levels. A single dose (300 mg/kg) of bovine lactoferrin administered to healthy mice resulted in a 3-fold increase in circulating IL18, and an increase of a similar order of magnitude in the

intestinal mucosa (Iigo, Shimamura et al. 2004). A similar effect on mucosal IL18 levels was reported for administration of recombinant human lactoferrin (3x 300 mg/kg) to healthy mice (Varadhachary, Wolf et al. 2004).

In the human studies cited, cytokine stimulating effects of oral lactoferrin are quite modest. The oral administration of human lactoferrin (between 1.5 and 9 g of lactoferrin per day for up to 28 days) to adult humans with solid tumors (Hayes, Falchook et al. 2005) gave only a 15% increase in circulating IL18. It is also noteworthy that oral administration of bovine lactoferrin (3g/day for 6 months) to HIV+ve children produced only a very modest immunological effect, *viz*. an increase of CD4+ cell counts from 21.5% to 25.4% (Zuccotti, Salvini et al. 2006). The concentrations of IL18 shown to be important as a contributing factor in inflammation range above 10ng/mL, more often 100 ng/mL, well above the values seen following oral lactoferrin exposure. Thus the modest 15% increase in serum IL18 reported following oral administration of human lactoferrin to cancer patients (Hayes, Falchook et al. 2005) seems far too insignificant to potentiate any pro-inflammatory effect. In addition, the induction of oral tolerance is classically associated with a decrease, not an increase, in pro-inflammatory cytokines. This is correlated with the production of anti-inflammatory/immunosuppressive cytokines such as TGFβ and IL10.

Thus oral administration of lactoferrin to non-infant humans seems to have a less immunostimulatory effect than that observed in mice. Lactoferrin can modulate immune responses, but this is true of both human and bovine lactoferrin, with the latter being if anything more active. In conclusion, it seems highly unlikely that reports of harmful lactoferrin-mediated immunomodulatory effects are relevant to ingestion of lactoferrin by adult humans.

Effects of Glycosylation on Immunogenicity of Lactoferrin

Concerns were raised that high level exposure to recombinant lactoferrin could lead to possible breakdown of tolerance due to the plant-based glycosylation of this self-protein. Indeed, there are reports in the literature that propose a role for differences in post-translational modification in the development of auto-immunity.

In a recent review (Anderton 2004) it is pointed out that many potential targets for self-reactive T cells are in fact glycoproteins. Collagen type II (CII) in collagen-induced arthritis is given as an example to illustrate that differences in glycosylation influence on reactivity of T cells (Michaelsson, Malmstrom et al. 1994; Fugger, Rothbard et al. 1996; Corthay, Backlund et al. 1998; Backlund, Carlsen et al. 2002; Backlund, Treschow et al. 2002).

The immuno-dominant T cell epitope of CII in H-2^q mice contains a 15 amino acid peptide containing a lysine that can be hydroxylated, allowing subsequent O-linked glycosylation. The presence of the resulting glycan, β-D-galactopyranosylhydroxylysine, was shown to be essential for recognition by a majority of T cells reactive with this epitope. In the absence of glycosylation T cell stimulation was ineffective.

The glycopeptide CII epitope is presented by the A^q major histocompatibility complex (MHC) class II molecule. Interestingly, the peptide-binding pocket of A^q in H-2^q mice is very similar to those of human DR4(DR1*0401) and DR1(DRB1*0101), allowing them to bind the same collagen-derived T cell epitope (Fugger, Rothbard et al. 1996). In rheumatoid arthritis patients it was demonstrated that 5/7 patients with T cells specific for the same peptide, only reacted to the glycosylated version of the epitope (Backlund, Carlsen et al. 2002).

In addition to a direct effect on the recognition of epitopes by the TCR (T cell [antigen] receptor), glycosylation can also influence the processing of antigens by APC (antigen presenting cells). N-glycosylation of influenza A nucleoprotein for example has been shown to inhibit generation of distal CTL (cytotoxic T lymphocyte) epitopes (Wood and Elliott 1998).

In a second review (Cobb and Kasper 2005) the relevance of glycosylation for the development of autoimmune disease is discussed in greater detail. In a rat model of autoimmune glomerulonephritis the disease only develops if the kidney glycoprotein megalin is glycosylated (Tramontano and Makker 2004). A non-glycosylated recombinant version of megalin failed to induce the autoimmune syndrome.

The relevance of glycosylation of self-proteins in human autoimmune disease is illustrated by marked differences in concanavalin A staining on blots of α2-macroglobulin between patients with various autoimmune diseases (SLE, RA, mixed connective tissue disease, scleroderma) and healthy subjects (Saso, Silvestrini et al. 1993). Additionally, RA patients were shown to have autoantibodies reacting with glycosylated autoantigen p68 but not with deglycosylated autoantigens (Blass, Meier et al. 1998).

Finally, this review describes the most recent data published on the role of glycosylation of the dominant T cell epitope of collagen type II. The importance of differences in glycosylation for the development of arthritis are illustrated by the observation that normal tissue contains primarily glycosylated CII whereas arthritic samples contain a mixture of glycosylated and non-glycosylated CII (Dzhambazov, Holmdahl et al. 2005).

Although these examples illustrate the important role glycosylation can have in autoimmune disease, in all of these examples the key is presence of absence of glycosylation. Human lactoferrin carries typical mammalian N-glycans. On the rice-derived molecule these are replaced by typical plant-like xylose/fucose containing complex N-glycans. Clearly, this is a completely different scenario from the studies described in both reviews. In all these studies, differences in glycosylation refer to absence or presence of glycosylation, not structural differences between substituted glycans. These observations are therefore not to be interpreted as a risk factor for application of rice-derived human lactoferrin.

There is also a theoretical possibility that the altered glycosylation pattern of rice lactoferrin might lead to exposure of epitopes that could result in removal of active suppression of existing lactoferrin-reactive T and B cells. Nothing is known about the epitopes recognized by anti-lactoferrin autoantibodies, nor is there any information on lactoferrin T cell epitopes. However, the concept of 'hidden epitopes' is controversial, and since the plant glycans are of a similar size and linked at the same glycosylation sites as native lactoferrin glycans, it is unlikely that any such putative new epitopes would be exposed. Moreover, any such epitopes would not really be self epitopes, in which case a reaction to rice lactoferrin would have occurred in the Ventria feeding study. None was observed.

Conjugate vaccines that are being developed against *Streptococcus pneumoniae* are based on conjugation of different serotypes of polysaccharides to a carrier protein. In a recent study, it is reported that different serotypes have different degrees of immunogenicity (Leonard, Canaday et al. 2003). This is shown to be caused by differences in the efficiency of antigen processing by APCs. Although these observations suggest that differences in glycans (serotype) cause differences in antigen processing and subsequent production of antibodies (immunogenicity), the authors favor another explanation. The conjugation of the different serotypes to the carrier protein is random and the positions for substitution differ per serotype. The observed differences in antigen processing are proposed to be caused by differences in the proximity of polysaccharide structures to the MHC-binding region of the carrier protein.

In fact, different proximity to the MHC-binding region can be interpreted as absence or presence of glycans as well. To the best of our knowledge there is no evidence in the literature that would support that antigen processing would be influenced by the minor differences in glycan structure observed between native and rice-derived recombinant human lactoferrin.

Recent studies have suggested that certain organisms, tumors or whole allergens (e.g. peanut) contain glycans that serve as natural adjuvants directing a Th2 skewed response (Atochina, Daly-Engel et al. 2001; Okano, Satoskar et al. 2001; Thomas and Harn Jr 2004). Many of these glycans bind to ligands on dendritic cells, promoting DC2 differentiation (promoting a Th2 response). While there is limited evidence to support the existence of rice allergy, glycans present in rice may, in combination with a potent antigen, alter the immune response to that antigen. We are cognizant of this possibility with rhLF but feel that this is unlikely given the rarity of rice allergy and the ubiquitous exposure to this foodstuff. In addition the product will be labeled as lactoferrin (human) from rice.

In summary, there is evidence supporting a role for differences in glycosylation in the development of autoimmune disease and in the immunogenicity of glycoproteins. These differences, however, are associated with the absence or presence of glycans rather than small differences in glycan structure.

Based on the data presented above and the intended uses of lactoferrin (human) from rice in GRN 000162, it is unlikely the consumption of the intended levels of lactoferrin would result in the breakdown of tolerance through mucosal exposure, the initiation of an autoimmune response, or immunogenic recognition of the small variations associated with the plant glycans. In fact, at the proposed intake levels, the response is more likely to be in support of oral tolerance to lactoferrin via the mechanisms of deletional tolerance.

FDA/CFSAN asked "autoantibodies were produced in response to presumably low levels of endogenous lactoferrin in patients that had already had autoimmune responses. Consumption of large doses of lactoferrin, in particularly lactoferrin that differs slightly from the endogenous form with respect to small sequence or conformational differences, could theoretically act as a trigger to autoimmune responses for those genetically predisposed."

In theory, anti-lactoferrin autoantibodies might have some pathological effect, either in a general way (eg formation of immune complexes) or by interfering with lactoferrin function. It is obvious that lactoferrin autoantibodies have the potential to interfere with the biological and physiological functions of lactoferrin. Anti-lactoferrin antibodies can interfere with iron-binding by lactoferrin (Britigan, Hassett et al. 1989; Audrain, Gourbil et al. 1996; Brock, Lamont et al. 1998), but this was only observed with hyperimmune antisera, human autoantibodies being inactive (Audrain, Gourbil et al. 1996); JH Brock, unpubl data). This suggests that human autoantibodies to lactoferrin are of relatively low affinity, and to our knowledge no derangement of iron metabolism or other events that could be related to impaired lactoferrin function have been reported in patients with anti-lactoferrin autoantibodies.

It has been suggested that ANCA may play a role in vasculitis due to neutrophil activation (Malenica, Rudolf et al. 2004). It has also been reported that anti-lactoferrin antibodies can cause neutrophil activation due to binding to lactoferrin on the neutrophil surface (Peen, Sundqvist et al. 1996). Again, the data referred to hyperimmune antisera; it was stated that human autoantibodies had a similar effect but no supporting data were presented in the paper. Neutrophils from lactoferrin-ANCA+ve ulcerative colitis patients had normal antimicrobial activity (Caradonna, Amati et al. 2000), suggesting no significant disruption of neutrophil function. Administration of human lactoferrin followed by anti-lactoferrin antibody increased albumin extravasation in a rat model compared to lactoferrin alone (Erga, Peen et al. 2000), but again hyperimmune serum rather than autoantibody was used. Thus there is no direct evidence that anti-lactoferrin autoantibodies cause neutrophil activation or are involved in vasculitis.

As mentioned above, the likely source of antigenic stimulation is apoptotic neutrophils, and it has been concluded that anti-lactoferrin autoantibodies probably represent a secondary or bystander response to inflammation and antigen release (Czaja and Norman 2003). While there is speculation as to a possible pathogenic role for anti-lactoferrin autoantibodies in several of the large number of reports on lactoferrin ANCA, none, with the exception of the work on autoimmune pancreatitis, discussed below, present any experimental evidence for such a role. Many of the studies consider these antibodies to be an epiphenomenon with no role in disease pathology.

The one condition in which experimental data relating anti-lactoferrin autoantibodies to disease pathology has been presented concerns immune-mediated pancreatitis. This is an uncommon disease, with about 150 cases reported, mainly in Japan (Ketikoglou and Moulakakis 2005). Okazaki (Okazaki, Uchida et al. 2000) proposed a mechanism involving an aberrant recognition of HLA class I or II in conjunction with autoantigenic

peptides from lactoferrin or carbonic anhydrase II on pancreatic cells. An experimental model was developed in which neonatally thymectomised BALB/c mice were immunised with lactoferrin or carbonic anhydrase and the spleen cells transferred to nude mice (Uchida, Okazaki et al. 2002). However, bovine rather than autologous murine lactoferrin was used as immunising antigen, and it was claimed (data not shown) that a murine pancreatic extract reacted with sera from animals immunised with bovine lactoferrin. Since there is no immunological cross-reactivity between murine and bovine lactoferrins (JH Brock, unpublished observations) it seems unlikely that this was a truly autoimmune type of reaction against murine lactoferrin induced by immunisation with the bovine protein. Indeed, the authors admit that the model does not necessarily represent an autoimmune phenomenon.

Since gene polymorphisms occur in lactoferrin (Liu, Gladwell et al. 2002) it could be argued that there could be minor differences in the polypetide sequence of orally ingested recombinant lactoferrin compared to the individual's native lactoferrin. These could result in functional or immunological differences. As far as we are aware the only reported functional significance of lactoferrin polymorphisms is a possible role in susceptibility to periodontitis (Jordan, Eskdale et al. 2005). Since any effect of lactoferrin in this condition is likely to occur in the gingival crevice, it would be expected that the individual's own native lactoferrin rather than ingested lactoferrin would be involved. To our knowledge there are no reports of immune responses to lactoferrin recognizing structural differences resulting from gene polymorphisms (Thaler, Labarrere et al. 1999)

In conclusion, the vast majority of the extensive literature on autoimmunity to lactoferrin provides no evidence that it is involved in disease pathology. In the case of autoimmune pancreatitis, the relevance of the animal model designed to investigate such a relationship is highly questionable. There is no evidence that gene polymorphisms could lead to heightened immunogenicity.

Based on the data presented above there is no evidence that in intake of large doses of lactoferrin from the intended uses in GRN 000162 would result in an adverse response in individuals with antibodies to lactoferrin due to a previously existing autoimmune condition. It is also unlikely that small sequence or conformation differences would trigger an autoimmune response following oral intake of lactoferrin.

FDA/CFSAN asked "is there published data on other products that would address the consumption of other human (or relevant model animal) N-linked glycoprotein produced in plants that have been consumed by humans (or those animals) at high levels?"

To our knowledge there is limited published data on high level consumption of human N-linked glycoprotein produced in plants. Data on the intake of recombinant human lactoferrin in combination with recombinant human lysozyme added to oral rehydration solution have been present in abstract form (Zavaleta, Figueroa et al. 2006). There is also a 28-day rat oral study in press (Cerven, Bethell et al. In Press).

There are two examples of consumption of lower doses of recombinant glycoprotein with plant N-linked glycan. One study used 100 mg of recombinant human lactoferrin from Ventria Bioscience saturated with iron (^{59}Fe) in adult women (Lonnerdal and Bryant 2006). There were no adverse events in the study. A second study used a plant produced secretory IgA monoclonal antibody against *Streptococcus mutans* as preventative therapy. The antibody was applied to teeth and gums twice a week for three weeks, requiring about 22.5 mg antibody per course of treatment. In this study a number of clinical parameters were assessed at regular intervals to monitor for adverse events, including immunogenic responses to the antibody or the plant glycans. There were no adverse events in the study and the pre- and post- treatment sera exhibited no change in antibody titers suggesting that the plant preparation containing purified antibodies was not immunogenic when given orally (Ma, Hikmat et al. 1998). The authors stated that given the daily intake of foreign plant and mammalian proteins the absence of adverse effects was not surprising.

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VENTRIA BIOSCIENCE

November 16, 2006

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Re: GRN 000162

Dear Dr. Merker,

In light of our discussions, Ventria hereby withdraws its GRAS Notice GRN No. 000162 for lactoferrin (human) purified from rice, and asks that FDA cease review of the GRAS Notice upon receipt of this letter. Ventria makes this withdrawal without prejudice to future GRAS notice submissions for use of lactoferrin.

Thank you for your time.

Regards,

Delia Bethell, Ph.D.
Vice President for Clinical Development

Cc: Scott Deeter, President & CEO
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