ISSN 0026-2617, Microbiology, 2012, Vol. 81, No. 3, pp. 324–331. © Pleiades Publishing, Ltd., 2012.
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EXPERIMENTAL ARTICLES

# Lipopolysaccharide of *Escherichia coli* M-17

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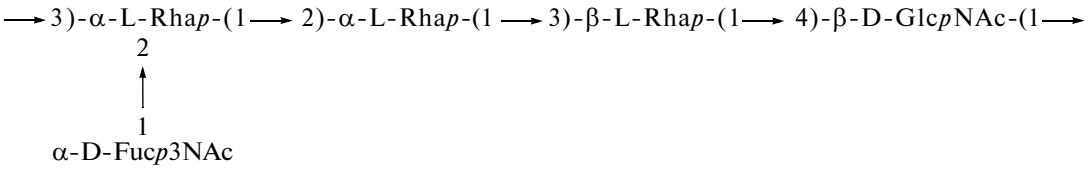
Received April 11, 2011

**Abstract**—The lipopolysaccharide (LPS) of *Escherichia coli* M-17 was isolated, studied, chemically identified, and shown to be an apyrogenic compound of low toxicity. Investigation of the effect of this LPS on T- and B-lymphocytes suggests that it can be used as a mitogen in blast transformation reactions, as it is only slightly less active than the commercial preparation. Double immunodiffusion in agar by Ouchterlony revealed that the LPS of *E. coli* M-17 in a homologous system exhibited an antigenic activity and did not interact with the antisera against representatives of other *Enterobacteriaceae* species (*Budvicia aquatica, Rahnella aquatilis*, and *Pragia fontium*) in serological cross-reactions. Mild acid hydrolysis yielded the following structural components of the lipopolysaccharide molecule: lipid A, core oligosaccharide, and O-specific polysaccharide. The structure of the O-specific polysaccharide determined using the data on the monosaccharide composition and the $^{1}H$ and $^{13}C$NMR spectra was found to be typical of representatives of the *E. coli* serogroup O2:




*Keywords: Escherichia coli*, lipopolysaccharide, biological activity, O-specific polysaccharide, structure.

**DOI:** 10.1134/S0026261712020154

Lipopolysaccharides (LPS) are specific components of the cell envelope of gram-negative bacteria, located at the external surface of their outer membrane and performing a number of important physicochemical and biological functions: they play a significant role in the maintenance of membrane integrity, regulate its permeability for various substances, and participate in the contacts of the microorganism with other micro- and macroorganisms. Due to the surface location and structural properties, LPS determine the O-antigenic specificity of bacteria [1].

An LPS molecule consists of three structural elements: an O-specific polysaccharide (OPS), a core oligosaccharide (OG-core), and a lipid A. Lipid A, the most conservative part of the LPS molecule, contains unusual fatty acids; their composition is rather stable within a species and, therefore, can be used as one of additional chemotaxonomic criteria. Lipid A is bound to the core oligosaccharide, a less conservative part of the LPS molecule, which contains specific monosaccharides such as 2-keto-3-deoxyoctonic acid (KDO) and heptoses [2].

The O-specific polysaccharide chains, the most variable part of LPS molecules varying in composition and structure in different bacterial strains, are responsible for the antigen specificity of bacterial cells. Since they are easily recognized by host antibodies, bacteria modify the OPS structure in order to avoid the response of the host immune system.

Since LPS are the endotoxins of a microbial cell, their presence in the organisms of higher animals and humans causes a wide range of endotoxic activities, which may result in septic shock. Due to these properties, LPS contribute to the pathogenic potential of gram-negative bacteria and are therefore among the factors of development of severe infectious diseases.

At the same time, LPS are known to be able to activate B- and T-lymphocytes, granulocytes, and mononuclear cells [3]; hence, they are considered as potential immunomodulators. A number of companies (e.g., Sigma, Serva) produce commercial LPS preparations, mainly *Escherichia coli* LPS; however, these preparations are rather expensive.

The goal of the present work was chemical characterization of the *E. coli* M-17 lipopolysaccharide and assessment of its biological activity.

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## MATERIALS AND METHODS

**Research object** was the strain *E. coli* M-17 (a component of the Bificol and Colibacterin probiotics). Bacteria were grown on meat infusion agar for 24 h at 28–30°C. The cells were harvested by centrifugation (20 min, 5000*g*) and dried with acetone and ether.

**LPS isolation.** LPS was extracted from dried cells with 45% aqueous phenol solution at 65–68°C. Aqueous fractions were dialyzed against tap and then against distilled water for phenol removal [4].

**Determination of the content of carbohydrates, nucleic acids and protein.** The amount of carbohydrates was determined by the method of Dubois [4]. The results were assessed spectrophotometrically (490 nm) by color change resulting from the reaction of phenol with sulfuric acid. The content of carbohydrates was determined in accordance with the standard calibration curves plotted for glucose.

The content of nucleic acids and proteins was assessed by the methods of Spirin [4] and Lowry with the Folin reagent [4], respectively.

**Identification of neutral monosaccharides** was carried out after hydrolysis of the preparations in 2 N HCl (5 h, 100°C). Monosaccharides were analyzed as alditol acetates [5] on an Agilent 6890/5973N chromatography–mass spectrometry system with a DB-225mS column (30 m × 0.25 mm × 0.25 μm), with helium as a carrier gas, and a flow through the column of 1 mL/min. The temperatures of the evaporator, interface and thermostat were 250, 280, and 220°C, respectively (isothermal mode). Monosaccharides were identified by comparing the retention time of alditol acetates in the experimental and standard samples and using the ChemStation database. The quantitative ratios of individual monosaccharides were expressed as percentage of the total peak area.

**Fatty acid composition** was determined upon sample hydrolysis in 1.5% acetyl chloride solution in methanol (100°C, 4 h); fatty acid methyl esters were analyzed on an Agilent 6890N/5973 inert chromatomass spectrometric system with a HP-5MS column (30 m × 0.25 mm × 0.25 μm), at the temperature mode of 150–250°C and temperature gradient, 4°C, with helium as the carrier gas at the flow rate of 1.2 mL/min). The evaporator temperature was 250°C; flow distribution was 1 : 100. Fatty acids were identified using the PC database and the standard mixture of fatty acid methyl esters.

**NMR spectroscopy.** NMR spectra were recorded for the OPS solution in 99.95 $D_2O$ at 30°C on a Bruker Avance II 600 spectrometer (Germany) using Ethyl-3-(trimethylsilyl)propionate-2,2,3,3-$d_4$ (δH 0 ppm) and acetone (δC 31.45 ppm) as internal standards. Before spectrum recording, the samples were lyophilized from 99.5% $D_2O$.

**LPS pyrogenicity** was determined in rabbits weighing 2.0–3.5 kg [4]. Thermometry was performed with an electronic thermometer (Omron Matsusaka Co. Ltd., Japan), which was inserted into the rectum to a depth of 5–7 cm (depending on the weight of a rabbit). All rabbits were pretested for immunoreactivity by intravenous injection of 10 mL/kg of 0.9% sterile apyrogenic sodium chloride solution. The tested LPS preparations were dissolved in a sterile apyrogenic isotonic solution, incubated for 10 min at 37°C before the injection, and introduced intravenously (1 mL/kg of animal weight). The minimal pyrogenic dose of LPS preparations was determined in a series of dilutions from 0.5 to $1.0 \times 10^{-2}$ mg/mL. Each series of the solutions was tested in 3 rabbits of similar weight (at a difference not exceeding 0.5 kg).

Prior to injection of the LPS solution, the temperature of the rabbits was measured twice with a 30-min interval. Since the difference between temperature indices should not exceed 0.2°C, the animals not satisfying this criterion were not used for investigation. The result of the last measurement was taken as the initial temperature. LPS solution was injected not later than 15–20 min after the last temperature measurement. After the injection, the measurements were made three times with 1-h intervals. The tested LPS solution was considered apyrogenic if the total temperature increase during 3 h did not exceed 1.4°C.

**LPS toxicity was determined** in healthy white nonpedigreed mice of both sexes weighing 19–21 g, not used previously for any experiments and sensibilized with galactosamine. For this purpose, 0.5 mL of 3.2% D-galactosamine hydrochloride solution in apyrogenic sterile 0.9% NaCl solution was injected intraperitoneally into all mice (both control and experimental ones). Immediately after that, 0.2 mL of LPS in isotonic sterile apyrogenic saline solution heated to 37°C was injected intraperitoneally at a rate of 0.1 mL/s. In the series of LPS dilutions, the preparation dose causing death of 50% tested animals ($LD_{50}$) was determined and its value was used for assessment of LPS toxicity. The control and experimental groups consisted of 10 mice each. The control mice were injected with 0.2 mL of sterile 0.9% NaCl solution. The animals were observed for 48 h [4].

**Immunological studies.** O-antiserum was obtained to heated *E. coli* cells (2.5 h, boiling water bath). The rabbits were immunized intravenously five times with 4-day intervals; cell concentration was $2 \times 10^9$/mL (0.1 to 1 mL).

The antigenic activity of LPS was studied by the method of double immunodiffusion in agar according to Ouchterlony [6].

The effect of LPS on different lymphocyte populations was assessed by the level of expression of $CD_3$ and $CD_{22}$ receptors on T and B lymphocytes by the reaction of rosette formation with the monoclonal antibodies to the above receptors. CD-diagnosticum for the antibodies was introduced into round-bottom wells of immunological multiwell plate by 0.25 mL (25 μL), supplemented by an equal volume of lym-

**Table 1.** Fatty acid composition of the LPS from *E. coli* M-17

| Acid | Amount (% of the total peak area) |
|---|---|
| $C_{12:0}$ | 14.5 |
| $C_{14:0}$ | 21.6 |
| 3-OH-$C_{14:0}$ | 46.6 |
| *i*-$C_{16:0}$ | 1.3 |
| $C_{16:1}$ | 2.0 |
| $C_{16:0}$ | 9.6 |
| *c*-$C_{18:1}$ | – |
| *t*-$C_{18:1}$ | 3.0 |
| $C_{18:0}$ | 1.4 |

Note: "–" not revealed.

phocyte mixture, incubated for 25 min at 37°C, centrifuged at 500–1000*g* for 3 min, and placed into a refrigerator for 1 h at 4°C. Lymphocyte precipitates treated with the monoclonal antibodies were dried, fixed with alcohol, and stained by Romanowsky–Giemza so that the nuclei of the lymphocytes were clearly visible.

For the reaction of blast transformation of human blood lymphocytes (blast transformation is a transition of small lymphocytes from a dormant state into blast forms capable of proliferation and further differentiation), LPS of the tested strains was used as a mitogen in the following dilutions: 1 mL of LPS per 10 mL, and 1 mg of LPS per 100 mL of sterile saline solution. The reaction was performed with 0.2 mL each of the LPS preparation, medium 199 [7], antibiotic preparation, bovine serum, and human blood; the mixture was incubated for 2 days at 37°C. After incubation, acetic acid (10% solution) was added for precipitation of lymphoblasts, which were separated by centrifugation. The pellet was separated from the supernatant, transferred onto slides, and stained by Romanowsky–Giemza; the cells were counted by direct microscopy [7].

**Statistic studies.** The experimental results were statistically processed by the method proposed for pharmaceutical preparations [7].

## RESULTS AND DISCUSSION

The studied LPS of *E. coli* isolated by the phenol–water method was characterized by a relatively high content of nucleic acids, which may be due to the method of isolation. LPS was purified from nucleic acids by ultracentrifugation (4 h, 105000*g*, 3 times). The purified LPS contained 52% carbohydrates and 7.5% nucleic acids; protein was not detected. The yield of LPS was 3.5%, being less than the average values typical of other representatives of *Enterobacteriaceae* (5%).

Analysis of the monosaccharide composition demonstrated that the predominant monosaccharides of the LPS from *E. coli* M-17 were rhamnose (45.4%), glucose (18.9%), and ribose (17.8%). The typical LPS components of gram-negative bacteria, heptose and KDO, were 7.9 and 0.25%, respectively.

Analysis of the fatty acid composition of the lipids A of the LPS (Table 1) showed the presence of fatty acids with 12 to 18 carbon atoms in their chains. Among hydroxy acids, only 3-hydroxy tetradecanoic acid was detected (46.2%); tetradecanoic (21.4%), dodecanoic (14.3%), and hexadecanoic (9.4%) acids were present as well.

The presence of only one hydroxy acid (3-hydroxy tetradecanoic acid, which acylates both amino and hydroxyl groups of glucosamine residues) is a characteristic feature of the whole family *Enterobacteriaceae*.

The generally known and best-studied role of LPS is its functioning as the main thermostable antigen of microbial cells. LPS introduction into the organisms of higher animals and humans results in production of complementary antibodies directed against specific structural regions in the LPS molecule is used for immunization [4]. The working titer of O-antisera to the heated culture of the tested strain *E. coli* M-17 determined by the ring precipitation reaction was 1 : 40000.

During the double immunodiffusion in agar by Ouchterlony, LPS of the tested *E. coli* strain in a homologous system exhibited an antigenic activity (Fig. 1). In cross-reactions, LPS of the tested strain did not interact with the antisera to representatives of other *Enterobacteriaceae* species: *Budvicia aquatica, Rahnella aquatilis*, and *Pragia fontium* (Fig. 2), which indicated the absence of common antigenic determinants.

Since O-specific polysaccharide is the molecular basis of serological affinity, its structure was investigated. O-specific polysaccharide was isolated by mild acid hydrolysis of the LPS.

The study of OPS monosaccharide composition showed the presence of rhamnose (Rha), N-acetylglucosamine (GlcNAc), and Fuc3NAc.

The $^{13}$NMR spectrum of the OPS (Fig. 3) contained the signals of five anomeric carbon atoms at 98.4–103.9 ppm, one hydroxymethyl group (C-6 GlcNAc) at 62.5 ppm, methyl groups at 18.0 ppm

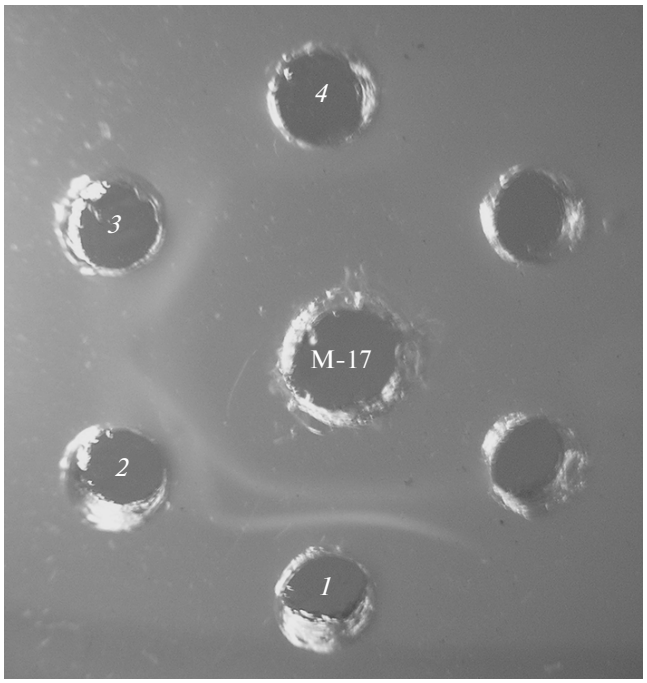


**Fig. 1.** Reaction of double immunodiffusion in agar by Ouchterlony between the LPS of *E. coli* M-17 and homologous O-antiserum. LPS dilutions: 1 : 100 (*1*), 1 : 5000 (*2*), 1 : 10000 (*3*), and 1 : 20000 (*4*).

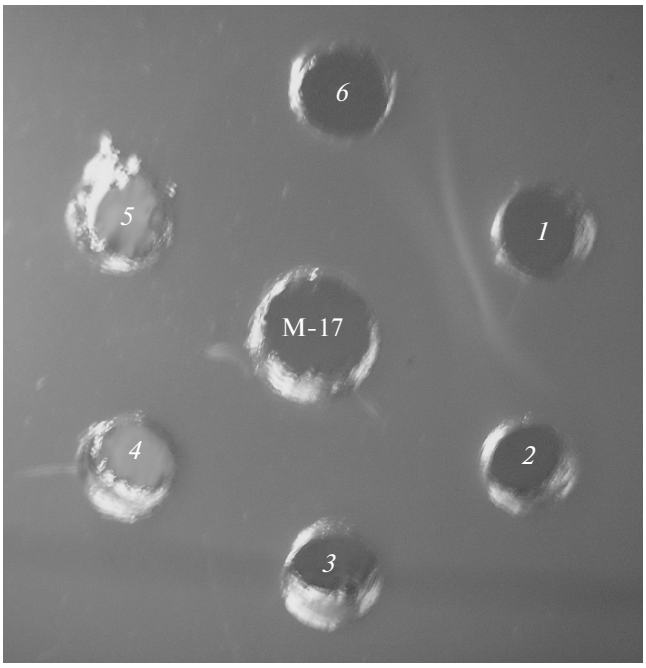


**Fig. 2.** Reaction of double immunodiffusion in agar by Ouchterlony between the LPS of *E. coli* M-17 (*1*), *E. coli* F-50 (*2*), *B. aquatica* 23270 (*3*), *B. aquatica* 124 (*4*), *P. fontium* 20125 (*5*), *R. aquitilis* 33071 (*6*), and O-antisera to *E. coli* M-17.

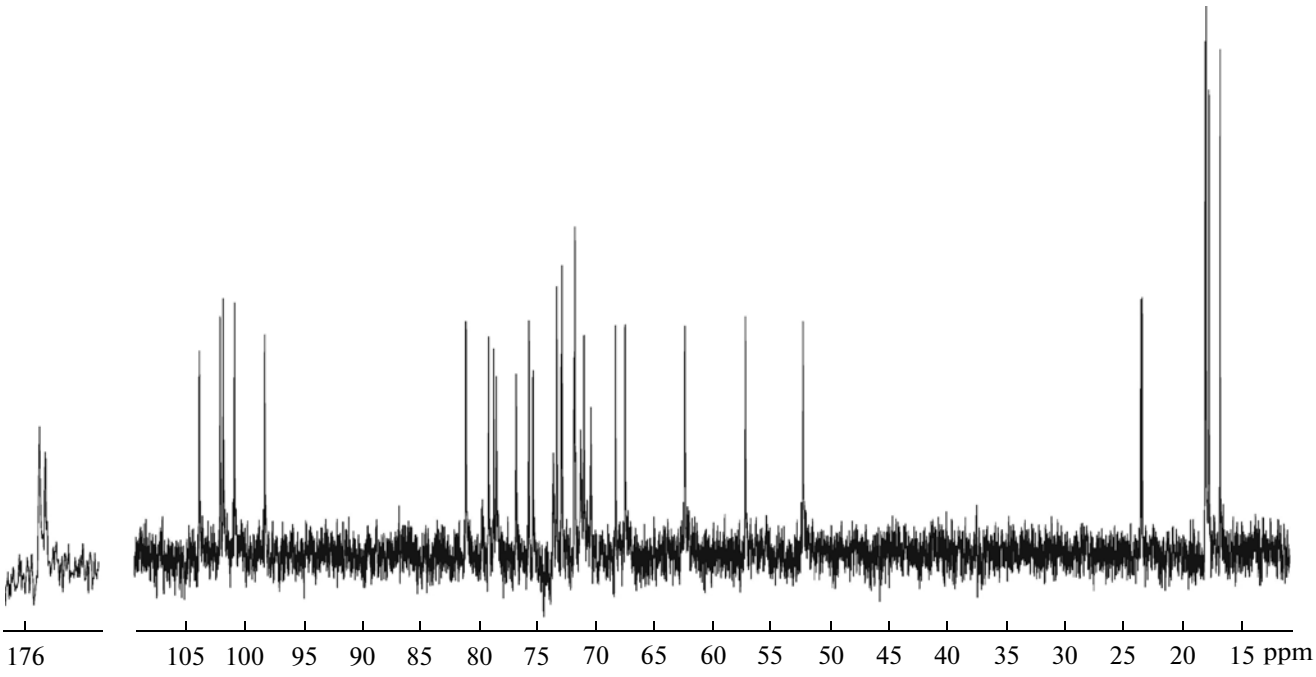


**Fig. 3.** The $^{13}$C-NMR spectrum of the OPS from *E. coli* M-17.

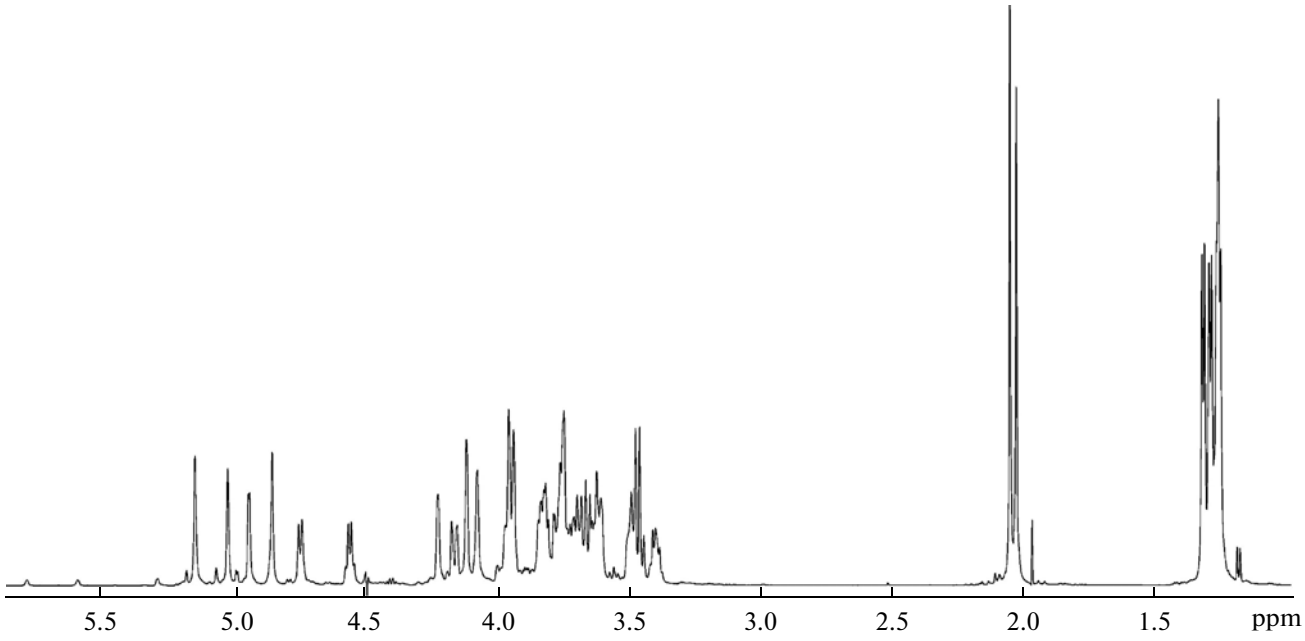


**Fig. 4.** The $^{1}$H-NMR spectrum of the OPS from *E. coli* M-17.

(C-6 Rha) and 16.8 ppm (C-6 Fuc3NAc), two nitrogen-bound carbon atoms (C-2 GlcNAc and C-3 Fuc3NAc) at 56.0 ppm and 52.5 ppm, and other carbon atoms of monosaccharide cycles at 67.5–81.3 ppm. The $^{1}$H NMR spectrum of OPS (Fig. 4) contained the signals of five anomeric protons 4.76–5.16 ppm, the protons of methyl groups (H-6 Rha and Fuc3NAc) at 1.26–1.32 ppm, and other protons of sugars at 3.41–4.23 ppm. The absence of signals in the region of 83–88 ppm in the $^{13}$C NMR spectrum suggested a conclusion that all monosaccharides were in the pyranose form [8].

Comparison of the monosaccharide composition and NMR spectra of the OPS with the data for O-specific polysaccharides of other tested *E. coli* strains showed that OPS had the following structure typical of representatives of the *E. coli* serogroup O2 [9]:

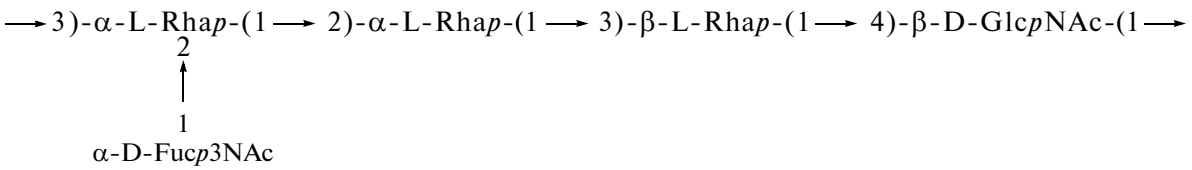


**Table 2.** Effect of the LPS from *E. coli* M-17 in different dilutions on the quantity (%) of blast-transformed lymphocytes

| Preparation | 1 : 10 | 1 : 100 |
|---|---|---|
| LPS of *E. coli* M-17 | 45.6 ± 2.6 | 56 ± 4.2 |
| Commercial LPS | 62.6 ± 2.0 | 63.8 ± 1.7 |

LPS are biological signal molecules able to activate the innate and acquired protective systems of the organism. It is known that T-lymphocytes play a key role in the regulation of practically all elements of immunity. In particular, they synthesize pro- and anti-inflammatory cytokines and regulate their synthesis by other cells. The ratio of these cytokines determines the direction of metabolic processes in the cells, their resistance to the action of apoptogenic factors, and many other physiological functions of the organism, which have a direct effect on the reparative processes in the tissues. B cells are a functional type of lymphocytes playing an important role in providing humoral specific immunity. The functional activity of T and B cells is assessed by the results of the reaction of lymphocyte blast transformation (RBTL). Introduction of

**Table 3.** Effect of the LPS from *E. coli* M-17 on the expression of $CD_3$ receptors on T lymphocytes and $C_{22}$ receptors on B lymphocytes

| Preparation | Indices of expression of $CD_3$ receptors | Indices of expression of $CD_{22}$ receptors |
|---|---|---|
| | LSP dilution 1 : 10 | |
| LPS of *E. coli* M-17 | 45.2 ± 0.8 | 24 ± 0.8 |
| Control (intact lymphocytes) | 50.6 ± 2.7 | 12 ± 0.5 |
| | LSP dilution 1 : 100 | |
| LPS of *E. coli* M-17 | 34.2 ± 1.3 | 10.7 ± 0.6 |
| Control (intact lymphocytes) | 38.6 ± 1.7 | 12 ± 0.5 |

mitogens or antigens into a culture of lymphocytes causes a series of morphological and biochemical changes in the cells. These include DNA synthesis and transformation of lymphocytes into blast forms (large dividing cells). RBTL determines the proliferative potential of the cells under study. Phytohemagglutinin, concanavalin A, bacterial lipopolysaccharides, enzymes, etc. are used as RBTL activators (mitogens).

The mitogenic activity of the LPS of the tested *E. coli* strain was studied in comparison with the commercial LPS preparation produced by the Diagnosticum Company at the Lviv Institute of Hematology and Blood Transfusion. The results showed (Table 2) that the commercial LPS preparation, irrespective of concentration, stimulated the formation of lymphoblasts to a somewhat higher extent. The tested LPS at a 1 : 100 dilution was more active than the LPS diluted 1 : 10.

The characteristic feature of B- and T-lymphocytes is the presence of a number of antigenic markers, in particular, $CD_{22}$ and $CD_3$ receptors, respectively. It is known that the $CD_3$ protein complex associated with an antigen-specific T cell receptor is a major functional marker of T lymphocytes. It promotes signal transduction from the cell membrane to the cytoplasm. Our studies have shown (Table 3) that the expression of $CD_3$ receptors on T lymphocytes decreased in the presence of the LPS from *E. coli* M-17 in 1 : 10 and 1 : 100 dilutions.

The B lymphocyte receptor $CD_{22}$ is a carbohydrate-binding transmembrane protein from the lectin family. It is a regulatory molecule, which prevents over-activation of the immune system and development of autoimmune processes. Analysis of the results presented in Table 3 demonstrates that in the 1 : 10 dilution of the LPS of the tested *E. coli* strain, a two-

**Table 4.** Determination of $LD_{50}$ for *E. coli* LPS

| LPS of the strain | $LD_{50}$ | |
|---|---|---|
| | μg/mouse | μg/kg |
| *E. coli* M-17 | 40.0 | 2000 |
| *E. coli* O55:B5 | 0.14 | 7.0 |

**Table 5.** Pyrogenic activity of *E. coli* LPS

| LPS of the strain | Pyrogenic dose of LPS, μg/rabbit | Total temperature rise (°C) after LPS injection during 3 h |
|---|---|---|
| *E. coli* M-17 | 0.021 | 1.97 |
| LPS of *Shigella typhi* (pyrogenal) | 0.022 | 1.99 |

fold enhancement of the expression of $CD_{22}$ receptors on B lymphocytes occurred. At the same time, the LPS from *E. coli* M-17 diluted 1 : 100 suppressed the expression of $CD_{22}$ receptors on B lymphocytes by more than 80%.

Thus, the expression of $CD_3$ and $CD_{22}$ receptors on T and B lymphocytes depended on the LPS dilution.

Although LPS, as is known [2], show an immunomodulating effect, their application as therapeutic preparations is prevented by their properties such as toxicity and pyrogenicity.

Our experiments with nonpedigreed mice showed that the LPS of *E. coli* M-17 was characterized by a much lower toxic effect than the LPS from *E. coli* O55:B5 (Table 4). Moreover, the toxicity of LPS of the tested strains was lower by an order of magnitude than the toxicity of the LPS of the new *Enterobacteriaceae* species: *R. aquatilis* [10] and *P. fontium* [11].

The study of pyrogenicity (Table 5, Fig. 5) showed that the LPS of *E. coli* M-17 lost its pyrogenic properties after 2 h of the experiment.

Thus, the LPS was isolated from *E. coli* M-17, chemically identified, and shown to be an apyrogenic compound of low toxicity. It is not surprising, since this strain is a component of the Bificol and Colibacterin probiotics. Investigation of the effect of the tested LPS on T and B lymphocytes suggests that it can be used as a mitogen in blast transformation reaction,

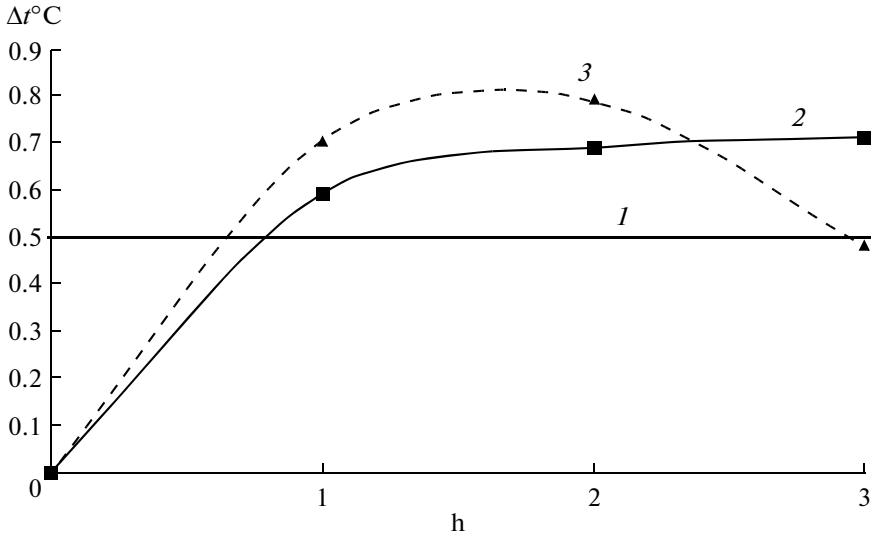


**Fig. 5.** Pyrogenic activity of the LPS from *E. coli* M-17: the time after LPS introduction, X-axis; the mean deviations of temperatures, Y-axis; pyrogenicity threshold (*1*), pyrogenal (*2*), LPS from *E. coli* M-17 (*3*).

because its activity is only insignificantly lower than that of the commercial preparation.

## ACKNOWLEDGMENTS

The authors are grateful to A.S. Shashkov for recording the NMR spectra of the polysaccharide and to Yu.A. Knirel for critical comments.

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