ISSN 0026-2617, Microbiology, 2008, Vol. 77, No. 2, pp. 186–191. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © O.V. Bukharin, A.O. Plotnikov, N.V. Nemtseva, L.V. Kovbyk, 2008, published in Mikrobiologiya, 2008, Vol. 77, No. 2, pp. 219–225.

EXPERIMENTAL ARTICLES

Histone–Antihistone Interactions in the *Escherichia coli–Tetrahymena pyriformis* Association

O. V. Bukharin[a], A. O. Plotnikov[a,1], N. V. Nemtseva[a], and L. V. Kovbyk[b]

[a] *Institute of Cellular and Intracellular Symbiosis, Urals Division, Russian Academy of Sciences, Orenburg, Russia*
[b] *Orenburg State Medical Academy, Ministry of Health, Russian Federation*

Received December 28, 2006; revised May 7, 2007

Abstract—Using the *Escherichia coli–Tetrahymena pyriformis* model system, we revealed the involvement of bacterial antihistone activity and protozoan histones in interactions between pro- and eukaryotic microorganisms. Antihistone activity enhanced the viability of *E. coli* in association with *T. pyriformis*, according to our data on the dynamics of *E. coli* cell numbers. The strain with antihistone activity induced incomplete phagocytosis in the infusorians, resulting in cytological changes and ultrastructural alterations that indicated the retention of bacterial cells in phagosomes. Bacteria with antihistone activity located in the *T. pyriformis* cytoplasm influenced the eukaryotic nucleus. This was accompanied by in macronucleus decompactization and a decrease in the average histone content in the population of infusorians. The data obtained suggest that protozoan histone inactivation by bacteria is one of the mechanisms involved in prokaryote persistence in associations with eukaryotic microorganisms.

Key words: protozoa, bacteria, histones, persistence, *Escherichia coli, Tetrahymena pyriformis.*

DOI: 10.1134/S0026261708020112

The role of cell metabolites in the functioning of microbial communities remains one of the most controversial issues in microbial ecology. Cationic proteins and peptides (lysozymes, histones, defensins, amebapores, etc.) are essential for interactions between pro- and eukaryotic microorganisms because of their versatility and antibacterial activity [1]. Among cationic proteins, intranuclear histones forming unique bonds with DNA during its packing in chromosomes are of paramount importance [2].

Recent studies on histones have made it possible to elucidate their structure and main functions [3]. In prokaryotes, histonelike proteins were revealed that are structurally similar to histones [4]. Mechanisms of spatial DNA–histone interactions were clarified [5], the involvement of histones in expressing hereditary information via transcription regulation [6] was established, and their antibacterial activity was detected [7]. Histones released in an inflamed tissue by destroyed leucocytes were found to facilitate the elimination of the pathogen due to their antibacterial effect [8]. A capacity of bacteria to inhibit the antibacterial effect of histones (termed antihistone activity) was detected [9]. It was suggested that the *E. coli–T. pyriformis* system should be used as a model to investigate the role of cationic proteins and their inhibitors in intermicrobial interactions [10].

[1] Corresponding author; e-mail: protoz@mail.ru

Despite the progress made in histone research, the role of histones in the interactions between pro- and eukaryotic microorganisms is as yet insufficiently understood. The goal of this work was to conduct an experimental study on the interactions between protozoan histones and bacterial antihistone activity, using the *E. coli – T. pyriformis* association as a model.

MATERIALS AND METHODS

This study used an axenic culture of *Tetrahymena pyriformis* GL infusorians kindly donated by V.I. Puskareva (Gamaleya Research Institute of Immunology, Epidemiology, and Microbiology); isogenic clones of *Escherichia coli* were obtained using the replica plating technique [11]. The (AHA+) clone of *E. coli* exhibited an antihistone activity (AHA) level of 8.2 μg/ml, while the (AHA–) clone lacked this activity. The antihistone activity of bacteria was determined photometrically by monitoring growth deceleration in the indicator strain *Micrococcus luteus* (No. 2665, Tarasevich State Research Institute of Seed-producing Crops) after incubating it with a supernatant of the tested culture supplemented with histones [12].

Prior to cocultivation, *E. coli* was grown for one day on nutrient agar. The infusorians were grown for three days on a medium containing nutrient broth, yeast autolysate, and horse serum. A suspension of bacterial

cells in sterile 0.9% NaCl solution was then prepared; its turbidity was adjusted to 10 arbitrary units (10^9 CFU/ml) according to the turbidity standard. The protozoa were separated from the cultivation medium by centrifugation three times for 10 min; they were resuspended in fresh medium after each centrifugation.

T. pyriformis–E. coli cocultivation was carried out in test tubes with 5 ml of the protozoan nutrient medium. The infusorian culture and the bacterial suspension were added to each test tube to final concentrations of 10^5 and 10^8 cells/ml, respectively. The test tubes were incubated at 25°C. In experimental test tubes, *T. pyriformis* was cocultivated with *E. coli* cultures that differed in antihistone activity. Pure infusorian and bacterial cultures grown on the protozoan medium served as the control samples.

During cocultivation, the *E. coli* cell number was monitored by plating decimal dilutions of the *E. coli* culture (0.1 ml) on nutrient agar using a spreading rod. *T. pyriformis* cells were counted in a Goryaev chamber.

The phagocytosis of bacteria by protozoa was estimated from the Hamburger index (the percentage of actively phagocytizing cells with bacteria-containing phagosomes) and the Wright index (the average phagosome number per infusorian), which were determined by a modification of the technique described in [13]. The sediment obtained by centrifuging the culture was used to prepare smears. They were dried in the air, fixed in Lilly fluid (a mixture of glacial acetic acid and 96% ethanol at a 1 : 2 ratio), stained with Manson blue, and studied in a microscope at a magnification of ×1000.

The histone content in infusorian cells was estimated by the cytochemical Olfert–Geschwind technique modified by Erenpreis [14] using the amido black 10B dye. The results of the cytochemical test for histones were evaluated visually, by scaling the nucleus coloration as follows: 0, no color; 1, light blue color; 2, intense blue color; and 3, manifest dark-blue color. The histone content in the infusorian population was expressed as the mean cytochemical coefficient (MCC) value [15] calculated after examining 100 cells in a smear according to the formula

$$\mathrm{MCC} = \frac{3 \times a + 2 \times b + c}{100},$$

where a, b, and c are the numbers of cells with nucleus coloration intensities of 3, 2, and 1, respectively.

The ultrastructural changes in *T. pyriformis* interacting with *E. coli* were monitored by examining them in an electron microscope after 1, 3, 6, 18, and 24 h of cocultivation. The pellets obtained by centrifuging the tested cultures were fixed with 4% glutaraldehyde for 60 min and subsequently with 1% osmium tetroxide for 60 min. The sediment samples were dehydrated in acetone solutions with increasing concentrations (30, 50, and 100% acetone) and embedded in Epon-812. Ultrathin sections were obtained with an LKB-5 ultramicrotome and double-stained with uranyl acetate and lead citrate solutions. The ultrathin sections were examined in an EMV-100 AK electron microscope, and micrographs of selected sections were prepared.

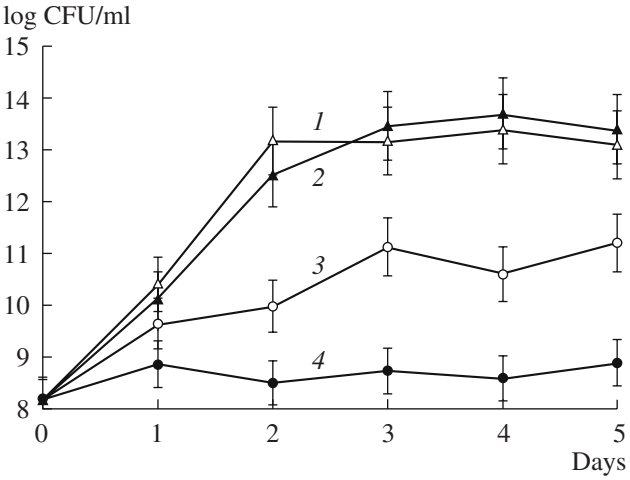

Fig. 1. Bacterial cell numbers in pure culture (*1, 2*) and in association with *T. pyriformis* (*3, 4*). *1* and *3*, *E. coli* (AHA+); *2* and *4*, *E. coli* (AHA–).

RESULTS

Population dynamics of microbial cell numbers. Cell numbers in pure *E. coli* (AHA+ and AHA–) cultures increased according to the physiological growth curve of a bacterial population in a liquid medium (Fig. 1). A logarithmic increase in the number of viable cells (by almost 5 orders of magnitude) continued for two days after the onset of the experiment, which was followed by a stationary phase characterized by the stabilization of bacterial cell numbers.

The bacteria that interacted with infusorians displayed a different population dynamics. Active consumption of bacterial cells by infusorians prevented any appreciable increase in *E. coli* (AHA–) cell numbers, and the growth curve was characterized by insignificant oscillations (Fig. 1). For example, the *E. coli* (AHA–) concentration increased fivefold after one day of cultivation, amounting to $0.72 \pm 0.08 \times 10^9$ CFU/ml. Afterwards, bacterial cell numbers did not change significantly. They remained at a level of 10^8–10^9 CFU/ml until the end of the experiment.

In the test tubes containing *T. pyriformis* and *E. coli* (AHA+), bacterial cell numbers increased significantly. However, they did not reach the values observed in the control (pure *E. coli* culture) (Fig. 1). The *E. coli* concentration was $0.16 \pm 0.03 \times 10^9$ CFU/ml at the beginning of the experiment and increased 30-fold and by nearly three orders of magnitude after one and two days, respectively. By day 3 it was $129.60 \pm 6.5 \times 10^9$ CFU/ml. Subsequently, the *E. coli* (AHA+) concentration remained at a level of 10^{10}–10^{11} CFU/ml until the end of the experiment. This dynamics of bacterial

Changes in the mean cytochemical coefficient and in the *T. pyriformis* population heterogeneity in terms of histone content

	Control population	Experimental population cocultivated with *E. coli* (AHA+)			
		0 h	6 h	18 h	24 h
Mean cytochemical coefficient (arbitrary units)	2.92 ± 0.19	2.92 ± 0.19	2.78 ± 0.18	2.20 ± 0.16	1.36 ± 0.14
Percentage of cells with different histone contents					
High (3 points)	92	92	78	32	12
Average (2 points)	8	8	22	56	25
Low (1 point)	0	0	0	12	50
Histones lacking (0 points)	0	0	0	0	13

cell numbers in an association with *T. pyriformis* suggests intense elimination of *E. coli* (AHA–) by infusorians and a comparatively high resistance of *E. coli* (AHA+) to phagocytosis.

The dynamics of the numbers of infusorians interacting with bacteria displayed no drastic changes that could result from the cytotoxic effects of the bacteria. The *T. pyriformis* numbers in test tubes varied within the physiological oscillation range $(1.0 \pm 0.05–1.3 \pm 0.07) \times 10^5$ cells/ml during cocultivation with *E. coli* (AHA+). In contrast, this number increased almost twofold (from $1.0 \pm 0.05 \times 10^5$ to $1.9 \pm 0.10 \times 10^5$ cells/ml) in an association with *E. coli* (AHA–). In the control sample (without bacteria), the infusorian concentration did not change significantly and remained within the physiological oscillation range $(1.0 \pm 0.05–1.1 \pm 0.05) \times 10^5$ cells/ml).

Dynamics of the phagocytosis activity of the infusorians. Light microscopy revealed differences in the dynamics of phagocytosis of bacteria with and without antihistone activity. One to five vacuoles (three on average) formed during the first hour of *T. pyriformis* cocultivation with *E. coli* (AHA–). The Hamburger index was 27%. The Wright index was 6 ± 2. After three hours, the phagocytic vacuole number increased to 8 ± 3. The Hamburger and the Wright indices reached 42% and 13 ± 2, respectively.

In association with *E. coli* (AHA+), the number of digestive vacuoles in the infusorian cytoplasm increased to 1–21 (15 on average) after one hour of cultivation; the Hamburger and the Wright indices were 65% and 10 ± 3, respectively. After two hours, the phagocytosis-related values changed. The Hamburger and Wright indices reached 69% and 23 ± 2, respectively. Some of the infusorian cells contained giant phagosomes enclosing dozens of bacterial cells.

The differences in the dynamics of the phagocytosis activity of the infusoria that were cocultivated with the isogenic *E. coli* clones (AHA+ and AHA–) were due to an incomplete phagocytosis of bacteria possessing antihistone activity. This resulted in an increase in *E. coli* (AHA+) cell numbers within the infusorian phagosomes, and the phagocytosis-related values exceeded those with *E. coli* (AHA–).

Dynamics of the infusorian population heterogeneity in terms of histone content. Taking into account the capacity of one of the tested *E. coli* clones to relieve the antibacterial effect of histones, we assessed histone content changes in infusorian populations. During the whole experiment, no changes occurred in the mean cytochemical coefficient of the histone content in *T. pyriformis* cells that were cocultivated with *E. coli* (AHA–). Like pure tetrahymena cultures, this culture was characterized by an MCC value of 2.92 ± 0.09. In contrast, the MCC value of histone content in the nuclei in the infusorian population that interacted with *E. coli* (AHA+) decreased to 1.36 ± 0.14 after 24 h of cultivation (table).

Apart from determining the mean cytochemical coefficient of histone content in the infusorian nuclei, we revealed changes in the heterogeneity of the infusorian population in terms of this coefficient. In the intact population, about 90% of the cell nuclei were rich in histones (3 points) and about 10% contained average (2 points) or low (1 point) amounts. Infusorians in association with *E. coli* (AHA–) did not differ in terms of population heterogeneity from intact infusorians. However, changes in the heterogeneity of histone contents occurred in *T. pyriformis* cocultivated with *E. coli* (AHA+). After 6 h of cocultivation, the histone content in 14% of the cells decreased from 3 to 2 points. After 18 h, cells with average histone contents (2 points) dominated the population. After 24 h, half of the population consisted of the cells with low histone content (1 point). The results obtained indicate a reduction in the histone content in protozoan nuclei in the process of interactions between protozoans and bacteria with antihistone activity.

Dynamics of ultrastructural changes in microorganisms. During the first hours of cultivation, interactions between protozoa and bacteria followed the same pattern, regardless of bacterial antihistone activity. After 1 h, phagosomes appeared in the infusorian cytoplasm in the peristome area. The phagosomes con-

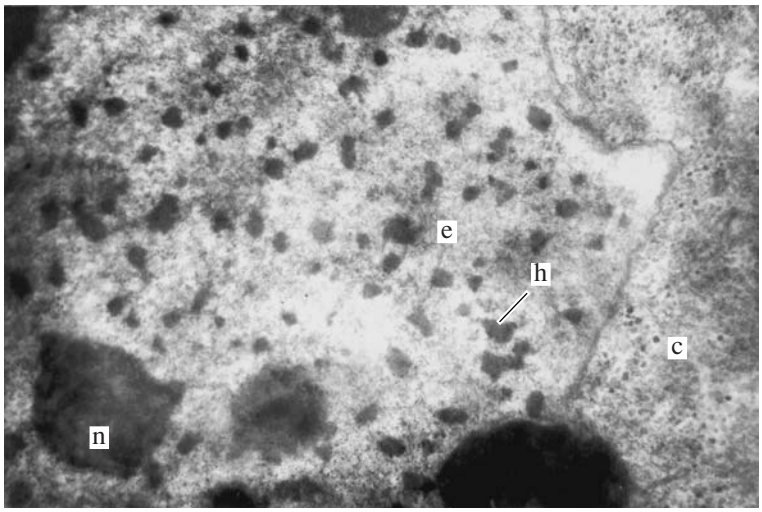

Fig. 2. A fragment of a macronucleus of *T. pyriformis* that interacts with *E. coli* (AHA–). Magnification, ×15000. h, heterochromatin (chromatin bodies); e, euchromatin; n, nucleolus; c, cytoplasm.

tained solitary bacterial cells. No significant ultrastructural changes were detected in the protozoa.

After 3 h of cocultivation, the phagosome number in the cytoplasm increased and reached 10–12 in some of the infusorian cells. After 6 h, the infusorians displayed changes characteristic of active phagocytosis. Primary lysosomes and mitochondria aggregated around phagosomes, indicative of an active process of digesting bacterial cells. The endoplasm of the infusorians cocultivated with *E. coli* (AHA+) contained enlarged granular ER cisterns and hypertrophied mitochondria characterized by an unusually big size. The mitochondria of the infusorians that were cocultivated with *E. coli* (AHA–) did not undergo such changes. The (AHA+) bacteria located in the phagosomes were degraded and demonstrated changes similar to those of the cells transformed into spheroplasts. Some cells only became denser. Interestingly, at this stage individual bacteria exited the phagosomes and were located directly in the cytoplasm.

After 18 h, (AHA+) bacteria were digested by most of the infusorians. However, individual bacteria without ultrastructural signs of degradation were detected in some of the phagosomes. The cytoplasm of a small number of infusorians contained lamellar myelin-like structures. Remnants of degraded bacteria were found in digestive vacuoles of the infusorians that interacted with *E. coli* (AHA–).

After 24 h of cocultivation with *E. coli* (AHA+), we revealed changes in the infusorian macronucleus. In a *T. pyriformis–E. coli* (AHA–) system, the macronucleus retained its native structure (100–200 nm chromatin bodies) (Fig. 2), while the macronucleus of most infusorians interacting with *E. coli* (AHA+) decompactized, the heterochromatin content drastically decreased, and the chromatin bodies disappeared (Fig. 3).

Hence an analysis of ultrastructural changes in the infusorians during their cocultivation with bacteria with and without antihistone activity revealed incomplete phagocytosis of *E. coli* (AHA+) by the infusorians. In addition, we detected structural responses of the macronucleus (chromatin decompactization) and the hypertrophy of the mitochondria with the formation of lamellar bodies in the cytoplasm.

DISCUSSION

Histone–antihistone interactions between protozoa and bacteria seem feasible because (i) histones occur in the nuclei of a large number of protozoa [16] and (ii) prokaryotes are capable of intranuclear symbiosis with eukaryotes [17]. The *E. coli–T. pyriformis* model provided evidence of the involvement of protozoan histones and bacterial antihistone activity in microbial interactions. Presumably, these interactions are based on the universal properties of microorganisms that manifest themselves in a uniform mechanism of formation of protozoan–bacterial associations, similar to lysozyme–antilysozyme interactions [10].

It was established earlier that the capacity to inactivate lysozyme (antilysozyme activity) is widespread among the bacteria that can persist in the human organism and eukaryotic hydrobionts [18]. This activity is based on a secretable factor involved in prokaryote persistence. It was revealed that antilysozyme activity promotes the survival of prokaryotes both in the human organism [19] and in protozoan cells [10]. The results of our research indicate that the survival of bacteria in association with protozoa may be due to the inactivation of histones, and not only lysozyme. This idea is confirmed by the fact that in association with *T. pyriformis*, the cell number of *E. coli* (AHA+) increased,

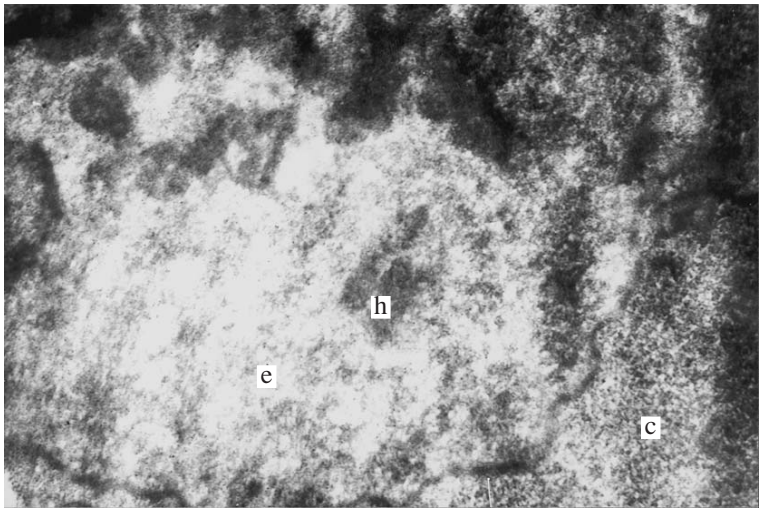

Fig. 3. A fragment of a macronucleus of *T. pyriformis* that interacts with *E. coli* (AHA+). Magnification, ×15 000. See Fig. 2 for designations.

whereas the isogenic clone *E. coli* (AHA–) was grazed by the infusorians, and its cell number did not increase.

It was shown earlier that retention of prokaryotes with antilysozyme activity within the protozoan cells is associated with incomplete phagocytosis of bacteria [10]. It turned out that a similar effect is caused by bacteria with antihistone activity. This was confirmed by light and electron microscopy. The observations indicate that phagosomes were accumulated and morphologically intact bacteria persisted in the infusorian cells. However, unlike lysozyme–antilysozyme interactions, histone–antihistone interactions do not confine themselves to changes in the infusorian cytoplasm; they affect the nucleus (macronucleus) as well.

Electron microscopy revealed disruptions in the chromosome organization of *T. pyriformis* macronuclei. Predominantly, infusorial nuclei are structurally organized as "blobs" or chromatin bodies consisting of DNA loops that contain transcriptionally inactive sections of the genome [20, 21]. The "blobs" disappeared, chromatine was decompactized, and the heterochromatin content was drastically reduced in the *T. pyriformis* nucleus after interaction with *E. coli* (AHA+). The ultrastructural alterations in the nucleus were accompanied by changes in the heterogeneity of the protozoan population associated with *E. coli* (AHA+), resulting in a decrease in its histone content. It seems likely that chromatin decompactization and a decrease in the histone content of infusorian nuclei are due to the antihistone activity of intracellular bacteria. Histone inactivation and chromatin decompactization may be accompanied by the transition of the DNA to the transcriptionally active state. This was shown in the in vitro DNA–histones–RNA polymerase model system [6, 22] and in the in vivo in the culture of *Drosophila* cells and in rat liver cells [23, 24].

Taken together, the results of the studies conducted by us significantly improve our understanding of the functional relations within protozoan–bacterial associations and provide evidence that inactivation of protozoan cationic proteins by bacteria is one of mechanisms involved in the persistence of prokaryotes in associations with eukaryotic microorganisms.

ACKNOWLEDGMENTS

This work was supported by the Russian Foundation for Basic Research (project no. 05-04-49870) and the Basic Research Program of the Presidium of the Russian Academy of Sciences "Scientific Foundations of Russia's Biodiversity Protection" (project no. BR-9-04).

REFERENCES

1. Kokryakov, V.N., *Biologiya antibiotikov zhivotnogo proiskhozhdeniya* (Biology of Antibiotics of Animal Origin), St. Petersburg: Nauka, 1999.
2. Metzler, D.E., *Biochemistry: The Chemical Reactions of Living Cells*, New York: Academic, 1977, vol. 3.
3. Lewin, B., *Genes V.*, Oxford: Oxford Univ. Press, 1272.
4. Romashkin, V.I. and Oparin, A.I., Histonelike Proteins in Cyanobacteria, *Dokl. Akad. Nauk SSSR*, 1981, vol. 259, no. 3, pp. 750–752.
5. Zbarskii, I.B., *Organizatsiya kletochnogo yadra* (Organization of Cell Nucleus), Moscow: Meditsina, 1988.
6. Pospelov, V.A., Histones and the Role of DNA Conformational Transitions in Transcription Regulation, *Tsitologiya*, 1973, vol. 15, no. 11, pp. 1327–1337.
7. Ashmarin, I.P. and Zhdan-Pushkina, S.M., Influence of Histones on Microorganisms, *Zh. Mikrobiol. Epidemiol. Immunobiol.*, 1966, no. 9, pp. 103–108.
8. Miller, T.E. and Watson, D.W., Biochemical Characterization of the Antimicrobial Histone Released by Deox-

yribonuclease and Lactic Acid, *Proc. Soc. Exp. Biol. Med.*, 1969, vol. 131, pp. 339–342.

9. Sokolov, V.Yu., Antihistone Activity in Bacteria, *Byull. Eksp. Biol.*, 1993, no. 2, pp. 180–183.
10. Bukharin, O.V. and Nemtseva, N.V., Investigation of Lysozyme-Antilysozyme Interactions in a Model *Tetrahymena–Escherichia* Community, *Mikrobiologiya*, 2001, vol. 70, no. 5, pp. 656–661 [*Microbiology* (Engl. Transl.), vol. 70, no. 5, pp. 564–569].
11. Lederberg, J. and Lederberg, E.M., Replica Plating and Indirect Selection of Bacterial Mutants, *J. Bacteriol.*, 1952, vol. 63, pp. 399–406.
12. Bukharin, O.V., Plotnikov, A.O., Nemtseva, N.V., and Sgibnev, A.V., Method of Determination of Antihistone Activity of Microorganisms, RF Patent No. 2203956, *Byul. Izobret.*, 2003, no. 13.
13. Pushkareva, V.I., Method of Experimental Study of the Hosts and Circulation of Infectious Agents in Aquatic (Soil) Ecosystems, in *Patogennye bakterii v soobshchestvakh (mekhanizmy i formy sushchestvovaniya). Sb. nauchnykh trudov NIIEM im. N.F. Gamalei*, (Pathogenic Bacteria in Communities (Mechanisms and Forms of Existence). Proc. Gamaleya Res. Inst. Exp. Medicine), Moscow, 1994, pp. 35–42.
14. Erenpreis, Ya.G., Cytochemical Study of Basic Cellular Proteins, *Arkh. Anat., Gistol. I Embriol.*, 1965, vol. 49, no. 12, pp. 3–8.
15. Mayanskaya, N.N., Nikolaev, Yu.A., Shurgaya, A.M., and Gefarova, Z.M., Cytochemical Determination of the Lysosomal Cationic Proteins in Blood Neutrophiles in Cases of Hypertrophic Cardiomyopathy, *Lab. Delo*, 1991, no. 5, pp. 15–18.
16. Lukashenko, N.P. and Rybakova, Z.I., *Struktura i funktsiya genomov prosteishikh* (Structure and Function of Protozoan Genomes), Moscow: Nauka, 1991.
17. Osipov, D.V. and Podlipaev, S.A., Theoretical and Practical Aspects of the Interactions between Protozoa and Microorganisms, in *Vzaimootnosheniya prosteishikh s virusami. Seriya "Protozoologiya". Vyp. 6* (Interactions between Protozoa and Viruses, iss. 6), Leningrad: Nauka, 1981, pp. 5–30.
18. Bukharin, O.V. and Nemtseva, N.V., The "Lysozyme–Antilysozyme" System and its Role in the Symbiotic Relations of Hydrobionts, *Usp. Sovrem. Biol.,* 2002, vol. 122, no. 4, pp. 326–333.
19. Bukharin, O.V., Gintsburg, A.L., Romanova, Yu.M., and El'-Registan, G.I., *Mekhanizmy vyzhivaniya bakterii* (Mechanisms of Bacterial Survival), Moscow: Meditsina, 2005.
20. Raikov, I.B., *Yadro prosteishikh. Morfologiya i evolyutsiya* (Protozoan Nucleus. Morphology and Evolution), Leningrad: Nauka, 1978.
21. Martynkina, L.P., Vengerov, Yu.Yu., Bespalova, I.A., Sergeeva, G.I., and Tikhonenko, A.S., Structure of the Interphase Chromatin of the Macronucleus of the Infusorian *Bursaria truncatella.* II. Loop Organization of Inactive Chromatine Lumps, *Mol. Biol.*, 1984, vol. 18, no. 1, pp. 272–276.
22. Kraevskii, V.A., Panin, V.M., and Razin, S.V., Histone Acylation in vitro Causes Chromatin Decomopactization, *Biofizika*, 1994, vol. 39, no. 4, pp. 613–618.
23. Preobrazhenskaya, O.V., Karpov, V.L., Nagorskaya, T.V., and Mirzabekov, A.D., Structure of Transcriptionally Active Chromatin, *Mol. Biol.*, 1984, vol. 18, no. 1, pp. 8–20.
24. Levitskii, E.L., Gubskii, Yu.I., Chabannyi, V.N., Volkov, G.L., and Novikova, S.M., Biochemical Characterization of the Fractions of Transcriptionally Active and Repressed Chromatin in Rat liver, *Biopolim. Kletka*, 1993, vol. 9, no. 6, pp. 13–21.