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SEROLOGICAL AND BIOLOGICAL ACTIVITY OF LIPOPOLYSACCHARIDE FROM *Escherichia coli* L-19

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The lipopolysaccharide from *Escherichia coli* L-19 was isolated, chemically identified and its non-toxicity but pyrogenicity were shown. The investigations of lipopolysaccharide influence on T- and B-lymphocytes indicated that it might be used as a mitogen in blasttransformation reactions since it was only in insignificant degree less active in comparison with the commercial one. It was shown that in reactions of Ouchterlony double immunodiffusion in agar LPS from *E. coli* L-19 exerted an activity of antigen in homological system. There were not established serological cross reactions between antiserum to heated *E.coli* L-19 cells and LPS from another of *E. coli* strain M-17 and also the representatives of *Enterobacteriaceae* species such as *Budvicia aquatica, Rahnella aquatilis* and *Pragia fontium*. These results indicate the absence of common antigenic determinants between the tested strains.

Key words: *Escherichia coli* lipopolysaccharide, toxicity, pyrogenicity, mitogenic activity.

Ecologic catastrophes which took place in the recent years led to essential decrease of human organism resistance. It involves the changes of the formed microcenosis of different human body cavities. Thus genetically determined properties of pathogenecity and virulence of pathogenic microflora tended to increase according to their phenotypic development. In particular, in the course of evolution virulent microorganisms developed a special system of devices which is aimed at modulating of phagocytic activity of the organism. One of the system factors is a lipopolysaccharide (LPS), which is one of the main components of outer membrane of Gram negative bacteria. Being in close contact with membrane proteins LPS provide integrity, stability, and functional purpose of the outer membrane of microbial cells [1].Thanks their location on the outer membrane LPS play an important role in the processes of bacterial interaction with the environment and other organisms, in particular, they determine immunogenic properties of bacterial cells. LPS have endotoxin properties and provocate some pathological reactions, which can in certain cases lead to the lethal outcome [2]. On the other hand, LPS stimulate factors of nonspecific resistance of macroorganism. However, despite long-term intensive investigations of LPS from Gram negative bacteria, the mechanisms of their actions are still unclear. The complexity of LPS investigation is related with the multifactor action on the host organism, when it is impossible to define whether the observed effect is due to the direct action on bacterial cells or it is the result of the action of biologically active compounds for which LPS are synthesis inducer. In the last decade it was noticed the essential progress in estimation of the role of LPS/lipid A in recognition and signal system of mammalian phagocytes. According to the modern model, specific cellular recognition of agonistic LPS/lipid A is initiated by the cascade of three LPS receptors: LPS-binding protein, CD 14 receptor and the complex of Toll-like receptor 4 (TLR4) with transport protein MD-2 [3]. The estimation of structure-activity relationship in LPS and lipid A not only contributes in understanding of immunostimulation and toxic process but also promotes the development of new pharmacological and immunostimulating methods of prophylaxis and treatment of infectious and tumor diseases. Since LPS synthesis is determined by genes with diffferent

level of conservatism the data concerning structure and properties of these biologically active molecules can be used in taxonomy to clarify the phylogenetic relationship between bacteria. Immunomodulating properties of LPS are determined by their ability to activate B- and T-lymphocytes, granulocytes and monocytes [4].

The aim of this work was chemical and biological characteristic of the lipopolysaccharide from unstudied strain *Escherichia coli* L-19.

Materials and Methods

The object of investigation was strain of *Escherichia coli* L-19 isolated from a healthy patient at the epidemiological centre in Kyiv. Bacteria were grown on the solid medium MPA (meat-peptone agar) during 24 hours at 28–30 °C. Cells were collected by centrifugation (20 min, 5,000 g), after that they were dried with acetone and ether.

LPS isolation. LPS were extracted from dried cells by 45% phenol water solution at 65–68 °C. The obtained aqueous fractions were dialyzed against water, and then distilled water to remove phenol [5].

Determination of carbohydrates, nucleic acids and protein content. The amount of carbohydrates was determined by phenol-sulfuric method [5]. The results were evaluated by color change during the phenol reaction with sulfuric acid in a spectrophotometer at 490 nm. Carbohydrate content was determined in accordance to the calibration curve, which was built using glucose as a standard. The content of nucleic acid was determined by Spirin [5], the content of protein by Lowry [5].

Identification of neutral monosaccharides was carried out after hydrolysis of preparations in 2 M HCl (5 h, 100 °C). Monosaccharides were analyzed as acetates of polyols [6] on chromato-mass-spectrometer system Agilent 6890/5973N inert, column DB-225 mS (30 m×0.25 mm×0.25 μm), gas-carrier-helium, flow rate-1ml/min. The temperature of the evaporator was 250 °C, interface — 250 °C, thermostat — 220 °C (isothermal regime). Monosaccharides were identified comparing the time of retention of acetates of polyols with the standards and using computer data base ChemStation. Quantitative ratios of individual monosaccharides were expressed as % from total sum of peak squares.

Fatty acid composition was defined after hydrolysis of the sample in 1.5% acetyl cloride in methanol (100 °C, 4 h), methyl ethers of fatty acids were analyzed on chromato-mass-spectrometer system Agilent 6890/5973 inert, column HP-5MS, length was 30m, inner diameter — 0.25 mm, phase thickness-0.25 μm, temperature regime — 150–250 °C, temperature gradient — 4 °C, gas-carrier-helium, flow rate — 1.2 ml/min, the temperature of evaporator was 250 C, flow distribution 1:100. Fatty acids were identified using computer base data and standard mixture of methyl ethers of fatty acids [6].

LPS pyrogenicity was determined on rabbits with body weight 2.0–3.5 kg [5]. Thermometry was carried out using an electronic thermometer (Omron Matsusaka Co. Ltd, Japain), by rectal injection into 5–7 cm (depending on the rabbit weight). All rabbits were preliminarily subjected to immunoreactivity test by intravenous introduction of 0.9% sterile apyrogenic sodium chloride solution (10 ml per kg). The investigated preparations of LPS were solved in the same solution and were incubated at 37 °C for 10 min. After that they were administered intravenously (1 ml per kg of rabbit body weight). The minimal pyrogenic dose of LPS preparations was determined in a series of solutions from 0.5 to $1.0 \cdot 10^{-2}$ mg/ml. Each series of investigated solutions was tested in three rabbits of similar weight (the difference was not more than 0.5 kg). Before administration of LPS the rabbit temperature was measured twice every 30 min. The animals with temperature difference more than 0.2 °C were not used for further investigations. The result of the last temperature measurement was taken as the initial temperature. The solution of LPS was administered no later than 15–20 min after the last temperature measurement. The subsequent measurements were carried out after preparation administration three times every hour. The investigated LPS solution was considered as apyrogenic one if the total amount of temperature rise for 3 hours was less or equal 1.4 °C.

Toxicity of LPS was determined on white inbred mice with body weight 19–21 g of both sexes. These animals had not been used in the previous experiments and were sensitized by galactosamine. All animals were subjected to intraperitoneal injection of D-galactosamine hydrochloride (0.5 ml) in 0.9% NaCl apyrogenic sterile solution. Immediately thereafter preheated to 37 °C LPS in apyrogenic solution (0.2 ml) was administered intraperitoneally with the flow rate 0.1 ml/s. In dilution series of LPS we defined the dose that causes the death of

50% of the tested animals (LD_{50}). This value was used to determine toxicity. The control and test group contained 10 mice each. The control group was subjected to administration of D-galactosamine hydrochloride with 0.2 ml of 0.9% NaCl apyrogenic sterile solution.The animal observation was carried out for 48 h [5].

Immunological investigations. O-antiserum toward heated *E. coli* (boiling water bath, 2.5 h) was obtained. Rabbits were immunized intravenously five times with an interval of four days, cell concentration was $2 \cdot 10^9$/ml (from 0.1 to 1 ml). Antigen activity of LPS was investigated by the method of Ouchterlony double immunodiffusion in agar [7].

The influence of LPS on different lymphocyte populations was studied according to the level of expression of CD_3- and CD_{22}-receptors on the surface of T- and B-lymphocytes using rosetting reaction with monoclonal antibodies towards above mentioned receptors. 0.25 ml of CD diagnosticum was transferred into round holes of immunological plate or microtubes. Then an equal volume of the lymphocyte mixture was added. The obtained mixture was incubated at 37 °C for 25 min, centrifuged at 500–1,000 g for 3 min and placed at 4 °C into a refrigerator for 1 h. Lymphocyte pellets were treated with monoclonal antibodies, dried, fixed by ethanol and stained according to Romanovsky-Giemsa to observe clearly nucleus of lymphocytes.

During the reaction of blast transformation of human lymphocytes LPS of investigated strains were used as a mitogen; the dilution was following: 1 mg of LPS per 10 ml and 1mg of LPS per 100 ml of the sterile saline solution. To carry out the reaction LPS preparation, medium 199 [8], antibiotic preparation, bovine serum and human blood were taken by 0.2 ml each. The mixture was incubated at 37 °C for 2 days. To precipitate lymphoblasts after incubation acetic acid was added (10% solution) and the probes were centrifuged. The pellet was removed from supernatant, transferred to glass slides, stained according to Romanovsky-Giemsa and estimated by microscopy [8].

Statistical analysis. Results of investigations were statistically tested according to the method proposed for pharmaceutical preparations [8]. Each experiment was performed in 3–5 replicates. In all tables and graphics the mean values and their standard deviations were presented. The data were statistically tested by Student's t-test, $P < 0.05$ were considered to be statistically significant.

Results and Discussion

Enterobacteria are widespread in nature. Among them there are saprophytes, which are useful human symbionts, and causative agents of acute intestinal infections. The role of saprophyte microflora is not limited to its participation in digestion process. Endotoxin, which is released as a result of self-renewal of *E. coli* pool, partially enters the portal blood and provides antigenic stimulation. Besides, a small amount of endotoxin is also released by live Gram-negative bacteria. Taking into account the large population of intestinal bacteria it can lead to considerably high concentration of the endotoxin. Thus, the study of the structure and biological activity of new strains, which are found in human organism, has not only theoretical, but also practical importance.

LPS from *E. coli* L-19 is characterized by a relatively high content of nucleic acids that can be explained by peculiarities of the isolation using water-phenolic method. The purified preparation of LPS (triple ultracentrifugation at 105,000 g, 4 h) contained 40% of carbohydrates and 0.8% of nucleic acids whereas proteins were not detected. The yield of LPS was 2.0% that is less than it was shown for other representatives of *Enterobacteriaceae* (5%).

Monosaccharide composition analysis (Table 1) proved that predominant monosaccharides of LPS of *E. coli* L-19 were glucose (55.5%) and ribose (24.0%). Characteristic components of LPS of Gram-negative bacteria such as heptose and KDO (2-keto-3-deoxy-octanoic acid) accounted for 10.8% and 0.8% respectively.

LPS showed a broad spectrum of biological activity (endotoxicity) in mammals. It was found that endotoxic activity of LPS

Table 1. **Monosaccharide composition of LPS from *E. coli* L-19**

Monosaccharides	Amount
% from total sum of pike surfaces (as a control)	
Fucose	2.4±0.12*
Ribose	24.0±1.2*
Galactose	7.3±0.36*
Glucose	55.5±2.7*
Heptose	10.8±0.54*
% from dry weight of LPS	
KDO	0.8±0.04*

Note: here and after * — $P \leq 0.05$.

is determined by the lipid fragment of the molecule [9].

Analysis of fatty acid composition of lipid A of LPS from the investigated strains showed the presence of fatty acids with the number of carbon from 12 to 18 (Table 2). Among hydroxy acids 3-hydroxy-tetradecanoic (48.8%), tetradecanoic (22.7 %), dodecanoic (15.3%) and hexadecanoic (6.7%) acids were found. The presence only one hydroxy acid, i.e. 3-hydroxy-tetradecanoic, which acylates both amino- and hydroxy groups of glucosamine residues, is the attribute of *Enterobacteriaceae* family.

The functional role of LPS as the main thermostable antigen of microbial cells is well-known and the most studied. LPS administration into higher animals and humans leads to production of complementary antibodies against the certain structural sites of LPS molecule [5]. Polyclonal antiserum obtained to heated culture of *E. coli* L-19 was used as antibodies for serologic examinations. LPS purified from this strain was used as an antigen. For ring precipitation reaction the working titer of O-antiserum obtained to heated culture of *E. coli* L-19 was determined (1:40, 000).

It was found that LPS of the investigated strain *E. coli* L-19 possessed antigen activity in homologous system by the method of Ouchterlony double immunodiffusion (Fig. 1). It was not determined serological cross reactions between antiserum against *E. coli* L-19 cells and LPS from another strain *E. coli* M-17. The cross reaction was not also shown for other representatives of *Enterobacteriaceae* such as *Budvicia aquatica, Rahnella aquatilis* and *Pragia fontium*. It proves the absence of common antigenic determinants for these

Table 2. **Fatty acid composition of LPS from *E. coli* L-19**

Fatty acid	**Amount** (% from total sum of peaks surfaces which was taken as a control)
Dodecanoic $\mathbf{C_{12:0}}$	15.3 ± 0.76*
Tetradecanoic $\mathbf{C_{14:0}}$	22.7 ± 1.13*
3-oxytetradecanoic **3-OH-$C_{14:0}$**	48.8 ± 2.44*
Iso-hexadecanoic **i$C_{16:0}$**	1.5 ± 0.07*
Hexadecenoic $\mathbf{C_{16:1}}$	1.6 ± 0.08*
Hexadecanoic $\mathbf{C_{16:0}}$	6.7 ± 0.33*
Trans-octadecenoic **т$C_{18:1}$**	2.1 ± 0.11*
Octadecanoic $\mathbf{C_{18:0}}$	1.3 ± 0.06*

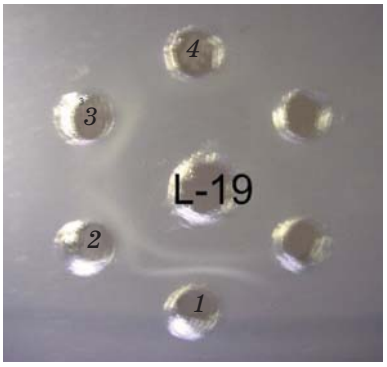

Fig. 1. **Ouchterlony double immunodiffusion in agar of LPS from *E. coli* L-19 with homologous O-antiserum.** LPS dilution: 1:100 (*1*), 1:5, 000 (*2*), 1:10, 000 (*3*), 1:20, 000 (*4*)

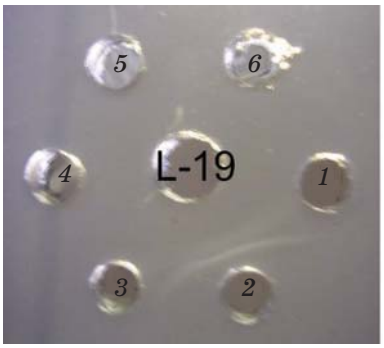

Fig. 2. **Ouchterlony double immunodiffusion in agar:** LPS from *E. coli* M-17 (*1*); *E. coli* L-19 (*2*); *B. aquatica* 23270 (*3*); *B. aquatica* 124 (*4*); *P. fontium* 20125 (*5*); *R. aqutilis* 33071 (*6*) with O-antiserum against *E. coli* L-19

strains (Fig. 2). The obtained results are in accordance with our previous investigations [10] where we showed the inability of such serological cross reactions, because the structure of O-specific polysaccharide of LPS from *E. coli* L-19 is unique and hasn't been found in any bacterial polysaccharides. The studied LPS did not interact with any known polyvalent antisera including O-antigenic forms of *E. coli* indicated as serogroups from O1 to O180. Moreover, *E. coli* L-19 did not interact with the individual antiserum against *E. coli* O43 which was the closest to *E. coli* L-19. It proves that the investigated strain belongs to a new serogroup of *E. coli* [10].

Among wide spectrum of biological activity of LPS the special attention is paid to its toxicity and ability to activate cells of immune systems. The result of specific interaction of LPS with the cells of macroorganisms is biosynthesis of biologically active mediators — cytokines, which at low concentration regulate the work of immune system and at high

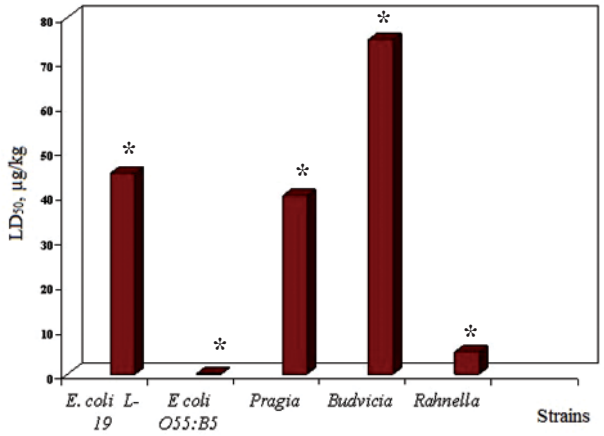

Fig. 3. **Toxicity of LPS from *E. coli* L-19 as well as new species of *Enterobacteriaceae* — *R. aquatilis*, *B. aquatica* and *P. fontium***

Control — 0%; the control group was subjected to administration of D-galactosamine hydrochloride with 0.2 ml of 0.9% NaCl sterile solution.

Here and after * — $P \leq 0.05$.

concentration lead to the development of toxic effects such as pyrogenicity, leukopenia and septic shock [2]. Experiments with inbred mice showed that LPS from *E. coli* L-19 was characterized by toxic action, which was significantly less than for LPS from *E. coli* O55:B5 (Fig. 3) and for new species of *Enterobacteriaceae* such as *R. aquatilis* [11] and *P. fontium* [12].

It was shown (Fig.4) that LPS from *E. coli* L-19 had a similar pyrogenicity as pharmaceutical preparation pirogenal.

It is known [4], that bacterial LPS are stimulators of inborn and acquired immunity in different eukaryotic species including insects and humans. T-lymphocytes take part in regulation of all parts of immunity in particular they synthesize and regulate the synthesis by other cells of pro- and anti-inflammatory cytokines. The ratio of these cytokines defines the ways of metabolic

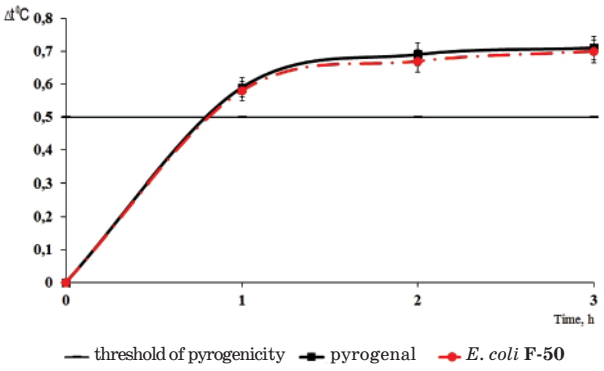

Fig. 4. **Pyrogenic activity of LPS from *E. coli* L-19**

processes in cells, their resistance to apoptotic factors and many other organism functions, which have direct influence on reparative processes in tissues. B-lymphocytes play an important role in providing humoral specific immunity. The results of lymphocyte blast transformation reaction (LBTR) provide information about functional activity of T- and B-lymphocytes. Introduction of mitogens or antigens in the lymphocyte culture induces the number of morphological and biochemical changes in cells. These changes include DNA synthesis and transformation of lymphocytes into blast forms (big dividing cells). LBTR determines a proliferative potential of studied cells. Phytohemagglutinin, concanavalin A, bacterial lipopolysaccharides, enzymes etc are used as activators (mitogens) of LBTR.

Mitogenic activity of LPS from *E. coli* L-19 was compared with the commercial preparation of LPS from firm "Diagnosticum", Lviv Research Institute of hematology and blood transfusion. According to the results the commercial preparation of LPS largely stimulated formation of lymphoblasts regardless of the concentration (Fig. 5). The studied preparation of LPS at a dilution 1:100 was more active in comparison with a 1:10 dilution.

Characteristic feature of B- and T-lymphocytes is the presence of some antigenic markers particularly CD_{22}- and CD_3-receptors respectively. It is known that CD_3-protein complex, which is associated with antigen-specific T-cellular receptor, is a functional marker of T-lymphocytes. It facilitates the transmission of activation signal from the membrane into the cytoplasm. The carried investigations showed the decrease of expression of CD_3- receptors on T-lymphocytes when the dilution of LPS from *E. coli* L-19 was

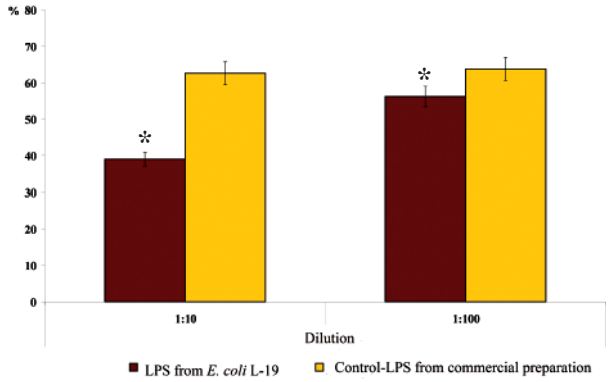

Fig. 5. **Influence of LPS from *E. coli* L-19 on the amount (%) of blast transformed lymphocytes**

1:10 and the increase of this expression when the dilution was 1:100 (Fig. 6).

The receptor of B-lymphocytes, CD_{22} is transmembrane protein which belongs to lectin family. This regulator molecule prevents overactivation of immune system and development of autoimmune processes. The obtained results (Fig. 7) showed the increase of expression of CD_{22}-receptors on B-lymphocytes (approximately 80% increase) when the dilution of LPS from *E. coli* L-19 was 1:10 and 1:100. So, the expression of CD_3- and CD_{22}-receptors on the surface of T- and B-lymphocytes correspondingly depends on LPS dilution.

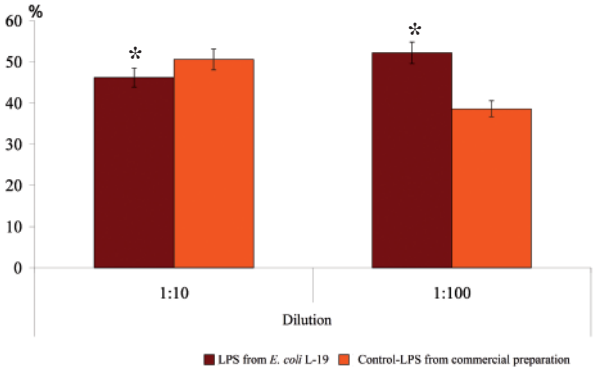

Fig. 6. **Influence of LPS from *E. coli* L-19 on the expression of CD_3-receptors on the surface of T-lymphocytes**

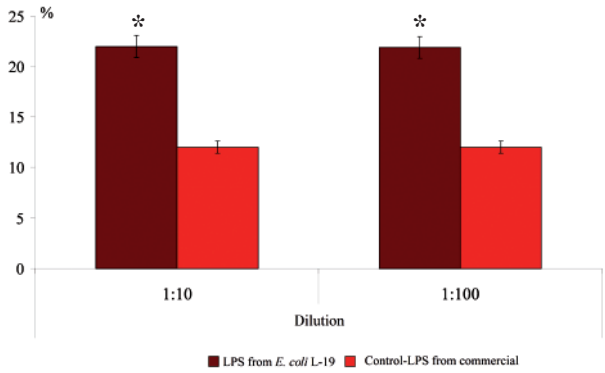

Fig. 7. **Influence of LPS from *E. coli* L-19 on the expression of CD_{22}-receptors on the surface of B-lymphocytes**

So, LPS from *E. coli* L-19 was isolated and its chemical identification was performed. It was shown that it is nontoxic but pyrogenic. The influence of LPS on T- and B-lymphocytes let us suggest that it can be used as a mitogen in the reaction of blast-transformation because its activity at a dilution 1:100 is slightly inferior to the commercial preparation from firm "Diagnosticum", Lviv Research Institute of hematology and blood transfusion.

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СЕРОЛОГІЧНА ТА БІОЛОГІЧНА АКТИВНІСТЬ ЛІПОПОЛІСАХАРИДУ *Escherichia coli* L-19

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Ізольовано ліпополісахарид *Escherichia coli* L-19, здійснено його хімічну ідентифікацію, показано, що він є малотоксичним, але пірогенним. Вивчення впливу досліджуваного ліпополісахариду на Т- і В-лімфоцити свідчить, що його можна використовувати як мітоген у реакціях бласттрансформації, оскільки за активністю він незначною мірою поступається комерційному препаратові ліпополісахариду. У реакціях подвійної імунодифузії в агарі за методом Оухтерлоні показано, що ліпополісахарид *E. coli* L-19 в гомологічній системі виявляє активність антигену. Не виявлено перехресних серологічних реакцій між антисироваткою до прогрітих клітин *E. coli* L-19 та ліпополісахаридом штаму *E. coli* M-17, а також представниками інших видів *Enterobacteriaceae — Budvicia aquatica*, *Rahnella aquatilis, Pragia fontium*, що свідчить про відсутність у них загальних антигенних детермінант.

Ключові слова: ліпополісахарид, *Escherichia coli*, токсичність, пірогенність, мітогенна активність.

СЕРОЛОГИЧЕСКАЯ И БИОЛОГИЧЕСКАЯ АКТИВНОСТЬ ЛИПОПОЛИСАХАРИДА *Escherichia coli* L-19

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Выделен липополисахарид *Escherichia coli* L -19, проведена его химическая идентификация, показано, что он является малотоксичным, но пирогенным. Изучение влияния исследуемого липополисахарида на Т- и В-лимфоциты дает основание предположить, что он может быть использован как митоген в реакциях бласттрансформации, поскольку по активности лишь в незначительной степени уступает коммерческому препарату. В реакциях двойной иммунодиффузии в агаре по методу Оухтерлони установлено, что липополисахарид *E. coli* L-19 в гомологичной системе проявляет активность антигена. Не выявлено перекрестных серологических реакций между антисывороткой к прогретым клеткам *E. coli* L-19 и липополисахаридом штамма *E. coli* M-17, а также представителями других видов *Enterobacteriaceae — Budvicia aquatica, Rahnella aquatilis, Pragia fontium*, что свидетельствует об отсутствии у них общих антигенных детерминант.

Ключевые слова: липополисахарид, *Escherichia coli*, токсичность, пирогенность, митогенная активность.