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Lactoferrin Is the Major Deoxyribonuclease of Human Milk

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Abstract—Lactoferrin is the major iron-transferring protein of human barrier fluids such as blood and milk. It is a polyfunctional protein capable of binding DNA exposed on the surface of various cells. Electrophoretically homogenous lactoferrin was prepared by sequential chromatography of human milk proteins on DEAE-cellulose, heparin-Sepharose, and Sepharose containing immobilized anti-lactoferrin antibodies. By subsequent chromatography on Blue Sepharose the resulting lactoferrin was fractionated into several subfractions with different affinity for the sorbent, and this was associated with separation of additional lactoferrin peaks with DNase activity from the main peak. By various techniques, in particular, by *in situ* testing the DNase activity of lactoferrin in a DNA-containing gel after SDS-electrophoresis, hydrolysis of DNA was for the first time shown to be an intrinsic property of lactoferrin. The substrate specificity of lactoferrin in hydrolysis of DNA was different from specificities of known human DNases. Hydrolysis of DNA was activated by bivalent metal ions and also by ATP and NAD. Unlike the main fraction of lactoferrin with the highest affinity for Blue Sepharose, all protein subfractions with DNase activity were cytotoxic and suppressed growth of human and mouse tumor cell lines.

Key words: lactoferrin, human milk, hydrolysis of DNA, allosteric effect

Lactoferrin, an iron-transferring glycoprotein, is present in high concentration (~1-2 mg/ml) in milk and other human epithelial secretions [1]. It has also been found in neutrophil granules and blood plasma [2, 3]. Lactoferrin (its molecular weight is ~76-80 kD) consists of a single polypeptide chain of 673 amino acid residues, which contains two sites of glycosylation [4]. X-Ray diffraction analysis shows that the lactoferrin molecule is coiled into two homologous domains, each with one iron-binding site [5]. The affinity of iron atoms for lactoferrin is extremely high (K_d 1 nM), but its saturation in milk is not higher than 10-15%.

Another fundamental feature of lactoferrin is its ability for binding polyanions such as heparin, DNA, RNA, polysaccharides, etc. The interaction of this protein with heparin and chondroitin sulfate is mainly contributed by its 33 N-terminal amino acid residues [6]. Two clusters, GRRRRS and RKVR, which are not directly involved in the interaction with the iron atom, play the dominant role in the binding of ligands.

Lactoferrin displays antibacterial properties, both iron-dependent and iron-independent. The iron-dependent properties are caused by the competition for iron ions between the protein and receptors of bacterial membranes. The nature of the iron-independent properties is not sufficiently clear, but it is known to depend on the structural region of amino acid residues 20-37 of the protein.

There are many data on the ability of lactoferrin to interact with eucaryotic cells and influence their functional properties [7-9]. The liver reticuloendothelial system and lung macrophages have a common site responsible for binding lactoferrin and other basic proteins of elastase type. Treatment of cells with DNase significantly decreases the interaction of lactoferrin with mononuclear cells and neutrophils; therefore, DNA is suggested to be the common cellular receptor. The ability of lactoferrin to penetrate into the nucleus [10, 11] and activate transcription is especially interesting; DNA sequences with increased affinity for lactoferrin have been found [11]. Some lactoferrin fractions with different affinities for Blue Sepharose display features of ribonuclease [12]. Biological features and specific behavior of the protein in *in vitro* and *in vivo* systems have been analyzed in more detail in reviews [13, 14].

Abbreviations: oxATP) 2′,3′-dialdehyde derivative of ATP; T_9U_{ox}) 2′,3′-dialdehyde analog of $d(pT)_9(pU)$; TNF-α) tumor necrosis factor α.

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We have earlier shown that, in addition to DNase II, human milk contains IgG sIgA-antibodies (abzymes) capable of effective degrading DNA and RNA [15-19]. When searching for other alternative milk nucleases unlike DNase II and abzymes, we found that DNase activity is associated with lactoferrin of human milk. The present work shows that the DNase activity is an intrinsic property of the protein, and specific activators of this activity have been found.

MATERIALS AND METHODS

Ribo- and deoxyribooligonucleotides, homogenous by data of ion-exchange and reverse-phase chromatography, were synthesized as described in [20].

Isolation of lactoferrin from milk. Milk was taken from healthy donors 0.5-3 months after the beginning of lactation. To isolate lactoferrin, the milk was centrifuged to remove the upper lipid fraction and cell precipitate, and the solution was applied onto a column (20 × 1 cm) with DEAE-cellulose. The fraction of proteins without affinity for the sorbent was collected and applied onto a column (10 × 1 cm) with heparin-Sepharose. The sorbent was washed with 20 mM Tris-HCl buffer (pH 7.5), then with the same buffer supplemented with 1% Triton X-100, and then with Triton-free buffer until the absorption at 280 nm disappeared. Lactoferrin was eluted with linear gradient of NaCl concentrations from 0 to 1 M in 20 mM Tris-HCl buffer. The lactoferrin peak was located by electrophoresis of the fractions. The isolated preparation of lactoferrin (LF) was additionally purified on a column (20 × 1 cm) with anti-lactoferrin-Sepharose pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). After the preparation was applied onto the column, the sorbent was washed with 20 mM Tris-HCl buffer (pH 7.5) until the disappearance of optical density at 280 nm. The elution was performed with 50 mM Gly-HCl buffer (pH 2.6). The isolated protein fractions were neutralized with 1 M Tris-HCl buffer (pH 7.5) The activity of lactoferrin was determined in hydrolysis of plasmid DNA using 2 μl of the eluate per 20 μl of the reaction mixture (see below). During all stages of the purification the homogeneity of LF preparations was tested by electrophoresis in 12% polyacrylamide gel containing 0.1% SDS as described in [21], and the proteins were stained with silver as described in [22].

Chromatography of lactoferrin on Blue Sepharose. Earlier, in work [12], lactoferrin preparations were separated by chromatography on Blue Sepharose into several subfractions, and one of them displayed RNase activity. We used this approach with some modifications. Homogenous preparations of LF were chromatographed on a column (1.5 × 0.5 cm) with Blue Sepharose equilibrated with 20 mM NaOAc buffer (pH 4.0). Before application, the pH of the lactoferrin solution was adjusted to 4.0 with 1 M NaOAc. The proteins were eluted first with 20 mM Tris-HCl buffer (pH 7.5) and then with a linear gradient of NaCl concentrations from 0 to 1 M in the same buffer. The fractions were dialyzed against 20 mM Tris-HCl buffer (pH 7.5) and tested for the DNase activity (1-3 μl of the solution per 20 μl of the reaction mixture, see below).

Conditions of DNA hydrolysis. Conditions of DNA hydrolysis catalyzed by lactoferrin were optimized by varying the pH of the mixture and concentrations of the components mentioned below. The DNase activity of lactoferrin preparations was tested under optimal conditions at 37°C. The reaction mixture (10-20 μl) contained 20 mM Tris-HCl buffer (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, and 150 ng DNA of phage λ (or plasmid pBluescript DNA) or 10^{-6} M oligonucleotide substrate. The reaction was initiated by addition of 0.05-0.1 μg lactoferrin. The reaction mixture was incubated for 2-4 h at 37°C. Accumulation of 5′-[^{32}P]oligonucleotide hydrolysis products was tested by electrophoresis in 20% polyacrylamide gel containing 7 M urea, or by TLC on Kieselgel 60 F_{254} plates (Merck, Germany) in the system dioxane–NH_4OH–water (5 : 1 : 4) with subsequent radioautography. Products of phage λ DNA hydrolysis were analyzed by electrophoresis in 1% agarose gel followed by staining of DNA with ethidium bromide [23].

All kinetic measurements were performed on linear portions of the reaction rate dependences on time and lactoferrin concentration. The radioactivity of hydrolysis products (in pieces of gel or on TLC plates) was determined by the Chernkov's method with a Minibeta counter (LKB, Sweden). Values of K_m and V_{max} were determined with a computer program by methods of nonlinear regression and diagrams as described in [24]. Errors in the determination of parameters were no more than 10-30%.

Testing of lactoferrin activity in gel. The DNase activity of lactoferrin was *in situ* determined by SDS-PAGE in 12% polyacrylamide gel containing polymerized DNA from calf thymus (20 μg/ml) [15, 16]. The gel was washed with 4 M urea for 1 h and then 10 times with water. To renature the lactoferrin activity, the gel was incubated for 16 h at 37°C in buffer (20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA) and stained with ethidium bromide. The gel region containing the cleaved DNA was revealed as a black spot on a homogenous fluorescent background. The protein bands were identified by staining the gel with Coomassie R-250.

Affinity modification of lactoferrin. 2′,3′-Dialdehyde derivatives of ATP (oxATP) and oligonucleotide [d(pT)$_9$]r(pU) (T_9U_{ox}) were synthesized as described in [25]. The reaction mixture (10 μl) for affinity modification of lactoferrin with these analogs contained 20 mM Tris-HCl buffer (pH 7.5), 10 pM α-[^{32}P]oxATP or [^{32}P]T_9U_{ox}, and 3-7 μg lactoferrin. The mixture was incubated for 30 min at 25°C; to reduce the resulting Schiff base, 1 μl of 0.01 M sodium borohydride in 50 mM NaOH was added, and the mixture was incubated again

for 30 min. The labeled lactoferrin was detected by SDS-PAGE of the protein in 12% polyacrylamide gel with subsequent radioautography of the gel.

Limited proteolysis of lactoferrin with trypsin. Proteolysis of lactoferrin modified with oxATP or T_9U_{ox} was performed as described in [26]. The reaction mixture (10 μl) contained 0.1 M Tris-HCl buffer (pH 8.2), 25 mM $CaCl_2$, 10-15 μg lactoferrin, and 0.1-0.15 μg trypsin. The mixtures were incubated for 4 h at 37°C and analyzed as described above (12% polyacrylamide gel).

Limited proteolysis of lactoferrin with cyanogen bromide. Proteolysis of the affinity-labeled lactoferrin at methionine residues was performed as described in [27]. The reaction mixture (10 μl) contained 25 mM HCl, 0.1% SDS, and 10-15 μg lactoferrin. The mixture was supplemented with 1.5 μl of 0.1 M BrCN in acetonitrile, incubated for 3-5 min at 20°C, boiled for 30-40 sec, and analyzed as described above (12% polyacrylamide gel).

Determination of cytotoxicity of lactoferrin. Cytotoxicity of the protein was determined using the model cell line mouse L-929 fibroblasts and human tumor cells HL-60. The cells were plated onto DMEM medium supplemented with 5% calf serum and grown in a CO_2-incubator until a monolayer was produced. Then the cells were treated with mitomycin (1 μg/ml) for 5 h, washed, and the medium was replaced with the same medium but containing lactoferrin (10^{-7}-10^{-8} M). As the control, inducer of cell death tumor necrosis factor (TNF-α) was used in the concentration of 10^{-8} M. The cells were grown in a CO_2-incubator for 12-48 h. The number of dead cells was determined every 12 h by staining with Trypan Blue [28]. Cytotoxicity of lactoferrin was determined by comparison to the level of natural cell death. Each experiment was repeated at least thrice.

Fragmentation of DNA and analysis of cell apoptosis. The above-mentioned cells were incubated for 12-24 h in the presence of various subfractions of lactoferrin (10-100 nM, Fig. 1) as described above, then subjected to lysis according to [29], centrifuged at 20,000*g*, and the supernatant was extracted with phenol–chloroform mixture [29]. The resulting solution of DNA was used for analysis of its fragmentation by electrophoresis in 1.2% agarose gel and staining with ethidium bromide [23].

Apoptosis of the cells incubated in the absence or in the presence of lactoferrin was analyzed using a standard Annexin-V-Fluorescein kit for testing cell apoptosis according to instruction of the producer (Boehringer Mannheim, Germany).

DNA of phage λ, DNA of plasmid pBR 322 (SibEnzyme, Russia), DNA of calf thymus (Sigma, USA), polynucleotide kinase of phage T4 (Fermentas, Lithuania), Triton X-100 (Ferak, Germany), trypsin (Merck, Germany), Tris and Blue Sepharose (Sigma, USA), heparin-Sepharose (Pharmacia, Sweden), Toyopearl HW-55 Fine (Toyo Soda, Japan) were used. Other reagents were of special purity.

RESULTS AND DISCUSSION

Isolation of lactoferrin. Lactoferrin was isolated from human milk by consecutive chromatography of their proteins on DEAE-cellulose, heparin-Sepharose, and anti-lactoferrin-Sepharose (Figs. 1 and 2). Data on chromatography of lactoferrin on heparin-Sepharose are presented in Fig. 1a. Lactoferrin was eluted from this sorbent as two peaks, but only protein of peak III (Fig. 1a) was nearly homogenous (lane *2* in Fig. 1b). Just this peak of lactoferrin was used for the further purification of the protein on a sorbent with immobilized antibodies to it (Fig. 2). At the last stage, homogenous preparations of lactoferrin were chromatographed on Blue Sepharose (Fig. 3a), which binds many enzymes possessing affinities for DNA and nucleotides. By chromatography, lactoferrin was separated by affinity for Blue Sepharose into three-or-four more or less pronounced protein peaks: I, II, III, and IV (Fig. 3a). The first protein peak had neither affinity for the sorbent nor catalytic activity. The fourth peak of lactoferrin (IV), which displayed the maximum affinity for Blue Sepharose, was also catalytically inactive. The maximum DNase activity was found in the second peak of the protein (II). The third protein peak (III) corresponded to two separate peaks of the DNA-hydrolyzing activity. None of the DNAse-active lactoferrin fractions contained a detectable amount of iron. The relative quantity of the protein in the peaks (Fig. 3a) and their relative DNA-hydrolyzing activities strongly depended on the milk donor, but all lactoferrin preparations from ten donors manifested all protein peaks and three peaks of the DNase activity.

Electrophoresis of lactoferrin preparations (under conditions of dissociation or non-dissociation) revealed only a single protein band corresponding to the protein monomer with molecular weight of ~80 kD (Figs. 1b and 2b).

High affinity of lactoferrin for DNA has been shown earlier [13, 14, 30]. We found that the major part of the protein preparations from any donor has increased affinity for Blue Sepharose and lacks the catalytic activity (Fig. 3a, peak IV). Only a minor part of the total pool of human milk lactoferrin can hydrolyze DNA. And the protein fraction with the DNase activity varied in different preparations, but in ten samples of milk under study this fraction was no more than 5-15% of the total amount of lactoferrin.

To show that the DNase activity belonged to lactoferrin and not to microcontaminations of concurrently isolated enzymes, we controlled this by some severe tests elaborated earlier for detection of the catalytic activity in the case of catalytically active antibodies [13-19]. Even very strong protein complexes are known to be destroyed in acidic buffers [17-19]. Lactoferrin did not lose the nuclease activity after its incubation in buffer with low pH (pH 2.3) and gel filtration under conditions of "acidic

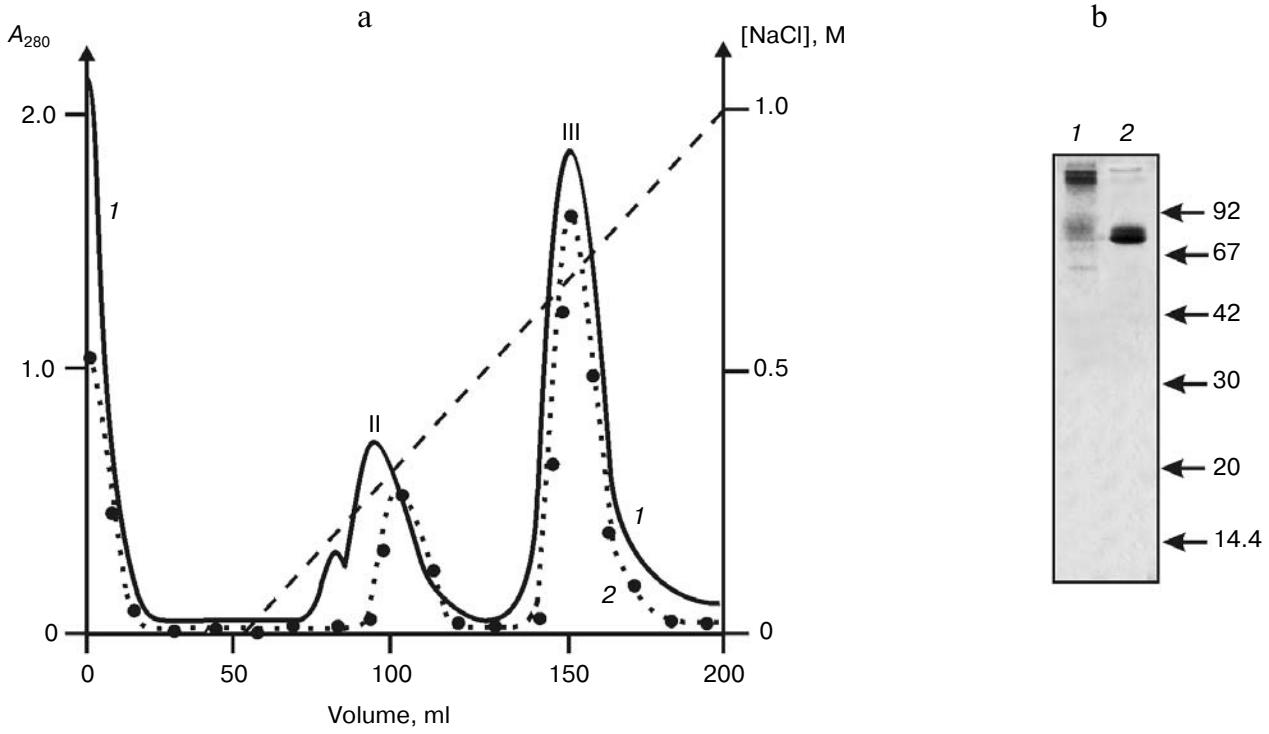

Fig. 1. a) Chromatographic separation on heparin-Sepharose of milk protein fractions affinity-free for DEAE-cellulose: *1*) absorption of the proteins at 280 nm; *2*) relative DNA-hydrolyzing activity (maximum relative activity is taken as 100%). b) Electrophoretic separation in the absence of 2-mercaptoethanol of proteins corresponding to the peaks in Fig. 1a: *1*) fraction II; *2*) fraction III. To the right, molecular weights of marker proteins are presented in kD.

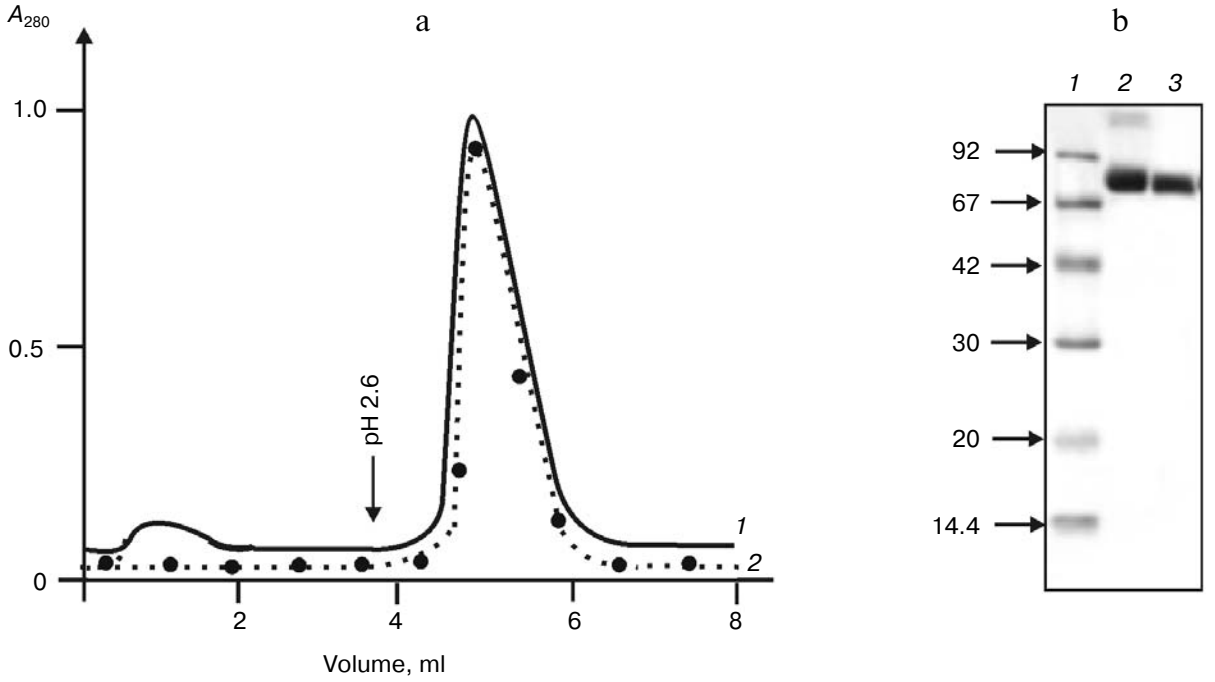

Fig. 2. a) Affinity chromatography of lactoferrin on anti-lactoferrin-Sepharose (lactoferrin corresponds to fraction III, Fig. 1a): *1*) absorption at 280 nm; *2*) relative DNA-hydrolyzing activity (maximum activity is taken as 100%). b) SDS-PAGE of the lactoferrin homogeneity in the presence of 2-mercaptoethanol: *2*) protein corresponding to fraction III after heparin-Sepharose (see Fig. 1a); *3*) lactoferrin after chromatography on anti-LF-Sepharose; *1*) positions of marker proteins (to the left, their molecular weights in kD are shown).

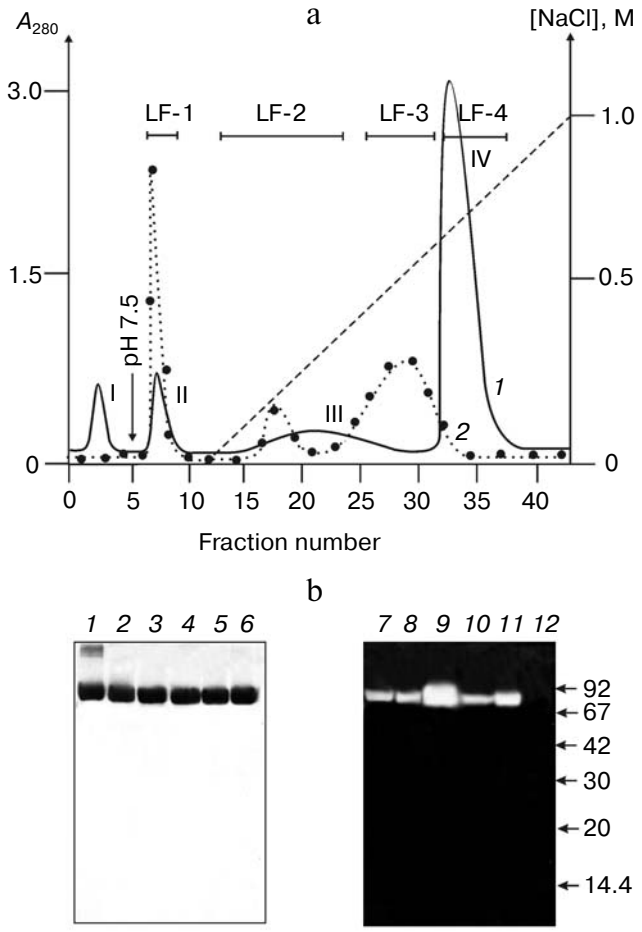

Fig. 3. a) Separation by chromatography on Blue Sepharose of lactoferrin fraction preliminary purified on anti-lactoferrin-Sepharose: *1*) absorption at 280 nm; *2*) relative DNA-hydrolyzing activity (maximum activity of fraction LF-1 is taken as 100%). b) Analysis of DNA-hydrolyzing activity of lactoferrin *in situ* after SDS-electrophoresis of the protein in DNA-containing polyacrylamide gel (under conditions of dissociation): *1*, *7*) fraction III after heparin-Sepharose (Fig. 1a); *2*, *8*) fraction of lactoferrin after isolation on anti-lactoferrin-Sepharose; *3*, *9*) fraction LF-1; *4*, *10*) fraction LF-2; *5*, *11*) fraction LF-3; *6*, *12*) fraction LF-4 after chromatography on Blue Sepharose; *1-6*) Coomassie-stained gel; *7-12*) gel after staining of DNA with ethidium bromide (the film negative is presented, the DNase activity is displayed as a light band on the background of homogenously fluorescent dark gel). To the right, molecular weights of marker proteins in kD are shown.

shock" (Fig. 4). Also, the protein peak coincided with the activity peak.

SDS-PAGE is one of the most severe methods for dissociating protein complexes [17-19]. The testing of enzymatic activity in the gel region corresponding to location of an individual protein after electrophoresis under the above-mentioned conditions is considered to be a stringent and reliable proof of enzymatic function of the protein [17-19]. To show the DNA-hydrolyzing activity of lactoferrin, we used SDS-PAGE in the presence of 2-mercaptoethanol; the gel for electrophoresis contained polymerized DNA. After the electrophoresis, activities of the protein bands were restored by removal of SDS and subsequent incubation of the gel in buffer for recovery of the enzymatic activity as described in [31]. Figure 3b shows that in the region of the protein corresponding to lactoferrin (after various stages of purification) DNA is hydrolyzed and not stained with ethidium bromide. The staining of DNA in other regions of the gel results in a homogenous fluorescence of the total surface of the gel. Thus, the ability of lactoferrin for hydrolyzing DNA was confirmed by one of the most reliable approaches.

DNA-hydrolyzing activity of lactoferrin. Then, to characterize the DNase activity of lactoferrin, preparations corresponding to the lactoferrin fraction 1 (LF-1) were used (Fig. 3a). The DNase activity of lactoferrin was determined using high-molecular-weight DNAs (DNA of phage λ and of plasmid pBR 322 or pBluescript) and oligonucleotides with various structure and length. The optimum pH of the reaction mixture during hydrolysis of polymeric double-stranded DNA was about 7.0-7.5 (Fig. 5a). During hydrolysis of single-stranded nucleotides, the pH optimum is displaced to alkalinity.

It was said above that the lactoferrin fraction under study did not contain iron. The saturation of the proteins with iron ions followed by removal of their excess by gel filtration did not change the enzymatic properties of the lactoferrin, and this suggested that the hydrolytic function of the protein should be Fe-independent.

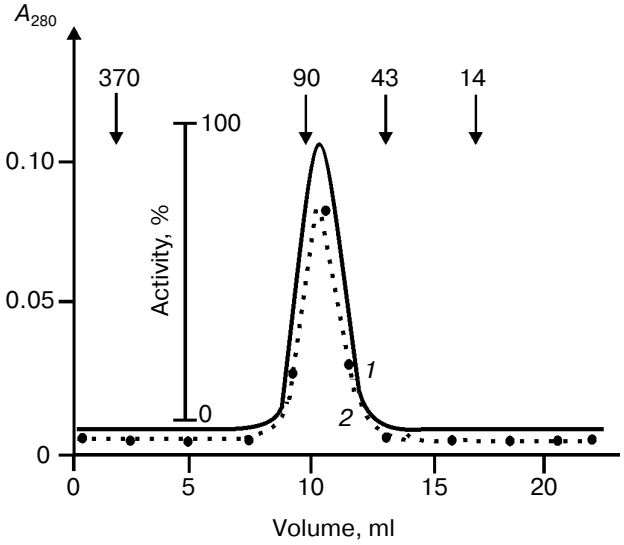

Fig. 4. Profiles of absorption (*1*) and DNase activity (*2*) of lactoferrin on its gel filtration on a column with Toyopearl HW-55 resin in 50 mM acidic glycine-HCl buffer (pH 2.3) containing 0.1 M NaCl. Previously to the gel filtration, lactoferrin was preincubated for 1 h at 37°C in the same buffer supplemented with 1 M NaCl. Above the arrows, the molecular weights of marker proteins are shown in kD.

While iron ions had no significant effect on the lactoferrin activity, an increase in the concentration of NaCl (to 100 mM) increased its activity (Fig. 5b). Moreover, Mn^{2+}, Mg^{2+}, and to the lesser degree Ca^{2+}, Cu^{2+}, and Zn^{2+} at the concentration of 5 mM activated the hydrolysis of DNA-substrates (Fig. 5c), and EDTA at the concentration of 10 mM and higher inhibited the reaction (data not presented). Oligonucleotides were hydrolyzed under the influence of lactoferrin significantly more slowly than DNA substrates. The lactoferrin-dependent degradation of DNA of phage λ and of plasmid DNAs occurred already after 20-30 min, whereas effective hydrolysis of oligonucleotide occurred only after incubation for 2-4 h.

The binding of lactoferrin to some DNA sequences, such as GGCACTT(G/A)C (ON_1), TAGA(A/G)GATCAAA (ON_2), and ACTACAGTCTACA (ON_3), is specific [12]. To more completely characterize the DNase function of the protein, we evaluated apparent thermodynamic and kinetic parameters of hydrolysis with the enzyme of $d(pT)_{10}$ and $d(pA)_{14}$, and also of specific ON_1, ON_2, and ON_3. Note that the oligonucleotides were hydrolyzed by two pathways depending on manifestation by lactoferrin of the DNase and phosphatase activities. The removal by lactoferrin of the 5′-terminal phosphate of oligonucleotide made difficult the determination of K_m and k_{cat} values which characterize the DNase activity of the protein. But the separation of the hydrolysis products by TLC allowed us to evaluate the contribution of each activity to the lactoferrin-dependent hydrolysis of the oligonucleotide. Values of K_m determined for the removal of the 5′-terminal phosphate were in the range of 60-95 μM independently of composition or length of the oligonucleotide. The relative efficiency of the phosphatase activity of the protein preparations varied from donor to donor and was 20-80% of their total DNase activity. To evaluate the direct DNA-hydrolyzing activity of lactoferrin, preparations with the minimal phosphatase activity (not higher than 30%) were chosen.

Note that K_m values for the lactoferrin-dependent hydrolysis of various oligonucleotides (3.5-9.8 μM) insignificantly depended on their structure and length and also were similar for the protein preparations from different donors (the table presents data for three lactoferrin preparations). Values of k_{cat} differed more markedly (see table). Because all K_m values were determined under conditions of quasi-first order reaction, they did not depend on content in the lactoferrin preparations of its non-hydrolyzing subfraction, and, consequently, characterized the true affinity of the oligonucleotide for the DNase site of the protein.

Blue Sepharose has affinity for numerous DNA-dependent proteins [32]. Because not only the DNAse-active lactoferrin can bind to the sorbent (Fig. 3, peak IV), fraction LF-1 separated on Blue Sepharose from the major protein, in addition to catalytically active lactofer-

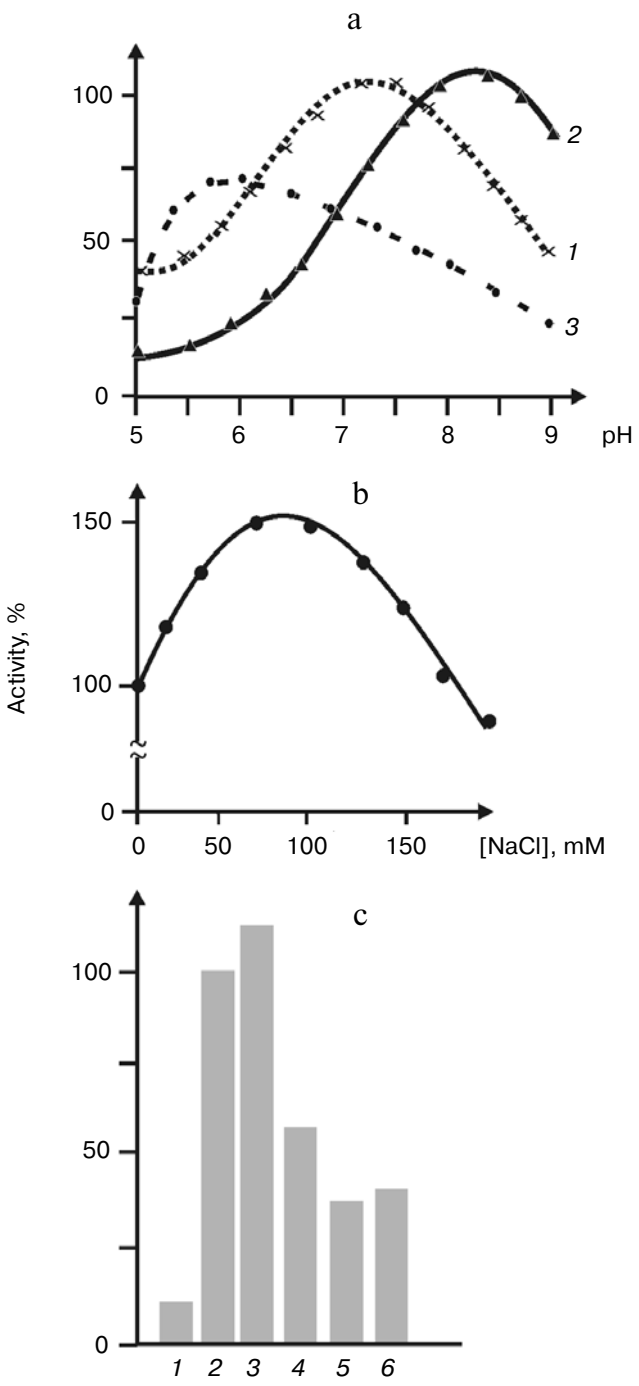

Fig. 5. a) pH dependence of the relative activity of lactoferrin in hydrolysis of double-stranded DNA of phage λ (*1*) and of single-stranded 5′-[^{32}P]$d(pT)_{10}$ (*2*) and also in the removal of the 5′-terminal phosphate from 5′-[^{32}P]$d(pT)_{10}$ (*3*) (phosphatase activity). b) Effect of concentration of NaCl and bivalent metal ions on the relative activity of lactoferrin in the hydrolysis of 5′-[^{32}P]$d(pT)_{10}$. c) Lactoferrin activity in the absence (*1*) and in the presence of 5 mM metal ions: Mg^{2+} (*2*), Mn^{2+} (*3*), Ca^{2+} (*4*), Cu^{2+} (*5*), Zn^{2+} (*6*). The lactoferrin activity in the presence of 5 mM $MgCl_2$ (2 h at 37°C) is taken as 100%.

Kinetic and thermodynamic characteristics of hydrolysis of oligonucleotides with the lactoferrin subfraction LF-1 (see Fig. 3)

Substrate	K_m*, μM	$k_{cat} \times 10^3$, min^{-1}	$k_{cat}/K_m \times 10^{-3}$, $M^{-1} \cdot min^{-1}$
d(pT)10	5.6 ± 2.0	42.8 ± 10.0	7.6
d(pA)10	4.2 ± 1.5	6.4 ± 2.0	1.5
(pC)10	5.0 ± 2.1	29.6 ± 9.1	5.9
d(pGpGpCpApCpTpTpApC)	7.2 ± 1.1	27.2 ± 5.7	3.8
d(pTpApGpApApGpApTpCpApApA)	3.7 ± 1.0	8.2 ± 2.5	2.2
d(pApCpTpApCpApGpTpCpTpApCpA)	5.3 ± 1.8	42.2 ± 4.1	8.0

* Mean values for three lactoferrin preparations from different donors are presented.

rin isoenzymes, can also contain DNA-binding catalytically inactive protein molecules. Probably, we have only partly "enriched" the LF-1 preparation with the DNA-hydrolyzing subfraction. Therefore, the question of the true values of k_{cat} for homogenous catalytically active lactoferrin isoforms from milk of different donors is still unclear.

It is interesting that K_m values for hydrolysis of oligonucleotides are an order of magnitude lower than values determined for the removal of the 5′-terminal phosphate. Considering significant changes in the relative specific DNase and phosphatase activities of lactoferrin preparations from different donors, these results are suggested to be caused by belonging of these functions to different isoforms of the protein. The presence of two active sites with different functions seems to be another explanation. The multiplicity of DNA-specific sites in the protein is supported by the presence of two anticooperative sites responsible for binding DNA and oligonucleotides [33]. Therefore, it is likely that the second DNA-binding site of lactoferrin, which has about an order of magnitude lower affinity for DNA, can also have phosphatase activity. Nevertheless, significantly varied ratios of the DNase and phosphatase activities in lactoferrin preparations from different donors, as well as displacement of the pH optimum of the phosphatase activity to acidity (Fig. 5a, pH 5.5-6.0) compared to the DNase activity (7.0-8.5) is more consistent with the assumption of the existence of different isoenzymes catalyzing these two reactions.

Activation of lactoferrin by nucleotides. We have earlier shown [34] that lactoferrin can bind ATP, and the interaction with the nucleotide changes its affinity for DNA and polysaccharides. With this in mind, we studied the effect of nucleotides on hydrolysis of DNA. The lactoferrin-dependent hydrolysis of DNA substrates was activated with ATP or dATP, and this activation was significantly stronger in the presence of NAD (Fig. 6a). To elucidate how hydrolysis of DNA occurs in the presence of ATP, we modified lactoferrin with an oligonucleotide derivative (T_9U_{ox}), with the subsequent cleavage of the modified protein.

A mild treatment of lactoferrin with trypsin results in hydrolysis of the protein molecule between the residues Lys283 and Ser284 into two N- and C-tryptic fragments with molecular weights of 30 and 50 kD, respectively [35]. The partial hydrolysis with trypsin of the $[^{32}P]T_9U_{ox}$-labeled lactoferrin results in these two polypeptides, but only the 30-kD polypeptide is radiolabeled (Fig. 6b, lane *4*). Consequently, the DNA-binding site of lactoferrin is located in the N-terminal domain of the protein. However, it has been earlier shown that the ATP-binding site is located in the C-terminal half of the molecule [34].

The interaction and modification with T_9U_{ox} of the DNase site of lactoferrin located in the N-terminal region of the protein molecule are also confirmed by the lactoferrin-dependent hydrolysis of this oligonucleotide and inhibition of hydrolysis of DNA substrates (oligonucleotides and DNA of phage λ) on addition of T_9U_{ox} (data not presented).

As the DNA- and ATP-binding sites are spatially separated, it is suggested that the ATP activation of the DNA hydrolysis should be allosteric.

Many DNA-hydrolyzing enzymes have protective functions in pro- and eucaryotic cells [15]. To elucidate the possible biological role of the DNase activity of lactoferrin, we compared its activity in the hydrolysis of DNA to activities of other DNases from human milk. According to the literature, human milk contains a single DNase with molecular weight of 42 kD, with catalytic properties similar to those of DNase II from human blood [36]. We also found in human milk only this classic DNase. Moreover, it has been recently shown that milk of healthy donors contains DNA-hydrolyzing subfractions of antibodies IgG (150 kD) and sIgA (360 kD) [15-19].

The DNase activity of milk proteins was studied after their separation (lipid-free milk plasma) by SDS-PAGE in the DNA-containing polyacrylamide gel. Figure 7 shows that the maximum DNase activity belongs to the

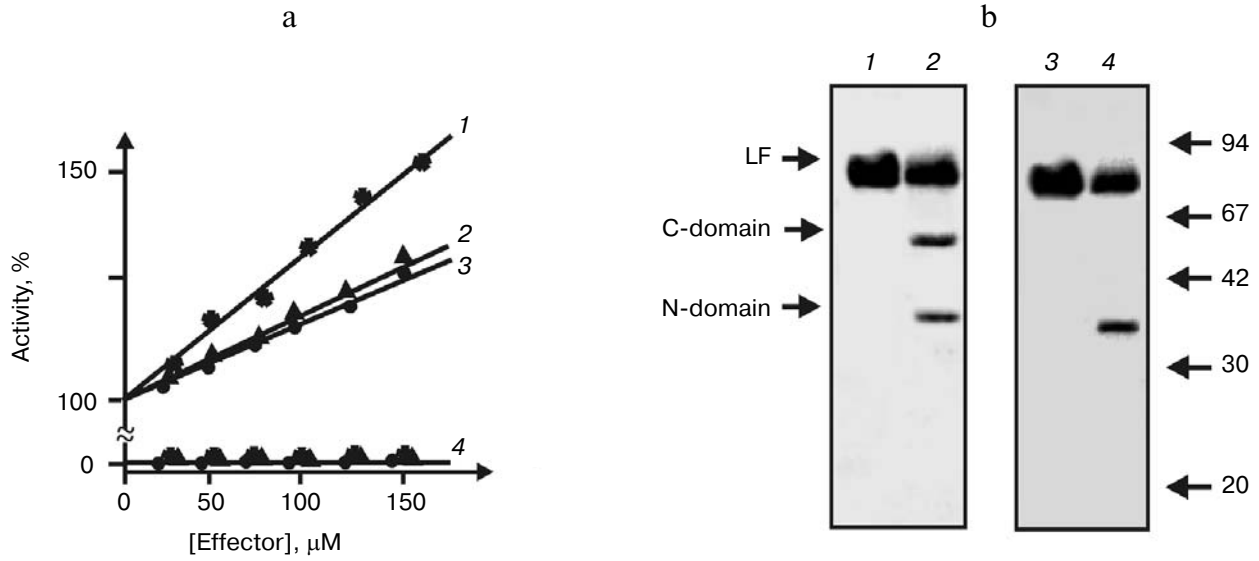

Fig. 6. a) Dependence of the relative DNA-hydrolyzing activity of lactoferrin in the hydrolysis of 5′-[^{32}P]d(pT)$_{10}$ on concentration of effector nucleotides (the relative activity of lactoferrin in the absence of effectors is taken as 100%): NAD (*1*), dATP (*2*), ATP (*3*), control (*4*). In the absence of lactoferrin no significant hydrolysis of oligonucleotides was noted before and after addition of the effectors (see the control). b) Determination of location of the DNA-binding site of lactoferrin by modification of lactoferrin with the affinity reagent [^{32}P]T$_9$U$_{ox}$ (*1*, *3*) and subsequent cleavage of lactoferrin-[^{32}P]T$_9$U$_{ox}$ with trypsin (*2*, *4*). *1*, *2*) Coomassie-stained gel; *3*, *4*) radioautograph of the gel. To the right, molecular weights of marker proteins in kD.

protein corresponding to lactoferrin by location in the gel and staining with horseradish peroxidase conjugated with anti-lactoferrin antibodies. Surprisingly, the DNase activity corresponding to DNase II (42 kD) was detected in only half of the 14 milk samples studied. The DNase activity of IgG antibodies was found in all 14 milk preparations, but in all cases, it was significantly lower than the activity of lactoferrin. According to the literature data and results presented in Fig. 7 (lane *1*), human milk contains a rather high concentration of lactoferrin. Our findings suggested that lactoferrin should be the major DNase of milk considering its total DNase activity. However, this conclusion should be tested by more careful comparison of the catalytic properties of lactoferrin and DNase II.

Lactoferrin is a protein of the acute phase and nonspecific human defense against various damages, including bacterial and viral [13, 14]. Every kind of damage, such as wounds or burns, activates synthesis of lactoferrin, and its concentration increases in human epithelial secretions and barrier fluids and directly in the region of damage. It has been mentioned above that the minor lactoferrin fractions can hydrolyze RNA. The presence of DNase and RNase activities in this protein seems to be important for strengthening its protective functions not only due to the binding but also to hydrolysis of nucleic acids of viral and bacterial origin.

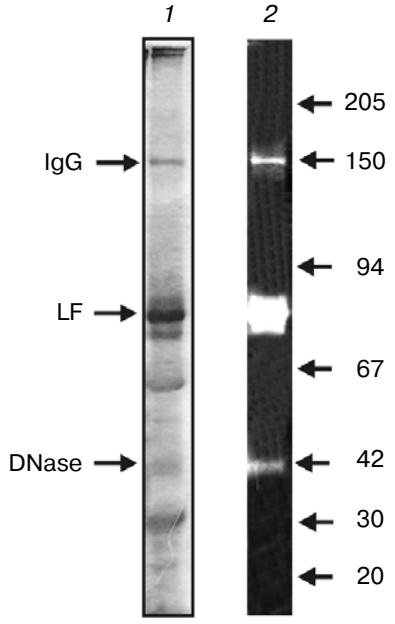

Fig. 7. Analysis of the DNase activity of human milk proteins *in situ* after SDS-PAGE separation of proteins in polyacrylamide gel containing DNA (5 μl of milk plasma): *1*) Coomassie-stained gel; *2*) gel after the staining of DNA with ethidium bromide. To the right, molecular weights of marker proteins in kD.

Cell apoptosis under the influence of lactoferrin. Human milk for a newborn is the source not only of nutrients but many antibacterial and antiviral agents. We have studied the effects of different catalytically active lactoferrin isoforms on the growth of mouse fibroblasts L929 and human promyelocytes HL-60. The major protein fraction prepared by chromatography on Blue Sepharose (Fig. 3, peak IV (LF-4)) had no catalytic activity and was not cytotoxic. However, fraction LF-1 (Fig. 3, peak II) which had the maximum DNase activity also manifested a high level of cytotoxicity, which was only ~1.5-1.7-fold lower than the cytotoxicity level of the tumor necrosis factor TNF-α (Fig. 8a). Other catalytically active lactoferrin fractions (Fig. 3, peak III)—LF-2 and LF-3—also displayed cytotoxicity which was ~10-50% lower than that of LF-1. The cytotoxicity levels of apo-lactoferrin and Fe-lactoferrin were similar. Because fraction LF-4 was neither catalytic nor cytotoxic, it was suggested that the cytotoxic effect of fractions LF-1, LF-2, and LF-3 should be associated with their DNase activity. The fragmentation of DNA of tumor cells L929 and HL-60 became significant when the cells were treated with fraction LF-1 at the concentration of 100 nM for 12-24 h (Fig. 8b). And the typical for apoptosis fragmentation of DNA to fragments of oligonucleosome size was observed [37, 38].

At present, apoptosis induced by various compounds can be reliably shown by morphological analysis of changes in the cells using a standard test system for apoptosis, which includes staining the cells with annexin. The cells treated with LF-1 bound to annexin V and displayed apoptosis-specific morphologic changes (data not presented). This shows once more that lactoferrin induces cell apoptosis.

For a long time lactoferrin attracted the attention of many researchers. It is rather well studied from many standpoints. This protein regulates concentrations of free iron ions in the blood and secretions, binds different DNAs (including specific ones), and can penetrate into the cell nuclei and activate transcription [11]; a lactoferrin isoenzyme hydrolyzes RNA [12, 39].

The present work has shown for the first time that human lactoferrin can also hydrolyze DNA and, thus, it is a unique nuclease activated by ATP and NAD. Our findings, combined with the literature data, suggest that this protein exists in both iron-dependent and iron-independent forms, different monomers and oligomers, and is also present in the cells as a number of isoenzymes, and all this provides its extremely wide polyfunctionality. Lactoferrin is believed to be the protein second in significance after lysozyme for nonspecific human defense against viruses and bacteria. It seems that just the polyfunctionality of lactoferrin determines its biological role as an effective nonspecific protector of cells.

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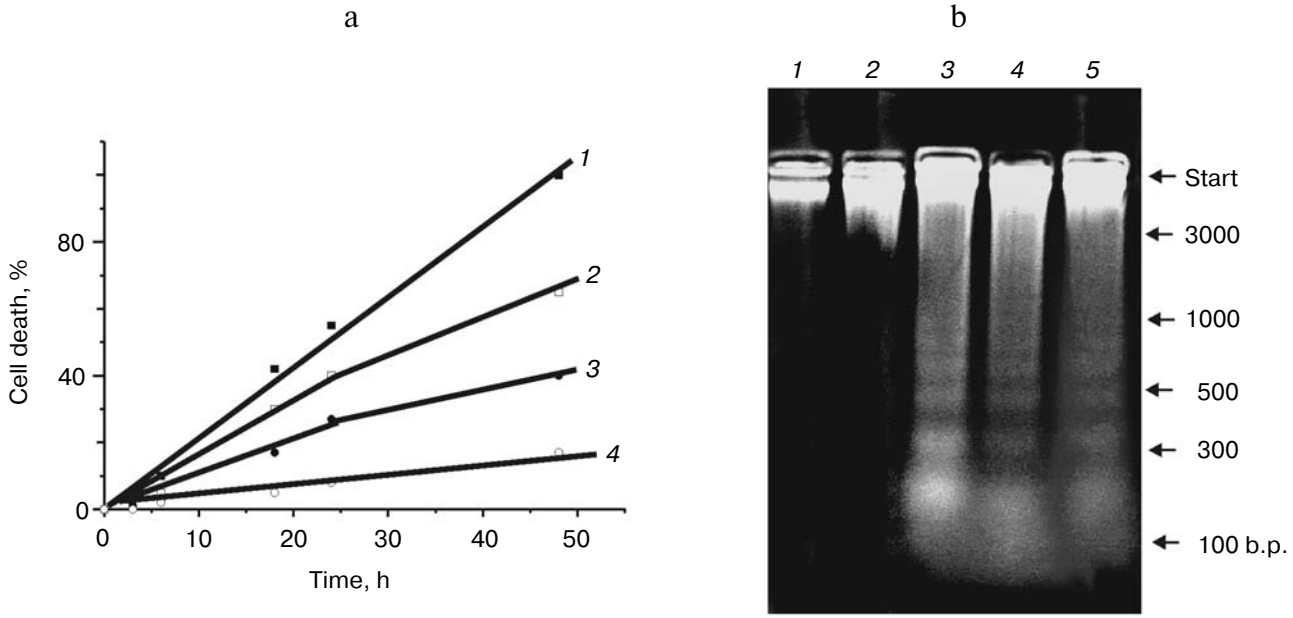

Fig. 8. a) Death of strain L929 cells after incubation with 10 nM tumor necrosis factor (*1*), 100 nM LF-1 (*2*), LF-4 (*3*); *4*) the natural cell death in the absence of agents. b) Fragmentation of DNA of L929 cell chromatin under the influence of lactoferrin. The cells were not treated with the protein (control) (*1*) or were treated with fraction LF-4 (5 mg/ml) (*2*) or LF-1 (100 nM) from three different donors of milk (*3-5*) for 18 h. DNA was separated in 1.2% agarose gel and stained with ethidium bromide. Lanes *1* and *2* show the absence of fragmentation, whereas addition of fraction LF-1 (lanes *3-5*) results in formation of a "ladder" of DNA fragments.

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REFERENCES

1. Ott, V. D., Dyukareva, S. V., and Mel'nikov, O. R. (1993) *Vopr. Pitaniya*, **1**, 6-13.
2. Birgens, H. (1985) *Scand. J. Haematol.*, **34**, 326-331.
3. Hansen, N. E., Malmquist, J., and Thorell, J. (1975) *Acta Med. Scand.*, **198**, 437-443.
4. Van Berkel, P. H., van Veen, H. A., Geerts, M. E., de Boer, H. A., and Nuijens, J. H. (1996) *Biochem. J.*, **319**, 117-122.
5. Jameson, G. B., Anderson, B. F., Norris, G. E., Thomas, D. H., and Baker, E. N. (1998) *Acta Crystallogr. D. Biol. Crystallogr.*, **54**, 1319-1335.
6. Mann, D. M., Romm, E., and Migliorini, M. (1994) *J. Biol. Chem.*, **269**, 23661-23667.
7. Levay, P. F., and Viljoen, M. (1995) *Haematologica*, **80**, 252-267.
8. Bennett, R. M., Davis, J., Campbell, S., and Portnoff, S. J. (1983) *Clin. Invest.*, **71**, 611-618.
9. Bennett, R. M., Merritt, M. M., and Gabor, G. (1986) *Br. J. Haematol.*, **63**, 105-117.
10. Fleet, J. C. (1995) *Nutr. Rev.*, **53**, 226-227.
11. He, J., and Furmanski, P. (1995) *Nature*, **373**, 721-724.
12. Furmanski, P., Li, Z. P., Fortuna, M. B., Swamy, C. V., and Das, M. R. (1989) *J. Exp. Med.*, **170**, 415-429.
13. Kanyshkova, T. G., Buneva, V. N., and Nevinsky, G. A. (2001) *Biochemistry (Moscow)*, **66**, 1-7.
14. Kanyshkova, T. G., Buneva, V. N., and Nevinsky, G. A. (2002) *Usp. Sovr. Biol.*, **122**, 259-271.
15. Nevinsky, G. A., Kanyshkova, T. G., Semenov, D. V., Vlassov, A. V., Gal'vita, A. V., and Buneva, V. N. (2000) *Appl. Biochem. Biotechnol.*, **83**, 115-130.
16. Vlassov, A., Florentz, C., Helm, M., Naumov, V., Buneva, V., Nevinsky, G., and Giege, R. (1998) *Nucleic Acids Res.*, **26**, 5243-5250.
17. Nevinsky, G. A., Kanyshkova, T. G., and Buneva, V. N. (2000) *Biochemistry (Moscow)*, **65**, 1245-1255.
18. Nevinsky, G. A., Favorova, O. O., and Buneva, V. N. (2002) in *Protein–Protein Interactions; a Molecular Cloning Manual* (Golemis, E., ed.) Cold Spring Harbor, N. Y., pp. 523-534.
19. Nevinsky, G. A., and Buneva, V. N. (2002) *Meth. Immunol.*, **269**, 235-249.
20. Levina, A. S., Nevinsky, G. A., and Lavrik, O. I. (1985) *Bioorg. Khim.*, **11**, 358-369.
21. Laemmli, U. K. (1970) *Nature*, **227**, 114-125.
22. Blum, H., Beier, N., and Giross, H. J. (1987) *Electrophoresis*, **8**, 93-99.
23. Zhivotovsky, B., Wade, D., Gahm, A., Orrenius, S., and Nicotera, P. (1994) *FEBS Lett.*, **351**, 150-154.
24. Cornish-Bowden, E. (1978) *Principles of Enzyme Kinetics* [Russian translation], Mir, Moscow, pp. 260-266.
25. Nevinsky, G. A., Gazaryants, M. G., and Mkrtchan, Z. S. (1983) *Bioorg. Khim.*, **9**, 487-495.
26. Legrand, D., Mazurier, J., Metz-Boutigue, M., Jolles, J., Jolles, P., Montreuil, J., and Spik, J. (1984) *Biochim. Biophys. Acta*, **787**, 90-96.
27. Mazurier, J., Spik, G., and Montreuil, J. (1974) *FEBS Lett.*, **48**, 262-265.
28. Buravlev, V. M., and Veprintsev, B. N. (1976) *Guidebook for Culture of Nervous Tissue. Approaches*, *Technique*, *Problems* [in Russian], Nauka, Moscow.
29. Hakasson, A., Zhivotovsky, B., Orrenius, A., Sabharwal, H., and Svanborg, C. (1995) *Proc. Natl. Acad. Sci. USA*, **92**, 8064-8068.
30. Bennett, R. M., and Davis, J. (1982) *J. Lab. Clin. Med.*, **99**, 127-138.
31. Rosenthal, A. L., and Lacks, S. A. (1977) *Analyt. Biochem.*, **80**, 76-82.
32. Bezwoda, W. R., and Mansoor, N. (1986) *Clin. Chim. Acta*, **157**, 89-93.
33. Kanyshkova, T. G., Semenov, D. V., Buneva, V. N., and Nevinsky, G. A. (1999) *FEBS Lett.*, **451**, 235-237.
34. Semenov, D. V., Kanyshkova, T. G., Akimzhanov, A. M., Buneva, V. N., and Nevinsky, G. A. (1998) *Biochemistry (Moscow)*, **63**, 944-951.
35. Mazurier, J., and Spik, G. (1980) *Biochim. Biophys. Acta*, **629**, 399-408.
36. Gudding, R. (1979) *Acta Vet. Scand.*, **20**, 404-416.
37. Schwartz, L. M., and Osborne, B. A. (1993) *Immunol. Today*, **14**, 582-590.
38. Wyllie, A. H. (1980) *Nature*, **284**, 555-556.
39. Devi, A. S., Das, M. R., and Pandit, M. W. (1994) *Biochim. Biophys. Acta*, **1205**, 275-281.