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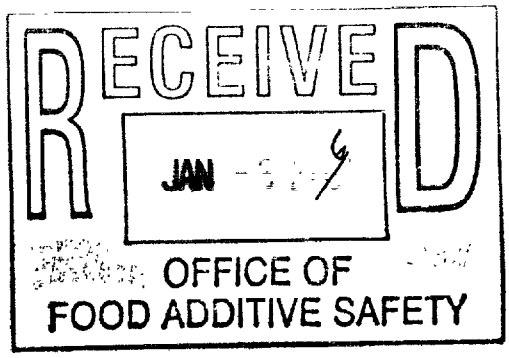
Law Offices Of
Morin & Associates

Suite 500
388 Market Street
San Francisco, California 94111
Telephone: (415) 957-0101 e-mail: charleslmorin@earthlink.net Facsimile: (415) 957-5905



December 29, 2005

Antonia Mattia, PhD (HFS-255)
Director
Division of Biotechnology and
GRAS Notice Review
Office of Food Additive Safety
Center for Food Safety and Applied
Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Notice of GRAS exemption for human lactoferrin derived from the milk of transgenic cows expressing a human gene encoding human lactoferrin

Dear Dr. Mattia:

Pursuant to proposed 21 CFR § 170.36 and on behalf of our client, i.e., Pharming Group N.V. (Archimedesweg 4, 2333 CN Leiden, The Netherlands), this submission – forwarded in triplicate – is intended to notify FDA of Pharming's and an expert panel's independent determinations – based primarily on scientific procedures, but also on common use in food, i.e., human exposure – that the above-referenced human lactoferrin is generally recognized as safe (i.e., GRAS) when used as an ingredient in sports and functional foods at a level of 100 mg of Pharming's hLF per product serving. Accordingly, and as with all GRAS substances, such human lactoferrin – when used as described above and in the attached information – is exempt from the premarket approval requirements

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applicable to food additives set forth in Section 409 of the Food, Drug, and Cosmetic Act and that section's implementing regulations.

In support of said notice, please find attached a summary discussion of the information relied upon by Pharming and others when determining that Pharming's human lactoferrin is GRAS. Such discussion is set forth in the format required by proposed 21 CFR § 170.36(c). (62 FR 18961 – 4/17/97). Please note that none of this discussion contains confidential business information.

After you and your colleagues have had an opportunity to review and consider the attached information, if you should have questions or need additional information, please let me know. All of the information that serves as the basis for this GRAS determination can be sent – upon request – to FDA and is available for review and copying at reasonable times.

Thank you in advance for your and your colleagues' efforts on behalf of Pharming's notice.

Sincerely.

Charles L. Morin

Cc: Frans de Loos, PhD
Project Director (rhLF)
Pharming Group N.V.

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Computer Technology Services, Inc.

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Pharming Group N.V.

Notice Of GRAS Exemption For Human Lactoferrin Derived From The Milk of Transgenic Cows Expressing A Human Gene Encoding Human Lactoferrin

Pharming Group N.V.
Archimedesweg 4
2333 CN Leiden
The Netherlands
(T): 011 31 71 524-7173
(F): 011 31 71 524-7445
(email): f.deloos@pharming.com

December 29, 2005

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Section I

I. INTRODUCTION

This GRAS Notification concerns the safe use of a transgenically produced form of a substance – commonly known as **human lactoferrin** – in human food. Human lactoferrin (or hLF) is an important, naturally-occurring protein that is native to many mammals, including humans. Indeed, with regard to humans, hLF is found naturally in numerous human biological fluids and mucus secretions – including in significant quantities in human milk. Human lactoferrin is of significant importance to humans because it serves to naturally promote and maintain a healthy homeostasis due to its significant involvement in host defense against infection and excessive inflammation. Accordingly, hLF has an inherent value for food use.

Pharming's hLF – as discussed in significant technical detail within this notification – is produced from the milk of transgenic cows. Briefly stated, such cows result from insertion of a human genomic hLF sequence under control of regulatory elements from the bovine αS_1 casein gene into the bovine germline. As a result of this remarkable achievement, such hLF – unlike the hLF that can be produced from samples of human milk – can now be produced in significant quantities and at a competitive price.

Given the availability of its hLF, Pharming – via this notification – seeks from CFSAN its determination that the agency has no questions at this time (i.e., the time after CFSAN's review of this notification) regarding Pharming's conclusion that its hLF is GRAS under the intended conditions of use, i.e., as an ingredient in sports and functional foods at a level of 100 mg of Pharming's hLF per product serving.

Following in Sections II through VII is a careful and thorough discussion of the information Pharming relied on – especially the considerable published scientific information – to reach its determination that its hLF is GRAS for the intended uses discussed in this notification. Also included in such discussion is a report from an expert panel of how it reached the same determination. In short, both entities concluded that Pharming's hLF is GRAS for its intended uses. Following, then, is the referenced discussion.

Section II

II. ADMINISTRATIVE INFORMATION

A. Claim regarding GRAS status

Via this GRAS Notification, Pharming notifies FDA that it believes that its human lactoferrin product – which is the subject of this GRAS Notification – when derived from the milk of transgenic cows expressing a human gene encoding human lactoferrin (as more specifically detailed within this Notification) and when used as an ingredient in sports and functional foods (as more specifically detailed herein) is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because the notifier, i.e., Pharming, has determined – as has an independent ex~~pert panel~~ – that such use is generally recognized as safe, i.e., GRAS.

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Date

Charles L. Morin
Attorney for the notifier
Morin & Associates
Suite 500
388 Market Street
San Francisco, CA 94111
(415) 957-0101
charleslmorin@earthlink.net

B. Name and address of notifier

Frans de Loos, PhD
Project Director (Pharming's hLF)
Pharming Group N.V.
Archimedesweg 4
2333 CN Leiden
The Netherlands
F.deLoos@pharming.com

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C. Common or usual name of the subject substance

Human lactoferrin

D. Conditions of use

Pharming's human lactoferrin is intended for use as an ingredient in sports and functional foods at a level of 100 mg of Pharming's hLF per product serving.

E. Basis for GRAS determination

The GRAS determinations – discussed at length below – pertinent to the subject substance are based primarily on scientific procedures, but also on experience based on common use in food.

F. Statement of availability of data and information

The data and information that are the basis for the notifier's and expert panel's determinations are available for review and copying at the office of Morin & Associates, 388 Market Street, Suite 500, San Francisco, CA 94111 **or** will be sent to FDA upon request.

Section III

III. IDENTITY OF NOTIFIED SUBSTANCE

A. Name

Human lactoferrin

B. Physical and chemical composition

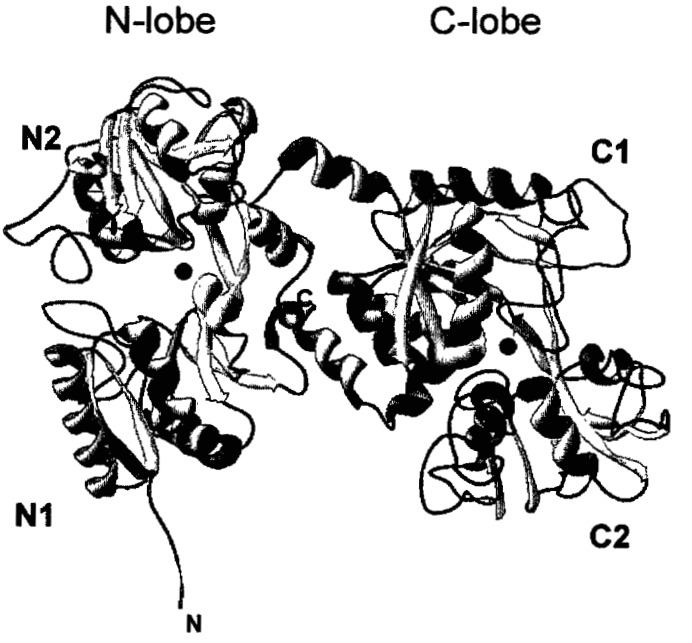
In physical appearance, Pharming's hLF is a salmon colored powder.

With regard to chemical composition, Pharming's human lactoferrin is an iron-binding glycoprotein of about 76 kDa. (Thomassen, 2005; van Berkel, 2002). The molecule – as does natural hLF (nhLF) from human milk - consists of a single polypeptide chain of 692 amino acids that is folded into two globular lobes, the N- and C-lobe, which are connected by an α-helix. (Thomassen, 2005). Each lobe consists of two domains (I and II), connected by a hinge region, creating a deep iron-binding cleft that can bind a single ferric ion with high affinity while simultaneously incorporating a suitable anion. (Thomassen, 2005; Anderson, 1989). (See Figure 1).

Pharming's hLF is, like natural hLF, N-glycosylated. (van Berkel, 2002). The glycosylation occurs at one site (Asn479), two sites (Asn138 and 479) or three sites (Asn138, 479, and 624) in about 5%, 85% and 10% of the molecules, respectively, which is virtually identical to nhLF. (van Veen, 2004; van Berkel 1996). Both Pharming's hLF and natural hLF have the same number of glycosylation sites (which glycosylation sites are in identical positions) and both utilize these glycosylation sites in the same fashion. (van Veen, 2004). In addition to bearing complex N-linked glycans, which are the only glycans found on natural hLF (Spik, 1982b), Pharming's hLF also bears oligomannose and/or hybrid-type glycans. (van Berkel, 2002).

Figure 1

Pharming's hLF: Three Dimensional Structure



The crystallographic structure of Pharming's hLF, in its iron-saturated conformation (Thomassen, 2005), shows the typical bilobal (N- and C-lobe), four domain (N1/N2, C1/C2) folding pattern which is characteristic for proteins of the transferrin family. (Baker, 2000). The α-helices and β-strands are indicated in blue and yellow, respectively. The iron ions are indicated by red dots.

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C. Manufacturing processes

1. Production of transgenic cattle

a. Founder generation

All transgenic founder animals (generation F_0) were generated using well-known and widely-used *in vitro* (embryo) production techniques, including microinjection. (van Berkel, 2002; Krimpenfort, 1991). In summary, a limited number of ovaries were collected in a local slaughterhouse[1] and transported to the laboratory. The follicular fluid containing the oocytes was subsequently harvested by aspiration of the follicles. The oocytes were separated from the follicular fluid, and then transferred to maturation medium and incubated overnight. The handlings of oocytes and embryos were done according to high quality standard of embryology practice aiming at minimizing contamination. Oocytes were fertilized *in vitro* using semen provided by commercial, EU-certified, semen collection stations.[2] Bulls were selected on the basis of their dairy merit and other desirable traits. The gene construct containing the human lactoferrin gene was microinjected into one of the pronuclei of the fertilized oocytes (i.e., zygotes). After *in vitro* culture, embryos that reached the morula or blastocyst stage on days 7 or 8 were selected and were transferred to hormonally synchronized heifers. After birth, calves were tested for transgenesis, using well-known and widely-used molecular biological techniques such as PCR analysis. (Krimpenfort, 1991).

b. Cloning and characterization of the transgene (construct)

As more fully described in Appendix 17, a unique human lactoferrin (hLF) expression vector was designed by using two different clones. These clones, derived from human gene banks (Blonden, 1989) and present in cosmid vector C2RB (Bates, 1983), were clone 13.2, which contained most

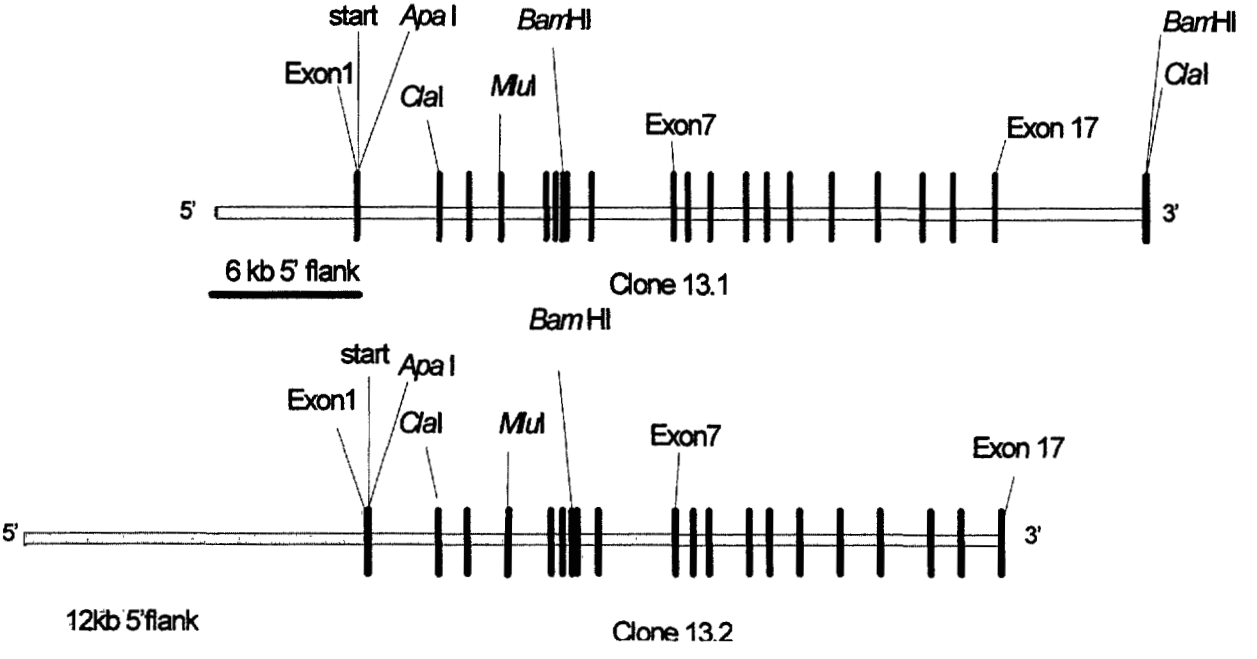
[1] All source animals came from herds in which BSE had not occurred and were inspected as to their health status. Only animals passing such inspection were admitted to the slaughterhouse and used as a source of ovaries.

[2] When materials from animal origin, such as fetal calf serum, were used in these procedures, such materials were obtained from New Zealand – a non-BSE country. With regard to the entire risk management program pertinent to pathogenic entities, including BSE, see Subsection IIIC8 (entitled: "Pathogenic entities").

of the 5' genomic hLF sequences (about 13 kb), and clone 13.1, which contained most of the 3' genomic sequences (about 20kb). (See also Figure 2).

Figure 2

Cosmids When Combined Contain The Entire hLF Gene



The 5' part of 13.2 was combined with the 3' part of 13.1. Briefly, the construction encompasses the linkage of 34.7 kb of the human genomic hLF sequence (exons 1 through 17 plus 6.7 kb of the 3' flanking region) to the regulatory sequences of the bovine αS1 casein promoter (6.3 kb). (See also Appendix 2 for a detailed sequence).

In detail, first, plasmid 8hLFgen9k was constructed (see Figure 3), in which the αS_1 sequences were fused to the hLF genomic sequences, from cosmid clone 13.2 via use of a synthetic linker. (See Figures 3 and 4).

Figure 3

Construction Of Plasmid 8hLFgen9k

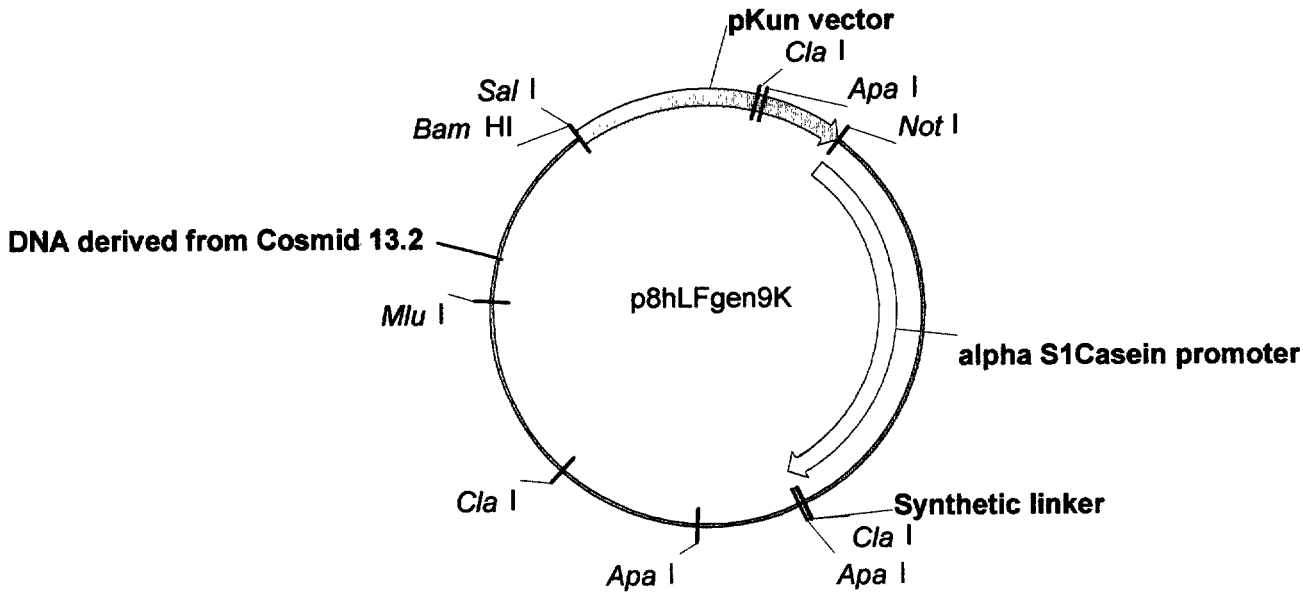


Figure 4

Synthetic linker

Translation initiation codon

ClaI ApaI

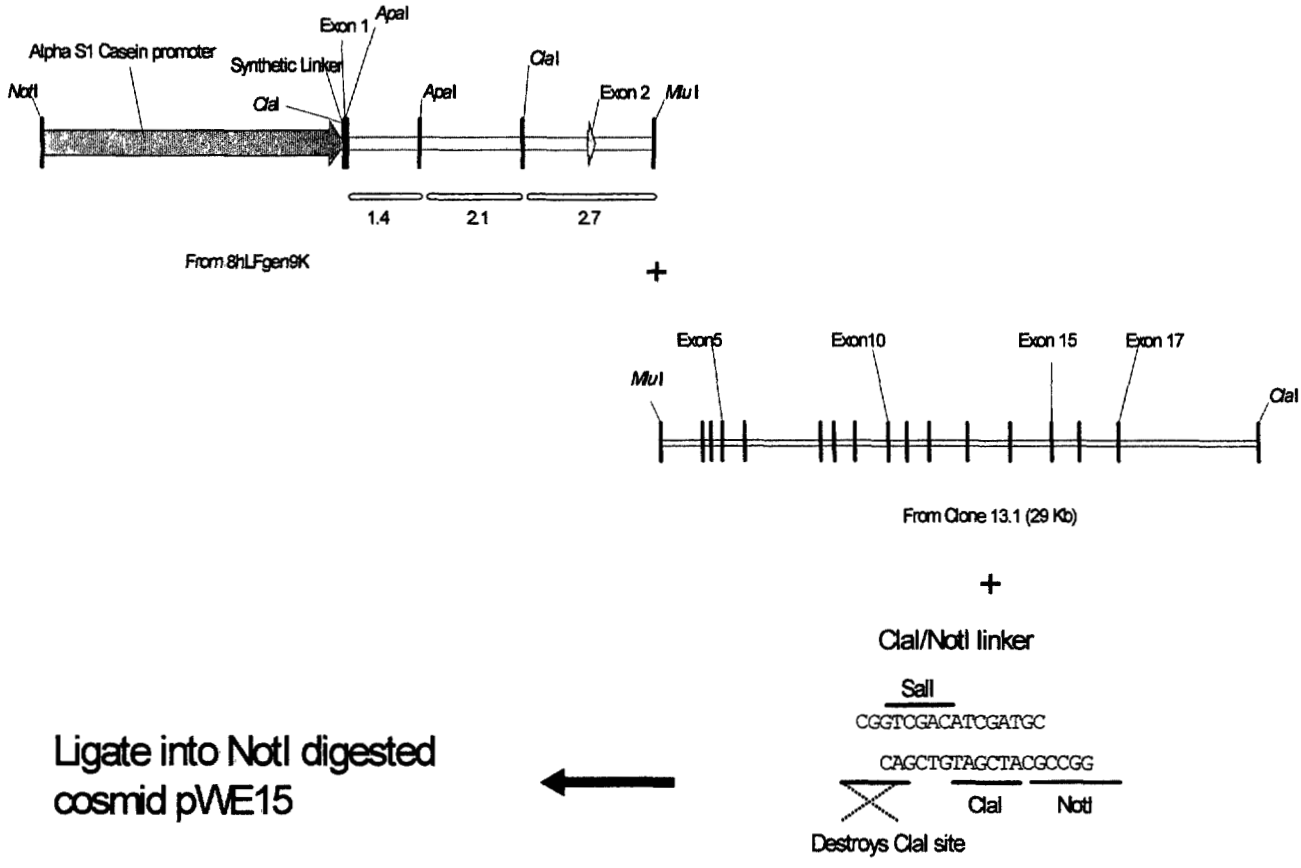
5'-CGATACCAAGTCGCCTCCAGACCGCAGACATGAAACTTGTCTTCCTCGTCCTGGTGTTCCTCGGGGCC-3'

TATGGTTCAGCGGAGGTCTGGCGTCTGTACTTTGAACAGAAGGAGCAGGACCACAAGGAGCC

The synthetic linker contains 24 bp of the hLF 5'-UTR and encodes for most of the hLF signal sequence. The αS1 casein sequences were derived from a plasmid harboring a αS1 promoter fragment from - 6.3 kb to +19 bp followed by a *Cla*I restriction site. (US patent 6,140,552, Appendix 17) A *Not*I*Mlu*I fragment from the construct 8hLFgen9k was then ligated to the 3'*Mlu*I-*Cla*I fragment from clone 13.1, combined with a *Cla*I-*Not*I linker. (See figure 5).

Figure 5

Generation Of 8hLF37 Gene



The cloning vector cosmid pWE15 (Clontech, Mountain View, CA USA) was used to harbor the complete hLF construct after deletion of the vector's internal *Cla*I and *Sal*I sites and subsequent digestion with *Not*I. Prior to micro-injection into the pronucleus of a fertilized oocyte, the 41.0 kb hLF DNA fragment was removed from the vector sequences using *Not*I and purified using gel electrophoresis. (For additional information, see Appendix 17).

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c. Breeding of production animals

From each male founder, offspring (generation F_1) was generated in order to carry out expression studies, evaluate transgene transmission, and provide milk for initial protein characterization and process development.

Out of four founder animals, ultimately one bull named "Max" (identification number NL11258785) was selected on the basis of copy number, hLF expression level in the offspring, health, fertility and general genetic merit. The semen from this bull was used to generate the second generation of transgenic animals – including three transgenic cows used for production of Pharming's hLF and a transgenic bull named Theo (NL112588214) which will be used for the generation of future production animals. (See Figure 6 for a graphic representation of the transgenic cattle founder generation and Figure 7 for the transgenic cattle herd generation from the MAX-line).

Figure 6

Graphic Representation Of Transgenic Cattle Founder Generation Via Microinjection Of A DNA Construct

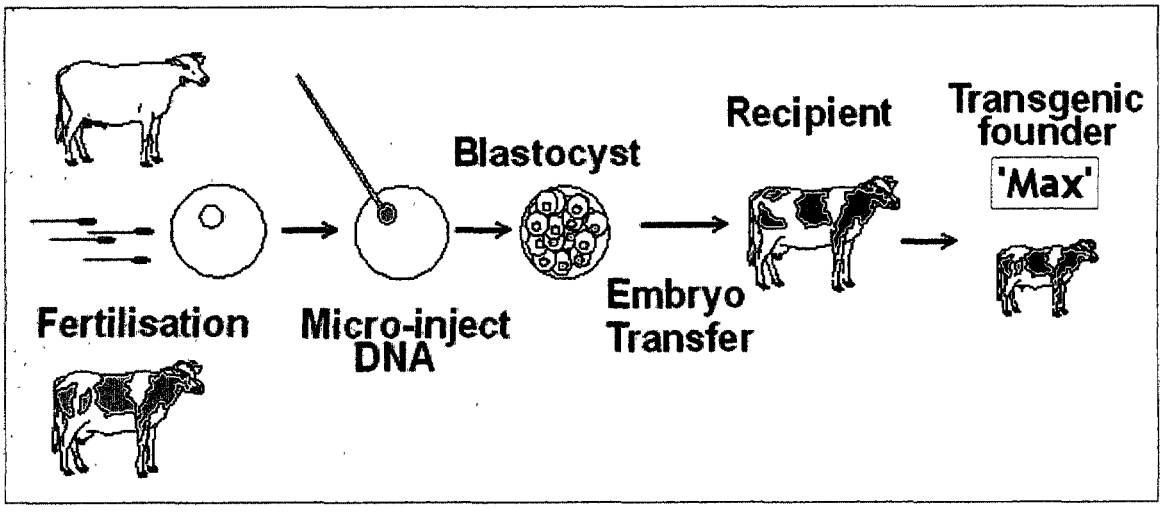

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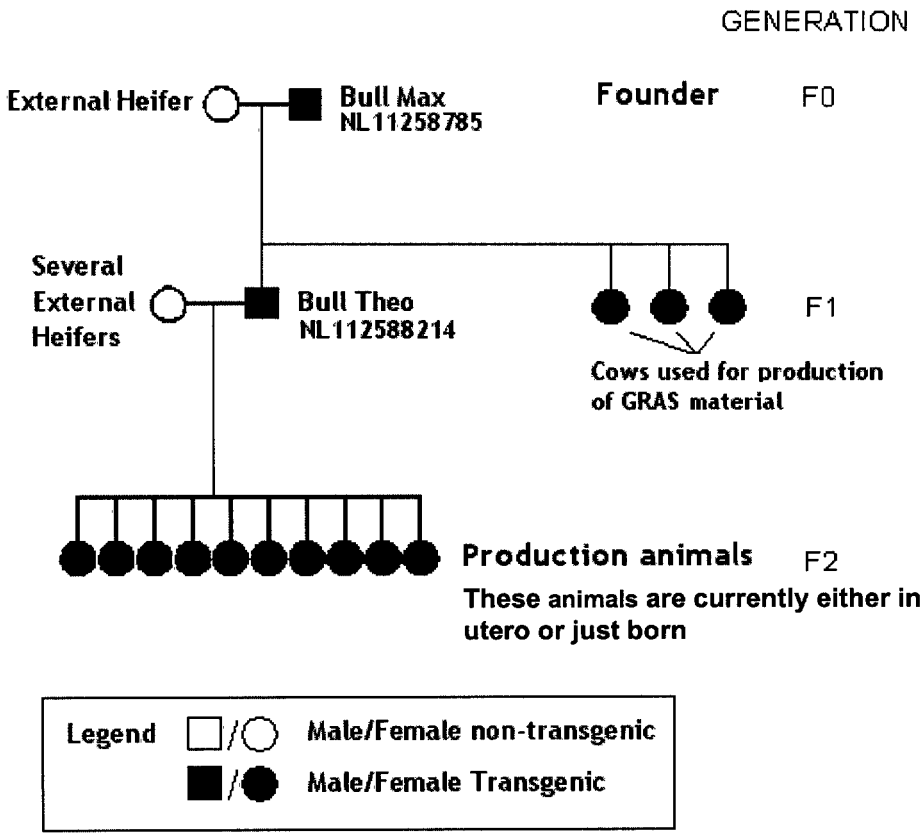
Figure 7

Generation of Transgenic Cattle Herd from The MAX-line



d. Transgene copy number and integration site

Since the process of exogenous DNA insertion into the germline of a host animal often involves integration of multiple copies of the DNA, usually into a single chromosomal site, the transgene copy number was determined and the number of independent integration sites was verified by using well-known and widely-used molecular biological techniques, such as fluorescent *in situ* hybridization (FISH). Molecular biological analysis revealed that in the MAX-line, i.e., the male founder line, the transgene is localized at **a single chromosomale site**. Furthermore, it was established that the founder bull MAX has multiple intact copies of the transgene.

e. Protein Identity

1.) Identity

The identity of Pharming's hLF was confirmed by analytical Mono S chromatography and N-terminal protein sequence analysis and crystallography. (van Berkel, 1996, Thomassen, 2005).

2.) Nucleotide sequence

The sequence of the entire transgene construct - including the αS1 casein promoter, the hLF exons and introns, and the 3' regulatory sequence - has been determined. The nucleotide sequence is presented in Appendix 2. Since the vector sequences are removed before injection, determination of the vector sequences was not necessary. Finally, the hLF mRNA sequence, which is expressed in mammary epithelial tissue of the transgenic line MAX has also been determined. From this sequence the amino-acid sequence is derived and presented in Appendix 1.

3.) Sequence comparison

The deduced amino acid sequence has been compared with ten different previously published hLF amino acid sequences derived from pubmed, no lBOLA, AAG48753, lN76A, AAN11304, lCB6A, lFCKA, AAA68565, CAA17316, NP002234, and P02708. This comparison is presented in Appendix 2. Alignments of the hLF amino acid sequences demonstrate 6 differences in total in the mature peptide. (See Table 1).

Table 1

Comparison Of Different Amino Acid Sequences

Sequence	AA4	AA11	AA14	AA29	AA413	AA561
Pharming	R	A	Q	R	A	D
lBOLA	-	T	Q	K	A	E
AAG48753	R	T	Q	R	A	D
lN76A	-	T	Q	R	A	D
AANl1304	R	T	Q	R	A	E
lCB6A	-	A	Q	K	A	E

lFCKA	R	A	N	K	A	E
AAA68565	R	A	Q	K	G	E
CAA17316	R	A	Q	K	A	E
NP002334	-	A	Q	K	A	E
P02708	R	A	Q	K	A	E

Extensive analysis of hLF sequences in the past revealed polymorphic sites in the coding sequence at amino acid position 4 (deletion of Arg), position 11 (Ala or Thr), position 29 (Arg or Lys) and position 561 (Asp of Glu). (van Veen, 2004). Pharming's hLF produced from the milk of transgenic cows has an Arginine at positions 4 and 29, an Alanine at position 11 and an Aspartic acid at position 561. (van Veen, 2004). Comparison of the amino acid sequence of Pharming's hLF with ten published amino acid sequences confirmed the above-mentioned polymorphic sites. The other two differences were found only in sequences lFCKA and AAA68565 and, since these have not been confirmed by any other group, they will not be discussed further. From this comparison it was concluded that hLF produced in the milk of transgenic cattle (i.e., Pharming's hLF) falls within the range of variation that can be found in a normal population.

2. Care and handling of transgenic cattle

In summary, the care and handling of transgenic cattle is done according to current good husbandry practices, especially as they relate to good dairy practices, food production, and use of transgenic animals. Following is a discussion of these practices in greater detail.

a. Facilities

Transgenic cattle are maintained within a facility where access is controlled and unintended mingling with other cattle or other animals is prevented. This minimizes the risk of introduction of pathogenic and adventitious agents, as well as unwanted breeding.

While local requirements pertinent to housing are used, significant additional efforts in the design of housing facilities have been implemented which are aimed at improving the health and welfare of the transgenic animals and facilitating early detection of abnormalities.

b. Monitoring the health of transgenic animals

Blood and fecal samples are collected on a regular basis to establish, along with other practices, the health status of the transgenic herd and detect – in timely fashion – any changes of the epidemiological status in the cattle population. The frequency and type of tests utilized (with regard to the referenced samples) vary depending on the epizootic situation of the region where the cattle are kept.[3] Pathogen testing, de-worming and vaccination programs are designed by the veterinarian responsible for the herd and are revised as needed.

c. Standard care of transgenic animals

1.) Partum and post partum care

Monitoring around calving includes the usual day and night checks for signs of imminent calving. The checks are intensified as the expected week of calving nears. In addition, calving will be assisted and, if necessary, obstetrical or surgical interventions initiated.

Post partum follow-up includes careful monitoring of the general condition of the animal. In particular, attention is paid to the uterine involution and return to reproductive cyclicity.

2.) Health monitoring of transgenic calves

Information is recorded during the neo-natal period of transgenic calves. Special attention is given to easiness of calving, physical integrity and weight. Any problems related to respiration, feeding locomotion, blood circulation, etc. is documented and addressed by the veterinarian. Growth is also monitored (via recorded weight and height measurements).

---

[3] The following are examples of infections that are routinely screened for (as determined by local requirements and other pertinent considerations): Leptospira hardjo; Infectious bovine rhinotracheitis/pustular vulvitas virus; Bovine leucosis virus; Brucella abortus; Bovine viral diarrhea virus; Salmonella spp.; Neospora caninum; and Mycobacterium paratuberculosis (only if older that 2 years).

3.) Health monitoring of transgenic cows during lactation

Daily general inspection is performed. It is conducted by animal caretakers and milking personnel. Such inspection is part of everyday care and consists of visual observation of the animal's gait, behavior and condition. Abnormalities are recorded and action is taken accordingly, including via instruction from or participation by the attending veterinarian. Such inspection also includes daily monitoring of the udder and milk, udder temperature, color of tissue and reaction of the animal to touch, as well as the physical integrity of the organ and, especially, the teats which are controlled every day.

d. Establishment of a reliable and continuous source of transgenic animals (founder strain); collection and storage of certified sperm.

Bulls must fulfill a number of criteria for acceptance as a semen provider. In addition to the EU health requirements that govern admitting bulls into bull stud status and the collection and freezing of semen, additional requirements pertaining to transgenesis are used. For example, in order for bull semen to be used, a transgenic bull must be genetically characterized by using well-known and widely-used molecular biological techniques, such as PCR analysis.

3. Milking of transgenic cattle

Milking is performed in a milking parlor and pursuant to current good dairy practices. Before the milking is started, the hind quarters and teats and udder of the cow are thoroughly cleaned and dried. Premilking is carried out for visual inspection of the milk characteristics. Further samples may be collected, if warranted, during the actual milking for cell count, bacterial count, hygiene and bacteriology. Alternatively, samples may be taken from the milk tank followed by purification of Pharming's hLF. Immediately after the milking, the raw milk is collected into a cooled milk tank and followed by purification of Pharming's hLF.

4. Purification of Pharming's hLF from cow's milk

Table 2 provides the process steps pertinent to purification of Pharming's hLF from the cooled milk of transgenic cows. These steps are

performed under appropriate quality standards to ensure a high quality hLF product. First, the milk is processed by a centrifugation and pasteurization step to remove fat and to reduce bacterial contaminants. Pharming's hLF is subsequently purified by ion-exchange chromatography, filtered through various filtration systems and spray- or freeze-dried. As shown by SDS-PAGE analysis of milk, SP Sepharose purified lactoferrin and three individual batches (Figure 8 and 9), the production method of Pharming's hLF yields, consistently, a highly pure lactoferrin product.[4]

Table 2

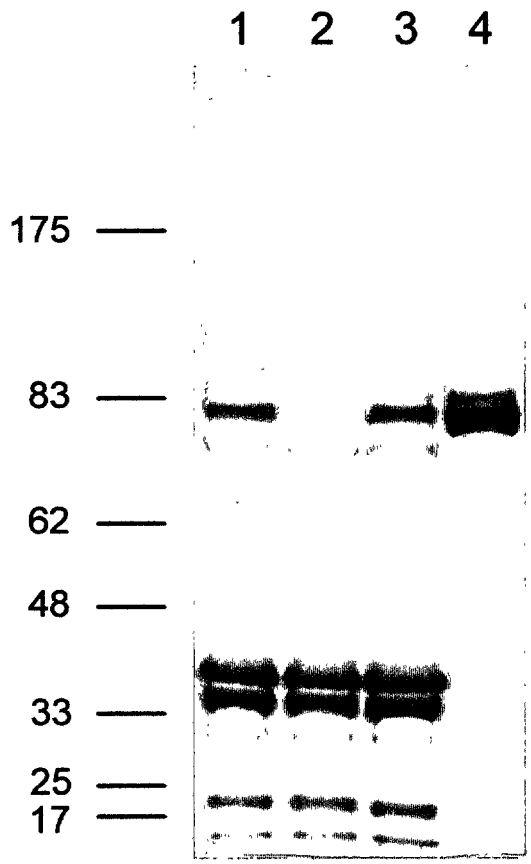
Production Process For Pharming's hLF

Step	Method
Transgenic milk containing Pharming's hLF ↓ Milk processing	Centrifugation / Pasteurization
↓ Lactoferrin purification	Ion-exchange chromatography
↓ Lactoferrin processing	Ultra- and micro-filtration / Spray- or Freeze-drying
↓ Pharming's hLF product	

[4] The doublet of protein bands of Pharming's hLF on SDS-PAGE (seen on Figure 8) results from heterogeneity in utilization of N-linked glycosylation sites. (van Berkel, 1996 and 2002). The utilization of N-linked glycosylation sites by Pharming's hLF and nhLF is virtually identical. (van Veen, 2004).

Figure 8

SDS-PAGE analysis of transgenic milk and purified lactoferrin



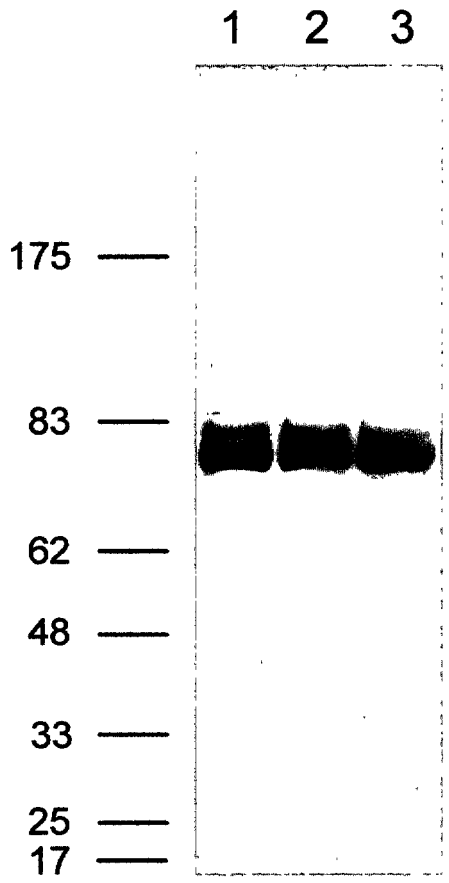
Milk samples (0.5 μl) and purified lactoferrin (4 μg) were subjected to non-reduced, boiled 4-12% SDS-PAGE. Lane 1: transgenic milk; lane 2: non-transgenic milk; lane 3: transgenic milk after defatting and pasteurization; lane 4: SP Sepharose purified lactoferrin. Proteins were visualized by Coomassie brilliant blue. Left-hand numbers (10^{-3} x Mr) indicate the migration of the protein standards.

With regard to the double protein band seen at approximately Mr 83,000 on the above Figure 8, lane 4, the double band on SDS-PAGE is a result of heterogeneity in utilization of N-linked glycosylation sites of hLF. (van Berkel, 1996 and 2002). The upper band (about 10% of total) consists of hLF molecules bearing 3 glycans (attached at Asn138, 479 and 624)

whereas the lower band (about 85%) represents hLF bearing two glycans (at Asn138 and 479). Both van Berkel, 2002 and van Veen, 2004 show that the SDS-PAGE profiles of Pharming's hLF and nhLF are virtually identical. The presence of bLF in the upper band cannot be excluded; however, the amount of bLF in Pharming's hLF is about 2.5-fold lower than the amount of hLF glycosylated at all three N-linked glycosylation sites. (Appel, in press).

Figure 9

SDS-PAGE analysis of Pharming's hLF product



Three batches of Pharming's hLF product (lane 1-3) were subjected to non-reduced, boiled 4-12% SDS-PAGE. Proteins (4.5 μg/lane) were visualized by Coomassie brilliant blue. Left-hand numbers (10^{-3} x Mr) indicate the migration of the protein standards.

5. Processing aids and chemicals used

All chemical substances and food contact surfaces used in Pharming's hLF production process – including sanitizing wash solutions, contact surfaces, cleaning agents, the cation-exchange resin, and the micro- and ultra-filters - have been approved (and are, otherwise safe and suitable) for their intended uses. None are derived or made from animal sources – either in part or whole.

Table 3 below provides the processing aids and chemicals used in (but not limited to) the purification of Pharming's hLF. The chemicals are all GRAS.

Table 3

Processing Aids And Chemicals

Demineralized water
Sodium citrate
Sodium chloride
Hydrochloric acid
Sodium hydroxide
Ion-exchange media (SP Sepharose): GE Healthcare, Uppsala, Sweden
Ultrafilters (PES[1]): Millipore, Bedford, MA, USA
Microfilters (CN/CA[2], PVDF[3], PP[4]): Millipore, Bedford, MA, USA

[1] Polyethersulfone

[2] Cellulose Nitrate / Cellulose Acetate

[3] Polyvinylidene fluoride

[4] Polypropylene

6. Finished product specifications

Table 3 below provides the acceptance criteria, i.e., specifications, pertinent to Pharming's hLF purified from the milk of transgenic cows.

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Table 4

Finished Product Specifications

Assay	Specification
Total protein	≥ 85%
hLF content	≥ 90%
Iron saturation of hLF	≤ 20%
Unsaturated iron binding capacity	≥ 70%
pH (10% in 0.9% NaCl)	6.5 ± 1.5
Color	Salmon colored
Moisture	≤ 8%
Solubility (10% in 0.9% NaCl)	Complete
Ash (550° C)	≤ 2%
Lead[5]	< 0.5 mg/kg
Plate count	Max. 1000 CFU/g
Escherichia coli	Not detected in 1 g
Enterobacteria	Max. 10 CFU/g
Yeast	Max. 10 CFU/g
Mold	Max. 10 CFU/g
Staphylococcus aureus	Not detected in 1g
Salmonella	Not detected in 5 g

The hLF content of Pharming's hLF product produced from the milk of transgenic cows is, at least, 90 percent. The main contaminant is bovine lactoferrin. (Appel, in press). The remaining contaminants are whey proteins. The iron-saturation content of Pharming's hLF is less than 20 percent and its capacity to bind iron is, at least, 70 percent. The spectrophotometric method for determination of the percentage iron-saturated lactoferrin in purified preparations and LF that has been saturated with iron in-vitro is as described in Nuijens, 1997a. Analytical cation-exchange chromatography (as discussed in Section IV and van Veen, 2002) is used to determine the purity of Pharming's human lactoferrin.

[5] **Please note** that up until recently the specifications list included heavy metals (as Pb) instead of lead. The heavy metals specification has been removed and replaced with a specification for lead.

7. Product Stability

Thus far, Pharming has produced several lots of its hLF product. Table 5 below provides the release results of three batches – all of which met all product specifications.

Table 5

Release Results Of Pharming's hLF Batches

Assay	Specifications	Batch		
		I	II	III
Total protein	≥85%	90	92	94
hLF content	≥90%	93	97	97
Iron saturation of hLF	≤ 20%	7	7	7
Unsaturated iron binding capacity	≥ 70%	89	87	90
pH (10% in 0.9% NaCl)	6.5 ± 1.5	6.8	6.3	6.3
Color	Salmon colored	Conform	Conform	Conform
Moisture	≤ 8%	4	3	3
Solubility (10% in 0.0% NaCl)	Complete	Conform	Conform	Conform
Ash (550°C)	≤ 2%	1%	1%	1%
Heavy metals (as Pb)[6]	< 20mg/kg	Conform	Conform	Conform
Lead	< 0.5mg/kg			
Plate count	Max. 1000 CFU/g	20 CFU/g	10 CFU/g	<10 CFU/g
Escherichia coli	Not detected in 1 g	Conform	Conform	Conform
Entereobacteria	Max. 10 CFU/g	< 10 CFU/g	< 10 CFU/g	< 10 CFU/g
Yeast	Max. 10 CFU/g	< 1 CFU/g	< 1 CFU/g	< 1 CFU/g
Mold	Max. 10 CFU/g	< 1 CFU/g	< 1 CFU/g	< 1 CFU/g
Staphylococcus aureus	Not detected in 1 g	Conform	Conform	Conform
Salmonella	Not detected in 5 g	Conform	Conform	Conform

These several lots have been and are being tested for product stability over time. To date, two evaluations have been completed. One study – by OctoPlus (located in Leiden, The Netherlands) – indicated that Pharming's hLF, a freeze-dried formulation, was stable after storage at 25° C or 4° C for

[6] **Please note** that on all future "release results" documents the specification for heavy metals will have been removed and replaced by a specification for lead.

6 weeks. The second study – also by OctoPlus – indicated that Pharming's hLF is stable after 4.4 years when stored at 2-8° C.,

In addition, Pharming's hLF is also being evaluated via ongoing accelerated and real time stability studies. Pharming anticipates that, by the time it is ready and permitted to introduce its product into commerce, it will have more than sufficient stability data to support the one year shelf life it expects to go to market with.

8. Pathogenic entities

Pharming recognizes the need to reduce the risk of its hLF product containing any pathogenic entity to, at least, that risk associated with producing milk from dairy cows by dairy farmers. To this end, Pharming has an extensive program to properly manage such risk. Such program includes all the many procedures and other efforts expressly described in Section III, Subpart C, sub-subparts 1, 2, 3, 4 (dealing specifically with product purification), 5 and 6. Such program yields Pharming's hLF product which has been shown via testing to be safe and suitable for use in human food.

Also of concern to Pharming with regard to the hLF production process here in question – since such process incorporates use of dairy cattle – is the potential for such cattle to transmit a transmissible spongiform encephalopathy – specifically Bovine Spongiform Encephalopathy (or BSE) – via bovine milk to one consuming Pharming's hLF product. Despite the fact that BSE is **not** transmitted via certain animal tissues – including bovine sperm, bovine oocytes, bovine colostrum, tissues of the bovine mammary gland, and bovine milk[7] - **and** the fact that authoritative risk assessments – performed by, for example, the European Commission and the WHO – have resulted in the above-referenced bovine tissues being classified in the category of "no detectable infectivity, i.e., the lowest possible risk category (Vetrugno, 2004), Pharming, nevertheless, engages in the following practices – consistant with European and other similar governmental TSE guidelines – to assure that the pertinent prionic agent that causes BSE is not present in the hLF product:

[7] Various studies have documented not only that bovine milk itself does **not** contain the agent for BSE but also that even the milk of BSE infected cows is **unable** to transmit the disease regardless of whether it is given orally, intracerebrally or intraperitineally. (Vetrugno, 2004; see also, Taylor, 1995 and Wilesmith, 1997). Also, please note that **no** BSE cases have occurred in offspring born to BSE-infected cows. (Wilesmith, 1997).

First, in sourcing animals, every effort is made to assure that animals do not come from herds wherein BSE has occurred;
Second, animals are not fed any animal feed which contains any animal tissue, including animal meat or bone meal;
Third, Pharming does not use any substance in its manufacturing process that is made from animal tissue;[8] and
Fourth, the cow's milk that Pharming uses to produce its hLF product is sourced from healthy animals and is in the same condition as is milk collected for human consumption.

Consequently, Pharming believes that the risk of the milk it uses to produce its hLF product being able to transmit BSE is, at worst, negligible and, at best, non-existent.

[8] Even certain reagents used in the reproductive portion of Pharming's activities (but not in the processing portion) are acquired from New Zealand – a non-BSE country.

Section IV

IV. ANALYTICAL METHODOLOGY

Early on, Pharming recognized that it would need a validated, analytical methodology to control and assure the quality of batches of its hLF made from the milk of transgenic cows. Such a methodology was developed and validated. It is known as high-performance, cation-exchange chromatography on Mono S. Such methodology has been demonstrated to be a useful and reliable tool for evaluating the identity, purity, and N-terminal integrity of Pharming's hLF. (For further information, please see the considerable write-up set forth in van Veen, 2002, a copy of which is attached as Appendix 3).

Section V

V. DETAILED SUMMARY OF THE BASIS FOR NOTIFIER'S GRAS DETERMINATION

A. Human lactoferrin

1. Introduction

Lactoferrin is a major, naturally-occurring protein of approximately 77kDa that is native to humans – as well as other mammals (Lönnerdal, 1995; Nuijens, 1996) and was first isolated from human milk in 1960. (Johanson, 1960). It is a member of the transferrin family of iron-binding glycoproteins. (Anderson, 1989). These proteins share the common function of controlling levels of free iron in the biological fluids through their ability to sequester and solubilize Fe^{3+}. Binding of iron by lactoferrin is associated with a significant conformational change – the molecule shifting from an open to a closed form and becoming markedly more compact. (Baker, 2000).

Human LF consists of a polypeptide chain of 692 amino acids, which is folded into two globular lobes. (Anderson, 1989; Nuijens, 1996). These lobes, designated the N- and C-lobe, share an internal amino acid identity of about 40 percent (Metz-Boutigue, 1984) and are connected by an α-helix. Each lobe folds into α-helix and β-sheet arrays to form domains I and II, respectively, which are connected by a hinge region, creating a deep iron-binding cleft within each lobe. Each cleft binds a single ferric ion with high affinity while simultaneously incorporating a bicarbonate ion. (Anderson, 1989; Brock, 2002). Crystallographic studies of hLF have shown that upon binding of iron, domain I of the N- and C-lobe rotates relative to its respective domain II by ~ 54° and ~20°, respectively, resulting in a more globular, closed, and stable conformation of the entire molecule. (Baker, 2000). Whereas some of the biological activities of hLF relate to iron-binding, others are mediated by a positively charged domain located in the N-terminus. This domain binds to negatively charged ligands such as the lipid A portion of LPS (Appelmelk, 1994), DNA (He, 1995), and heparin (Mann, 1994), as well as to other proteins such as lysozyme (van Berkel, 1995) and cell membrane molecules. (Ziere, 1993; Legrand, 1997).

Human LF contains three possible N-glycosylation sites, Asn^{138} in the N-lobe and Asn^{479} as well as Asn^{624} in the C-lobe (Rey, 1990), which are

utilized in about 94, 100 and 9% of the molecules, respectively. (van Berkel, 1996). The glycans of natural hLF from human milk are of the sialyl-N-acetyllactosamine type. (Spik, 1982b). However, carbohydrate moieties do not play a role in polypeptide conformation. (Sun, 1999; Thomassen, 2005), nor does glycosylation affect hLF's ability to bind iron. (van Berkel, 1995). The DNA and amino acid sequences of human lactoferrin (hLF) have been determined. (Metz-Boutigue, 1984; Rey, 1990). Gene polymorphisms do occur in human lactoferrin, but it is currently generally believed that there is but a single, functional human lactoferrin gene which is located in chromosome three, mapping to 3q21-q23. (Teng, 1987; Teng 2002).

2. Presence in human tissues

hLF is produced and released by mucosal epithelial cells and neutrophils. (Nuijens, 1996). It is virtually ubiquitous in the human body and found, for example, in human milk, tears, saliva, blood, and bronchial and intestinal secretions, as well as in the secondary granules of neutrophils. (Nuijens, 1996). The lactoferrins from human milk and neutrophils are identical except for their pattern of glycosylation as the glycans of milk-derived hLF contain, in contrast to the glycans of neutrophil-derived hLF, fucose residues. (Derisbourg, 1990; Moguilevsky, 1985).

3. Natural human exposure

Of greatest significance to this GRAS Notification in terms of human exposure to hLF is the **maximum**, natural exposure that a human can encounter, i.e., in this instance, the exposure resulting from infant consumption of mother's milk. Mammals have different levels of lactoferrin in their milk, with humans having the highest concentration. (For examples, see Table 6).

Table 6

Range Of Concentrations Of Lactoferrin In Milk From Various Species (Masson and Heremans, 1971)

>2mg/ml	0.2-2mg/ml	20-200 μg/ml	<50 μg/ml
Human	Guinea-pig Mouse Mare	Cow Goat Sow	Rat Rabbit Dog

The concentration, however, varies very significantly during the human period of lactation. (Nagasawa, 1972; Lönnerdal, 1976; Mathur, 1990; Nuijens, 1996). During the first three days postpartum – when human colostrum is being expressed – hLF concentration may approach 10 mg/ml by day two and then taper off by day seven to about 5 mg/ml. (Nuijens, 1996, Davidson, 1987). Thereafter, the concentration continues to decline during mid lactation, eventually persisting at about 1-2 mg/ml in mature milk. (Nuijens, 1996).

For purposes of determining the safety of the acceptable daily intake (ADI) corresponding to **maximum**, natural exposure to hLF during infancy (the time of highest natural exposure to hLF), let one assume the worst (or the highest exposure), i.e., that the concentration of hLF reaches 10 mg/ml in colostrum during the first few days of infancy. At this point, an average[9] infant's weight would be either approximately seven pounds (or 3.25 kilograms, assuming 1 kilogram per 2.2 pounds) or between approximately 16 pounds at six months and 21 pounds at 12 months (or 7.53-9.84 kilograms, assuming 1 kilogram per 2.2 pounds). (U.S. EPA, 2002). Such infant would consume per day an average of 1000 ml during early infancy and mid infancy (months 3-6). (U.S. EPA, 2002). As Table 7 below shows, this consumption could equate to a typical daily exposure (over the first year of an infant's life) of from 266-3077 mg hLF per kilogram of infant body weight.

Table 7

Naturally-Occurring Daily Infant Exposure to nhLF

Age	Weight	Human Milk Consump. (per day)[10]	Maximum Amt. Of hLF In Human Milk	Total hLF Potentially Consumed Per Day	Total hLF Consumed Per Day Per Kilogram Of Body Weight
Newborn	7.15 lbs. (3.25 k)	1000 ml	10 mg/ml	10,000 mg	3077 mg/k BW
Six months	16.57 lbs. (7.53 k)	1000 ml	2 mg/ml	2,000 mg	266 mg/k BW

[9] Average weight is used (instead of the 90th percentile weight) in order to establish **worst case**, i.e. reasonably highest, exposure.
[10] Consumption at the 90th percentile is used in order to establish **worst case**, i.e., reasonably highest, exposure.

As the foregoing table indicates, **natural,** daily, infant exposure to hLF – via consumption of human milk – is very significant, thus well establishing a **natural,** safe, high acceptable daily intake.

Both infants and adult humans also have a daily, naturally-occurring exposure to hLF via release of hLF from neutrophils and via mucosa as hLF is found in a large number of secretions, such as tears, seminal plasma and saliva. The mean adult plasma lactoferrin value amounts to about 122 ± 40 μg/L. (Scott, 1989). In infants, these plasma levels change in the course of their infancy but are the highest during the first three weeks of life, i.e., 385 ± 113 μg/L. (Scott, 1989). The hLF concentration in saliva of human adults is about 33 ± 14 μg/ml. (Tanida, 2003).

4. Natural purpose

A wide variety of functions have been attributed to lactoferrin, including modulation of iron absorption, regulation of gene transcription and various enzymatic activities, and promotion of bone growth. (Brock, 2002). However, the best established and most relevant functions concern its antimicrobial, anti-inflammatory and immunomodulatory properties.

Extensive in-vitro and in-vivo studies show hLF to have antibacterial, antifungal, antiviral and anti-inflammatory activities. On the basis of these activities, hLF is postulated to be involved in the innate host defense against infection and severe inflammation, most notably at mucosal surfaces such as those of the gastro-intestinal tract. (Nuijens, 1996). Antimicrobial activities of hLF include bacteriostasis by iron deprivation (Bullen, 1972; Reiter, 1975; Brock, 2002), bactericidal activity via destabilization of the cell-wall (Ellison III, 1988; Ellison III, 1991) and via hLF's ability to bind to porins (Erdei, 1994), and antiviral activity via inhibition of viral infection. (van der Strate, 2001; Brock, 2002). Anti-inflammatory actions of hLF include inhibition of hydroxyl-radical formation (Aruoma, 1987), of complement activation (Kijlstra, 1982) and of cytokine production (Zucali, 1989), as well as neutralization of lipopolysaccharide. (Mattsby-Baltzer, 1996; Kruzel, 1998; Lee, 1998). In addition to antimicrobial activity, hLF has been shown to promote the growth of Bifidobacterium species, the predominant bacteria of the intestinal flora of healthy breast-fed infants. (Petschow, 1991). In addition, hLF has been shown to promote the growth of intestinal cells both

in vitro (Nichols, 1987) as well as *in vivo* (Zhang, 2001), which may be mediated through binding to specific receptors. (Ashida, 2004).

Most of the biological actions of hLF are mediated by the sequestration of iron or by the previously mentioned positively charged domain located in the N-terminus. The release of a N-terminal fragment from hLF by pepsin action yields a potent bactericidal peptide (lactoferricin) against Gram-positive and -negative bacteria, yeast and molds. (Tomita, 1994).

5. Fate

The fate of hLF both in plasma and in the gastrointestinal tract is described below.

After ingestion of human milk, it is subjected to various digestion and absorption processes – each of which impacts on the fate of the inherent hLF. (Brock, 2002). Initially, in infants or adults with a pepsin-competent stomach, gastric digestion of milk lactoferrin, i.e., proteolysis, results in only some – probably a small amount – of the hLF possibly being converted to bio-active peptides, which may include lactoferricin. (Nuijens, 1996; Hamosh, 1998). Subsequently, gastric emptying is thought to be the primary mechanism via which the absorption kinetics of nitrogen from milk is controlled. (Tomé, 1998). After gastric emptying, hLF and its digestion products are further metabolized via the actions of pancreatic enzymes and peptidase – producing peptides and amino acids. Some of these resulting products are absorbed as they pass through the small intestine by intestinal mucosa in which brush border membranes peptidases and transport systems serve to transfer resulting amino acids into the blood. (Tomé, 1998).

The fate of hLF in the gastrointestinal tract is best illustrated by studies of breast-fed infants. Human lactoferrin is only slowly degraded (Tomé, 1998; Lindberg, 1998; Davidson, 1987, 1985; Spik, 1982a), thus significant amounts of hLF may reach the intestinal mucosa intact. Infants are known to excrete – via defecation – some hLF, about 5%, which is intact. (Davidson 1985; Davidson, 1987; and Spik, 1982a). However, the amount excreted varies significantly (it is greater early on when also the intake of hLF is higher) and decreases, due to increased proteolysis and lower intake, as the infant ages (Davidson, 1987). Fragments of hLF have been reported to be detected in the urine in human-milk fed preterm infants

(Hutchens, 1991 a and b) in which the immaturity of the gut may allow passage of larger amounts of intact hLF.

Plasma lactoferrin – as discussed above – is derived from neutrophils. (Nuijens, 1996). If hLF is given intravenously, it is rapidly cleared from the circulation via sequestration by the liver and spleen and eliminated via the urine. (Bennett, 1979). Such sequestered lactoferrin is quickly catabolized. (Bennett, 1979). The resulting iron, however, is not eliminated via the urine; rather, it is retained and slowly transferred to the bone marrow where it is eventually integrated into erythrocytes. (Bennet 1979).

6. Safety

The safety of **naturally-occurring** hLF has also been evaluated via preclinical and clinical studies. Relevant information – but not an overwhelming amount – was found via an extensive search of the available published, scientific literature. The search database included: Medline, the Hazardous Substances Data Bank, the Integrated Risk Information System, the Registry of Toxic Effects of Chemical Substances, Toxline and toxline special, the Development and Reproductive Toxicology Database, Gene-tox and the internet. The primary reason that few preclinical and clinical studies have been undertaken to evaluate the safety of **naturally-occurring** hLF is because there is a general consensus among experts that hLF has been shown to be so safe – via natural exposures – and at such high doses that no additional safety evaluation is necessary. Following is a summary of the safety evaluations that have been published.

Preclinical studies

Two reports are available on hLF in mature human milk fed to rhesus monkeys. (Davidson, 1990; Lindberg, 1997). These studies are summarized below (and more extensively at the end of Section V, i.e., this section).

Table 8

Preclinical Studies With Orally Administered nhLF

Ref.	Type of Animal	No. of Animals	Dose	Adverse Findings
Lindberg, 1997	Rhesus monkey			
	Preterm	10	20-40mg/kg BW/day for 1 day	None
	6-week	10	39-79mg/kg BW/day for 1 day	None
	7-month	10	25-50 mg/kg BW/day for 1 day	None
Davidson, 1990	Rhesus monkey			
	1 month	6	10mg/day for 1 day	None

Clinical studies

Various clinical studies involving oral administration of nhLF have been reported. Although all such studies involve human exposure to nhLF, not all were designed as safety studies. Nevertheless, all such studies are summarized below (and more extensively at the end of Section V, i.e., this section).

Table 9

Clinical Studies With Orally Administered nhLF

Ref.	No. Of Subjects	Infant or Adult	Dose of nhLF	Adverse Findings
Davidsson, 1994	4/4	I	Breast milk with/without ≥ 700 – 1000 mg hLF/day for 1 day	None

Goldblum, 1989	33	I	794-1504 mg/kg/day for 11 days	None
Trümpler, 1989	5	A	800 mg/day for 10 days	None
Davidson, 1987	3	I	1443-3537 mg/day for 1 day	None
Spik, 1982a	15	I	700 –1400 mg/day for 21 days	None
McMillan, 1977	8	A	100mg/day for 1 day	None

7. Acceptable Daily Intake For nhLF

In conclusion, the long-term, **natural** history of human consumption of hLF resulting from natural consumption of breast milk demonstrates that consumption levels equivalent to, at least, 266 (and perhaps as high as 3077) mg hLF/kg BW per day represent a safe – indeed, a natural, daily intake.[11] In addition, preclinical and clinical testing demonstrate that consumption of up to 3.5 grams of hLF per day (approximately 59 mg/kg BW/day for an adult) is safe.

B. Equivalence of Pharming's hLF and natural human lactoferrin

Published studies and other information demonstrate that Pharming's hLF is identical to nhLF, except with respect to some aspects of their respective pattern of glycosylation – which (as explained below) is expected and normal. More specifically, **both** Pharming's hLF and nhLF:

1. are the same metal-binding, glycoprotein, i.e., hLF (Thomassen, 2005; van Berkel, 2002; Anderson, 1989);
2. have the same amino acid sequence and composition based on the nucleic acid sequence (See subsection III(C)(1)(e));

[11] Similarly, to the extent that native **bovine lactoferrin** is similar to native hLF, the extensive safety data on bLF – as set forth and discussed in recent GRAS Notifications to CFSAN (see, e.g., GNs numbers 42, 67, especially 77, and 130) – has resulted in it being deemed GRAS for the same intended uses as are here in question.

3. have the same N-terminal protein sequence (van Berkel, 2002);
4. have the same protein structure (Thomassen, 2005);
5. are N-glycosylated (van Berkel, 2002);
6. have the same number and location of glycosylation sites (van Veen, 2004);
7. show the same chromatographic profiles upon analytical Mono S analysis (van Berkel, 2002);
8. have the same core-molecular weight (although overall molecular weight slightly differs – Pharming's hLF is slightly lower – due to the differences in the carbohydrate moieties attached to the LF core) (van Berkel, 2002);
9. show the same tryptic degradation kinetics, i.e., digestibility (van Veen ,2004);
10. have the same iron-binding and iron-release properties (van Berkel, 2002); and
11. are equally effective against experimental infections with multidrug-resistant S. aureus and K. pneumoniae in mice (van Berkel, 2002).

With respect to glycosylation, the utilization of N-linked glycosylation sites in hLF is similar for both molecules. (van Veen, 2004; van Berkel, 1996. However, natural hLF and Pharming's hLF have differences in the carbohydrate structures that are attached[12] – as one would expect, since the distribution and structures of attached glycans is species, tissue, cell type and protein-specific (James, 1995; Opdenakker, 1993) – as Pharming's hLF contains oligomannose- and/or hybrid-type N-linked glycans next to complex-type glycans (van Berkel, 2002), which are the only glycans found on natural hLF from human milk. (Spik, 1982b). In addition, the N-linked glycans of Pharming's hLF contain N-acetylgalactosamine next to galactose, which is typical for N-linked glycoproteins produced in bovine milk such as bovine lactoferrin. (Van den Nieuwenhof, 1999; Coddeville, 1992). Also, Pharming's hLF contains less fucose compared to the glycans of natural hLF. (van Berkel, 2002).

[12] Please note that hLF from milk and hLF from human neutrophils also differ in their respective glycosylation patterns . (Derisbourg, 1990). The glycan associated with neutrophil hLF is not fucosylated – thus, it resembles the glycan pattern of human serum transferrin. (Spik, 1994). However, such difference does **not** affect hLF's function with respect to molecular mass, isoelectric point, stability of the iron-saturated form, rate of clearance, or immunogenicity. (Moguilevsky, 1985; Derisbourg, 1990).

Finally, as a result of complex crystallography studies, it has been determined that – despite differences in N-linked glycosylation – the three-dimensional structure of Pharming's hLF and natural hLF are identical. (Thomassen, 2005).

C. Production organism

As the following subsections discuss in greater detail, there is nothing inherent in the production platform here in question, i.e., a platform that incorporates a dairy cow and the genetic construct described in Section III C (1), that increases any risk of any untowardness beyond that inherent in the dairy cow itself. Following are the details.

1. Host organism

Without in any way attempting to overstate or understate the obvious, experts indicate – based both on scientifically-founded determinations and common and long-term use for food – that cattle are safe as a good source of human food and that most bovine tissue (including those commonly-consumed meat, meat byproducts and dairy products are safe for human consumption **provided** such products are appropriately prepared, excessive consumption is not involved, and the cattle are born and raised pursuant to current good husbandry practices. Indeed, such tissues have been safely consumed for centuries – especially when and where the above-referenced practices are implemented and followed. Such practices – when modified in timely fashion – also assure safety even when new threats arise from time to time, such as in the case of BSE. Given such safe long-term history and the significant amount of technical information known by experts about this genus of ruminant animals, the dairy cow amounts to an excellent choice as a production platform – especially given the large amount of milk that can be produced by each animal per year, i.e., up to 10,000 liters, and thus the large amount of expected substance of choice that can be produced per year, i.e., in this instance 24kg. (van Berkel, 2002). Thus, provided the bovine representatives are born and raised pursuant to the above-referenced practices – which all animals used by Pharming are (as detailed especially in subsections of Section III in this notification) – such animals should be safe for use as a host and present no risk greater that the risks commonly associated with ordinary dairy cows – which risks can be and are adequately managed via compliance with the above-referenced practices.

2. Sources of DNA

Only two sources of DNA, i.e., DNA from cattle and a human, are involved in the genetic construct used here in the transgenic activities in question. The DNA of a fertilized oocyte eminated only from the semen of bulls and the oocytes of cows. The bovine casein-hLF transgenic fragment used for injection into each fertilized, bovine oocyte consists only of:

1. genomic hLF sequences, derived from genomic cosmid libraries (Blonden, 1989, see also subsection III(C)(1)(b));
2. which were fused to the **bovine**, αS_1-casein signal sequence (see subsection III(C)(1)(b));

(For a more detailed description, see US patent 6,140,552, Appendix 17). Since the bovine DNA here in question is found in cattle tissues long and widely consumed by humans and the human gene here in question is inherent in all humans, such gene sources should not pose any additional risk.

3. Introduced DNA sequences

The introduced DNA sequences used in the bovine casein-hLF transgene here in question are well-known, and have either been safely consumed by humans for centuries or are an inherent part of the human genome. Thus, such sequences are not believed to represent any additional risk of an untoward nature.

4. Production organism construction

Construction of the transgenic cows here in question is described in detail in Section III, subpart C, sub-subpart 1. Such construction is accomplished via use of only well-known and widely-used molecular biological techniques and common breeding techniques. Nothing is accomplished via such techniques that in any way increases the risk from such transgenic cows beyond the ordinary risk associated with ordinary dairy cows.

5. Stability of introduced DNA sequences

In order to test the stability of introduced genes, through generations, different molecular biology techniques can be used. At present, stability of introduced genes (i.e., the copy number) in cows is determined using PCR based techniques, namely Real-Time PCR. As compared to other molecular biology techniques, like fiber FISH, Real-Time PCR is more accurate in determining stability of copy numbers through different generations. Moreover, Real-Time PCR can also be used retrospectively which enables the determination and comparison of stability of copy numbers with the founder animal as a standard.

D. Notified substance identity

As indicated in extensive discussions appearing throughout this notification, especially those set forth in subsections III(A), III(B), III(C)(1)(e), IV, and V((B), the identity of the notified substance here in question is human lactoferrin.

E. Absence of production organisms in notified substance

Unlike the possible situation involving, for example, the production of some enzymes from recombinantly modified microorganisms, the notified substance here in question, i.e., human lactoferrin, does not contain any production organisms.

F. Absence of transformable DNA in notified substance

The transgenic cows here in question – used to produce the milk from which Pharming's human lactoferrin is produced – are secretors; thus, there is no transformable DNA in the notified substance.

G. Absence of antibiotic resistance genes

No antibiotic resistance genes are used in the production of the transgenic cows here in question and there are no antibiotics in the notified substance.

H. Safety testing

Pharming's hLF has been evaluated in numerous preclinical studies. Such studies are discussed below.

1. Preclinical Studies

a. Genotox studies

Pharming's human lactoferrin has been tested by TNO (an independent testing laboratory in Zeist, The Netherlands) to investigate its genotoxic potential in both *in vitro* and *in vivo* genotoxicity screening assays. These assays include a bacterial reverse mutation test (i.e., an Ames test), a micronucleus test in bone marrow cells of mice, and a mouse lymphoma forward mutation assay in salmonella. All studies were conducted pursuant to the OECD Principles of Good Laboratory Practice. Following – in summary - are the results:

Table 10

Results of Genotoxicity Testing of Pharming's hLF

Test system	Test object	Concentration of rhLF	Results
Ames test *	Salmonella typhimurium TA 1535, TA 1537, TA 98, TA 100 E. coli WP2 uvrA	Up to 5000 µg/plate	Negative
Mouse bone marrow micronucleus test	Male CD-1 mice	0-2000 mg/kg b.w.	Negative
Mouse lymphoma forward mutation assay *	L5178Y $TK^{+/-}$ cells	Up to 5000 µg/ml	Negative

** With and without S-9 metabolic activation*

As the foregoing results indicate, Pharming's hLF is **not** genotoxic. (For further details, see Appendicies 4, 5, and 6 and Ziere, 2005).

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b. Animal studies

The safety of Pharming's hLF has also been evaluated via oral tox studies. Following is a discussion of that testing and the results.

1. 14-day oral range-finding study in neonatal rats with Pharming's hLF

Methods: In a study conducted by Covance (Vienna, Virginia), Pharming's human lactoferrin was administered by oral gavage for 14 consecutive days to groups of 12 male and 12 female neonatal Sprague-Dawley rats. Five groups of rats (F1 pups) were treated from days 7-20 of lactation at dosage levels of 0, 10, 100, 1000 and 6000 mg/kg/day in t.i.d. (approximately 8 hours apart). Parameters evaluated included survival, clinical observations, body weights, clinical pathology, organ weights, and macro-and microscopic pathology. This study was conducted pursuant to FDA's GLP regulations and prior-approved by the IACUC at Covance.

Results: There were no treatment related findings based on body weights, organ weights, clinical pathology, and anatomic pathology. The mean body weight change values were significantly increased in the 1000 mg/kg/day rats, but not in the 6000 mg/kg/day rats. The new findings that were observed (i.e., change in organ weight ratio and clinical pathology) were not dose dependant and, thus, considered to be incidental and not treatment related.

In this study, all doses of Pharming's hLF were well-tolerated; therefore, the maximum tolerated dose level was determined to be **greater than 6000 mg/kg/day.** (For further details and information, please see Appendix 7 and Ziere, 2005).

2. Sub-chronic (13-week) oral toxicity study with rhLF in rats

Methods: In a study conducted at TNO (Zeist, The Netherlands), Pharming's human lactoferrin was administered daily by oral gavage for 13 consecutive weeks to 4 groups of 20 Wistar rats / sex. The control group received physiological saline and the three test groups received daily dosages of 200, 600 or 2000 mg Pharming's hLF/kg BW/day. Parameters evaluated included clinical observations, growth, food consumption, food conversion efficiency, water consumption, neurobehavioral testing, ophthalmoscopy, haematology, clinical chemistry, renal concentration test,

urinanalysis, organ weights and gross examination at necropsy and microscopic examination of various organs and tissues. This study was conducted pursuant to The Netherland's GLP Compliance Monitoring Principles of Good Laboratory Practice.

Results: Overall, no treatment-related, toxicologically significant changes were observed in clinical observations, growth, food consumption, food conversion efficiency, water consumption, neurobehavioral parameters, ophthalmoscopy, hematology, clinical chemistry, urinary volume and density, urinanalysis, organ weights and gross macroscopy and microscopic examination. The minor findings that were noted that were found in the high dose groups (i.e., lower cholesterol in females, higher urinary density in males, lower urinary pH in males and females, and very slightly higher kidney weight in females) were considered of no toxicological significance.

The no-observed-adverse-effect level (NOAEL) was considered to be **2000 mg/kg body weight/day.** (For further details, see Appendix 8; Ziere, 2005; and Appel, in press).

3. Related rhLF studies

Additional **preclinical studies** have evaluated the safety of hLF produced by entities other than Pharming. They are summarized below (and more extensively at the end of Section V, i.e., this section).

Table 11

Preclinical Studies Using Orally Administered Non-Pharming rhLF's

Ref.	Type Study	No. Of Animals	Dose	Adverse Findings
Humphrey, 2002	18-day	660 3-day old broiler chicks	0-5% rhlf/diet	None
GN No. 162, p. 23(2004)	28-day	Rats	1000mg/kg BW/day	None

GN No. 162, p. 23 (2004)	28-day	Rats	1800 mg/ kg BW/day	None
GN No. 162, p. 64 (2004)	4 mos.	Infant rehsus monkeys	0.5-0.8 g/kg BW/day	None
GN No. 162, p. 66 (2004)	28-day	Rats	100, 500 or 1000 mg/kg BW/day	None

The foregoing information indicates that consumption of hLF up to 1800 mg rhLF per kg BW per day does not result in any adverse findings.

c. Allergenicity studies

Pharming's human lactoferrin has also been tested by TNO in animals to investigate its allergenic potential in a maximization test in guinea pigs, a dermal irritation/corrosion test in rabbits, a eye irritation/corrosion test in rabbits and an acute inhalation limit test in rats. All studies were conducted pursuant to the OECD Principles of Good Laboratory Practice. Following are the details (for further details, see Appendicies 9, 10, 11 and 12):

1. **Maximization test** (in guinea pigs)

Methods: In this study, a sample of Pharming's hLF was tested for its possible sensitizing properties in an experiment, i.e., a maximization test, with 10 test animals and 5 controls (i.e., fifteen Dunkin Hartley guinea pigs). Prior to conducting the main study, preliminary experiments were conducted to determine the correct concentrations for intradermal injection, topical induction and topic challenge. In the main study, fifteen guinea pigs were randomly divided into two groups, i.e., one test group of 10 animals and one control group of 5 animals. All animals were site clipped, pair injected intradermally (to achieve induction) with 0.1 ml (see Table 12 below, and skin read after 24 hours. Six days after the intradermal injections, all animals were site clipped again, enhanced sensitivity treated (with a 10 percent solution of sodium lauryl saline, and one day later a topical application (containing 30 percent (w/v) of Pharming's hLF in saline) were applied. Skin readings were conducted 48 hours later. Finally, fourteen days later all animals were topically challenged with a saline solution containing either 30% or 10% Pharming's hLF. This study was executed

according to EEC Directive 92/69/EEC, method B.6, and OECD guideline No. 406.

Table 12

Induction Treatment: Intradermal Injections

	Test animals		Control animals	
	Test substance	Dilution	Test substance	Dilution
1	FCA:saline	1:1	FCA:saline	1:1
2	10% rhLF in saline (vehicle)	-	saline vehicle	-
3	10% rhLF in saline:FCA)	1:1	FCA:saline (vehicle)	1:1

FCA: Freunds Complete Adjuvant

Results: Skin readings were obtained (24 hours after the induction treatment, 28 hours after the topical induction and 24 and 48 hours after the topical challenge), evaluated and scored according to scale. Positive results were evaluated per EC standards. The challenge treatment with Pharming's hLF (at 30% and 10%) did not cause signs of sensitization. Per use of pertinent EC standards, the test article was deemed **not** to be a sensitizer.

2. **Dermal irritation/corrosion test** (in rabbits)

Methods: A sample of Pharming's human lactoferrin was tested for acute dermal irritating properties in an experiment with three male New Zealand White albino rabbits. One day prior to the start of the study, the hair was removed from the back and flanks of the animals using electric clippers. An amount of 1g of Pharming's hLF was distributed over a cotton gauze patch (2.5 x 2.5 cm) and fixed to the application site. After 4 hours of exposure, the test patch was removed and the test site was cleaned with water. Skin reactions were evaluated 1, 24, 48 and 72 hours after treatment.This study was executed according to EEC Directive 92/69/EEC, method B.4 and OECD guideline no. 404.

Results: At 24 hours after removal, one rabbit showed very slight erythema and very slight oedema. At 1, 48 and 72 hours after removal of the test item, no signs of skin irritation were observed in any of the three rabbits. According to the EC-standards (as published in the Official Journal of the European Communities, L 110 A, Volume 36, 4 May 1993), Pharming's hLF is **not irritating** to (human) skin.

3. **Eye irritation/corrosion test** (in rabbits)

Methods: Previous to this experiment, an *in vitro/ex vivo* Non-GLP Chicken Enucleated Eye test was executed which did not result in significant signs of irritation. In this study, a sample of Pharming's human lactoferrin was examined for acute eye irritating/corrosive properties in three male New Zealand White albino rabbits. One eye was treated with Pharming's hLF (0.012 g) and the other eye, which remained untreated, served as a control. The reactions of the eyes were judged at 1, 24, 48 and 72 hours after treatment. This study was executed according to EEC Directive 92/69/EEC, method B.5 and OECD guideline no. 405.

Results: Corneal opacity, iritis, redness and swelling of the conjunctivae of each rabbit were determined at 1, 24, 48 and 72 hours after removal of Pharming's hLF. No signs of irritation were observed in any of the three rabbits. According to the EC-standards (as published in the Official Journal of the European Communities, L 110 A, Volume 36, 4 May 1993), Pharming's hLF is **not irritating** to (human) eyes.

4. **Acute inhalation limit test** (in rats)

Methods: In this study, a sample of Pharming's hLF was tested for its possible inhalation toxicity via a nose-only exposure of one group of five male and five female SPF-reared Wistar rats. The test animals were subjected to a test atmosphere – over the standard 4-hour period – containing an aerosol of Pharming's hLF at the limit concentration of 5.0 g/m^3. The test material was delivered as flat crystals. To aerosol this material, it was first milled 10 times during 30 seconds using a universal mill and subsequently during 60 minutes using a ball mill. The test atmosphere was generated by passing the milled test material to an eductor using a dust feeder which was placed at the inlet of the exposure unit. The test material was delivered in a slip stream of dry air. The Mass Median Aerodynamic Diameter of the particles in the test atmosphere measured twice during exposure was 4.4 and 4.3 μm, respectively. During the experiment the concentration of rhLF was measured by gravimetric analysis every 15 minutes. After the 14-day observation period, the rats were necropsied and examined for gross pathological changes. This study was designed and executed according to the OECD Guidelines for Testing of Chemicals no. 403, Acute inhalation toxicity, adopted 12 May, 1981; the EEC Directive 92/69/EEC, Annex, Part B, Methods for the determination of toxicity, dated

31 July 1992; and the OECD Principles of Good Laboratory Practices (as revised in 1997), OECD, Paris.

Results: Clinical signs were monitored prior to, during and repeatedly after exposure. The only clinical sign noted occurred during exposure when all animals at all hourly observations time points exhibited "slightly decreased breathing rate". Such sign disappeared shortly after exposure and was not present during the 14-day observation period. Mortality did not occur. Body weight gain was considered to be within normal limits. No macroscopic changes (i.e., treatment related effects) were noted during necropsy. Thus, despite exposure to the limit concentration, Pharming's hLF was deemed **not** to induce any toxicity up to $5.23 g/m^3$.

Please note that all of the above-discussed pre-clinical investigations have been publically reported and published. (Ziere, 2005; and Appel, in press, with regard to the 90-day study).

2. Clinical Studies

Various clinical studies have investigated orally administered hLF formulations produced by Pharming and others. They are reported below.

a. Pharming Study

Pharming has conducted one **orally administered**, clinical study of natural hLF and its hLF product. The study was not designed as a safety study (but is included here in the spirit of full disclosure). Rather, the study was designed to show the biodistribution of human lactoferrin (both nhLF and PhLF). When delivered by a gastric resistant or a gastric non-resistant preparation. The study was a limited study both in dose, i.e., 52 mgs per exposure, and frequency, i.e., six subjects received two times the dose of nhLF or PhLF (but only 52 mgs in any one 24-hour period). Following are the details of the study.

Study of biodistribution of human lactoferrin in the gastrointestinal tract of healthy volunteers using gastric resistant and non-resistant preparations (study including hLF0101-01 with nhLF and hLF0101-02 with Pharming's hLF)

Study design and objective: This study was an open-label, two-period, two-way, cross-over study in normal healthy volunteers. The study was conducted in accordance with the International Conference on Harmonisation (ICH) Guidelines on Good Clinical Practice (GCP) and the Declaration of Helsinki. Six healthy volunteers received a **single Eudragit-coated tablet** containing 52 mg of Pharming's hLF during one period and **a hard gelatin- coated tablet** containing 52 mg of Pharming's hLF during the other period. The main objective of the study was to assess and compare gastrointestinal release, biodistribution, site, and extent of absorption and gastric emptying time of both formulations.

Conclusion: No differences were seen in the behavior of the natural hLF (study hLF0101-01) and Pharming's hLF (study hLF0101-02). Both formualtions behaved similarly in the GI tract. The only difference noted in the study was the time of passage through the stomach of the gastric-reisistant and non-resistant capsules – as was expected. Both nhLF and Pharming's hLF caused no adverse events nor raised any additional safety concerns. (For further information, see Appendix 13).

b. Other related non-Pharming rhLF clinical studies

Various other non-Pharming rhLF clinical studies have also been reported in the scientific press in recent years. They too are supportive of the safety of rhLF. They are summarized below.

Table 13

Clinical Studies Using Orally Administered Non-Pharming rhLF

Ref.	No. Of Subjects (Adults)	Dose	Adverse Findings
Wang, 2005	55	1.5 g/day for 35 days	None
Hayes, 2004	27	4.5 or 9 g/day for 14 days	None

Andersen, 2004	30	1.5 to 9g/day for 14 days	None
	210	0 to 9g/day for 14 days	None
	15	15g/day for 1 day	None
GN No. 162, p. 67 (2004)	5	4.8 gm/day for 42 days	None
Troost, 2003	15	15 g/day for 1 day	None
VAMC, 2003	30	1.5 g/day-9 g/day for 14 days	None
Guttner, 2003	6	5 g/day for 5 days	None
	3	5 g/day for 14 days	None
Hayes, 2003	33	1.5, 4.5 or 9 gm/day for 14 or 28 days	None
Troost, 2002	8	5 g/day for 1 day	None
Opekun, 1999	6	1.25 g/day for 1 day	None
	6	5 g/day for 1 day	None

As the foregoing information indicates, rhLF has been repeatedly orally evaluated – in at least 10 clinical studies (all of which are summarized at the end of Section V, i.e., this section) - via exposures of up to **15 grams** rhLF (within a 24-hour period). No adverse findings were reported at any of the dosages given, i.e., from 1.5 grams to 15 grams per day (or 250mg rhLF per kg BW per day for an average adult). Such results indicate that rhLF is safe at daily consumption levels far in excess of any EDI likely to result from the use of Pharming's hLF as indicated in this GRAS Notification.

3. Allergenicity/Immunogenicity

At the outset, Pharming is well aware of the communications between FDA (i.e., CFSAN) and the sponsors of GRAS Notification numbers 42, 67, 77 and 130 concerning the potential for bovine LF (bLF) to sensitize individuals who are not already allergic to (bLF) and/or induce /exacerbate autoimmune disorders. Even though the experts involved in those notifications indicated that the occurrence of either event was "highly unlikely", they recommended that food products containing bLF be "clearly labeled as containing a milk protein so that existing milk-allergic consumers can avoid these products." (See, e.g., Agency Response Letter to GRN000077, dated 8/14/01, at page 3). Since Pharming's hLF product may contain residues of proteins from native cow's milk (including possibly the major milk allergens such as casein, ß-lactoglobulin, and a-lactalbumin), Pharming's product will be intended for use with the same, above-referenced cautionary information on product labeling.[13]

Thus, the remaining discussion below deals with the theoretical potential of whether Pharming's hLF might be immunogenic or might induce any allergenic symptoms in those consuming Pharming's hLF in a food product.

WHO/FAO has devised an international decision tree approach for assessing/predicting the allergenic potential of food, including proteins, derived from biotechnology. (WHO/FAO, 2001). In this decision tree approach, attention is given to:

1. the **source of the gene**;
2. the **sequence homology** of amino acids of the recombinant protein with well-known food allergens;
3. the specific and targeted serum screen (to study, respectively, **specific and cross reactivity** of the new protein with sera of allergic patients);
4. the **process and digestive stability** of the new protein; and
5. the assessment of the potential allergenicity in **animal models** focused on sensitization.

[13] Such labeling is also mandated by the Food Allergen Labeling And Consumer Protection Act of 2004 (a U.S. Act) and by European Directive 2003/89/EC.

Each of these areas are discussed below as they relate to Phaming's hLF.

a. Source of the gene

Pharming's hLF emanates from a human gene (as does native human LF) and is, therefore, not derived from an allergenic source. Human LF is a major naturally-occurring protein that is present in human milk and various other human biological fluids including, saliva, tears, and mucosal secretions. The equivalence of Pharming's LF and natural LF is further clearly supported by the observation that their physical, (bio)chemical and biological properties are comparable, except for some aspects of their glycosylation. (See Section VB). In addition, the immunological identity of Pharming's hLF and nhLF has been demonstrated in various studies. For example, double antibody assays showed that Pharming's hLF – produced in mice – and nhLF were immunologically identical. This was demonstrated by identical slopes and maximal responses in the radioimmunoassay, which indicated that the antigenic determinants for monoclonal- and polyclonal anti-hLF antibodies in natural hLF and Pharming's hLF are equally well accessible. (Nuijens, 1997a).

Various clinical investigations that have specifically tested human lactoferrin (administered both topically or orally) for its potential allergenicity have never identified hLF as being an allergen. (E.g., see, Yamauchi, 1998; Griffiths, 2001; and Zimecki, 2001). Indeed, humans are considered to be tolerant for hLF and, therefore, when searched against databases of known allergens (e.g., SWISS-PROT and TrEMBL databases and FARRP databases (Pearson, 1988)), human lactoferrin is not identified as an allergen.

b. Sequence homology

In order to evaluate whether Pharming's hLF has the ability to induce allergy, Pharming contracted TNO (Zeist, The Netherlands) to perform a BLAST search comparing the entire sequence of Pharming's hLF against the Food Allergy Research and Resource Program (FARRP) database. (See Appendix 16). To this end, a complete set of 80-amino acid length sequences (N=613) – derived from Pharming's hLF and together spanning the entire protein – were prepared and individually compared with all of the amino acid sequences contained in the FARRP database. The FARRP database is a public database that is accessible at:

http://www.allergenonline.com/asp/public/About.asp and contains unique known and putative allergens (1191 sequence entries) that have been identified by searching (via Entrée search and retrieval) a variety of publicly available protein databases (including Genbank and EMBL version 119, PIR version 56, PRF, POB, the NRL3D version 56 of RCSB PBD, and SwissProt version 38) using the key words "allergen" and "celiac" and by searching Medline.

The FARRP database utilizes a sequence comparison routine, i.e., FASTA. (Pearson, 1988). This version of the FASTA search interface utilizes the FASTA3 (Pearson, 2000) algorithm to evaluate – in this instance – whether any of the hLF protein sequences are identical to or homologous with known or putative allergens and gliadins in the database. Alignments with high identity scores may indicate a potential for allergenic cross-reactions. However, there is not sufficient scientific data to establish a simple scoring boundary (E-score or percent identity), beyond which cross-reactivity is certain, or below which cross-reactivity is not possible. Based on historical data, cross-reactivity is not likely for proteins with less than 50% identity over the entire protein sequence, and is fairly common above 75% identity. (Aalberse, 2000). According to the WHO/FAO guidelines for allergenicity evaluation of foods derived from biotechnology, a query protein is potentially allergenic if it either has an identity of at least 6 contiguous amino acids or more than 35% sequence similarity over a window of 80 amino acids when compared with a known allergen. (WHO, 2001).

The 80-mer sliding window search revealed that hLF shares significant homology with two allergens described in the FARRP database, i.e., ovotransferrin precursor (chicken) and ovotransferrin (chicken). The best percentage identical amino acids (%ID) in any 80-mer sequence in both hits was about 67%. All 613 overlapping 80-mer from hLF showed >35% homology with ovotransferrin (precursor). The percentage identical amino acids in the full alignment (the whole protein, not just the 80-mer sequences) was 52% in both hits.

Despite this identity of more that 50% for chicken ovotransferrin precursor (52.2%) and chicken ovotransferrin (51.9%), it was clearly demonstrated (in GRAS Notification 162) that there is no immunological cross-reactivity between chicken ovotransferrin and human lactoferrin or recombinant human lactoferrin produced in rice. In *in vitro* studies using

Western blots, the cross-reactivity of a rabbit IgG antibody to human lactoferrin was compared against recombinant human lactoferrin from rice, human lactoferrin from milk, bovine lactoferrin, human transferrin, bovine transferrin and chicken egg ovotransferrin. Moreover, antibodies against bovine lactoferrin and bovine transferrin, as well as antibodies against human transferrin and chicken ovotransferrin were tested for cross-reactivity against human lactoferrin and recombinant human lactoferrin from rice. Apart from expected cross-reactivity between human and recombinant human lactoferrin antigens and their respective antibodies, no other cross-reactivity was observed among any of the combinations with related proteins.

The foregoing information indicates that – as antibodies against native human LF did not cross react with ovotransferrin, and antibodies to ovotransferrin did not cross react with human LF – the observed amino acid sequence homology of approximately 52% between Pharming's hLF and chicken ovotransferrin does not pose an allergenic safety issue.

c. Cross reactivity

As a 70% amino acid sequence homology exists between native hLF and bovine lactoferrin, cross reactivity might be suspected between IgE antibodies to bLF from cow's milk allergic patients and hLF or Pharming's hLF. However, based on various observations, this cross-reactivity is very unlikely to occur. First, in the studies with rabbit antibodies against hLF, no cross-reactivity was observed with bLF and antibodies to bLF did not cross-react with hLF. (GN 162, pp. 51-53, 2004). Second, a search against a database of known allergens does not identify bLF as an allergen as there is almost no clinical information that bovine lactoferrin is an allergen. Third, there are hardly any sera from cow's milk allergic individuals that are mono IgE sensitized for bLF. (Wal, 1998). Finally, the absence of cross-reactivity between antibodies to bLF and hLF is further supported by studies with 2 unique serum samples from individuals mono-sensitized for bLF which did not show any cross-reactivity with human LF. (See, GRAS Notification 162).

The foregoing information indicates that human sensitization to bLF is unlikely and that cross-reactivity of IgE antibodies to bLF with human LF is also unlikely. In any case, food products containing Pharming's hLF are

recommended to be labeled precautionary for milk allergic patients in order to avoid these products.

d. Glycosylation

It is known that glycosylation may affect the allergic potential of recombinant proteins – in particular if they are produced in plants – by changing susceptibility to protein processing and proteolysis or by changing epitope structure, e.g., by introducing glycan epitopes, which are known to be highly cross-reactive. Introduction of highly cross-reactive glycans epitopes will in particular be of importance for recombinant proteins produced in plants. In contrast, both Pharming's hLF and nhLF are both mammalian derived despite the differences in glycosylation between both molecules. (See Section VB; and van Berkel, 2002). Moreover, they do not differ in the number and location of the glycosylation sites. (van Veen, 2004). Finally, carbohydrate structures are not generally considered to be allergens. (Dr. Pennink, Appendix 18). Thus, with regard to the allergic potential of the glycosylation of Pharming's hLF, Dr. A. H. Penninks (an expert immunologist) indicates (See Appendix 18) that based on current scientific knowledge, it is "very unlikely that the bovine glycosylation of Pharming's hLF will result in clinical symptoms of allergy from the consumption of foods containing Pharming's hLF".

Based on these observations, the bovine glycosylation of Pharming's hLF should **not** be a safety issue with respect to its potential allergenicity (i.e., its sensitizing potential and/or cross-reactivity).

e. Human LF as a potential auto-immunogen

Since hLF is well-recognized as a target antigen for anti-neutrophil cytoplasmic antibodies (ANCA) in a variety of autoimmune diseases, the possibility is raised that hLF may exacerbate autoimmune diseases. As of this date, despite an extensive clinical literature on the existence of lactoferrin ANCA in a number of autoimmune diseases, there is so far no evidence that such antibodies play any role in the pathology of autoimmune disease. Thus, while there are theoretically reasons for considering that lactoferrin could prove to be a mediator of autoimmune disease, current scientific evidence and consensus indicate that such scenario is very unlikely.

f. Digestive stability

As several well-known food allergens are known to be resistant to processing and digestion, attention should be given to these aspects in the safety assessment of recombinant proteins. From various digestion studies, it is clear that the digestion of Pharming's hLF and natural LF is comparable; all dietary hLF is digested in the upper GI tract and does not reach the colon. (See, Section VA5; Van Veen, 2004; and Troost, 2002). Accordingly, the non-resistance of Pharming's hLF to digestion should **not** pose a safety issue with respect to its potential allergenicity.

g. Immunological identity as demonstrated in animal models

To assess the potential allergenicity of (recombinant) proteins, additional information can be generated using available animal models. As human proteins will, in general, be immunogenic in rodents, for example, the available rodent models (which are still in development) cannot be used. An opportunity to study the sensitizing potential of a recombinant human protein in comparison with its natural product could be accomplished via use of transgenic animal models. In such models, e.g., with rodents, animals can be made transgenic to the natural human protein that will result in immunological tolerance to this human protein. Upon subsequent immunization with the recombinant human protein, this will not – in case of immunological identity of the recombinant human protein with the natural human protein – result in antibody production. However, if immunological differences exist between the two proteins, antibodies will be produced. Such studies can also be performed in animals made transgenic to the transgenic human protein and subsequent immunization with the natural human protein.

In mice made transgenic for Pharming's hLF, subsequent repeated immunizations with natural hLF did not result in antibody production against natural hLF. (Nuijens, 1997)a. As could be expected, a strong antibody response was observed against natural hLF in non-transgenic mice immunized with natural hLF. (Nuijens, 1997)a. These results clearly indicate that natural hLF is recognized as "self" by the immune system in the Pharming's hLF transgenic mice, and that Pharming's hLF is immunologically identical to hLF.

Finally, based on a weight of evidence approach using the foregoing information, it is clear that Pharming's hLF, as well as natural hLF, will not provoke an allergic or any other harmful immunological response.

I. Applications and intake estimate

1. Proposed applications in foods

Pharming's hLF is proposed for use as an ingredient in sports and functional foods. More specifically, such hLF is proposed for use in the foods in question at 100 mg per product serving.

2. Estimated daily intake of human lactoferrin by the U.S. population from proposed food-uses[14]

Intended use of hLF and levels of use of foods

Pharming intends to market its human lactoferrin as a food ingredient in a variety of foods products in the United States.[15] Food codes representative of each proposed food-use were chosen from the CSFII 1994-1996, 1998 (USDA, 2000) and grouped in food-use categories according to Title 21, Section §170.3 of the Code of Federal Regulations (U.S. FDA, 2005a). Serving sizes were assigned according to Title 21, Section §101.12, Reference Amounts Customarily Consumed Per Eating Occasion (RACC) (U.S. FDA, 2005b). Product-specific adjustment factors were developed based on data provided in the standard recipe file for the CSFII 1994-1996, 1998 survey (USDA, 2000). The individual proposed food uses and use levels of hLF from all proposed food-uses are summarized in Table 14.

[14] The information referenced and discussed in this subsection was generated and prepared by CANTOX Health Services International (which is located in Mississauga, Ontario, Canada).

[15] Such food products are all contained within six food groups. These food groups include "meal replacements" (dry, fluid and solid) (which group includes 56 food items), "yogurts, including frozen" (which group includes 42 food items), "Popsicles (dairy containing only)" (which group includes 9 food items), and "sports beverages" (which group includes 1 food item). All six food groups contain 108 food items.

Table 14

Summary of the Individual Proposed Food-Uses and Use-Levels for Human Lactoferrin in the United States

Food Category	Proposed Food-Use	Use-Level (mg/RACC*)	Use-Level (%)
Beverages and Beverage Bases	Meal Replacements (Not-Milk Based)	100	0.042
	Sports Beverages	100	0.042
Frozen Dairy Desserts and Mixes	Frozen Yogurt	100	0.083
	Ice Cream and Other Frozen Milk Desserts	100	0.083
Grain Products and Pastas	Cereal, Energy and Health Bars	100	0.250
Milk Products	Milk-Based Meal Replacements	100	0.042
	Yogurt	100	0.044

RACC – Reference Amounts Customarily Consumed Per Eating Occasion (21 CFR §101.12).

Estimated consumption of Pharming's human lactoferrin from proposed food-uses.

The consumption of Pharming's hLF from all proposed food-uses was estimated using the USDA 1994-1996 Continuing Survey of Food Intakes by Individuals (USDA CSFII 1994-1996) and the 1998 Supplemental Children's Survey (USDA CSFII 1998) (USDA, 2000). Combined, these surveys provide the most appropriate data for evaluating food-use and food consumption patterns in the U.S., containing 4 years of data on individuals selected *via* stratified, multistage area probability sampling of American households within all 50 states. USDA CSFII (1994-1996, 1998) survey data were collected from individuals and households *via* 24-hour dietary recalls administered on 2 non-consecutive days (day 1 and day 2) throughout all 4 seasons of the year. Data was collected in-person, a minimum of 3 days apart, on different days of the week, to achieve the desired degree of statistical independence. USDA CSFII (1994-1996) contains 2-day dietary food consumption data for more than 15,000 individuals of all ages, and 1-

day data for 16,103 individuals. USDA CSFII (1998) contributes consumption data from an additional 5,559 children from birth through 9 years of age to data reported for 4,253 children of the same ages within USDA CSFII (1994-1996). The overall USDA CSFII (1994-1996, 1998) response rate for individuals selected for participation in surveys was 81.5 and 77.5% for day 1 and day 2, respectively.

In addition to collecting information on the types and quantities of foods being consumed, USDA CSFII (1994-1996, 1998) collected physiological and demographic information from individual participants in the survey, such as sex, age, self-reported height and weight, and other variables useful in characterizing consumption. The inclusion of this information allows for further assessment of food intake based on consumption by specific population groups of interest within the total population. USDA sample weights were developed and incorporated with USDA CSFII (1994-1996, 1998) data to correct for potential under-representation of intake that results from variability in samples due to survey design, non-response, or other factors.

Estimates for the daily intake of Pharming's hLF represent projected averages over 2 days (day 1 and day 2) of USDA CSFII (1994-1996, 1998) data. Individual consumption data were collated by computer and the resulting distributions were analyzed statistically. All-person intake refers to the intake of Pharming's hLF averaged over all individuals surveyed regardless of whether they consumed food products formulated with Pharming's hLF, hence the 'all-person' designation. All-user intake refers to the intake of Pharming's hLF by individuals consuming food products formulated with Pharming's hLF, hence the 'all-user' designation. Individuals were considered users if they consumed 1 or more food products formulated with Pharming's hLF on either day 1 or day 2 of the survey.

Calculations for the mean and 90th percentile all-person and all-user intakes, and percent consuming were performed for each of the following population groups:

1. infants, ages 0 to 2;
2. children, ages 3 to 11;
3. female teenagers, ages 12 to 19;
4. male teenagers, ages 12 to 19;
5. female adults, ages 20 and up;

6. male adults, ages 20 and up; and
7. total population (all population and gender groups combined).

The estimated total consumption of hLF from all proposed food-uses is summarized in Tables 15 and 16 on a mg/person and mg per kg body weight basis, respectively.

Table 15

Summary of the Estimated Daily Intake of Human Lactoferrin in the United States by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (mg)	90th Percentile (mg)	Mean (mg)	90th Percentile (mg)
Infant	0-2	15.6	558	9.07	37.40	52.00	100.80
Children	3-11	20.3	1,277	12.60	49.90	64.14	122.34
Female Teenager	12-19	14.8	104	14.10	46.25	91.88	176.53
Male Teenager	12-19	17.0	118	24.21	97.50	136.74	252.00
Female Adult	20 and Up	16.7	764	16.97	53.90	95.42	206.25
Male Adult	20 and Up	13.4	638	19.66	53.16	135.69	294.15
Total Population	All Ages	16.8	3,459	17.18	52.92	102.42	214.20

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Table 16

Summary of the Estimated Daily Per Kilogram Body Weight Intake of Human Lactoferrin in the United States by Population Group (1994-1996, 1998 USDA CSFII Data)

Population Group	Age Group (Years)	% Users	Actual # of Total Users	All-Person Consumption		All-Users Consumption	
				Mean (mg/kg)	90th Percentile (mg/kg)	Mean (mg/kg)	90th Percentile (mg/kg)
Infant	0-2	15.6	558	0.77	2.94	4.32	8.47
Children	3-11	20.3	1,277	0.53	1.98	2.65	5.50
Female Teenager	12-19	14.8	104	0.25	0.81	1.57	2.89
Male Teenager	12-19	17.0	118	0.39	1.39	2.19	4.17
Female Adult	20 and Up	16.7	764	0.26	0.87	1.48	3.39
Male Adult	20 and Up	13.4	638	0.24	0.65	1.67	3.55
Total Population	All Ages	16.8	3,459	0.32	1.00	1.91	3.95

Approximately 16.8 % of the total U.S. population was identified as potential consumers of Pharming's hLF from the proposed food-uses (3,459 actual users identified). Consumption of these types of foods by the total U.S. population resulted in estimated mean all-person and all-user intakes of Pharming's hLF of 17.18 mg/person/day (0.32 mg/kg body weight/day) and 102.42 mg/person/day (1.91 mg/kg body weight/day), respectively (Tables 2-1 and 2-2). All-person and all-user 90th percentile intakes of Pharming's hLF from all proposed food-uses by the total population were estimated to be 52.92 mg/person/day (1.00 mg/kg body weight/day) and 214.20 mg/person/day (3.95 mg/kg body weight/day), respectively.

Of the individual population groups, children were estimated to comprise the greatest percentage of users at 20.3%. The greatest mean all-person and all-user intakes of Pharming's hLF on an absolute basis were

determined in male teenagers, at 24.21 mg/person/day (0.39 mg/kg body weight/day) and 136.74 mg/person/day (2.19 mg/kg body weight/day), respectively. Infants had the lowest intakes of Pharming's hLF on an absolute basis, with mean all-person and all-users intakes of 9.07 and 52.00 mg/person/day, respectively. On a body weight basis, mean all-person and all-user intakes of Pharming's hLF were highest in infants (0.77 and 4.32 mg/kg body weight/day), and lowest in male (0.24 and 1.67 mg/kg body weight/day) and female (0.26 and 1.48 mg/kg body weight/day) adults (Table 2-2).

When heavy consumers (90th percentile) were assessed, all-person and all-user intakes of Pharming's hLF from all proposed food-uses also were determined to be greatest in male teenagers (97.50 and 252.00 mg/person/day) and male adults (53.16 and 294.15 mg/person/day) and lowest in infants (37.40 and 100.80 mg/person/day, respectively) on an absolute basis (Table 2-1). On a body weight basis, infants were determined to have the greatest all-person and all-user 90th percentile intakes of Pharming's hLF at 2.94 and 8.47 mg/kg body weight/day, respectively (Table 2-2). The lowest all-person and all-user 90th percentile intakes of Pharming's hLF on a body weight basis were observed in male adults (0.65 and 3.55 mg/kg body weight/day, respectively) and female teenagers (0.81 and 2.89 mg/kg body weight/day, respectively).

Conclusion

Consumption data and information pertaining to the individual proposed food-uses of Pharming's hLF were used to estimate the all-person and all-user intakes of Pharming's hLF for specific demographic groups and for the total U.S. population. This type of intake methodology is generally considered to be worst case as a result of several conservative assumptions made in the consumption estimates. For example, it is often assumed that all food products within a food category contain the ingredient at the maximum specified level of use. In addition, it is well established that the length of a dietary survey affects the estimated consumption of individual users. Short-term surveys, such as the typical 2- or 3-day dietary surveys, overestimate the consumption of food products that are consumed relatively infrequently; however, adequately indicate the consumption of food products that are consumed frequently.

In summary, on an all-user basis, the mean intake of Pharming's hLF by the total U.S. population from all proposed food-uses was estimated to be 102.42 mg/person/day or 1.91 mg/kg body weight/day. The heavy consumer (90th percentile) all-user intake of Pharming's hLF by the total U.S. population from all proposed food-uses was estimated to be 214.20 mg/person/day or 3.95 mg/kg body weight/day. The greatest mean and 90th percentile all-user intakes of Pharming's hLF were estimated to occur in male teenagers, at 136.74 and 252.00 mg/person/day, respectively (2.19 and 4.17 mg/kg body weight/day, respectively) and in male adults, at 135.69 and 294.15 mg/person/day, respectively (1.67 and 3.55 mg/kg body weight/day, respectively).

Based on a NOAEL of 2000 mg of Pharming's hLF per kg BW (resulting from the preclinical testing of Pharming's hLF as discussed above), the above-referenced exposures are all well under 1/100th of such NOAEL – even at the 90th percentile consumption level. Accordingly, such consumption levels should not pose any safety risk to any such consumer. Such safety level is even more pronounced for any consumer below the 90th percentile. Moreover, such safety level is fully supported by the safety levels emanating from the natural exposure to native hLF and from the preclinical and clinical studies of various hLF products (as discussed in subsections V(H)(1)(b)(3) and V(H)(2)(b)).

Summaries of Preclinical And Clinical Studies

With nhLF And rhLF

Andersen, 2004 In this clinical study 15 healthy volunteers were initially administered 5 g of rh lactoferrin, followed 15 h later by 5 g of rh lactoferrin and 75 g of indomethacin. After an overnight fast, individuals were administered 5 g of rh lactoferrin and 50 g of indomethacin, and 1 hour later a lactulose/rhamnose gut permeability test was performed. Indomethacin increased small intestinal permeability by approximately 2- to 3-fold at doses similar to those used in the study. The results demonstrated that concomitant rh lactoferrin administration with indomethacin ingestion significantly attenuated the urinary lactulose/rhamnose ratio compared with placebo (water) plus indomethacin from 0.036 to 0.028, indicating that the drug attenuated indomethacin-induced intestinal permeability and appeared to protect against NSAID-induced enteropathy. The exact mechanism of action of rh lactoferrin in attenuating enteropathy remains to be elucidated.

Andersen, 2004 This clinical study involves a phase I/II, open label, safety and efficacy trial of oral rh lactoferrin in the treatment of patients with solid tumors who have failed chemotherapy. Patients were to receive four different doses of rh lactoferrin, ranging from 1.5 to 9 g/day for either 14 days or in two cycles of 14 days with a 14-day gap. In the safety study, 30 patients were enrolled, of which 29 completed dosing, and one patient withdrew from treatment due to progressive disease. Results of the safety study were promising, and the efficacy trial is ongoing. Results from 19 evaluable patients demonstrated that ten out of 19 (53%) had stable disease, three of whom showed some tumor shrinkage. The most encouraging results to date, although in a small number of patients, were observed in renal cell cancer and included a confirmed and durable partial response, a dramatic reduction in the rate of tumor growth, and a high incidence of stable disease (83%).

Andersen, 2004 In this preclinical study mice were fed rhLF for 30 days daily up to 1000mg/kg. No evidence of toxicity was found.

Davidson, 1987 This clinical investigation examined the effect of development of the infant's digestive system (i.e., the extent of maturity) on

the stability (i.e., intact survival) of lactoferrin (and other proteins). Three infants – all fed only human milk – were evaluated. The three consumed from 1443-3537 mg nhLF in a 24-hour period. Fecal samples were collected from the term infants. Soluble proteins in the feces were extracted and analyzed for total proteins, nitrogen, lactoferrin, secretory IgA, serum albumin and lysozyme. Significant amounts of lactoferrin and secretory IgA were excreted by the infants and this excretion decreased throughout the study period in a trend similar to the decreasing milk concentrations of these proteins. Gel filtration demonstrated excreted lactoferrin and secretory IgA to be intact. No serum albumin or lysozyme was detected in the fecal extracts. Crossed immunoelectrophoresis showed three human milk proteins to be present in the feces – the third was identified as α_1-antitrypsin. Excretion of these proteins indicates the total protein content of human milk is an over-estimation of the protein nutritionally available to the infant.

Davidson, 1990 This preclinical study was initiated to evaluate whether the presence of hLF affects iron retention from human milk and milk-based infant formula (IF). Both were evaluated with and without supplemental ferrous sulfate, and IF was also evaluated after being supplemented with either human or bovine lactoferrin (LF). All five diets were fed (but not at the same time) to six infant rhesus monkeys. The exchange of ^{59}Fe (III) Cl_3 between the whey, casein, and fat fractions required up to 72 h to reach the same distribution as intrinsic iron, depending on the type of diet. Infant monkeys were intubated with labeled human milk or IF and counted in a whole body counter. Each infant received all five diets and was also intubated with a reference dose of ^{59}Fe (II) ascorbate. There was no significant difference in iron retention (means ± SEM) from experimental diets; human milk 32.5 LF ± 5.1%; IF 32.1 ± 8.0%; IF + Fe 23.0 ± 3.9%; IF + human LF 23.5 ± 3.3%; IF + bovine LF 22.7 ± 4.9%. Therefore, infant monkeys absorb and retain iron similarly from human milk and infant formula. Supplementation of infant formula with human or bovine LF resulted in similar iron retention to that of ferrous sulfate-supplemented infant formula.

Davidsson, 1994 This clinical investigation was conducted to examine the influence on iron absorption from human milk in infants. The potential effect of hLF on Fe absorption was investigated by measuring Fe absorption in four infants fed breast milk (with its native content of LF) and four infants fed the same milk from which LF had been remove (>97%) by treatment with heparin-Sepharose. Eight breast-fed infants (2-10 mo, mean age 5

mon) were fed 700 to 1000 g of each milk in a randomized, cross-over design with each child acting as his/her own control. The milk was labeled with 8.6 μmol (0.5 mg) of ^{58}Fe and Fe absorption was measured by quantifying the incorporation of the isotope into red blood cells 14 d after intake using thermal ionization mass spectrometry. Fractional Fe absorption was significantly lower ($p < 0.05$) from breast milk than from LF-free breast milk. The geometric means (range) was 11.8% (3.4% - 37.4%) for breast milk and 19.8% (8.4% - 72.8%) for LF-free breast milk. These results do not support a direct role for LF in the enhancement of Fe absorption from human milk at this age. In addition, Fe absorption (11.8%) from human milk fed over several feeds was lower than that previously reported for single feed studies.

GN No. 162, p. 23 (2004) In this preclinical study – briefly reported in GN 162 – Ventria sponsored a 28-day feeding study in rats being fed its rhLF. This study resulted in a NOAEL for the maximum dose, i.e., 1000 mg/kg BW/day.

GN No. 162, p. 23 (2004) In this preclinical study – briefly reported in GN 162 – Ventria sponsored a 28-day feeding study in rats being fed its rhLF. This study resulted in a NOAEL for the maximum dose, i.e., 1800mg/kg BW/day.

GN No. 162, p. 64 (2004) In this preclinical study – as reported by Ventria – "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."

GN No. 162, p. 66 (2004) In this preclinical study – as reported by Ventria – "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 conjugated antibody. ELISA were run on all sera and low levels of IgG antibody to lactoferrin were detected in all groups, including control animals, 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 in a 1:4 dilution for the presence of IgE antibodies. 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."

GN No. 162, p. 69 (2004) In this clinical study – as reported by Ventria – "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."

Goldblum, 1989 This clinical investigation was conducted to examine the effects of fortified human milk feedings on the urinary excretion of lactoferrin, lysozyme, secretory component, IgA and secretory IgA antibodies to *Escherichia coli* O antigens in very low birth weight infants. Infants were maintained on either a human milk (N=33) or a cow's milk

preparation (N=20). The amounts of each immune factor that were ingested and excreted were quantified during balance studies conducted at 2.5 and 5 wk of age. Serum levels of these immune factors were similar to both feeding groups. The urinary excretion of all factors except lysozyme was 7- to 150- fold greater in infants fed human milk than in those fed cow's milk formula. IgA was the only factor for which the amount of the factor excreted correlated with the amount ingested. Fragments as well as whole molecules of lactoferrin were found in the urine of the infants fed human milk, but the molecule sizes of the excreted proteins exceeded those normally filtered by the kidneys. Therefore, the genesis of the enhanced levels of host defense factors in the urine of infants fed human milk is not clear. Gastrointestinal absorption and subsequent renal excretion as well as enhanced production of immune factors in the infant's urinary tract are possible explanations.

Guttner, 2003 The authors indicate that this clinical study was conducted to determine the efficacy, safety and tolerability of recombinant human lactoferrin as a therapy in suppressing or eliminating *H. pylori* infection in subjects with minimal upper gastrointestinal symptoms who have not previously been treated. Nine healthy subjects with minimal upper gastrointestinal symptoms and a positive urea breath test were recruited. None of the volunteers had previously been treated for *H. pylori.* Subjects received 5 x 1.0 g human recombinant lactoferrin daily for 5 or 14 days. Breath tests were repeated during therapy and shortly after to check for eradication The safety and tolerability of the drug were assessed by physical examination, by monitoring adverse events, and clinical laboratory evaluation.

No conversions of the urea breath test from positive to negative was observed and there was no consistent change in urea breath test counter to indicate a possible suppression of *H. pylori.* Thus, the authors concluded that lactoferrin, given as a single agent, does not eradicate *H. pylori* infection.

Hayes, 2003 Because the authors believed that "there is extensive animal data showing inhibition of tumor growth and metastases by lactoferrin" and that "rhLF appears to be safe and well tolerated, having been administered orally to over 160 patients/subjects without a drug-related serious adverse event," they evaluate the safety and efficacy of rhLF in patients with solid tumors that failed chemotherapy. 33 evaluable patients are receiving open

label rhLF in two 14-day cycles separated by a 2-week gap. The first nine patients receive rhLF doses (1.5, 4.5 and 9g/day) in cohorts of 3 to establish safety, with the rest randomized between the two highest doses. The authors are also evaluating 8-week and 16-week radiological tumor response, tumor markers and biological markers of rhLF activity, including increases in circulating CD40+ cells and immunomodulatory cytokines. To date, they have enrolled seven patients; five have completed both cycles without a dose limiting toxicity. While no clinical or radiological responses has been observed so far, serum CEA levels in both colon cancer patients stabilized after rising in the preceding months. The authors also observed an immunostimulatory effect in all patients – increases in CD40+ and decreases in Annexin V staining in peripheral blood cells. Annexin V is an apoptotic marker whose decrease is associated with enhanced survival of circulating immune cells. CD40+ antigen-presenting cells are critical to development of a tumor specific T-cell response. The authors indicate that they are encouraged by seeing tumor marker stabilization and immunostimulation in low dose cohort patients by a novel and well-tolerated oral drug.

Hayes, 2004 In this clinical study patients with advanced solid tumors that had progressed on previous chemotherapy regimens, or who had no effective chemotherapy regimens available were scheduled to receive oral rhLF monotherapy in cycles of 2 weeks on, two weeks off, until tumor progression. Following an initial dose escalation, patients were randomized between the two highest rhLF doses (4.5 or 9 g/day). Tumor response was followed by periodic CT scans using RECIST criteria.

To date, the authors have enrolled 27 patients (9 with NSCLC, 4 each with renal and colon cancer, and 3 or fewer with seven other tumor types). RhLF was safe and well tolerated, with no drug-related SAEs or DLTs. A total of 22 patients were evaluated radiologically. The authors observed one (5%) partial response and 12 (55%) stable disease. In NSCLC patients, the largest group, 5 patients (53%) had stable disease. In patients with renal cancer, they had one (25%) partial response and 3 (75%) stable disease. Colon cancer patients had 50% stable disease. The authors observed a substantial decrease in the growth rate of target lesions before and after start of rhLF. The average tumor growth rate dropped by 68% ($p<0.05$) from 5.2% to 1.7% per week. A reduction in tumor growth rate could potentially result in increased survival. The NSCLC patients (88% tumor rate reduction; $p< 0.05$) already appear to demonstrate a survival benefit. The mean survival to date among the 9 NSCLC patients is 8 months (with only

one death), which compares favorably to the 4.6 months expected median for pretreated patients with metastic lung cancer. The authors conclude that rhLF is safe and well tolerated, shows promise in salvage therapy of NSCLC and renal cancer, and should be evaluated in larger Phase II trials.

Humphry, 2002 In order to find a viable substitute for use of antibiotics in poultry, two preclinical experiments were conducted to determine whether rice that has been genetically produced to express human lactoferrin (LF) or lysozyme (LZ) protects the intestinal tract similarly to subtherapeutic antibiotics (bacitracin + roxarsone). Experiment 1 compared 10 corn-soy diets containing 20% of various proportions of LF, LZ or conventional rice (CONV). 300 3-day old chicks fed 5% LF + 10% LZ + 5% CONV had significantly better feed efficiency and thinner lamina propria in the duodenum than those fed 20% CONV. Experiment 2 compared five corn-soy diets containing experimental rice combinations totaling 15% rice. 300 3-day old chicks fed 10% LZ + 5% CONV or 5% LF + 10% LZ had significantly lower feed intake and significantly better feed efficiency than those fed 15% CONV. Chicks fed 10% LZ + 5% CONV, 5% LF + 10% LZ or antibiotics had significantly greater villous height in the duodenum compared with chicks fed 15% CONV. The lamina propria of the ileum was thinner and contained fewer leukocytes in chicks fed 10% LZ + 5% CONV or antibiotics compared with those fed 15% CONV. The results from these experiments demonstrate, the author concluded, a potential of genetically produced LF and LZ rice to be used as a substitute for antibiotics in broiler diets.

Lindberg, 1997 In this preclinical (*in vivo*, digestion) study, investigators examined the capacity of the newborn (in this case, preterm, 6 weeks old, and 7 months old rhesus monkeys) to digest various human and bovine milk proteins. Preterm infant monkeys were given 2.5 ml of Presemp-fortified human milk (N=6) or Similac Special Care (N=4) via an orogastric tube, 30 or 60 min before death. Blood samples were drawn immediately before death and serum was stored at –20°C. Gastric, duodenal, and jejunal contents were collected at necropsy (immediately after death), frozen on dry ice, and stored at –20°C. Nursing and weaned infant monkeys were fasted for 2 and 3 hours, respectively. During anesthesia (ketamin, 10mg/kg body weight, i.m.), a tube was placed with the tip in the ascending part of the duodenum (fluoroscopic control). During the sampling period the infants were sedated with diazepam (1 mg/kg body weight, i.m.) or with a repeated dose of ketamin. The localization of the tube was always checked

by fluoroscopy at the end of the sampling period and occasionally in the middle of the period. In all instances it was located in the transversal or ascending part of the duodenum. The test meal (human milk (N=3), fortified human milk (N=4), or Similac Special Care (N=3)) was given via an orogastric tube. The duodenal content was collected continuously by siphonage on crushed ice and immediately frozen on dry ice before and 15, 30, 60, and 90 min (± 5 min) after the meal. The amount of retention in the stomach was in all instances measured after the sampling period. Venous blood samples were taken at 0, 30, 60, and 90 min. Duodenal samples and serum were stored at -20°C until analysis, which was performed within 8 months.

This study demonstrated, among other things, an age-dependent capacity to hydrolyze bovine and human whey proteins in the monkey gut lumen. Such outcome possibly depends upon an immaturity of the exocrine pancreas of suckling rhesus monkeys to respond to secretogogues.

McMillan, 1977 Studies from the investigator's laboratory have shown that iron is better absorbed from human milk than from cow milk and that human milk can provide sufficient iron for infants during their first year. Therefore, in this clinical study the authors compared iron availability from human milk with that from other formulas and determined the factors responsible for its superiority. Eight, healthy, iron-sufficient adults were fed 100 ml of human milk, simulated human milk, simulated human milk containing added lactoferrin, two commercial formulas containing iron, 12 mg/qt, and human milk that had been boiled. The simulated human milk resembled human milk in concentration of protein, fat, carbohydrates, iron, total minerals, calcium, and phosphorus. Iron 59 was added to each feeding and iron incorporation into RBCs was determined 14 days after each feeding. Percent iron absorption was highest from human milk and lowest from the commercial formulas. The simulated human milk supported a 9.0% absorption; addition of lactoferrin reduced this to 4.7%. Net iron absorption was 0.12 mg/liter from human milk and 0.40 and 0.37 mg/liter from the iron-enriched commercial formulas. Absorption of iron from boiled human milk was the same as from the unboiled milk.

This study confirms the unique ability of human milk to promote iron absorption. Simple manipulation of the protein fat, lactose, calcium, phosphorus, or lactoferrin content of proprietary milk did not reproduce the iron absorption demonstrated with human milk.

Opekun, 1999 This clinical study was conducted to evaluate orally administered novel therapies, including use of rhLF, for the treatment of *H. pylori* infection. Healthy *H. pylori* infected volunteers received: (a) hyperimmune bovine colostral immune globulins (N=15), (b) an oligosaccharide containing an *H. pylori* adhesion target. Neu5Aca2-3Galb1-4Glc-(3'-sialyllactose) (N=6), or (c) recombinant human lactoferrin (N=12). Outcome was assessed by urea breath test or histological assessment of the number of *H. pylori* present. Subjects receiving rhLF received either 1.25 or 5.0 grams of orally administered rhLF. None of the novel therapies appeared effective and no adverse events occurred. Thus, the authors concluded, although *in vitro* data appeared promising, *in vivo* results were disappointing. Higher doses, longer duration of therapy, adjunctive acid suppression, or a combination could possibly yield better results.

Spik, 1982a This clinical investigation was intended to examine and characterize the remaining forms of lactoferrin after passage through the infant's intestine. Human lactoferrin was characterized in the fecal extracts from (among others) fifteen breast-fed babies. Quantitative determination of human copro-lactoferrin from birth for a period of up to three weeks showed that the daily elimination decreased from 35 to 5 mg. The amount of copro-lactoferrin corresponded to the endogenous secretion was calculated to be from 0.5 g to 1 mg per day. When a cow's milk diet supplemented by partially or completely iron-saturated human or bovine lactoferrin was fed to the babies, the amounts of copro-lactoferrin excreted depended on the origin and on the iron saturation of the lactoferrin. In particular, the amount of bovine copro-lactoferrin in the feces averaged 200 mg per day. The human and bovine copro-lactoferrin were isolated by ion-exchange chromatography or by affinity chromatography and were still able to bind iron. The fingerprints of native human and bovine lactoferrin hydrolyzed in vitro by infant's gastric or duodenal secretions showed that both proteins were not entirely digested. This demonstrates that these lactoferrins ingested by babies are not completely destroyed and keep their ability to bind iron and ,thus, may supplement the bacteriostatic effects of the endogenous lactoferrin in the intestinal tract.

Troost, 2002 The aim of this clinical study was to determine the survival rate of orally administered recombinant human lactoferrin (rhLF) in the upper gastrointestinal (GI) tract and in the small intestine *in vivo* in humans. Female ileostomy patients (N=8; median age 44 (43-57) y) ingested a test

beverage containin 5 g rhLF and collected full ileostomy output for 34 h. The passage of the test beverage was monitored using radio-opaque markers. Intact LF in ileal effluent was quantified by ELISA. The entire test beverage passed the small bowel during the 24-h ileal effluent collection period. LF was excreted at a low but consistent rate (0.35 (0.28-0.47) μg LF/2 h). Gastrointestinal passage of the test beverage and LF excretion over time in the ileal effluent were not correlated. In total, a median of 4 μg LF was excreted over the 24-h collection period, whereas 5 g rhLF was ingested. Hence, dietary rhLF is digested in the upper GI tract and does not reach the colon. Intact LF appearing in ileostomy effluent is likely to originate from endogenous source.

Troost, 2003 The purpose of this clinical study was to determine whether recombinant human lactoferrin ingestion inhibits nonsteroidal anti-inflammatory drugs (NSAID)-induced gastroenteropathy *in vivo* in healthy volunteers as a model for disorders associated with a rise in permeability of the stomach and small intestine. This study was a randomized crossover dietary intervention. In all, 15 healthy volunteers (age 23 ± 1.4 y) were tested. A sucrose and a lactulose/rhamnose (L/R) permeability test was performed to assess gastroduodenal and small intestine permeability as indicator of NSAID-induced gastroenteropathy. All subjects consumed standardized meals for 2 days. On the second day at time = - 24 h each subject ingested a drink containing 5 g recombinant human lactoferrin or placebo during breakfast. At t = -9 h, subjects ingested the same drink with 75 mg of the NSAID indomethacin and after an overnight fast at t = -1 h subjects consumed the drink and 50 mg indomethacin. After 1h at t = 0, a permeability test was performed.

The results indicate that small intestine permeability after indomethacin and placebo was significantly higher (L/R ratio = 0.036; 0.014-0.092, $P<0.05$) compared to the permeability observed after ingestion of indometnacin and lactoferrin (0.028; 0.015-0.056), whereas gastroduodenal permeability did not differ between the two interventions ($P=0.3$). Thus, the authors concluded that oral recombinant human lactoferrin supplementation during a short-term indomethacin challenge reduced the NSAID-mediated increase in small intestinal permeability and hence may provide a nutritional tool in the treatment of hyperpermeability-associated disorders.

Trümpler, 1989 This clinical investigation examined whether lactoferrin, after enteral administration, is able to prevent, postpone or alleviate enterogenic infection in patients with chemotherapy-induced neutropenia. Five qualified patients received (4 x 200 mg/day, p.o., days 1 through 10 of neutropenia) natural lactoferrin. The investigators described the lactoferrin as being "non-toxic" and, thus, a good alternative treatment. Lactoferrin treatment did not significantly prolong the infection-free interval, but did, however, significantly shorten the duration of the first infection. The authors concluded that the results suggest that growth and/or systemic spread of Enterobacteriaceae may indeed be inhibited in the gut lumen by iron-deficient lactoferrin depriving these organisms of iron and, thus, acting as a bacteriostatic agent.

VAMC, 2003 Ventria reported in GN No. 162 that: "Thirty patients were enrolled in the safety phase of the trials, and administered one or four different doses of rhLF, ranging from 1.5g/day to 9 g/day, for either 14 days (South America) or in cycles of 14 days with a 14 day gap (VAMC). Patients were observed for adverse events, and tumor size was measured radiologically at baseline and 8 weeks following start of therapy. Of the 30 patients enrolled, 29 completed dosing, with one patient withdrawing prior to completion due to progressive disease. The drug, which was administered **orally** was well tolerated without a single drug-related serious adverse event or a drug-related laboratory abnormality greater than Grade 2. Safety results were presented in an abstract submitted to the 2003 annual meeting of the American Society of Clinical Oncology."

Wang, 2005 Because the authors believed that recombinant human lactoferrin (rhLF) is a novel immunostimulatory agent with demonstrated anti-cancer activity in animals and NSCLC patients in PhaseI/II trials, they evaluated oral rhLF in a combination with standard chemotherapy in first line NSCLC patients in this clinical trial. 110 chemo-naïve patients with advanced or metastic NSCLC from 11 cancer centers were randomized to receive standard therapy (carboplatin + paclitaxel; C/P) plus either rhLF or placebo. C/P was administered in 3-week cycles. RhLF (or placebo) was administered orally, 1.5 b B.I.D., in 35-day cycles until radiological progression for a maximum of three cycles, starting the day following C/P dosing in cycles 1,3 and 5. The primary end point is Best Overall Response (BOR) by CT using RECIST criteria. Secondary end points included Progression Free Survival (PFS) and Overall Survival by Kaplan-Meier curves and Quality of Life Assessments (EORTC QLQ C30, QLC LC 13) at

weeks 0, 6, 12, and 18. Data will be unblended for preliminary analysis in February 2005.

All 100 patients have been enrolled for over ten weeks. Study drug appears to be safe and well tolerated, with only one Grade3/4 toxicity (abdominal pain) that is possibly rhLF/placebo related. 100 patients have had at least one post-therapy CT, and are evaluable for response rate (RR). So far, there have been 37 (37%) partial responses (PR) and an additional 53 patients (53%) had stable disease on their 6-week CT or later. Median PFS has not been reached. The five major published randomized trials evaluating 3-weekly first line C/P in Stage IIIB/IV NSCLC report an average RR of 25.9%. The 37% blended response rate observed in this trial is significantly better than that expected in this patient population.

The authors concluded that oral rhLF is a safe and well-tolerated immunostimulatory drug. If the response rate of placebo patients in this study is consistent with the published literature, the blinded data suggest that rhLF may significantly enhance the response to first line C/P chemotherapy in advanced NSCLC without increasing toxicity.

Section VI

VI. SCIENTIFIC EXPERT PANEL DETERMINATION

Pharming convened a panel of independent scientific experts – none of which currently serve or has served as a consultant to Pharming with regard to hLF, but all of whom were paid for their service on the panel – each of which had the appropriate education, training and experience. The expert panel consisted of:

1. Jeremy H. Brock, ScD, PhD, Msc, a chemist/ micro - biologist/immunologist who is currently a Senior Research Fellow at the University of Glasgow in the Department of Immunology and who has a distinguished research career which includes the study and publication of much of what is known about lactoferrin;

2. Ian C. Munro, PhD, FRCPath, MSc, (**Panel Chair**), a toxicologist/pharmacologist who is currently a Professor at the University of Toronto in the Department of Nutritional Science and with CANTOX Health Sciences International (consultants in health and environmental sciences) who has a long and distinguished career in virtually all food safety matters;

3. F. Jay Murray, PhD, DABT, a toxicologist who is currently an independent consultant in toxicology and risk assessment at Murray & Associates with a distinguished career in analyzing the safety of chemical substances and foods who served on the State of California's first Governor's Scientific Advisory Panel pertinent to Proposition 65 (i.e., the Safe Drinking Water and Toxic Enforcement Act of 1986) and who serves as an Assistant Clinical Professor of Medicine, Department of Occupational Medicine, UCSF School of Medicine; and

4. Jorge A. Piedrahita, PhD, MSc, an animal scientist/cell and development biologist/molecular geneticist who is currently Professor of Genomics, Department of Moleular Biomedical Sciences, College of Veterinary Medicine, NC State University and who has distinguished himself via his

> extensive knowledge and research experience in the functional genomics, cell biology and embryology pertinent to transgenic animals.

(for additional information, see attached curricula vitae, Appendix 14). The panel had the aggregate expert capability to deal with all of the food safety issues inherent in this GRAS Notification. Such aggregate capability includes expertise in, among other areas, food safety, toxicology, allergenicity and immunology, use of recombinant technology, molecular biology and dairy matters.

Panel members were given a copy of this GRAS Notification **and** access to all information (including references and appendicies) in support of such Notification – i.e., the same aggregate information relied on by Pharming in reaching its GRAS determination. The panel members reviewed such information, met to discuss such information and had their questions answered, and then concluded – based on all such information – that Pharming's human lactoferrin – derived from transgenic cows expressing a human gene encoding human lactoferrin – is GRAS, based on scientific procedures and common use in food, for its intended use. More specifically, the panel concluded:

> Based on our independent collective critical and in-depth evaluation of the available pertinent, scientific (both published and unpublished) and other information, we conclude that Pharming's human lactoferrin – which is derived from the milk of transgenic dairy cattle carrying and expressing a human lactoferrin gene – is manufactured in accordance with good dairy practices and cGMPs, meets the relevant food grade specifications and, based primarily on scientific procedures, is Generally Recognized As Safe (i.e., GRAS) for use in food as described within the GRAS Notification. (Expert Panel Report, page 3; see Appendix 15).

Section VII

VII. CONCLUSIONS

When determining whether its hLF product was generally recognized as safe under the conditions of its intended uses set forth in this GRAS Notification, Pharming was ever mindful that its determination had to be supported to a reasonable certainty by experts:

1. who were qualified by scientific training and experience to evaluate such safety;
2. who were in agreement in consensus fashion;
3. who based their determination primarily on scientific procedures (and also on experience based on common use in food, if applicable); and
4. who relied on common knowledge, i.e., public information – especially that published in peer-reviewed scientific sources.

Accordingly, in order to satisfy these regulatory criteria, Pharming:

1. gathered together all relevant scientific information it could locate and obtain from both private (i.e., unpublished) and published sources (over 1000 scientific articles were identified and considered);
2. had all such information organized as it related to all aspects of the production of Pharming's hLF product (which aspects included research, development, product identity, physical and chemical composition, all aspects of the manufacturing process (including production of transgenic cattle, care/handling/milking of transgenic cattle, purification, substances used during manufacturing, product specifications and stability and safety), analytical process, information on hLF, investigations of product safety, and the safety of specific product exposures to humans); and
3. had all such information carefully and thoroughly evaluated – over a period of more than a year – by numerous (both inside and outside) qualified experts.

Based on these evaluations, Pharming first determined what information:

1. was believed in consensus fashion;
2. was supported by adequate scientific information; and
3. was published.

Pharming then determined that the experts believed that there was sufficient published scientific information to more than support the experts consensus view that to a reasonable certainty that Pharming's hLF was GRAS under the conditions of its intended uses (as set forth in this GRAS Notification). Accordingly, Pharming determined its product was GRAS for such intended use.

Not satisfied, Pharming then had an independent expert panel review its determination and the information supporting such determination all in an effort to determine whether such panel agreed or disagreed with Pharming's determination. The expert panel determined that it agreed with Pharming's determination.

Accordingly, Pharming is confident that FDA'as review of the extensive information summarized in the GRAS Notification will permit FDA to indicate to Pharming – at the conclusion of its review – that it has no questions at the time in question about Pharming's determination.

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Appendicies

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SUBMISSION END