MINIREVIEW / MINISYNTHÈSE

Production of human lactoferrin in animal milk[1]

I.L. Goldman, S.G. Georgieva, Ya.G. Gurskiy, A.N. Krasnov, A.V. Deykin, A.N. Popov, T.G. Ermolkevich, A.I. Budzevich, A.D. Chernousov, and E.R. Sadchikova

Abstract: Genetic constructs containing the human lactoferrin (*hLf*) gene were created within a joint program of Russian and Belorussian scientists. Using these constructs, transgenic mice were bred (the maximum hLf concentration in their milk was 160 g/L), and transgenic goats were also generated (up to 10 g/L hLf in their milk). Experimental goatherds that produced hLf in their milk were also bred, and the recombinant hLf was found to be identical to the natural protein in its physical and chemical properties. These properties included electrophoretic mobility, isoelectric point, recognition by polyclonal and monoclonal antibodies, circular dichroic spectra, interaction with natural ligands (DNA, lipopolysaccharides, and heparin), the binding of iron ions, the sequence of the 7 terminal amino acids, and its biological activity. The latter was assessed by the agglutination of *Micrococcus luteus* protoplasts, bactericidal activity against *Escherichia coli* and *Listeria monocytogenes*, and fungicidal activity against *Candida albicans*. We also demonstrated a significant increase in the activity of antibiotics when used in combination with Lf.

Key words: human lactoferrin, gene construction, transgenic animals.

Résumé : Des constructions comportant le gène de la lactoferrine humaine (*hLf*) ont été créées dans le contexte d'un programme conjoint regroupant des scientifiques russes et bélarussiens. Des souris transgéniques générées à partir de ces constructions ont servi à la reproduction (la concentration maximale de hLf du lait était de 160 g/L) et des chèvres transgéniques ont aussi été générées (jusqu'à 10 g/L de hLf dans le lait). Les chèvres transgéniques sécrétant de la hLf dans le lait ont servi à la reproduction, et la hLf recombinante s'est montrée identique à la protéine naturelle au plan de ses propriétés physiques et chimiques. Ces propriétés incluaient la mobilité électrophorétique, le point isoélectrique, la reconnaissance par des anticorps polyclonaux et monoclonaux, les spectres de dichroïsme circulaire, l'interaction avec des ligands naturels (ADN, lipopolysaccharides (LPS) et héparine), la liaison des ions du fer, la séquence des sept acides aminés terminaux et l'activité biologique. Cette dernière a été évaluée par l'agglutination de protoplastes de *Micrococcus luteus*, l'activité bactéricide envers *Escherichia coli* et *Listeria monocytogenes*, et l'activité fongicide envers *Candida albicans*. Nous avons aussi démontré une augmentation significative de l'activité d'antibiotiques utilisés en combinaison avec la lactoferrine.

Mots-clés : lactoferrine humaine, construction génique, animaux transgéniques.

[Traduit par la Rédaction]

Introduction

Lactoferrin (Lf) could be used as a dietary supplement for functional nutrition and as a component of a new generation of highly efficient and biologically safe drugs with antiinfection properties in humans. Accordingly, this need for Lf protein presents a challenge for the pharmaceutical industry to meet the demand for industrial production of this bioactive protein. In many countries, extensive studies are being performed to develop biotechnological methods of obtaining human lactoferrin (hLf) from transgenic plants (Suzuki et al. 2003), fungi (Ward et al. 1995), and the milk of various agricultural animals (Bai et al. 2010; van Berkel et al. 2002; Li et

Received 2 August 2011. Revision received 16 November 2011. Accepted 1 December 2011. Published at www.nrcresearchpress.com/bcb on 23 February 2012.

I.L. Goldman, A.V. Deykin, and T.G. Ermolkevich. Institute of Gene Biology, Russian Academy of Sciences, 119334, Vavilova str, 34/5, Moscow, Russia; "Transgenebank" of IGB RAS, Vavilova str, 34/5, Moscow, 119334, Russia.
S.G. Georgieva and R. Sadchikova. Institute of Gene Biology, Russian Academy of Sciences, 119334, Vavilova str, 34/5, Moscow, Russia; "Transgenepharm LLC" Skolkovo Innovation HUB Development Foundation, 2-th Baumanskaya str, 5, Moscow, 105005, Russia.
Y.G. Gurskiy, A.N. Krasnov, A.N. Popov, and A.D. Chernousov. Institute of Gene Biology, Russian Academy of Sciences, 119334, Vavilova str, 34/5, Moscow, Russia.
A.I. Budzevich. Scientific and Practical Centre of the National Academy of Sciences of Belarus on Animal Husbandry, Frunze Str. 11, Zhodino, Minsk region, 222160, Belarus.

Corresponding author: E.R. Sadchikova (e-mail: ers@igb.ac.ru).

[1]This article is part of a Special Issue entitled Lactoferrin and has undergone the Journal's usual peer review process.

al. 2006; Han et al. 2007; Zhang et al. 2008). To this end, we are developing a method of obtaining hLf in the milk of dairy goats. In addition, we have sought to produce a highly active and biologically safe hLf that is identical to the natural protein by applying economical technologies. This report presents the main results of our study and perspectives of hLf industrial production.

Materials and methods

Expression constructs

A collection of genetic constructs containing the *hLf* gene was created at the Institute of Gene Biology of the Russian Academy of Sciences. The constructs were created on the basis of the pBC1 vector from the pBC1 Milk Expression Vector kit (Invitrogen). The hLf2 construct, representing the 2100 bp Lf cDNA, was cloned into the pBC1 vector at the *Xho*I restriction site. The hLf3 construct, a hybrid construct containing the Lf gene, the first part of which was the genomic copy comprising exons 1–7 (14 479 bp long, from the ATG codon to the *Sma*I site) and the second part was cDNA (1331 bp, from the *Sma*I site to the stop codon), was cloned into the pBC1 vector at the *Xho*I site. These 2 parts were linked at the *Sma*I site located at exon 7. The hLf5 construct, the genomic Lf sequence 35 013 bp long starting from the ATG codon, was cloned into the pBC1 vector at sites *Xho*I and *Not*I. The hLf7 construct, the genomic Lf sequence 28 672 bp long starting from the ATG codon, was cloned into the pBC1 vector at the *Xho*I site. The hLf8 construct was derived from hLf7, from which the insulators upstream of the β-casein promoter were deleted (Fig. 1).

Transgenic mice

To obtain primary transgenic animals, DNA (5 ng/μL in 0.1 mmol/L EDTA and 10 mmol/L Tris (pH 7.4)) was injected into the male pronuclei of F1 fertilized ova (CBA × C57BL/6) mice, and the surviving microinjected zygotes were implanted into pseudopregnant recipients. The ova were obtained by inducing superovulation (see Zvezdova et al. (2010) for further details).

Transgenic goats

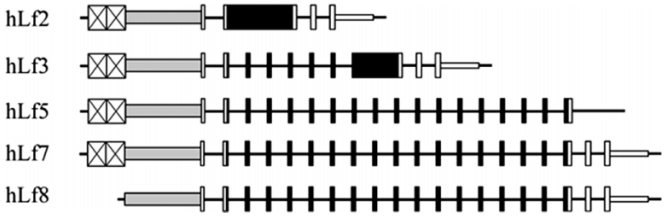
Transgenic goats (Saanen breed) were obtained by microinjecting the construct into the zygotic male pronucleus. The animals (donors and recipients) were treated with hormones to induce polyovulation and the synchronization of sexual cycles (Goldman et al. 2002). Washout and transplantation of the zygotes was carried out surgically (Youn et al. 1997). Up to 3 zygotes injected with 1–5 ng/μL DNA (hLf3 or hLf5) were implanted per recipient.

DNA and RNA assays

Transgenic animals were confirmed by PCR with the following primers: forward, TTGTCAGCTATCTCCCCAAGCTC (the intron upstream of hLf exon 7); and reverse, GCCACAACGGCATGAGAAGGGACC (exon 7).

This PCR yielded a 237 bp product and was assessed on a 2% Tris–acetate–EDTA (TAE) agarose gel. Human genomic DNA served as a positive control; the negative controls were wild-type murine and goat genomic DNA.

Fig. 1. The expression constructs of the human lactoferrin (*hLf*) gene that were used for producing transgenic animals. Two copies of the insulator are indicated with crosses; the β-casein promoter is shown in gray; the coding and untranslated regions of the *Lf* gene are shown in black and white, respectively.



The copy number was determined by real-time PCR using human genomic DNA as the reference (assumed to contain 2 copies). The PCR assay used 100 ng of DNA, and the DNA concentration was measured using a Qubit fluorimeter (Invitrogen).

Tissue specificity was assessed by real-time (RT) – PCR with cDNA synthesized from the RNA from different organs and tissues. The RNA was isolated using a Qiagen RNeasy mini kit. Reverse transcription using an oligo(dT) primer was performed with Invitrogen SuperScript III with 2 μg of RNA. The hLf and G3PDH genes were PCR amplified with the following primer pairs: hLf forward, GCAGACATGAAACTTGTCTTCCTCGT and reverse, GCCACAACGGCATGAGAAGGGACC; and G3PDH forward, ACCACAGTCCATGCCATCAC and reverse, TCCACCACCCTGTTGCTGTA.

The RT–PCR was performed using a Chrom4 RT–PCR system and Opticon Monitor 3.1.32 (Bio-Rad).

Animal breeding and milk production

The milk content of the recombinant hLf obtained from the primary transgenic females was tested during the first pregnancy upon crossing with wild-type males. The transgenic males were evaluated by the hLf content in the milk of their daughters from wild-type females.

Protein assays

We purified hLF from acid whey or skimmed milk using a 3-step chromatographic procedure. First, the samples were passed over SP-Sepharose and dialyzed, followed by a negative DEAE step with 0.25 mol/L NaCl, with a final purification on SP-Sepharose followed by dialysis and freeze-drying (Liang et al. 2011; Fee and Chand 2006).

The recombinant Lf was identified with rabbit polyclonal antibodies against hLf by Western blot (Kanyshkova et al. 2001). Due to the pronounced expression, hLf was observed as a novel band upon resolution of the total milk protein by denaturing PAGE and Coomassie staining (Sokolov et al. 2006).

The degree of Lf saturation with iron ions was assessed using the extinction coefficients for $(Fe^{3+})_2$-Lf at 460 and 280 nm (Zakharova et al. 2000)

Circular dichroic spectra were recorded in a buffer containing 10 mmol/L HEPES–NaOH (pH 7.4) and 0.1 mol/L NaCl at 25 °C with a JASCO 750 instrument at the laboratory of G.V. Gurskii (Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow).

The interaction of recombinant hLf with natural ligands was examined in a model, as in Zakharova et al. (2000).

Sequencing of hLF was performed according to van Veen (2002).

Mouse tissue-specific transgene expression

A comparison of the recombinant protein expression in the tissues of transgenic mice was performed by RT–PCR based on the cDNA generated from the reverse transcription of RNA from tissue lysates. The level of expression was normalized to that of the animals' muscle for which the expression was set as 1.

The biological activity of hLf

The agglutination of *Micrococcus luteus* protoplasts with recombinant hLf was demonstrated using the model system described in Perraudin and Prieels (1982).

The bactericidal activity against *Escherichia coli* and *Listeria monocytogenes* and the fungicidal activity against *Candida albicans* were detected by radial diffusion in an agarose gel after PAGE in the presence of urea and acetic acid (Lehrer et al. 1991).

The hLf biological activity was assayed against ordinary and antibiotic-resistant strains of microorganisms obtained from patients with chronic upper respiratory tract infections by the Immunology Institute clinic (Russia).

Pure microbial cultures were defined from pathological strains according to commonly used procedures (Reisner et al. 1999). Microorganisms were identified using the BBL CRYSTAL Identification Systems Enteric/Nonfennenter ID kit (Becton Dickinson). The strains were stored in slant tubes in a refrigerator at 2–8 °C and subcultured weekly.

The susceptibility of the defined strains of *Staphylococcus aureus* to vancomycin, ciprofloxacin, cefoperazone 75, azithromycin, amoxiclav, chloramphenicol, ampicillin, oxacillin, cefazolin, ciprofloxacin, cefuroxime, erythromycin, clindamycin, co-trimoxazole, gentamicin, mupirocin, doxycycline, moxifloxacin, levofloxacin, clarithromycin, and amikacin was detected using the Kirby-Bauer disk diffusion test and in accordance with National Committee for Clinical Laboratory Standards (NCCLS 2001, Ml 00-Sll [M2], Disk Diffusion 11th informational Suppl.). A 1-day culture of microorganisms was used for the preparation of the inocula; the culture suspension was prepared in accordance with the 0.5 McFarland turbidity standard.

When detecting hLf antibacterial activity, a freeze-dehydrated hLf sample was dissolved in physiological phosphate-buffered saline (PBS), pH 7.2, and applied onto the disks with or without the antibiotics for which resistance had been detected. The inhibition halos were then measured using an automated analyzer (OSIRIS IMAGER 88 333; Bio-Rad). The results obtained were considered individually for each antibiotic accordingto the following 3-point scale: resistant (R), relatively resistant (I), and sensitive (S). In all cases, a preliminary visual count was performed, and, where necessary, the inhibition halos were measured directly.

Results

The hLf protein was expressed and detected in the milk of transgenic mice harboring 5 of the genetic constructs generated (Fig. 1).

Table 1. Human lactoferrin (hLf) contents in the milk of 18 transgenic female founder mice.

Gene construction	Founder No.	Level of hLf (g/L)
hLf2	221	4.0
hLf3	277	6.1
	1118	8.7
	385	14.0
	263	10.2
	230	0.9
hLf5	112	1.5
	115	4.8
	116	33.0
	146	40.0
hLf7	705	4.2
	787	5.6
	790	8.1
	793	3.7
	809	7.4
hLf8	1252	3.3
	1238	0.2
	1174	1.5

Experiments with mice

The expression level of hLf in the milk of transgenic females (Table 1) and daughters of transgenic male founders (Table 2) varied depending on the gene structure and individual mice characteristics.

The production of hLf in the milk of the transgenic mice occurred tissue specifically in the mammary gland. For our analysis, samples were obtained from the tissues and organs of mice with the transgenic construction LTF5 (Table 3). Trace amounts of *hLf*-RNA were found in different organs, which can be explained by the presence of insulators in the constructs.

As characterized by the stable production of hLf in the milk (the average production of hLf varied by 15.3 g/L), 54 transgenic mouse lines were generated (up to F12) through the reproduction of the initial transgenic female mice with high levels of expression.

We managed to create several lines of transgenic mice that maximally produced 160 g/L hLf in their milk.

When performing the histological studies of the tissues and organs from the experimental and control animals, we studied the following mouse internal organs and tissues: brain, spinal cord, spleen, mesenterial lymph nodes, thymus, lungs, heart, kidneys, uterus and ovaries (in lactating females), appendages, prostate and testicles (in males), skin, mammary gland skin, bicep thigh muscle, thyroid gland, bladder, pancreas, liver, stomach, and intestines (duodenum, nestis, ileum, caecum, colon, and rectum).

Analysis of the histological preparations of the brain, spinal cord, intramural ganglia, and nerves revealed no pathological changes in the experimental and control animals. Similarly, normal morphological patterns were observed in the experimental and control animal gonads (testicles and ovaries), skin derivatives (hair and glands), adrenal glands, organs of the cardiovascular system, lungs, and organs of the excretory system. The morphological patterns of the skeletal

Table 2. The level of human lactoferrin (hLf) production in the milk of the transgenic offspring's per 2 gene constructions hLf3 and hLf5.

Construction	Male No.	Daughter No.	hLf in the milk (g/L)	Average
hLf3	281	285	12.3	14.6
		295	12.6	
		395	14.6	
		1152	16	
		293	16.3	
		1150	18.1	
		294	20.7	
		1304	5.8	
hLf3	373	718	8.7	11.6
		609	20.2	
		1212	6	
hLf3	382	2436	12.4	12.4
hLf3	398	1216	10.4	10.4
hLf3	1112	148	3.7	6.6
		2633	7.1	
		2599	4.7	
		151	8.3	
		2633	7.8	
		149	9.9	
		2629	4.8	
hLf5	113	121	13	23.4
		164	16.4	
		127	32.4	
		185	22.3	
		175	33	
hLf5	138	636	10	12.4
		641	11.2	
		631	12	
		633	14	
		634	15	
hLf5	145	49	15.7	16.2
		672	4.9	
		678	20.3	
		684	24	

muscle tissue from the bicep muscle of the thigh, skeletal muscle tissue, skin, and spinal cord were similar and unchanged in both the experimental and control groups. In the bicep muscle of the thigh of 1 animal, we observed a zone in which the partial lysis of several muscle fibers and the simultaneous well-expressed formation of new young fibers (myotubule stage) were observed. The discovered zone may correspond to the posttraumatic regeneration of the skeletal muscle fiber. A study of the digestive glands revealed similar morphological patterns in the animals of both groups. We also detected small local zones in the exocrine sections of the pancreas of 1 experimental and 1 control animal that contained cells with vacuolized cytoplasm. The hematopoietic and immunogenic organs had similar morphological parameters in the experimental and control animals. Of note is the relative increase in the germ centres of lymphatic nodes of both groups of animals.

The lungs of the experimental and control animals had similar airway and respiratory morphologies, except for a zone of the subpleural atelectasis with an unclear etiology, which was observed in 1 of the control animals.

Table 3. The results of the analysis of transgene expression (construction hLf5).

Organ/tissue	Level of expression
Mammary gland	4470
Liver	0
Spleen	1
Ovaries	5
Kidneys	1
Pancreas	0
Heart	5
Lungs	1
Thymus	0
Brain	0
Muscle	1

Note: Values were normalized to the expression level in muscle.

The results of the population studies in 54 primary transgenic mice carrying the *hLf* gene and approximately 5800 offspring to 12 generations allow us to make a general conclusion that the transgenic mice are in accordance with the physiological norms for this species, as the single changes detected by the histological preparations were equally characteristic of both the experimental and control animals. Importantly, the reproductive function was retained and a high level of hLf was detected in the milk of the transgenic animals (Deykin et al. 2009)

Experiments with goats

In the fall of 2007, the joint experiments of Russian and Belorussian scientists led to the production of transgenic goats: 32 progenies were born and 2 hLf transgenic founders (Lac-1 on hLf5 construction and Lac-2 on hLf3 construction) were obtained.

Verification of the presence of the *hLf* gene in the goats' genome (blood sample, and when kids were at 8 months of age, in the first portions of the sperm) showed that the founders contained the transgene in their genomes, Lac-1 and Lac-2 have approximately 1 copy of *hLf* in their genome (Fig. 2).

In 2009, at biotechnological farms in Belarus and Russia, the Lac-1 and Lac-2 founders produced 2 healthy transgenic progeny.

The 38 daughters of the transgenic bucks produced hLf, ranging from 1.5 to 10.8 g/L. A sperm bank of transgenic goats was created.

The milk production varied negligibly during the animals' entire lactation period.

The physical and chemical properties of hLf

The recombinant hLf we isolated was 50% saturated with iron ions, on average, in contrast to the recombinant hLf obtained by Nuijens et al. (1997) which was 90% saturated. In breast milk, hLf is represented primarily (75%–88%) by the apo-form (Farnaud and Evans 2003). The apo-form of the recombinant hLf was obtained by decreasing the pH of the solution to 3.0 in the presence of 50 mmol/L EDTA and

Fig. 2. Verification of human lactoferrin DNA in the sperm of 2 transgenic founders. K–, nontransgenic goat; K+, human DNA; and Tr 1, 2, transgenic goat Lac-1 and Lac-2.

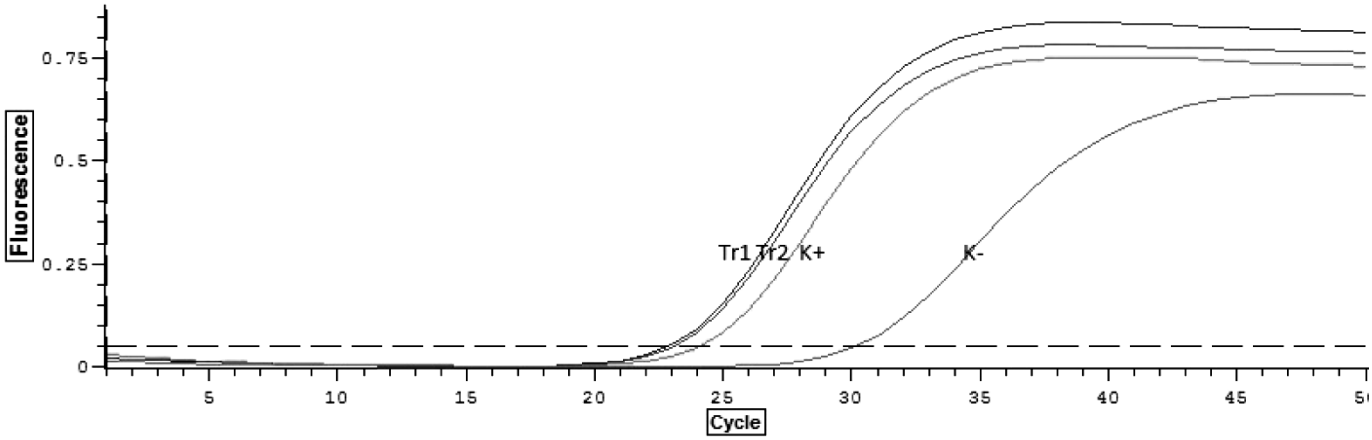
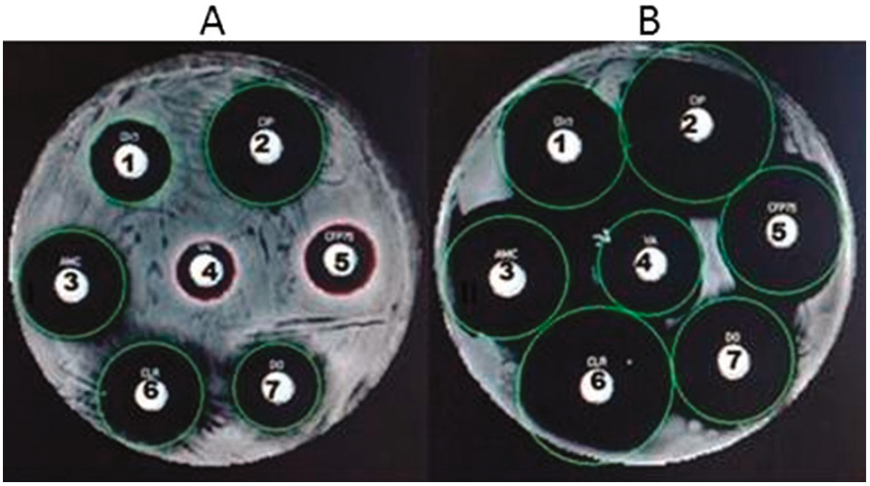


Fig. 3. An increase in antibacterial activity for the standard antibiotic set (1–7) by human lactoferrin obtained from the milk of goats against a clinical isolate of *Staphylococcus aureus* (clinical isolate No. 12) resistant to vancomycin (4) and cefoperazone (5). (A) The antibacterial activity of only antibiotics. Diluting fluid (50 μL; control) was applied to the disks. 1, Disk with oxacillin (18 mm*); 2, disk with ciprofloxacin (25 mm*); 3, disk with amoxicillin/clavulanic acid (23 mm*); 4, disk with vancomycin (12 mm*); 5, disk with cefoperazone (15 mm*); 6, disk with clarithromycin (22 mm*); and 7, disk with doxycycline (19 mm*). (B) Antibacterial activity of antibiotics in the presence of human lactoferrin. 1–7, Human lactoferrin obtained from the milk of goats was applied to the disks with the corresponding antibiotic as in (A) (50 μL of solution at a concentration 3 mg/mL). 1,25 mm*; 2,33 mm*; 3,26 mm*; 4,23 mm*; 5,28 mm*; 6,34 mm*; and 7,26 mm*. *, Diameter of zones of growth inhibition.



0.1 mol/L NaCl. The ability of the recombinant hLf to bind iron ions was studied using buffers containing citric acid and Na_2HPO_4 in the pH range of 2.0–6.0. The A460/A280 ratio, which was used to assess the degree of saturation of the protein, was the same for the hLf and recombinant hLf in the specified pH range and close to previously reported values (Baker and Baker 2004).

In the circular dichroic spectra of the hLf and recombinant hLf completely saturated with Fe^{3+} and their apo-forms, the α-helix content was determined to be 0.40, 0.2 β-sheets, and 0.18 β-turns. These values are similar to the values previously published (Anderson et al. 1989), which testifies to the native conformation of the recombinant hLf and its apo-form.

Protein sequencing of the N-terminal 7 amino acids showed that the hLF from goat milk is identical to native hLF.

Biological activity of hLf

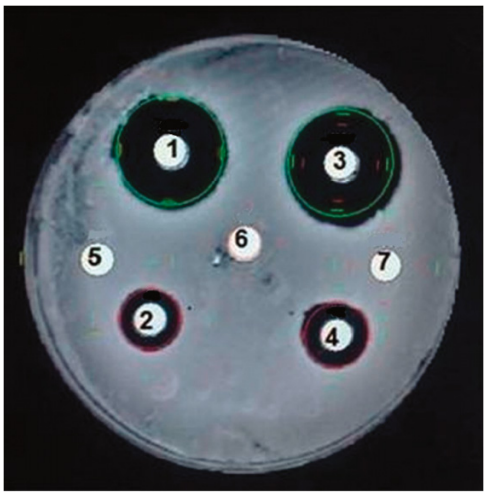
hLf has an antimicrobial activity that affects antibiotic-resistant pathogenic microorganisms and the microorganisms excreted from patients with depressed immunity. Swabs from tonsils were taken from the pharynxes of 421 patients with chronic inflammatory diseases from different parts of the nasopharynx, and the activity of the Lf samples was assessed by the disk diffusion method.

When using hLf combined with antibiotics, an increased antibiotic activity was observed.

Enlarged zones of growth inhibition of *S. aureus* were observed with hLf using standard antibiotic sets with both initial sensitivity and initial resistance (Fig. 3).

We determined that the antifungal activity of Lf and its derivatives manifests as an inactivation of sporozoids, which were found to be incapable of infecting cells, or by the direct

Fig. 4. An increase in the antifungal activity of (1) nystatin and (3) amphotericin by human lactoferrin obtained from the milk of goats against a clinical isolate of *Candida albicans* (clinical isolate No. 29.10.10) resistant to (2) nystatin and (4) amphotericin. Lactoferrin (50 μL at a concentration 3 mg/mL) was applied to disks with the corresponding antifungal (1. 21 mm*; and 3, 23 mm*); 2, disk with nystatin (12 mm*); 4, disk with amphotericin (14 mm*); and 5, 6, and 7, 50 μL of diluting buffer was applied to the disks without antifungals (controls) (0 mm*). *, Diameter of zones of growth inhibition.



destruction of the phytopathogen cell wall, leading to its death (Xu et al. 1999; Valenti et al. 1986; Nikawa et al. 1993, 1995).

By destroying pathogenic microflora, viruses, and fungi, Lf does not cause microorganism resistance as occurs with antibiotics (Flores-Villaseñor et al. 2010). The Lf that we obtained from the milk of goats increased the antifungal activity of antimycotic agents (Fig. 4).

Conclusion

At the Institute of Gene Biology of the Russian Academy of Sciences, we created genetic constructs to allow for the economically significant production of hLf from a series of mice and goat generations. Our work has been focused on resolving the fundamental issue of increasing the expression of foreign genes, which is a key problem for a number of practical issues when using genetically modified animals. Population studies were performed to demonstrate the importance of choosing transgenic animal producers with high levels of hLf production as the founders of industrial animal herds. We proposed technology for using the initial transgenic males, which would allow for the creation of herds of animal producers for medicinal human proteins, while preserving the economically significant production of a protein in a series of generations.

Acknowledgement

This study was supported by the Transgenebank and Federal programs of the Union State of Russia and Belarus.

References

Anderson, B.F., Baker, H.M., Norris, G.E., Rice, D.W., and Baker, E.N. 1989. Structure of human lactoferrin: crystallographic structure analysis and refinement at 2.8 A resolution. J. Mol. Biol. **209**(4): 711–734. doi:10.1016/0022-2836(89)90602-5. PMID:2585506.

Bai, Q., Zhang, Y., Wang, Y., Luo, J., Li, Y., Huang, Y., et al. 2010. [Purification and characterization of recombinant human lactoferrin expressed in a cattle mammary bioreactor]. Sheng Wu Gong Cheng Xue Bao, **26**(11): 1576–1583. [in Chinese.] PMID: 21284219.

Baker, H.M., and Baker, E.N. 2004. Lactoferrin and iron: structural and dynamic aspects of binding and release. Biometals, **17**(3): 209–216. doi:10.1023/B:BIOM.0000027694.40260.70. PMID: 15222467.

Deykin, A.V., Ermolkevich, T.G., Gursky, Y.G., Krasnov, A.N., Georgieva, S.G., Kuznetsov, S.L., et al. 2009. [The state of health and the reproductive potential of transgenic mice secreting recombinant human lactoferrin in milk]. Dokl. Biochem. Biophys. **427**(1): 195–198. [Original in Russian.] doi:10.1134/S1607672909040073. PMID:19817135.

Farnaud, S., and Evans, R.W. 2003. Lactoferrin–a multifunctional protein with antimicrobial properties. Mol. Immunol. **40**(7): 395–405. doi:10.1016/S0161-5890(03)00152-4. PMID:14568385.

Fee, C.J., and Chand, A. 2006. Capture of lactoferrin and lactoperoxidase from raw whole milk by cation exchange chromatography. Separ. Purif. Tech. **48**(2): 143–149. doi:10.1016/j.seppur.2005.07.011.

Flores-Villaseñor, H., Canizalez-Román, A., Reyes-Lopez, M., Nazmi, K., de la Garza, M., Zazueta-Beltrán, J., et al. 2010. Bactericidal effect of bovine lactoferrin, LFcin, LFampin and LFchimera on antibiotic-resistant *Staphylococcus aureus* and *Escherichia coli*. Biometals, **23**(3): 569–578. doi:10.1007/s10534-010-9306-4. PMID:20195887.

Goldman, I.L., Sadtchikova, E.L., Kadulin, S.G., and Gnuchev, N.V. 2002. Technology of obtaining goat zygotes with known time of formation suitable for microinjection of recombinant DNA in order to create transgenic animals. Dokl. Biol. Sci. **384**(1/6): 195–198. doi:10.1023/A:1016001004381. PMID:12134482.

Han, Z.S., Li, Q.W., Zhang, Z.Y., Xiao, B., Gao, D.W., Wu, S.Y., et al. 2007. High-level expression of human lactoferrin in the milk of goats by using replication-defective adenoviral vectors. Protein Expr. Purif. **53**(1): 225–231. doi:10.1016/j.pep.2006.11.019. PMID:17208010.

Kanyshkova, T.G., Buneva, V.N., and Nevinsky, G.A. 2001. Lactoferrin and its biological functions. Biochemistry (Mosc.), **66**(1): 1–7. [Original in Russian.] doi:10.1023/A:1002817226110. PMID:11240386.

Lehrer, R.I., Rosenman, M., Harwig, S.S., Jackson, R., and Eisenhauer, P. 1991. Ultrasensitive assays for endogenous antimicrobial polypeptides. J. Immunol. Methods, **137**(2): 167–173. doi:10.1016/0022-1759(91)90021-7. PMID:1901580.

Li, L., Shen, W., Min, L., Dong, H., Sun, Y., and Pan, Q. 2006. Human lactoferrin transgenic rabbits produced efficiently using dimethylsulfoxide-sperm-mediated gene transfer. Reprod. Fertil. Dev. **18**(6): 689–695. doi:10.1071/RD06001. PMID:16930515.

Liang, Y., Wang, X., Wu, M., and Zhu, W. 2011. Simultaneous isolation of lactoferrin and lactoperoxidase from bovine colostrum by SPEC 70 SLS cation exchange resin. Int. J. Environ. Res. Public Health, **8**(9): 3764–3776. doi:10.3390/ijerph8093764. PMID:22016715.

Nikawa, H., Samaranayake, L.P., Tenovuo, J., Pang, K.M., and Hamada, T. 1993. The fungicidal effect of human lactoferrin on *Candida albicans* and *Candida krusei*. Arch. Oral Biol. **38**(12):

1057–1063. doi:10.1016/0003-9969(93)90167-K. PMID: 8141667.

Nikawa, H., Samaranayake, L.P., and Hamada, T. 1995. Modulation of the anti-Candida activity of apo-lactoferrin by dietary sucrose and tunicamycin in vitro. Arch. Oral Biol. **40**(6): 581–584. doi:10.1016/0003-9969(94)00195-H. PMID:7677606.

Nuijens, J.H., van Berkel, P.H., Geerts, M.E., Hartevelt, P.P., de Boer, H.A., van Veen, H.A., and Pieper, F.R. 1997. Characterization of recombinant human lactoferrin secreted in milk of transgenic mice. J. Biol. Chem. **272**(13): 8802–8807. doi:10.1074/jbc.272.13.8802. PMID:9079716.

Perraudin, J.P., and Prieels, J.P. 1982. Lactoferrin binding to lysozyme-treated Micrococcus luteus. Biochim. Biophys. Acta, **718**(1): 42–48. doi:10.1016/0304-4165(82)90007-1. PMID: 7138905.

Reisner, B.S., Woods, G.L., Thomson, R.B., Larone, D.H., Garcia, L.S., and Shimizu, R.Y. 1999. Specimen Processing. *In* Manual of Clinical Microbiology. *Edited by* P.R. Murruy, E.J. Baron, V.A. Pfaller, et al. 7th ed. Washington. pp. 64–104.

Sokolov, A.V., Pulina, M.O., Kristiyan, A.V., Zakharova, E.T., Runova, O.L., Vasil'ev, V.B., et al. 2006. A study of recombinant human lactoferrin secreted in milk of transgenic mice. Dokl. Biochem. Biophys. **411**(1): 336–338. [Original in Russian.] doi:10.1134/S1607672906060032. PMID:17396575.

Suzuki, Y.A., Kelleher, S.L., Yalda, D., Wu, L., Huang, J., Huang, N., and Lönnerdal, B. 2003. Expression, characterization, and biologic activity of recombinant human lactoferrin in rice. J. Pediatr. Gastroenterol. Nutr. **36**(2): 190–199. doi:10.1097/00005176-200302000-00007. PMID:12548053.

Valenti, P., Visca, P., Antonini, G., and Orsi, N. 1986. Interaction between lactoferrin and ovotransferrin and Candida cells. FEMS Microbiol. Lett. **33**(2–3): 271–275. doi:10.1111/j.1574-6968.1986.tb01285.x.

van Berkel, P.H., Welling, M.M., Geerts, M., van Veen, H.A., Ravensbergen, B., Salaheddine, M., et al. 2002. Large scale production of recombinant human lactoferrin in the milk of transgenic cows. Nat. Biotechnol. **20**(5): 484–487. doi:10.1038/nbt0502-484. PMID:11981562.

van Veen, H.A., Geerts, M.E.J., van Berkel, P.H.C., and Nuijens, J.H. 2002. Analytical cation-exchange chromatography to assess the identity, purity, and N-terminal integrity of human lactoferrin. Analytical Biochemistry, **309**: 60–66. PMID:12381362.

Ward, P.P., Piddington, C.S., Cunningham, G.A., Zhou, X., Wyatt, R.D., and Conneely, O.M. 1995. A system for production of commercial quantities of human lactoferrin: a broad spectrum natural antibiotic. Biotechnology (N.Y.), **13**(5): 498–503. doi:10.1038/nbt0595-498. PMID:9634791.

Xu, Y.Y., Samaranayake, Y.H., Samaranayake, L.P., and Nikawa, H. 1999. In vitro susceptibility of Candida species to lactoferrin. Med. Mycol. **37**(1): 35–41. PMID:10200932.

Youn, W.S., Lee, C.S., Goldman, I., Fang, N.Z., Koo, D.B., Han, Y.M., et al. 1997. Studies on the superovulation and collection of microinjectable embryos in Korean native goats (Capra hircus aegagrus). Korean J. Anim. Reprod. **21**: 373–379. [in Korean.]

Zakharova, E.T., Shavlovski, M.M., Bass, M.G., Gridasova, A.A., Pulina, M.O., De Filippis, V., et al. 2000. Interaction of lactoferrin with ceruloplasmin. Arch. Biochem. Biophys. **374**(2): 222–228. doi:10.1006/abbi.1999.1559. PMID:10666301.

Zhang, J., Li, L., Cai, Y., Xu, X., Chen, J., Wu, Y., et al. 2008. Expression of active recombinant human lactoferrin in the milk of transgenic goats. Protein Expr. Purif. **57**(2): 127–135. doi:10.1016/j.pep.2007.10.015. PMID:18054499.

Zvezdova, E.S., Silaeva, Yu.Yu., Vagida, M.S., Maryukhnich, E.V., Deikin, A.V., Ermolkevich, T.G., et al. 2010. [Generation of transgenic animals expressing the α and β chains of the autoreactive T-cell receptor]. Mol. Biol. **44**(2): 277–286 . [Original in Russian.] doi:10.1134/S0026893310020135.