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Lactoferrin from Canine Neutrophils: Isolation and Physicochemical and Antimicrobial Properties

M. N. Berlov[1,2], E. S. Korableva[1], Yu. V. Andreeva[2], T. V. Ovchinnikova[3], and V. N. Kokryakov[1,2]*

[1]*Ukhtomsky Institute of Physiology of St. Petersburg State University, Universitetskaya Nab. 7/9, 199034 St. Petersburg, Russia; fax: (812) 234-9493; E-mail: kokryak@VK5270.spb.edu*
[2]*Institute for Experimental Medicine, Russian Academy of Medical Sciences, ul. Akademika Pavlova 12, 197376 St. Petersburg, Russia*
[3]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 111997 Moscow, Russia; fax: (495) 336-4333; E-mail: ovch@ibch.ru*

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Abstract—Lactoferrin has been isolated from canine leukocytes for the first time. Lactoferrin was identified by N-terminal amino acid sequence and by capability to capture ferric cations resulting in a complex with absorbance maximum at 460-470 nm. It is demonstrated that canine lactoferrin resembles the human homolog in some physicochemical properties, i.e. molecular weight, carbohydrate presence, and conditions of protein–iron complex dissociation. Bactericidal activity of dog lactoferrin was demonstrated on the gram-negative bacterium *Escherichia coli* and gram-positive bacterium *Listeria monocytogenes*. Bactericidal activity of canine lactoferrin is similar to that of human lactoferrin.

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Lactoferrin (Lf) is a marker protein of specific granules of human and other mammalian neutrophils [1]. It was first isolated from bovine milk [2] and later found in secretions of surface epithelia and of many exocrine glands of mammals. Lactoferrins from milk and neutrophils are structurally identical [3, 4].

Current opinion on the structure and biological functions of Lf is stated in detail in a number of reviews [5-8]. The protein is a member of the transferrin family and is able to bind and to transport ferric cations as well as cations of other metals with variable valence (for example, copper and zinc). Binding of ferric cation is coupled with bicarbonate anion binding and leads to formation of a red colored complex with absorbance maximum at 460-470 nm.

All investigated Lfs are glycoproteins containing intramolecular disulfide bonds with molecular weights in the range 75-80 kD. The polypeptide chain of Lf forms two globular domains called the N- and C-lobes joined by α-helix. Each lobe contains an iron-binding center. The tertiary structures of Lf lacking iron (apoLf) and Lf holding iron (Fe^{3+}–Lf) are different. In particular, binding of iron is a prerequisite for the bacteriostatic and antioxidant activities of Lf.

An important function of Lf is its action as an antimicrobial substance that may be realized in the phagolysosomal vacuole of neutrophils as well as on the surface of mucous epithelia. Antimicrobial activity of Lf is related both with microbiostatic and microbicidal actions of this protein.

Besides its antimicrobial action, Lf was shown to possess many other biological properties. Namely, the protein could be immunomodulator, antioxidant and anti-inflammatory agent, transcription factor, and presumable participant of iron metabolism [5-8].

Comparative investigations are highly important in research of biological activities of Lf. Thus, the investigation of the structure and functions of Lf from different sources is significant. Interestingly, there was no direct information about Lf presence in granular apparatus of canine neutrophils to date. Data about Lf in dog neutrophils were obtained indirectly resulting from experiments on radioactive Fe^{3+} binding with protein material

Abbreviations: CTAB) cetyltrimethylammonium bromide; CFU) colony forming unit; Lf) lactoferrin; LPS) lipopolysaccharide; TSB) trypticase soy broth.

* To whom correspondence should be addressed.

of neutrophil granule extract. The protein was not purified and was not even estimated by molecular weight [9]. The first information about canine Lf structure in freely accessible databases appeared in 2005. Upon determination of nucleotide sequence of the 20th dog chromosome, a gene was revealed coding a potential protein product homologous to Lfs of other mammals (the link is http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=full_report&list_uids=484787).

The purpose of present study was to isolate Lf from canine neutrophils and to characterize its principal physicochemical and antimicrobial properties.

MATERIALS AND METHODS

Leukocytes of 16-18-h exudates of outbred male and female dogs (*Canis familiaris*) were utilized for investigation. The exudation was induced by anesthetized (30 μg of ketamine per kg of body weight intravenously) intraperitoneal injection of saline solution (0.85% NaCl) containing 0.3% starch. The cells were washed with saline by centrifugation for 10 min at 1000*g* and suspended in H_2O. Neutrophils constituted 80-90% of the cells by microscopic data. The cell suspension was stored at −20°C. For a single preparative experiment, the material from 4-6 animals was combined.

Lactoferrin isolation. The lactoferrin isolation procedure consisted of three principal steps: cationic protein extraction from the whole leukocyte cells, ion-exchange chromatography, and gel filtration. Purified preparations were subjected to concentration in an ultrafiltration cell (Amicon, The Netherlands) using YM-10 membranes and were stored at −20°C. Lf was revealed in samples electrophoretically based on the suggestion that the electrophoretic mobility of canine Lf is similar to that of human Lf. The final identification of purified protein by spectrophotometric methods and by N-terminal amino acid sequencing confirmed our suggestion.

Cationic proteins were extracted from the leukocytes with 0.05 M sodium acetate buffer, pH 4.5 (further referred as acetate buffer), containing 0.3% cetyltrimethylammonium bromide (CTAB) using a glass-Teflon homogenizer. The homogenate was centrifuged for 40-60 min at 10,000*g* at 4°C. The pellet was collected, and the extraction procedure was repeated. Thus, 10 sequential extracts were prepared. Extracts with maximal Lf content (from 6th to 10th for the typical experiment described below) were combined for further purification.

Ion-exchange chromatography was performed on a CM-cellulose column (2.4 × 25 cm) equilibrated with acetate buffer containing 0.05 M NaCl. After sample loading, the column was washed with acetate buffer containing 0.1 M NaCl until no absorption at 280 nm was detected in the eluate. During this step, CTAB was removed from the column. The Lf was eluted with 0.2 M NaCl in acetate buffer at 20 ml/h. Fractions with maximal Lf content were combined.

Gel filtration was performed on a Sephadex G-150 column (2.5 × 75 cm) equilibrated with acetate buffer containing 1 M NaCl. Before applying to the column, the samples were concentrated to 3-5 ml in an ultrafiltration cell (Amicon) using YM-10 membranes. The proteins were eluted with the starting buffer at 16 ml/h.

All procedures of Lf isolation and purification were performed at 4°C.

Human Lf was isolated from milk as described previously [3].

Electrophoretic methods. Protein spectra in the samples as well as homogeneity of purified preparations were estimated by electrophoresis in an acid buffer system according to Panyim and Chalkley [10] in gels containing 12.5% acrylamide and 6.25 M urea. Carbohydrate components in proteins were revealed after the electrophoresis by staining the gel with Schiff's reagent [11].

The molecular weight of Lf was determined by SDS electrophoresis according to Schagger and von Jagow [12] in 8% polyacrylamide gel. Molecular weight was also estimated by gel filtration.

Immunocytochemistry. For immunocytochemical analysis, we used polyclonal serum obtained by immunization of rabbits with purified canine Lf.

Dog blood samples were applied on object-plates, then were dried and fixed with cold (−20°C) acetone for 10 min. In the following step, endogenous peroxidase activity was inhibited by incubation in methanol containing 0.3% H_2O_2 for 20 min. Then the preparations were treated with blocking solution (1% BSA in 0.01 M sodium phosphate buffer containing 0.9% NaCl, pH 7.4) for 1 h. Polyclonal immune serum against canine Lf was diluted 100-fold in blocking solution and incubated with preparations for 1 h. Then the preparations were incubated with peroxidase-conjugated goat polyclonal antibodies against rabbit immunoglobulins G (400-fold dilution in blocking solution) for 1 h. Finally, the preparations were treated with peroxidase substrate solution (0.06% diaminobenzidine, 0.03% H_2O_2 in 50 mM Tris-HCl buffer, pH 7.4) until desirable coloration developed (about 10 min). The reaction was stopped by washing with distilled water. The preparations were dried and analyzed microscopically. All incubations were performed at room temperature; between incubations, the preparations were washed with 0.01 M sodium phosphate buffer containing 0.9% NaCl, pH 7.4.

N-Terminal amino acid sequencing. The N-terminal amino acid sequence of Lf was determined by automatic degradation according to Edman on a Procise cLC 491 protein sequencing system (PE Applied Biosystems, USA) followed by detection of phenylthiohydantoin derivatives of amino acids on a 120A PTH Analyzer (PE Applied Biosystems).

Investigation of binding of Lf with iron. The spectrophotometric properties of Lf were measured using a Beckman DU50 spectrophotometer (USA).

The level of Lf saturation with iron was determined by optical density value of protein solution at 460 nm. Iron saturation of Lf was expressed as a percentage. Iron-free Lf (apoLf) was prepared by dialysis of Lf preparation against solution containing 0.2 M sodium citrate and 0.2 M sodium phosphate, pH 3.0-3.1 ([13] with modifications). Lf saturated with iron (Fe^{3+}–Lf) was prepared by dialysis of Lf preparation against solution containing 0.1 M sodium citrate and 0.05 M sodium bicarbonate, pH 8.4-8.5, followed by dialysis against the same solution containing $FeCl_3$ in 10-20-fold excess for complete saturation of Lf. Finally, Fe^{3+}–Lf was dialyzed against 0.15 M NaCl to remove unbound iron ([13] with modifications).

Dissociation of Lf–iron complex was studied in two ways.

1. In the first case, dissociation of Fe^{3+}–Lf was analyzed in 3 mM sodium citrate buffer containing 0.15 M NaCl with varying pH values (3.0-5.0). Samples were incubated overnight at 20°C.

2. In the second variant, dissociation of Fe^{3+}–Lf was analyzed in buffer containing 0.05 M sodium citrate, 0.2 M sodium phosphate, and 0.15 M NaCl with varying pH values (3.0-8.5) ([13] with modifications). Samples were incubated overnight at 20°C. Phosphate and citrate anions possess chelating properties stimulating complex dissociation even at high pH values. This method allows observing of Fe^{3+} release from the two iron-binding sites of Lf separately.

The final Lf concentration was 3-5 mg/ml in both cases. Protein content in samples was determined by the Lowry method [14].

Lf bactericidal activity. The bactericidal action of Lf was estimated by colony counting assay. The following microorganism cultures were used as targets for determination of antimicrobial action of proteins: *Escherichia coli*, ML-35p strain (gram-negative bacterium); *Listeria monocytogenes*, EGD strain (gram-positive bacterium). Microorganisms in logarithmic growth phase were used in experiments [15].

The sample of total volume 20 μl contained Lf diluted in 0.01 M sodium phosphate buffer, pH 7.4, and bacterial suspension in the same buffer in concentration $2 \cdot 10^4$ CFU/ml. The samples were incubated on a shaker at 37°C for 1 h. After incubation, 10-μl aliquots were withdrawn from the samples and rapidly mixed with 2 ml of solution (thermostatted at 42°C) containing 1% TSB (trypticase soy broth) and 1% melted agarose. The resulting mixture was poured onto a 40-mm Petri dish. After agarose gelation, the dishes were incubated overnight at 37°C. Then bacterial growth was stopped by adding 5% acetic acid, and the number of colonies grown was counted. Microbicidal activity was expressed as $\log_{10}(N_1/N_2)$, where N_1 is number of colonies in the control sample (typically 100-200 counts per dish) and N_2 is number of colonies in the Lf-containing sample.

In this work we used CTAB, BSA, acrylamide, protein markers for gel filtration, polyclonal goat antibodies to rabbit IgGs conjugated with peroxidase, and diaminobenzidine from Sigma (USA); SDS from Fischer (USA); CM-cellulose from Whatman (Great Britain); Sephadex G-150 from Pharmacia (Sweden); protein markers for SDS electrophoresis from Serva (Germany); TSB from Becton Dickinson (USA); agarose from Gibco BRL/Life Technologies (USA).

RESULTS

We performed five preparative experiments on Lf isolation from dog neutrophils; here we present results of a typical experiment.

Lf content was maximal in the 6-10th of the ten extracts prepared, so they were used for its isolation. Combined extracts were subjected to ion-exchange chromatography on CM-cellulose. Lf was eluted from column by buffer with 0.2 M NaCl. This salt concentration led to a single eluted protein peak comprising both Lf and other proteins (not shown). Prevalent protein material not containing Lf still remains bound on the column at this NaCl concentration (data not shown).

Lf-containing fractions were combined and concentrated to a volume of 3-5 ml using an ultrafiltration cell. While concentrating, the samples were purified from components of molecular weight below 10 kD.

The final step of Lf purification utilized gel filtration. Results are presented in Fig. 1. While running through Sephadex G-150, Lf was separated at least from three

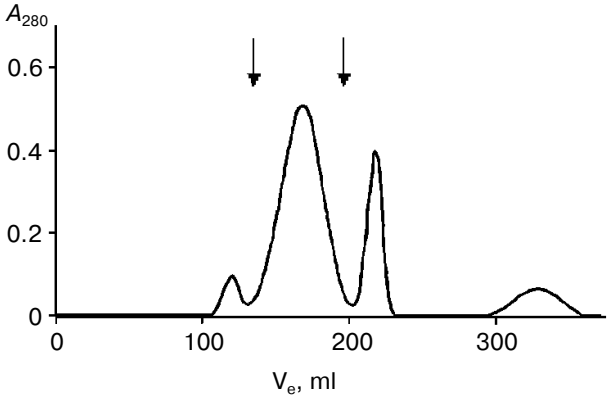

Fig. 1. Gel filtration of the Lf-containing protein fraction on a Sephadex G-150 column. This fraction was obtained by running of combined CTAB extracts with maximal Lf content through the CM-cellulose column, utilizing buffer with 0.2 M NaCl for elution. The abscissa axis represents elution volume (ml). The ordinate axis represents absorbance at 280 nm. The arrows show Lf elution area revealed electrophoretically.

protein impurities. One of the separated components has greater molecular weight (lesser elution volume) compared with Lf, while two others have lesser molecular weights. One of these two low molecular weight components (3rd peak on Fig. 1) represents neutrophil elastase as revealed by its enzymatic activity (data not shown). The shape of the Lf peak implies a homogenous component. Fractions with maximal Lf content were combined and concentrated in an ultrafiltration cell using YM-10 membrane.

Homogeneity of purified preparation was estimated electrophoretically (Fig. 2). For comparison, Fig. 2a represents also results of electrophoretic run of combined extracts employed for Lf isolation.

Data of immunocytochemical assay utilizing specific polyclonal serum to isolated dog Lf revealed its localization exactly in neutrophils (Fig. 3). One can see on the figure that cells with segmented nuclei typical for neutrophils are colored on the dog blood preparation.

Results of N-terminal amino acid sequencing of isolated canine Lf revealed the PRKNVRW sequence for the initial seven residues. The molecular weight of the protein was estimated as 79 kD by SDS-PAGE (Fig. 2b) and 77 kD by gel filtration. The molecular weight of human Lf determined by the same methods is in the same range, 77-79 kD. Schiff's reagent revealed a carbohydrate component in the structure of canine Lf.

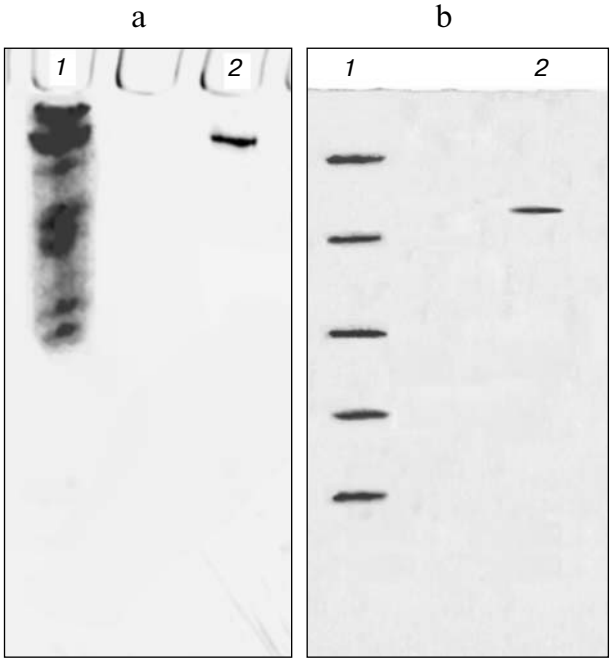

Fig. 2. Results of electrophoresis of canine Lf in polyacrylamide gels by two methods. a) Electrophoresis in acid buffer in the presence of urea: *1*) combined extracts 6-10; *2*) Lf. b) Electrophoresis under denaturing conditions in the presence of SDS: *1*) molecular weight protein markers (20, 30, 45, 66, and 97 kD starting from bottom); *2*) Lf.

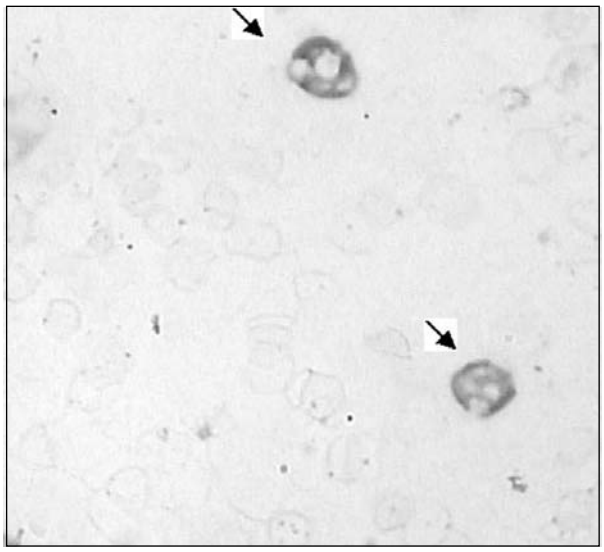

Fig. 3. Immunocytochemical staining of dog blood sample with specific polyclonal immune serum to canine Lf. The arrows indicate neutrophils with stained cytoplasm containing granules with Lf, and with unstained segmented nuclei typical for neutrophils.

In the experiments on iron binding by Lf as well as on antimicrobial activity of Lf, the canine and human proteins were compared. Both methods of dissociation of Lf–iron complex give no differences for canine and human Lfs (Fig. 4). In the first case, no dissociation is observed down to pH 4.0. Between pH 3.0 and 4.0 about 80% of Fe^{3+} is released from Lf (Fig. 4a). In the presence of high concentrations of chelating agents (citrate, phosphate) separate desaturation of iron from two Fe^{3+}-binding sites is achieved (Fig. 4b). In this case, release of Fe^{3+} from the site with lower affinity occurs already at pH values more than 8.0, and release of Fe^{3+} from the site with higher affinity is observed at pH 3.5-5.0.

Data of experiments on antimicrobial activity of canine and human apoLfs are presented in the table. Lf saturated with iron did not reveal antimicrobial activity. Based on the presented data, the antimicrobial activity of canine Lf is similar to that of human Lf. Both Lfs are active at concentrations of 20 μg/ml and more, and activity against *E. coli* is slightly higher than against *L. monocytogenes.*

DISCUSSION

To isolate Lf from canine neutrophils, we utilized a method comprising protein extraction with CTAB in the first step. Originally, CTAB was utilized for leukocyte cationic proteins isolation by Desser et al. for the case of peroxidase [16]. Subsequently, our group successfully modified this method for isolation of the variety of antimicrobial neutrophil proteins [3, 17, 18]. CTAB has

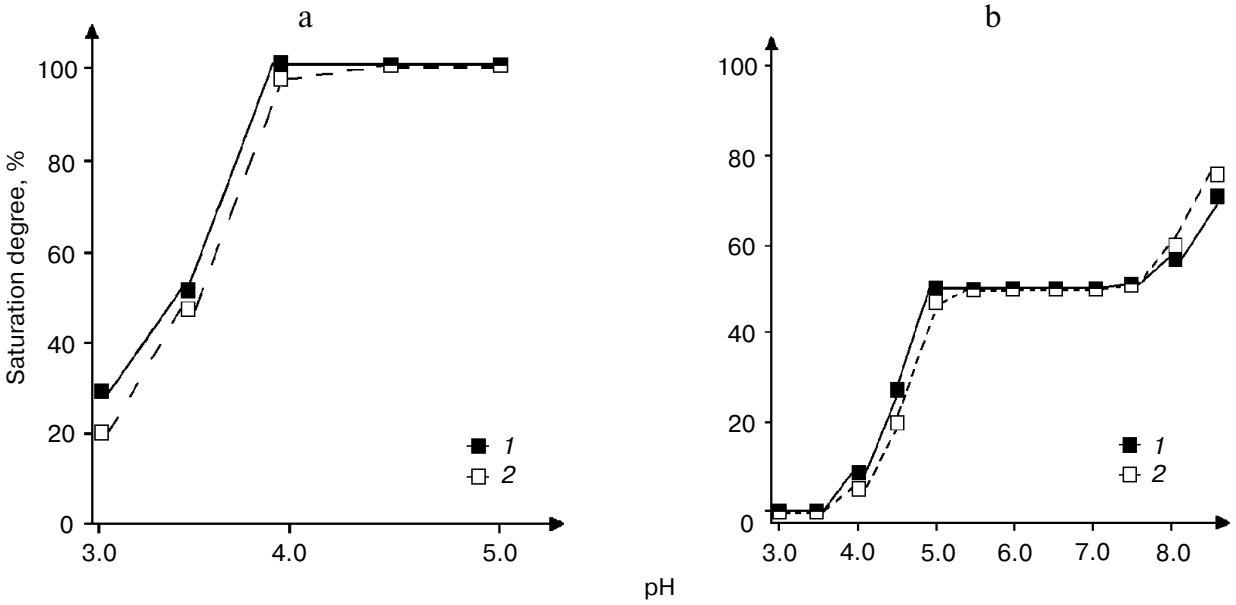

Fig. 4. The pH-dependent dissociation of Lf–Fe^{3+} complex under different conditions: a) in 3 mM sodium citrate buffer; b) in the presence of high concentrations of chelating agents (0.05 M sodium citrate and 0.2 M sodium phosphate); *1*) canine Lf; *2*) human Lf.

some advantages in comparison with other extractants. First, CTAB extracts preferentially cationic proteins providing partial separation of proteins in the initial stage of the isolation procedure. Due to the high efficiency of the extraction of cationic proteins by CTAB, it became possible to omit the step of granule isolation from leukocytes. Taking whole cells as material for extraction avoids protein loss in the step of granule isolation. The second advantage is the possibility to collect and to keep material as cells, while granule isolation from frozen cells is impossible. Another feature of our method is preparation of sequential extracts providing a partial separation of proteins at the extraction step. Summarizing, this method is rather effective to isolate homogenous canine Lf in amounts sufficient for investigation.

While there are some data indicating Lf presence in canine neutrophils published earlier [9, 19], we first isolated and purified the protein in this work. It is of interest that, according to one of the early works, canine milk contains no Lf [20]. According to our own data, Lf is present in canine milk but in low level compared with that in human milk, namely about 40 μg/ml (Berlov, Korableva, Andreeva, Kokryakov, unpublished).

The N-terminal amino acid sequence of isolated protein we determined completely coincides with the known N-terminal sequence of bovine Lf, APRKNVRW [21], with a single residue shift. Thus, the first amino acid of canine Lf corresponds to the second amino acid of bovine Lf. Recently, upon sequencing the canine genome there was a gene revealed on the 20th chromosome encoding a protein homologous to mammalian lactoferrins [10]. Analyzing the data presented, one can readily observe that the mature protein generated by cleavage of the 19 amino acid signal peptide should bear alanine on the N-terminus followed by the seven amino acid sequence determined. In such case the eight amino acid N-terminal sequence of canine Lf gives 100% agreement with that of bovine Lf. The reason for the difference in our data with expected results is a challenge for the future. Probably, after signal peptide cleavage a restricted N-terminal proteolysis of Lf can occur. By the data of van

Antimicrobial activity of canine and human apoLfs against *E. coli* and *L. monocytogenes*

	Lf concentration, μg/ml	$\log_{10}(N_1/N_2)$	
		E. coli	*L. monocytogenes*
Canine Lf	10	0.05	0
	20	0.31	0.05
	40	1.11	0.84
	80	>2	1.82
Human Lf	10	0.03	0
	20	0.33	0.05
	40	1.08	0.80
	80	>2	1.77

Note: Antimicrobial activity was estimated by colony counting assay and is expressed as $\log_{10}(N_1/N_2)$, where N_1 is number of colonies in the absence of Lf and N_2 is number of colonies in the presence of Lf. Median values of three experiments are presented in the table.

Berkel et al. [22], commercial human Lf preparations often include isoforms lacking two or three N-terminal amino acids. However, for human Lf a peptide bond between two arginines is cleaved, whereas to remove the N-terminal amino acid from canine Lf the breakage of the peptide bond between alanine and proline is necessary. It is difficult to say whether the N-terminal amino acid of canine Lf is removed *in vivo* or under the purification procedure. Nevertheless, it is likely that absence of alanine on the N-terminus should not be critical for exposition of physical, chemical, and antimicrobial properties studied here. The report about the structure of dog gene encoding the protein homological to Lfs appeared in database in January 2005, when the N-terminal amino acid sequence of Lf we isolated had already been determined. Later proposed mRNA and protein structures were replaced with novel refined data with prediction of the seven variants of potential transcripts coding distinct Lf isoforms [10]. The homology of canine Lf with the human protein is about 74%.

In view of prospective clinical application of Lf as an anti-inflammatory or antioxidant agent, it is of importance to perform comparative investigations of protein properties, in particular of its iron-binding capacity, since this can vary for Lfs from different species. Regarding this point, it is interesting that Lf is extremely more effective in iron binding than serum transferrin despite the identical structure of the iron-binding sites. In our investigation, we revealed no differences between canine and human Lfs in iron-holding capacity (Fig. 4).

Antimicrobial activity of Lf is one of the main physiological functions of the protein. According to initial views, the action of Lf on bacteria is limited to microbiostatic effect [23-27]. ApoLf restrains the growth and reproduction of microorganisms by means of withholding of metal cations (Fe^{3+}, Cu^{2+}, Mn^{2+}, Zn^{2+}), which are necessary for microbial protein functions, from medium and from surface structures of the microbial cell wall. Lf saturated with iron does not exhibit bacteriostatic activity.

However, later a direct microbicidal action of Lf on some gram-positive and gram-negative bacteria as well on the fungus *Candida albicans* was established [28-32]. The mechanism of this action is not quite clear, but like microbiostatic effect the microbicidal action is associated only with iron free Lf (i.e. with apoLf), which may be related with distinct tertiary structures of the two forms of the protein.

It is considered that one of the mechanisms of antimicrobial action of human Lf on gram-negative bacteria relies on its ability to bind lipopolysaccharide (LPS) with high affinity and to withdraw it from the outer bacterial membrane [33, 34]. The release of LPS from bacterial envelope stimulated by Lf is inhibited by Ca^{2+} and Mg^{2+} cations, which take part in stabilization of LPS structure [34]. Subsequently, in experiments with bovine Lf instead of the human protein, it was demonstrated that this glycoprotein is able to function as chelate binding Ca^{2+} cations with sialic acid residues localized in the carbohydrate part of the molecule. This binding also leads to LPS release from gram-negative bacterial envelope even without physical contact of Lf with microbes. However, under physiological conditions Lf is likely to be saturated with calcium, thus being unable to exhibit its chelating properties toward this cation [35].

Other mechanisms responsible for antimicrobial activity of Lf might be generation of cationic antimicrobial peptides (lactoferricins) from the N-terminal part of the protein [36], and the capacity of Lf itself to function as a protease cleaving a number of surface or secretory bacterial proteins, which contribute to the infectious process for some microbes [37, 38].

Based on the results presented in the table, the antimicrobial activity of canine Lf is similar to that of human Lf. The results of our experiments confirm the data about direct bactericidal action of Lf on some gram-positive and gram-negative bacterial species. It should be noted that bacterial cell concentration in our experiments was equal to $2 \cdot 10^4$ CFU/ml, whereas most researchers use 10^6-10^8. Evidently, under these conditions a higher Lf concentration is necessary to reveal its microbicidal action. Thus, it cannot be excluded that authors failing to observe microbicidal activity of Lf simply did not reach required protein concentration. In addition, susceptibility of microorganisms to Lf action can significantly vary for different species and different strains of the same species. Moreover, it was demonstrated that even for the same strain the sensitivity of bacteria to Lf can differ depending on cultivation conditions [29].

In our experiments, only iron-free Lf exhibited antimicrobial activity, in accordance to the literature. However, our results do not agree with data indicating that microbicidal activity of Lf is greatly decreased on enhancing pH value to more than 6 as well as in the presence of phosphate anions [30]. In our experiments, canine and human Lfs displayed evident antimicrobial activity in phosphate buffer with pH 7.4.

It is interesting to note that canine Lf, unlike human Lf, does not contain the N-terminal motif consisting of four arginines one after another that is crucial for LPS binding [39, 40] and therefore may be important for bactericidal activity against gram-negative bacteria. In canine Lf, this motif is replaced by a five amino acid sequence (in 2-6 position by our data or 3-7 according to predicted structure) including three positively charged residues and two neutral ones. In spite of this fact, the bactericidal activity of canine Lf against *E. coli* was similar to that of human Lf.

In summary, the results of our investigations indicate that canine Lf is rather similar to human Lf in basic physicochemical (molecular weight, carbohydrate content, interaction with ferric ions) and antimicrobial properties.

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