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SYNTHESIS OF A PROTEIN THAT IS IMMUNOCHEMICALLY SIMILAR TO HUMAN LACTOFERRIN BY KLEBSIELLA PNEUMONIAE

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ABSTRACT — Nowadays, great importance is attached to the role of iron in infection processes, since pathogenic bacteria produce Fe^{3+} cholinating compounds with the ability to absorb Fe^{3+} and remove it from the siderophores of the host. This ability is considered as a pathogenicity factor, enabling the pathogen to propagate in the macroorganism. Many bacterial species are known to increase virulence with iron ions present. Therefore, the sideroform system of bacteria can influence the disease severity by binding Fe^{3+} ions and reducing their content in tissues. THE STUDY PURPOSE was to determine the ability of *K. pneumonia* to synthesize a protein, immunochemically similar to human lactoferrin (hereinafter — microbial-derived lactoferrin, MdLF), and the resistance of these microorganisms to the damaging effect of human lactoferrin (LF). METHODS. The study was conducted on archival strains of *K.pneumoniae*, formerly isolated from patients with intestinal bacterial overgrowth syndrome (n=140), and from the control group (n=70). The supposed presence of MdLF in the samples was confirmed through an ELISA assay using *Hycult Biotech* test kits (Netherlands). RESULTS. The ability to synthesize MdLF, which directly depends on the presence of lactoferrin in the culture medium, was revealed in 100% of the strains. MdLF synthesis significantly decreased if LF was present. The patient strains synthesized MdLF less than those taken from the healthy people, but they were more resistant to human lactoferrin, which suggests that the criterion of microbial resistance to LF, taken at 400 ng/ml, should be used as a diagnostic criterion. *Klebsiellae* bacteria that formed single colonies after beef-extract agar inoculation synthesized MdLF the most and had the highest resistance to LF, which increases their epidemiological importance. CONCLUSION. The study established the diagnostic value of the human LF — microorganism MdLF system for predicting infectious process development and assessing the epidemiological danger from particular strains.

KEYWORDS — siderophores; protein immunochemically similar to human lactoferrin; *K.pneumoniae*; chromatography; electrophoresis.

INTRODUCTION

Bacteria that are capable of propagating in vivo are intangled in a complex competitive relationship both with the human organism and with other microorganisms at the metabolic level. Modern research suggests that iron is a universal growth factor for microorganisms; all microorganisms need iron to a different extent, depending on their taxonomy. Iron is exposed to oxidation and hydrolysis processes in the environment, which results in a decrease in the concentration of free Fe^{2+} and Fe^{3+} ions to 10^{-9}–10^{-18} M, which is not enough for adequate life activity of most microorganisms. Similarly, iron is bound to iron-binding proteins in mammals and is unavailable to microorganisms. Therefore, microorganisms have evolved ways of getting iron under iron-deficiency conditions. Synthesis of siderophores is one of the best-studied ways [1, 2, 3, 5, 12].

Siderophores are low-molecular substances that chelate Fe^{3+} ions released by microorganisms and plants under iron-deficiency conditions in the environment. The key function of siderophores is to convert iron that is bound to proteins or water-insoluble compounds into the Fe^{3+} ionic form available to microorganisms. Most of aerobic and facultative anaerobic microorganisms synthesize at least one siderophore. A relationship of siderophores to the virulence of microorganisms has been proven, and approaches for their clinical usage are being developed. Indeed, the loss of the ability to synthesize siderophores correlates with the loss of virulence, which is seen in many bacterial species — *Erwinia chrysanthemi*, *Pseudomonas aeruginosa*, *Vibrio anguillarum*, *Yersinia enterocolitica*, *Escherichia coli*, etc. [7, 11].

Active studies of siderophores started in the 1990s; since then, siderophores of different groups of microorganisms have been isolated and described. Siderophores can be divided into five classes depending on their chemical nature — catecholates and phenolates (aryl caps), hydroxamates (α-oxycarboxylic acids), carboxylates (dicarboxylic and tricarboxylic acids) and siderophores of mixed type. Mixed-type siderophores correspond to two classes by their structure, that is why they were classified separately [5].

At the same time, there are bacteria that are classified as non-siderophore-forming microorganisms.

These include, for example, *Neisseria* and *Moraxella*. They do not synthesize siderophores to utilize iron from the medium but utilize iron by means of special protein receptors on the surface of the outer membrane. Iron regulatory proteins are supposed to be such receptors. Enhanced expression of these proteins *in vitro* is observed under conditions of a limited amount of iron in the culture medium, in particular when chemical compounds (chelators) that form strong chelating complexes with iron are introduced into the medium. In particular, eleven iron regulatory proteins are known for *Neisseria meningitidis*, whereas only five have been reported for *Moraxella* so far. Basically, the iron regulatory proteins of *Neisseria meningitidis* are minor proteins under normal laboratory culture conditions and begin to express intensively only when the medium is iron-deficient. Cop B, which is one of the major proteins of *Moraxella catarrhalis* with a molecular mass of 81 kD, is quite sensitive to the iron content in the culture medium: its expression sharply increased under the conditions of limited iron content in the medium and, on the contrary, decreased in case of an increase in the Fe^{3+} ion content in the medium. For in vitro growth, the mentioned Cop B is able to utilize iron from different sources — iron citrate, transferrin, lactoferrin and heme-containing proteins. It was found that increased expression of iron regulatory proteins, capable of specific binding of transferrin and human lactoferrin, was observed once the iron chelator EDDA was introduced into the *Moraxella* culture medium. Other studies have shown that a 37-kD iron regulatory protein that is typical of most *Neisseria* but is not expressed by *Moraxella* and non-pathogenic *Neisseria*, is localized mainly in the periplasmic space and is involved into the iron transfer from transferrin to the cytoplasmic membrane through the periplasm. An analogy has also been established between the active centre of this protein and the one of invertebrate transferrin [6, 12].

Lactoferrin belongs to the transferrin family and is capable of active binding and transporting of Fe^{3+} and other mixed-valent metals. Previously examined lacroferrins are glycoproteins with the molecular masses within the range of 75–80 kDa, containing intramolecular disulfide bonds. A polypeptide chain of lactoferrin forms two globular domains called M- and C-lobes, connected with an alpha helix. Each lobe has an iron-binding centre. The tertiary structures of apo-lactoferrin (iron-free lactoferrin) and iron-binding lactoferrin differ [5, 7, 8, 9, 10]. For example, it is found that Fe^{3+} binding plays an essential role in implementing the antioxidant and bacteriostatic function of lactoferrin. Along with antimicrobial activity, lactoferrin also has other biological properties — it can act as an immunomodulatory agent, antioxidant and anti-inflammatory agent and transcriptional factor and definitely participates in iron metabolism [2, 4, 8, 9, 10].

The purpose of the study is to determine the ability of *K. pneumonia* bacteria to synthesize protein, immunochemically similar to human lactoferrin, and the resistance of these microorganisms to the damaging effect of human lactoferrin (LF).

MATERIALS AND METHODS

The culture under study was taken from a local centre for culture collection of microorganisms *Klebsiella pneumoniae*, isolated from the patients with the small intestinal bacterial overgrowth syndrome (n=140) and from the control group (n=70) and were identified as *Klebsiella pneumoniae* after performing standard microbiological tests and using the polymerase chain reaction (PCR) method. Later on, they were kept in a semisolid agar for microorganism cultures.

Determining the production of the protein immunochemically similar to human lactoferrin

The production of the protein that is immunochemically similar to human lactoferrin was determined in the following steps:

Lactoferrin solution (400 ng/ml concentration in the nutrient (beef-extract) broth) was poured into 0.2 ml tubes. Then a bacteriological loop was used to inoculate *K.pneumoniae* in the agar culture tubes.

At the same time, the authors took control samples with the beef-extract broth that did not contain lactoferrin, with *K.pneumoniae* being inoculated, as it was done with the test samples.

Once all the tubes were incubated at t=37° C for 24 hours, their content was centrifuged (5,000 rpm for 45 min). The supposed presence of MdLF in the samples was confirmed through an ELISA assay using *Hycult Biotech* test kits (Netherlands).

Determining K.pneumoniae resistance to human lactoferrin

The microbial number of *K.pneumoniae* culture in normal saline was adjusted to a concentration of 10^{20} CFU/ml by the turbidity standard (Scientific Centre for Expert Evaluation of Medicinal Products named after L.A. Tarasevich). After that, 0.3-ml suspension of *Klebsiellae* was added to two tubes; there were two tubes per strain.

Then 0.2 ml of lactoferrin apoform solution were added to the test sample (so that its final concentration was 400 ng/ml).

0.2 ml of normal saline were added to the control sample instead of lactoferrin.

Once the samples were incubated (t=37° C for 30 min), the reaction was stopped by adding 5 ml of broth to all the samples with subsequent measurement of optical density (OD1).

The samples were again incubated at t=37° C for one hour to propagate lactoferrin-resistant *Klebsiellae*, and the optical density of the solutions was measured again (OD2).

The bactericidal effect of lactoferrin was calculated by the formula:

ΔODt – ΔODc/ΔODt, where

ΔODc *is the changes in the optical density of the control samples*;

ΔODt *is the changes in the optical density of the test samples*.

The authors made an attempt to isolate and purify the protein that is immunologically similar to human lactoferrin (MdLF) from *Klebsiella* culture. MLF presence in the samples of the pre-centrifuged (5,000 rpm during 45 minutes) liquid culture of *K.pneumoniae* (meat-peptone broth, incubation time — 24 hours at t=37° C) was assumed on the basis of EIA results with the use of *Hycult biotech* test kits (Netherlands) [3].

STATISTICAL ANALYSIS

The results were processed with standard variance statistics methods using Statistica 12 software. The statistical significance was assessed with the Wilcoxon–Mann–Whitney test at a significance level of $p < 0.05$.

RESULTS

The intensity of MdLF synthesis by *Klebsiellae* directly depended on the presence of lactoferrin in the medium ($p<0.05$). Interestingly, all *K.pneumoniae* cultures produced MdLF both in the presence of human LF in the culture medium and in its absence. However, the average concentration (M±m) of MdLF in the non-lactoferrin medium was 24.6±2.34 ng/ml, while it was 37.5% lower in the medium containing lactoferrin (only 15.35±1.7 ng/ml).

K.pneumoniae that were isolated from the patients and the healthy group also demonstrated different levels of MdLF production: MdLF synthesis in the strains taken from the patients with SIBO averaged 9.62±1.1 ng/ml, which is $p<0.05$ reliably lower than in the strains isolated from the healthy group (17.98±2.21 ng/ml).

Binding iron ions, lactoferrin largely provides the bactericidal activity of serum and many other biological fluids. *K.pneumoniae* isolated from the patients and the healthy group differed in their resistance to it. The number of the survived microorganisms (in %) after their contact with lactoferrin at a concentration of 400 ng/ml was 98.03±7.2 in the strains isolated from the patients with SIBO and 69.31±4.91 ($p<0.05$) in the healthy group, which allows to recommend the lactoferrin resistance criterion as a prognostic one.

Therefore, one can speak of the existence of a kind of a human LF — microorganism MdLF system. The authors also revealed significant differences (Table 1) when considering this system among *Klebsiellae* with different growth rates on dense nutrient agar after inoculation with 1 ml of broth culture of *K.pneumoniae*.

The least significant production of MdLF was in the *Klebsiellae* with pronounced growth. At the same time, the lawn-growing *Klebsiellae*, i.e. suppressing all other bacteria most successfully, produced MdLF significantly higher ($p<0.05$). The lawn-growing *Klebsiellae* also had higher resistance to lactoferrin.

However, the most active synthesis of MdLF was noted in the *Klebsiellae* forming single colonies on dense medium ($p<0.05$ compared with the first group). The very representatives of this *Klebsiella* group proved to be the most resistant to lactoferrin — almost 100% of the cells remained viable.

DISCUSSION

Microorganisms have evolved ways of getting iron in iron-deficiency conditions, which is important for optimal development in the environment and, in certain cases, may be critical for survival of the population. In this case, microbial strategies for getting iron can be considered as one of the factors that determine the formation and stability of microbial associations, playing an important role in the functioning of normal and pathological microbiocenoses in the host.

Interpreting the results obtained in the first part of the study, in particular the increased content of MdLF in the control samples against the test samples, the authors found that LF and MdLF had much in common in their structure and are similar in immunochemical terms. Therefore, it seems clear that synthesis of MdLF by *Klebsiellae* in the nutrient media containing human LF is inhibited on the principle of negative feedback. The same negative feedback mechanism also explains the reduced production of MdLF by strains isolated from the patients with the small intestinal bacterial overgrowth syndrome against the strains obtained from the healthy group, which was identified by the authors in further studies, since an increase in the concentration of LF in bacterial infections has been proven and described in numerous articles and reviews.

Parasites constantly face problems of survival in the adaptively changing environment of the host where they parasitize and where humans control the infec-

Table 1. *MdLF synthesis by microorganisms and their resistance to human LF (M±m)*

K.pneumoniae growth rate	n	MdLF concentration in culture medium (ng/ml)	Number of *K.pneumoniae* bacteria (in %) survived after LF incubation (concentration – 400 ng/ml)
1. Lawn growth	30	33.942±2.4	78.0±3.6
2. Pronounced growth; *Klebsiellae* form isolated colonies	80	15.612±6.3	62.0±3.1
3. Single colonies when inoculated with beef-extract agar	100	42.810±7.1	98.0±4.4

tion; in this regard, the increased resistance to LF of the patient strains identified by the authors also seems quite logical.

Analyzing the production of MdLF and the resistance to LF depending on the intensity of growth, it is safe to say that in terms of predicting the infection development and determining the potential danger of inoculated microorganisms, it is important to evaluate not only their number but also the pathogenic potential of individual strains. These were *Klebsiellae*, forming single colonies in the authors' studies, that not only had almost 100% resistance to human LF but also actively produced MdLF themselves, which allows them both to successfully survive in the host organism and to compete with microorganisms of other taxonomic groups. It seems clear that such bacteria are most often the cause of bacteria carrying and pose a serious epidemiological danger.

CONCLUSION

The study established the existence of a system that includes synthesis of the protein immunochemically similar to human lactoferrin by microorganisms and, consequently, of microorganisms' resistance to the antibacterial action of human lactoferrin. This confirms the significance of both iron itself and biomolecules determining its level in the human organism under the pathogen persistence.

The study established the diagnostic value of the human LF — microorganism MdLF system for predicting infectious process development and assessing the epidemiological danger from particular strains.

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