Production and characterization of recombinant human lactoferrin

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Chapter 5

Large scale production of recombinant human lactoferrin in the milk of transgenic cows

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The limited capacity of current bioreactors has led the biopharmaceutical industry to investigate alternative protein expression systems. The milk of transgenic cattle may provide an attractive vehicle for large-scale production of biopharmaceuticals, but there have been no reports on the characteristics of such recombinant proteins. Here we describe the production of recombinant human lactoferrin (rhLF), an iron-binding glycoprotein involved in innate host defense, at gram per liter concentrations in bovine milk. Natural hLF from human milk and rhLF had identical iron-binding and -release properties. Although natural hLF and rhLF underwent differential N-linked glycosylation, they were equally effective in three different in vivo infection models employing immunocompetent and leukocytopenic mice, and showed similar localization at sites of infection. Taken together, the results illustrate the potential of transgenic cattle in the large-scale production of biopharmaceuticals.

Lactoferrin, a single-chain, metal-binding glycoprotein of 77 kDa, is a component of the innate host defense of mammals and is produced and released by mucosal epithelial cells and neutrophils[1]. It consists of two highly homologous lobes, designated the N and C lobes, each of which can bind a single ferric ion concomitantly with one bicarbonate[2]. Human lactoferrin (hLF) has antibacterial[3], antifungal[4], anti-endotoxin[5], and antiviral[6] activities. In addition, hLF interacts with host cells to modulate the inflammatory process and innate host defense[1]. Finally, lactoferrin in milk might play a role in iron absorption and/or excretion in newborns, as well as in promotion of intestinal cell growth[1].

On the basis of the many biological activities of hLF, researchers have considered a wide variety of possible applications in human health care, such as prophylaxis and treatment of infectious and inflammatory diseases. For these purposes, however, a recombinant source of hLF is required that avoids the limitations of bioreactor capacity[7]. Large-scale production of therapeutic proteins in the milk of transgenic cattle may be a cost-effective alternative to production of recombinant proteins through cell culture or fermentation. Here we report on the generation of transgenic cows expressing recombinant human lactoferrin (rhLF) in their milk, and on the properties of rhLF as compared with natural hLF from human milk.

Results and discussion

Recombinant hLF is expressed at high concentrations in bovine milk. We obtained, by microinjection[8], transgenic cattle lines 8778, 8783, 8785, and 8793 harboring the genomic hLF gene under regulatory control of the bovine αS1-casein promoter[9]. Expression levels in lactation-induced milk of the various lines are listed in Table 1. We detected similar levels, of about 0.8, 0.4, 3.0, and 2.0 g/l, in calving-induced milk of 8778, 8783, 8785, and 8793, respectively. Expression levels of recombinant hLF remained consistent throughout the lactation period of 280 days. Milk parameters such as total milk output, cell counts, and total protein, fat, and lactose were within the normal ranges and appeared unaffected by the expression of the transgene (data not shown).

Recombinant and natural hLF are structurally and functionally similar. Information about the functional and structural features of transgenic proteins obtained from the milk of larger mammals is limited, except for a few reports describing protein C (ref. 10) and antithrombin III (ref. 11) production. Recombinant hLF purified from bovine milk was subjected to analytical Mono S chromatography[9], which discriminates between hLF and bovine lactoferrin (bLF). The rhLF preparation was essentially free of bLF (Fig. 1A). Both rhLF and natural hLF eluted from the column at 0.7 M NaCl, whereas purified bLF eluted at 0.8 M. Enzyme-linked immunosorbent assay (ELISA) analysis indicated that rhLF preparations contained less than 0.1% (w/w) bLF. N-terminal sequencing confirmed the identity of rhLF and showed that the N-terminus of the protein was intact. Comparison of rhLF and natural hLF on SDS–PAGE showed that the relative molecular mass of rhLF was about 1–2 kDa smaller than that of natural hLF (Fig. 1B). After full deglycosylation[9] with N-glycosidase F, we saw no difference in molecular mass and found that only rhLF was sensitive to digestion with endoglycosidase H (Fig. 1B). Monosaccharide composition analysis of the two preparations indicated that rhLF contained relatively more mannose than did natural hLF (Table 2). Hence, the difference in molecular mass between rhLF and natural hLF resides in differential N-linked glycosylation: natural hLF contains only complex-type glycans, whereas rhLF also contains oligomannose- and/or hybrid-type glycans. Monosaccharide analysis also showed that the N-linked gly-

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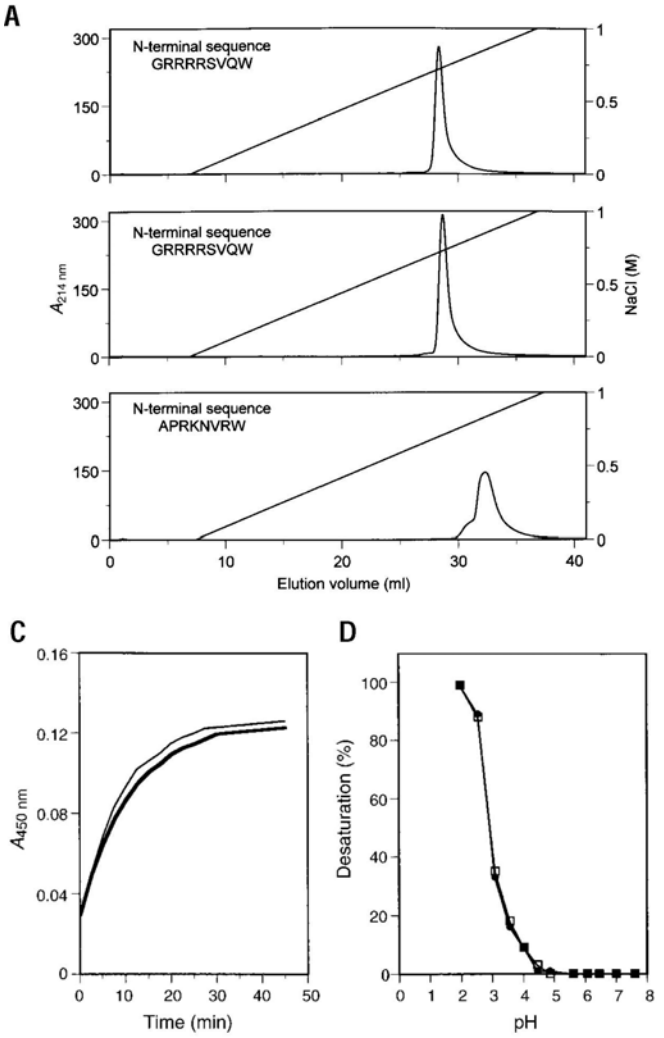

Figure 1. Characterization of rhLF isolated from bovine milk. (A) Mono S chromatography of purified lactoferrins. Purified rhLF (top), natural hLF (middle), and bLF (bottom) (100 µg each) were applied to Mono S HR 5/5 as described[14]. The left and right abscissa indicate the absorption at 214 nm and the NaCl molar concentration (M), respectively. N-terminal protein sequencing results are shown using the standard one-letter codes for amino acids. (B) SDS–PAGE of rhLF and natural hLF. Natural hLF (lanes 1–3) and rhLF (lanes 4–6) were analyzed untreated (lanes 1 and 4) and after treatment with N-glycosidase F (lanes 2 and 5) or endoglycosidase H (lanes 3 and 6) on 7.5% (w/v) SDS–PAGE. All lanes contain 10 µg of protein. Numbers on the right (kDa) indicate the migration of protein standards. (C) Iron-binding kinetics of rhLF (thin line) and natural hLF (thick line). Results are means of three independent experiments (s.d. < 5%). (D) pH-dependent desaturation of iron-saturated rhLF (●) and natural hLF (□). The percentage desaturation was calculated by reference to iron-saturated hLF diluted in 0.15 M NaCl.

cans of rhLF contain N-acetylgalactosamine next to galactose, and less sialic acid and fucose than do the glycans of natural hLF. Substitution of galactose with N-acetylgalactosamine has also been observed in human antithrombin III produced in goat milk[11].

The two most prominent functional activities of hLF, iron binding and release and antibacterial activity[1], were investigated for rhLF and natural hLF. The iron-binding (Fig. 1C) and iron-release characteristics (Fig. 1D) of rhLF and natural hLF were highly similar, in agreement with our previous observation of rhLF isolated from the milk of transgenic mice[9].

Table 1. Expression of rhLF in milk of distinct transgenic cattle lines

Line	Sex F_0	rhLF expression in F_0 (g/l)	Offspring (F_1)	rhLF expression in F_1 (g/l)
8778	Female	0.8	8811	0.9
8783	Male	–	8812	0.4
			8818	0.3
8785	Male	–	710	2.8
			712	2.5
			714	2.3
			715	2.3
8793	Male	–	883	1.6
			887	1.6
			888	2.0

Transgenic cows (founders (F_0) or offspring (F_1)) were induced to lactate by hormone treatment. Milk samples were collected for the analysis of rhLF concentration by ELISA. Expression levels represent the mean of at least five milkings during a lactation period of at least 14 days.

Table 2. Monosaccharide composition of rhLF and natural hLF

Monosaccharide	rhLF		Natural hLF	
	Batch 1	Batch 2	Batch 1	Batch 2
Fuc	0.3	0.2	1.8	1.8
Man	3.0	3.0	3.0	3.0
Gal	0.2	0.2	1.8	2.0
GlcNAc	1.6	1.7	2.8	3.3
GalNAc	0.5	0.6	–	–
Neu5Ac	0.2	0.1	0.9	1.0

Results are expressed relatively to the amount of mannose, which was arbitrarily set at 3.0. Fuc, fucose; Man, mannose; Gal, galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; Neu5Ac, N-acetyl-neuraminic acid.

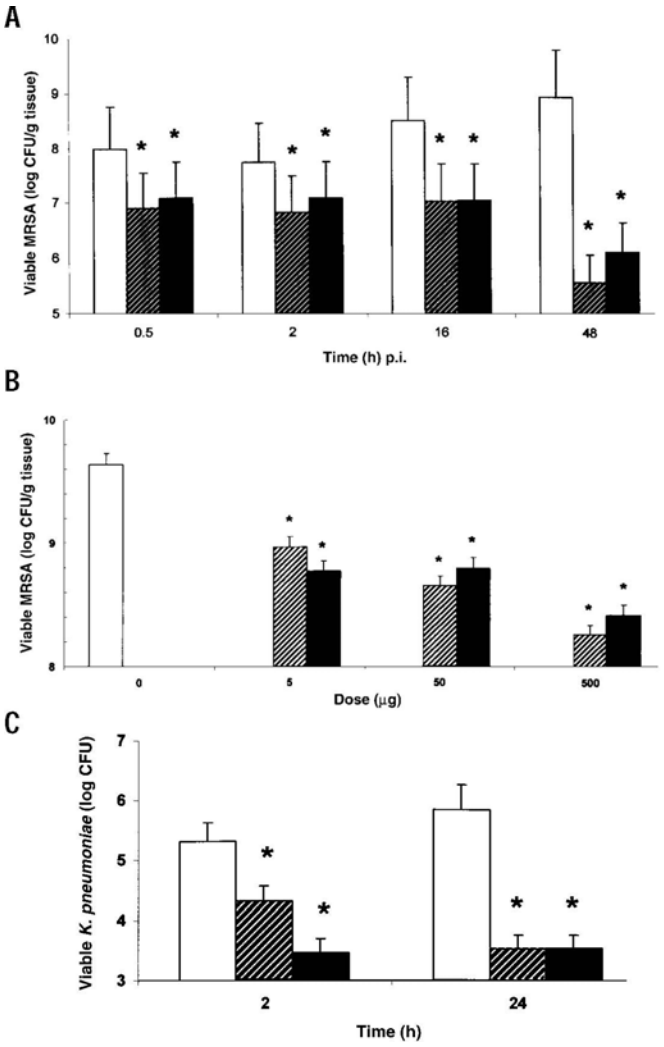

Figure 2. Effect of rhLF and natural hLF on experimentally induced infections in mice. (A) Mice infected with multidrug-resistant *S. aureus* were injected with 50 µg rhLF (hatched bars), natural hLF (black bars), or BSA (white bars). At the indicated time points, the number of CFU in the thigh muscle homogenates was determined microbiologically. Values are means (± s.d.) of CFU per gram of thigh muscle (n = 3). (B) Cyclophosphamide-treated mice, infected with multidrug-resistant *S. aureus*, were injected with various amounts (range: 0–500 µg/mouse) of rhLF (hatched bars), natural hLF (black bars) or BSA (white bars). After 24 h, the number of viable bacteria was determined microbiologically. Values are means (± s.d.) of CFU per gram of thigh muscle (n = 4). (C) Mice, intraperitoneally infected with *K. pneumonia*, were injected with 50 µg of rhLF (hatched bars), natural hLF (black bars), or BSA (white bars). At 2 and 24 h after injection of these proteins, the number of viable bacteria in the peritoneal cavity was determined microbiologically. Values are means (± s.d.) of CFU per lavage (n = 4). An asterisk indicates a significant difference of $P < 0.05$ in Figure 2A, B, and $P < 0.01$ in Figure 2C, between mice injected with rhLF or natural hLF and mice injected with BSA.

We have recently reported that natural hLF was highly effective against experimental infections with multidrug-resistant *S. aureus* in mice[3]. Comparison of rhLF and natural hLF in this assay showed that both reduced the number of viable bacteria equally well (Fig. 2A). A significant (Mann-Whitney U-test, $P < 0.05$) bactericidal effect became

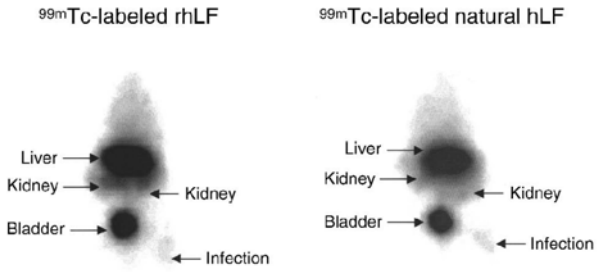

Figure 3. Scintigrams for ^{99m}Tc-labeled rhLF and ^{99m}Tc-labeled natural hLF in mice infected (thigh muscle) with multidrug-resistant *S. aureus*. Mice infected with *S. aureus* were injected with 10 µg of ^{99m}Tc-labeled hLF, and 60 min later, scintigrams were prepared using a planar low energy general purpose camera (Toshiba, Tokyo, Japan)[3,15].

apparent at 30 min after a single injection of 50 µg of rhLF or natural hLF per mouse; maximum effects of more than 99% reduction in the percentage of viable bacteria were reached at 24–48 h. We also compared the in vivo antibacterial activity of rhLF and natural hLF in two models in which hLF had not been tested before: a leukocytopenic model and a peritoneal infection model. In leukocytopenic mice with a tenfold-reduced number of circulating leukocytes, we saw maximum bactericidal effects 24 h after a single injection with 500 µg of hLF per mouse (Fig. 2B). This indicates that hLF is also effective against experimental bacterial infections in immunocompromised hosts. Furthermore, a single intravenous injection with 50 µg of rhLF or natural hLF rapidly reduced the number of viable bacteria in the peritoneal cavity of immunocompetent mice infected with *K. pneumoniae* (Fig. 2C); again, we saw no difference between the bactericidal effects of rhLF and natural hLF.

Pharmacological studies using radiolabeled proteins showed that ^{99m}Tc-labeled rhLF and natural hLF were rapidly removed from the circulation of multidrug-resistant, *S. aureus*–infected mice with a $t_{1/2}$ value of 24 ± 3 min (n = 4) and 21 ± 3 min (n = 4), respectively. The two hLF preparations were removed from the circulation both via the liver and intestines and via the kidneys and bladder (Fig. 3). Within 5 min after injection, we observed a significant amount of radioactivity (~2% of the injected dose) at the site of infection. After 60 min, we still detected similar amounts of radiolabeled rhLF and natural hLF at the site of infection (Fig. 3).

Conclusions

We describe here the expression of rhLF in the milk of transgenic cows at concentrations of up to 3 g/l. The protein was highly similar to natural hLF when analyzed for its two most prominent biological activities, iron binding and release and antibacterial activity. The differential N-linked glycosylation of rhLF and natural hLF apparently did not affect the proteins' biological activities either in vitro or in vivo. One might have theoretically predicted differences in vivo, considering the relatively low degree of sialylation and the presence of exposed mannose on rhLF, which might

result in faster clearance and reduced in vivo efficacy.

This report illustrates the potential of transgenic cattle as bioreactors in terms of capacity. With the observed expression level and an annual output of 8,000 liters of milk per cow, one cow of line 8785 produces 24 kg crude rhLF per year. Hence, a few hundred animals could supply thousands of kilograms of the product annually, and changes in dosing regimes, market projections, and additional applications of the biopharmaceutical can easily be met by expansion of the herd. Until recently, the limitations of using transgenic cattle as bioreactors were the inefficiency of microinjection to generate founders[8] and the lengthy time lines. These issues have been markedly improved by the introduction of nuclear transfer (NT) as a means to generate transgenic cattle[12]. NT is much more efficient than microinjection and allows the sex of founders to be predetermined. Thus, mini-herds consisting of genetically identical transgenic cows can rapidly be generated, providing quick access to large quantities of milk, especially when milk is obtained from young animals after induction with hormones. These data collectively provide strong arguments for the use of transgenic cattle as a serious alternative vehicle for biopharmaceutical protein production.

Experimental protocol

Proteins. Natural hLF was purified from human milk as described[9]. BSA was obtained from Sigma (St. Louis, MO).

Induction of lactation. Lactation was induced at the age of six to eight months by subcutaneous injections with 25 mg/kg/day medroxyprogesterone acetate (Vétoquinol, Lure Cedex, France) and 7.5 mg/kg/day estradiol benzoate (Intervet, Boxmeer, The Netherlands) for seven days. This course was followed by a two-day period of rest after which 4 mg/day dexamethasone (Intervet) was given intramuscularly for three days. Milking attempts were initiated on the second day of dexamethasone treatment. Cows were milked twice daily. When milking was stopped, intramuscular injections of spiramycine were administered for three days (30 mg/kg) to prevent mastitis. Genetic modification studies on cows were approved by the Committee for Ethical Evaluation of Genetic Modification of Animals (Schroten Committee), installed by the Dutch government (The Hague, The Netherlands) and chaired by E. Schroten (University of Utrecht, The Netherlands), permission number P1322.

Purification of rhLF from bovine milk. The lactoferrin fraction from transgenic bovine skimmed milk, containing rhLF and bLF, was captured on S Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). After extensive washing, lactoferrin was eluted with high salt, concentrated, buffer exchanged to 20 mM sodium phosphate pH 7.5 + 0.1 M NaCl, and filtered (0.22 μm). Then, Tween 80 (1%) and tri-N-butylphosphate (0.3%) were added for 15 ± 3 h at 25 ± 1°C to inactivate enveloped viruses. Next, rhLF was separated from added chemicals and bLF by cation-exchange chromatography on MacroPrep High S Support (Bio-Rad Laboratories, Hercules, CA) by stepwise elution with increased NaCl. Fractions containing only rhLF were pooled, concentrated, and filtered through two Planova hollow 15-nm fibers placed in series (Asahi, Tokyo, Japan). The final rhLF solution was concentrated, diafiltered against saline (0.9% NaCl), filtered (0.1 μm), and stored in vials. Analytical Mono S (Amersham Pharmacia Biotech) chromatography of purified rhLF was performed as described[9].

ELISA of hLF and bLF. Recombinant hLF and natural hLF were quantified by hLF-specific ELISA[13] . The ELISA specific for bLF was done similarly using affinity-purified rabbit anti-bLF and horseradish peroxidase–conjugated affinity-purified rabbit anti-bLF as coating and conjugate, respectively. Affinity-purified anti-bLF was absorbed with hLF–Sepharose to remove hLF crossreacting antibodies.

SDS–PAGE of rhLF and natural hLF. Samples of rhLF and natural hLF were digested either with N-glycosidase F, as described previously[9], or with endoglycosidase H (2 milliunits; Roche, Almere, The Netherlands) for 16 h at 37°C in 100 mM sodium citrate, pH 5.5, 0.05% wt/vol SDS. Treated and untreated (control) samples were boiled for 5 min in nonreducing sample buffer and then analyzed on 7.5% (w/v) SDS–PAGE. Proteins were stained with Coomassie brilliant blue.

Iron-binding and iron-desaturation studies on rhLF and natural hLF. To study iron binding, a freshly prepared iron citrate solution was added to rhLF and natural hLF, which were both 8% saturated with iron. The increase in absorbance at 450 nm (A450 nm) was measured every 30 s for 45 min, at which time saturation with iron was essentially complete (>98%). To study pH-dependent desaturation of iron-saturated rhLF and natural hLF, these preparations were diluted in buffers of varying pH and the A465 nm was measured after incubation for 21 h at 20°C (ref. 9).

Monosaccharide composition of rhLF and natural hLF. Proteins were lyophilized in the presence of 100 nmol mannitol and methanolized for 24 h at 85°C in 1.0 M HCl (ref. 16). Samples were neutralized with silver carbonate and N-acetylated with acetic anhydride. After centrifugation (5 min, 20,000g, 25°C), the supernatant was evaporated and dried. The methylglycosides were tri-methylsilylated and analyzed by gas–liquid chromatography with a CP Sil 5CB column (Chrompack, Walton on Thames, UK) and flame-ionization detection.

Bacteria. A multidrug-resistant strain of *Staphylococcus aureus* described previously[3] (a clinical isolate; Department of Infectious Diseases, Leiden University Medical Center) was used in this study. *Klebsiella pneumoniae* 43816 was purchased from the American Type Culture Collection (Rockville, MD). Bacteria were cultured overnight in nutrient broth at 37°C, diluted in brain–heart infusion broth, and cultured for an additional 2 h in a shaking water bath at 37°C. Stocks of bacteria (1×10^8 colony-forming units (CFU)/ml) were then prepared and stored at –20°C.

Effect of rhLF and natural hLF on experimentally induced infections. Specific pathogen-free male Swiss mice weighting 20–25 g (Broekman Institute, Someren, The Netherlands) were infected by intramuscular injection with approximately 1×10^6 CFU of multidrug-resistant *S. aureus*; 24 h thereafter, 50 μg rhLF, natural hLF, or BSA was injected intravenously. At 0.5, 2, 16, and 48 h after injection of proteins, mice were killed and their thigh muscles removed, weighed, and then homogenized. Next, the number of CFU in the homogenates was determined microbiologically as described in detail elsewhere[3]. Where indicated, experiments were carried out in mice injected intraperitoneally with 200 mg cyclophosphamide (Endoxan-ASTA, Dagra, Utrecht, The Netherlands) three days prior to the induction of leukocytopenia. Blood leukocyte counts were taken from randomly selected mice to confirm leukocytopenia. Cyclophosphamide-treated mice were infected intra-muscularly with approximately 1×10^6 CFU of multidrug-resistant *S. aureus*; 24 h thereafter, mice were injected with various amounts (range: 0–500 μg per mouse) of rhLF, natural hLF, or BSA. After 24 h, the number of viable bacteria was determined microbiologically. In the

K. pneumoniae model, mice were infected intraperitoneally with approximately 1×10^6 CFU of *K. pneumoniae*; 24 h thereafter, 50 µg of rhLF, natural hLF, or BSA was injected intravenously. At 2 and 24 h after injection of these proteins, mice were killed and their peritoneal cavities were lavaged with 4 ml of ice-cold PBS supplemented with 50 units/ml heparin. The number of viable bacteria was determined microbiologically. All mouse studies were done in compliance with Dutch laws relating to the conduct of animal experiments and were approved by the Dutch Experimental Animal Committees DEC 98120 and DEC 99097, Leiden University (Secretary, P.G.J. Reuzel, P.O. Box 2081, Leiden, The Netherlands).

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (http://biotech.nature.com) for details.

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References

1. Nuijens, J.H., van Berkel, P.H.C. & Schanbacher, F.L. Structure and biological actions of lactoferrin. J. Mammary Gland Biol. Neoplasia 1, 285–295 (1996).

2. Anderson, B.F., Baker, H.M., Norris, G.E., Rice, D.W. & Baker, E.N. Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 Å resolution. J. Mol. Biol. 209, 711–734 (1989).

3. Nibbering, P.H. et al. Human lactoferrin and peptides derived from its N terminus are highly effective against infections with antibiotic-resistant bacteria. Infect. Immun. 69, 1469–1476 (2001).

4. Soukka, T., Tenovuo, J. & Lenander, L.M. Fungicidal effect of human lactoferrin against Candida albicans. FEMS Microbiol. Lett. 69, 223–228 (1992).

5. Zhang, G.H., Mann, D.M. & Tsai, C.M. Neutralization of endotoxin in vitro and in vivo by a human lactoferrin-derived peptide. Infect. Immun. 67, 1353–1358 (1999).

6. Hasegawa, K., Motsuchi, W., Tanaka, S. & Dosako, S. Inhibition with lactoferrin of in vitro infection with human herpes virus. Jpn. J. Med. Sci. Biol. 47, 73–85 (1994).

7. Garber, K. Biotech industry faces new bottleneck. Nat. Biotechnol. 19, 184–185 (2001).

8. Krimpenfort, P. et al. Generation of transgenic dairy cattle using 'in vitro' embryo production. Biotechnology (NY) 9, 844–847 (1991).

9. Nuijens, J.H. et al. Characterization of recombinant human lactoferrin secreted in milk of transgenic mice. J. Biol. Chem. 272, 8802–8807 (1997).

10. Velander, W.H. et al. High-level expression of a heterologous protein in the milk of transgenic swine using the cDNA encoding human protein C. Proc. Natl. Acad. Sci. USA 89, 12003–12007 (1992).

11. Edmunds, T. et al. Transgenically produced human antithrombin: structural and functional comparison to human plasma-derived antithrombin. Blood 91, 4561–4571 (1998).

12. Brink, M.F., Bishop, M.D. & Pieper, F.R. Developing efficient strategies for the generation of transgenic cattle which produce biopharmaceuticals in milk. Theriologenology 53, 139–148 (2000).

13. van Berkel, P.H., van Veen, H.A., Geerts, M.E., de Boer, H.A. & Nuijens, J.H. Heterogeneity in utilization of N-glycosylation sites Asn624 and Asn138 in human lactoferrin: a study with glycosylation-site mutants. Biochem. J. 319, 117–122 (1996).

14. van Berkel, P.H.C. et al. Glycosylated and unglycosylated human lactoferrins can both bind iron and have identical affinities towards human lysozyme and bacterial lipopolysaccharide, but differ in their susceptibility towards tryptic proteolysis. Biochem. J. 312, 107–114 (1995).

15. Welling, M.M., Paulusma-Annema, A., Balter, H.S., Pauwels, E.K. & Nibbering, P.H. Technetium-99m labelled antimicrobial peptides discriminate between bacterial infections and sterile inflammations. Eur. J. Nucl. Med. 27, 292–301 (2000).

16. Kamerling, J.P. & Vliegenthart, J.F.G. Carbohydrates. In Mass spectrometry. (ed. Lawson, A.M.) 176–263 (Walter de Gruyter, Berlin; 1989).