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Structural Diversity and Endotoxic Activity of the Lipopolysaccharide of *Yersinia pestis*

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Abstract—The endotoxic activity of the lipopolysaccharides (LPS) with defined chemical structure from *Yersinia pestis* strains of various subspecies differing in their epidemic potential was studied. The LPS of two strains of *Y. pestis* ssp. *caucasica* and ssp. *altaica*, whose structures have not been studied earlier, were analyzed by high-resolution mass spectrometry. In addition to reported structural changes, an increase in the degree of LPS phosphorylation was observed when strain I-2377 (ssp. *altaica*) was cultivated at an elevated temperature. A high tumor necrosis factor α (TNF-α)-inducing activity observed for LPS samples from *Y. pestis* cultures grown at 25°C correlated with an increased degree of lipid A acylation, particularly, with the presence of the hexaacyl form of lipid A, which was absent from the LPS when bacteria were cultivated at 37°C. No correlation was found between the lethal toxicity of the LPS *in vivo* and its ability to induce TNF-α production *in vitro*.

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Key words: lipopolysaccharide, lipid A, endotoxin, tumor necrosis factor, cytokine-inducing activity, *Yersinia pestis*

Yersinia pestis, the causative agent of plague, is a Gram-negative bacterium from the *Enterobacteriaceae* family. Currently, *Y. pestis* is circulating in populations of more than 200 species of wild rodents and lagomorphs inhabiting natural plague foci on all continents save Australia and Antarctica, whereas more than 80 flea species serve as vectors. Human infections are reported for epizootic exacerbations and are caused by bites of infected flea, direct contact with infected animals' tissues, consumption of insufficiently heat-treated meat, or inhaling aerosol respiratory excretions of animals with pneumonic form of infection [1-6]. Three major clinical types of plague—bubonic, septic and pneumonic—differ in epidemiological characteristic, disease pattern, and autopsy changes [5, 6], but in all cases pathogenesis includes development of the endotoxic shock syndrome. The pathohistological changes in the internals typical of guinea pigs taken off by plague are reproduced upon introduction of the *Y. pestis* lipopolysaccharide (LPS) preparations into animals [7].

In natural plague foci in the former Soviet Union, *Y. pestis* strains differ in the main host, epidemic potential, and virulence for various animal species [1, 3]. Recently, we have determined LPS structures in various *Y. pestis* subspecies grown at different temperatures [8-10] and revealed a correlation between the LPS structure and the susceptibility of *Y. pestis* strains to a cationic peptide polymyxin B and normal human serum [11]. However, it remains unclear whether structural variations in the LPS isolated from various *Y. pestis* strains affect their endotoxic activity. In this work, we studied the cytokine-inducing activity and lethal toxicity of LPS from *Y. pestis* strains representing various subspecies and analyzed correlation

Abbreviations: Ara4N) 4-amino-4-deoxyarabinose; DD-Hep, LD-Hep) D-*glycero*-, L-*glycero*-D-*manno*-heptose; EtN*P*) phosphoethanolamine; LD_{50}) dose causing 50% death of animals; LPS) lipopolysaccharides; Kdo) 3-deoxy-D-*manno*-oct-2-ulosonic acid; Ko) D-*glycero*-D-*talo*-oct-2-ulosonic acid; TNF-α) tumor necrosis factor α; 3HO14:0) 3-hydroxymyristoyl; 12:0) lauroyl; 16:0) palmitoyl; 16:1) palmitoleoyl.

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between the biological activity and the chemical structure of the LPS. The LPS structures in the majority of strains have been reported by us earlier [8]; for two *Y. pestis* strains that were not studied previously, structural analysis was performed in this work.

MATERIALS AND METHODS

Bacterial strains. *Yersinia pestis* strain KIMD1 (bv. *medievalis* ssp. *pestis*) was kindly provided by Dr. M. Skurnik (University of Helsinki, Helsinki, Finland). Other *Y. pestis* strains including KM218 (bv. *orientalis* ssp. *pestis*), EV11M (bv. *antiqua* ssp. *pestis*), KM260(11) (bv. *antiqua* ssp. *pestis*), 1146 (bv. *antiqua* ssp. *caucasica*), 1680p$^-$ (bv. *antiqua* ssp. *caucasica*), and I-2377 (bv. *medievalis* ssp. *altaica*) came from the Russian Research Anti-Plague Institute "Microbe" (Saratov, Russia). The biovar-subspecies relationships and characteristics of each subspecies have been surveyed [3].

Bacterial growth. For LPS isolation, *Y. pestis* strains were grown at 25 or 37°C in a New Brunswick Scientific 10-liter fermentor in a liquid aerated medium consisting of fish-flour hydrolyzate (20-30 g/liter), yeast autolyzate (10 g/liter), glucose (3-9 g/liter), K_2HPO_4 (6 g/liter), KH_2PO_4 (3 g/liter), and $MgSO_4$ (0.5 g/liter) at pH 6.9-7.1. After 48-h incubation, biomass was harvested by centrifugation, cooled to –70°C, and lyophilized.

LPS isolation. LPS preparations were extracted from lyophilized cells of *Y. pestis* strains KM218, KM260(11), KIMD1, 1146, 1680p$^-$, I-2377, and EV11M grown at 25 and 37°C (hereupon referred to as KM218-25, KM218-37, KM260(11)-25, KM260(11)-37, etc.) by a phenol–chloroform–light petroleum mixture [12] and purified by enzymatic digestion with nucleases and protease and repeated ultracentrifugation (105,000*g*, 4 h). The content of proteins in the LPS preparations was estimated by gel electrophoresis and that of nucleic acids as described [8]. The endotoxically inactive LPS of vaccine strain *Francisella tularensis* 15/10 isolated as described [13] was used as negative control for cytokine induction *in vitro*. *Escherichia coli* O55:B5 LPS (Sigma, USA) was used as positive control for cytokine induction *in vitro*.

Mass spectrometry of LPS. Electrospray ionization ion-cyclotron resonance Fourier transform mass spectrometry (ESI FTMS) of oligosaccharides was performed in the negative ion mode using an Apex II Instrument (Bruker Daltonics, USA) equipped with a 7 Tesla magnet and an Apollo ion source. Samples (~10 ng/μl) were dissolved in a 50 : 50 : 0.001 (v/v) 2-propanol–water–triethylamine mixture and sprayed at a flow rate of 2 μl/min. Capillary entrance voltage was set to 3.8 kV and drying gas temperature to 150°C. Mass scale calibration of the broadband spectra was performed externally with a similar compound of known structure. The spectra were charge deconvoluted and mass numbers given refer to monoisotopic molecular masses.

Laboratory animals. For experiments, 8-10-week-old female outbred white mice (Swiss Webster specific pathogen free (SPF) category, weight 19 ± 2 g) were used. All experiments were approved by the State Research Center for Applied Microbiology and Biotechnology Bioethics Committee (protocol No. P01-12/19.01.2001).

Cell cultures. Mouse macrophage-like cells line J774.1A were used as tumor necrosis factor α (TNF-α) producing cells [14]. The biological activity of TNF-α was tested by the cytotoxicity assay against actinomycin D-sensitized mouse fibroblast L929 cell lines [15]. Both cells lines came from the Russian Collection of Cell Cultures (Institute of Cytology, Russian Academy of Sciences, St. Petersburg).

***In vitro* TNF-α induction.** LPS preparations from *Y. pestis* strains grown at 25 and 37°C were added to a monolayer of J774A.1 cells at doses 3 to 300 ng/ml. After 24 h, the cell supernatant was collected and stored at –80°C for later usage in the TNF-α activity assay.

Determination of TNF-α activity in the supernatant of J774.A1 cells. The cytotoxic activity of TNF-α in the supernatant of stimulated J774.A1 cells was measured as described [16]. TNF-α activity units were calculated using the standard curve obtained with a recombinant mouse TNF-α preparation (Sigma).

Determination of endotoxic activity *in vivo*. Lethal toxicity of *Y. pestis* and *F. tularensis* LPS was determined by titration using actinomycin D-sensitized mice [17]. Each group consisted of eight animals. A 10-μg sample of actinomycin D (AppliChem GmbH, Germany) in 200 μl of apyrogenic 0.9% NaCl solution was introduced intraperitoneally together with 10-fold dilutions of LPS preparations (0.1 to 1000 μg/ml). Mortality rate was counted during the first seven days. All animals treated with actinomycin D without LPS survived. LD_{50} values and 95% confidence intervals were calculated by the modified Kärber method [18].

RESULTS

Structure and temperature-dependent variations of *Y. pestis* LPS. Structures of the LPS of *Y. pestis* strains KM218, KM260(11), KIMD1, 1146, and EV11M were determined earlier by chemical methods, high-resolution mass spectrometry, and NMR spectroscopy [8, 9]. Those studies resulted in elucidation of structures of the lipid A and core oligosaccharide, confirmed the lack of any O-antigen from the LPS, and revealed intraspecies differences in the LPS structures of various *Y. pestis* strains. The temperature-dependent variations were the most peculiar structural features of the *Y. pestis* LPS summarized in review [10]. For instance, strains KM218, KM260(11), and KIMD1 from the main subspecies (ssp.

pestis) grown at 37°C produced only one core glycoform 1 (Fig. 1) and a tetraacyl form of lipid A with four primary 3HO14:0 acid residues and a relatively low content of the cationic monosaccharide 4-amino-4-deoxyarabinose (Ara4N) at the phosphate groups. At 25°C, four core glycoforms (1-4) (Fig. 1) were synthesized, which differ in two terminal monosaccharide residues—ketodeoxyoctonic acid (Kdo) and D-*glycero*-D-*manno*-heptose (DD-Hep), that are typical of the high-temperature glycoform, are partially replaced with ketooctonic acid (Ko) and galactose. The low-temperature glycoforms are combined with a higher acylated lipid A (up to a hexaacyl form with two secondary 12:0 and 16:1 acid residues) containing up to two Ara4N residues (Table 1). Strain 1146 (ssp. *caucasica*) is unable to introduce DD-Hep residue into the core oligosaccharide and is distinguished by a low content of another terminal monosaccharide, GlcNAc (in the LPS of the main subspecies, this monosaccharide is present also in a non-stoichiometric but a much higher quantity) (Table 1). In addition, independently of the growth temperatures, lipid A of strain 1146 is characterized by a high content of Ara4N. *Yersinia pestis* ssp. *pestis* EV11M is a deep mutant strain with a core oligosaccharide limited to Kdo→Kdo and Ko→Kdo disaccharides. Its lipid A is represented by the same tetraacyl form as the parent strain and a pentaacyl form with an additional secondary 16:0 acid residue.

Our studies on *Y. pestis* LPS showed that high-resolution electrospray ionization mass spectrometry of the intact highly purified LPS is a powerful tool for gaining structural information on both core oligosaccharide and lipid A. In this work we have used the same approach to elucidate the LPS structure in two *Y. pestis* strains that were not studied earlier, namely 1680p$^-$ (ssp. *caucasica*) and I-2377 (ssp. *altaica*).

The most intense peak in the mass spectra of the LPS of strains 1680p$^-$ and I-2377 grown at 25°C (Fig. 2, a and b) belonged to glycoform 4 with terminal residues of

a

$$\begin{array}{l} \text{Sug}^2\text{-}(1\rightarrow7)\text{-L-}\alpha\text{-D-Hepp-}(1\rightarrow7)\text{┐}\quad \beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{┐}\quad \text{Sug}^1\text{-}(1\rightarrow4)\text{┐} \\ \textit{β-D-GlcpNAc}\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow5)\text{-}\alpha\text{-Kdo}p\text{-}(2\rightarrow \end{array}$$

1 $\text{Sug}^1 = \alpha\text{-Kdo}p$, $\text{Sug}^2 = \text{D-}\alpha\text{-D-Hep}p$
2 $\text{Sug}^1 = \alpha\text{-Kdo}p$, $\text{Sug}^2 = \beta\text{-D-Gal}p$
3 $\text{Sug}^1 = \alpha\text{-Ko}p$, $\text{Sug}^2 = \text{D-}\alpha\text{-D-Hep}p$
4 $\text{Sug}^1 = \alpha\text{-Ko}p$, $\text{Sug}^2 = \beta\text{-D-Gal}p$

$$\begin{array}{l} \text{L-}\alpha\text{-D-Hepp-}(1\rightarrow7)\text{┐}\quad \beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{┐}\quad \alpha\text{-Kdo}p\text{-}(1\rightarrow4)\text{┐} \\ \textit{β-D-GlcpNAc}\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow5)\text{-}\alpha\text{-Kdo}p\text{-}(2\rightarrow \end{array}$$

5

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \text{EtN}P\text{-}7\text{┐} \\ \beta\text{-D-Gal}p\text{-}(1\rightarrow7)\text{-L-}\alpha\text{-D-Hepp-}(1\rightarrow7)\text{┐}\quad \beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{┐}\quad \alpha\text{-Ko}p\text{-}(1\rightarrow4)\text{┐} \\ \textit{β-D-GlcpNAc}\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow3)\text{-L-}\alpha\text{-D-Hep}p\text{-}(1\rightarrow5)\text{-}\alpha\text{-Kdo}p\text{-}(2\rightarrow \end{array}$$

6

b

$$\begin{array}{l} \qquad\qquad\qquad\quad \text{R-3┐}\qquad\qquad\qquad \text{R-3┐} \\ \qquad\qquad\quad \rightarrow\!6)\text{-}\beta\text{-D-Glc}p\text{NR-}(1\rightarrow6)\text{-}\alpha\text{-D-Glc}p\text{NR-1-}P\text{-1-}\beta\text{-L-Ara}p\text{4N} \\ \beta\text{-L-Ara}p\text{4N-1-}P\text{-4}\text{┘} \end{array}$$

R = 3HO14:0

Fig. 1. Structural variants of the core 1-6 (a) and the tetraacyl form of lipid A with two Ara4N residues (b) of *Y. pestis* LPS. The GlcNAc residue shown in italics is present in a non-stoichiometric amount.

Table 1. Relative content (%) of the variable LPS components in various *Y. pestis* strains cultivated at 25 and 37°C (estimated based on the relative intensities of the molecular ion peaks in the mass spectra)

Variable LPS forms and components	ssp. *pestis*						ssp. *caucasica*				ssp. *altaica*	
	KM218		KIMD1		KM260(11)		1146		1680p⁻		I-2377	
	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C	25°C	37°C
Acyl variants of lipid A												
Tetraacyl form (4 × 3HO14:0)	70	100	85	81	95	100	52	93	88	n.d.	56	100
Pentaacyl form (tetraacyl form + C12:0)	15	trace	11	19	5	0	42	7	9	n.d.	35	0
Hexaacyl form (tetraacyl form + 12:0 + 16:1)	15	0	4	0	trace	0	6	0	3	n.d.	9	0
Variants with different content of Ara4N												
Two residues of Ara4N	92	27	72	16	76	43	93	94	91	n.d.	62	29
One residue of Ara4N	8	35	28	45	24	45	7	6	9	n.d.	38	47
No residues of Ara4N	0	38	0	39	0	12	0	0	0	n.d.	trace	24
Terminal core monosaccharides and glycine												
Kdo (glycoforms 1 and 2)	57	95	47	94	37	86	12	100	16	n.d.	38	100
Ko (glycoforms 3 and 4)	43	5	53	6	63	14	88	0	84	n.d.	62	0
Hep (glycoforms 1 and 3)	58	100	60	100	54	92	0	0	0	n.d.	0	0
Gal (glycoforms 2 and 4)	42	0	40	0	46	8	100	42	100	n.d.	100	10
GlcNAc	75	93	61	91	60	90	13	47	81	n.d.	73	83
Gly	20	10	n.d.	n.d.	n.d.	n.d.	38	28	19	n.d.	18	7

Note: n.d., not determined.

galactose and Ko and tetraacyl lipid A with two residues of the Ara4N (experimental molecular mass 3226.458 daltons; calculated molecular mass 3226.444 daltons). The spectra contained also a peak for minor glycoform 2 containing a Kdo residue instead of the Ko residue, whereas no peaks for glycoforms 1 and 3 with a terminal DD-Hep residue were observed. This pattern suggests that the LPS of both strains belong to the *Y. pestis* 1146 LPS structural type. Like the LPS of *Y. pestis* 1146-25, those of 1680p⁻-25 and I-2377-25 contain glycine (its position in the core oligosaccharide remains unknown), but the content of GlcNAc in them was substantially higher (Table 1). The main difference between the two LPS studied is a higher content of Kdo-containing glycoform 2 and a lower content of Ara4N in lipid A of strain I-2377-25 (however, even in this strain the molecules with two Ara4N residues were predominant). In addition, the LPS of I-2377-25 is distinguished by the presence of molecules (~13%) containing phosphoethanolamine (EtN*P*) (Fig. 2b), which has previously been reported only for the LPS core of strains KM218 [9] and 260(11) (authors' unpublished data) grown at 6°C. Following the data on the LPS of KM218-6 [9], structure 6 (Fig. 1) was proposed for this minor structural variant in the LPS of I-2377-25 too. Finally, the acylation pattern of I-2377-25 lipid A is typical of the low-temperature *Y. pestis* LPS (the predominant tetraacyl form with a rather high content of the pentaacyl form and the presence of the hexaacyl form), whereas lipid A of 1680p⁻-25 is represented mainly by the tetraacyl form with only a minor amount of the pentaacyl form (Table 1).

Variations in the LPS structure of strain I-2377 (ssp. *altaica*) observed when bacterial cells were grown at 37°C resembled those of both strain 1146 (ssp. *caucasica*) and

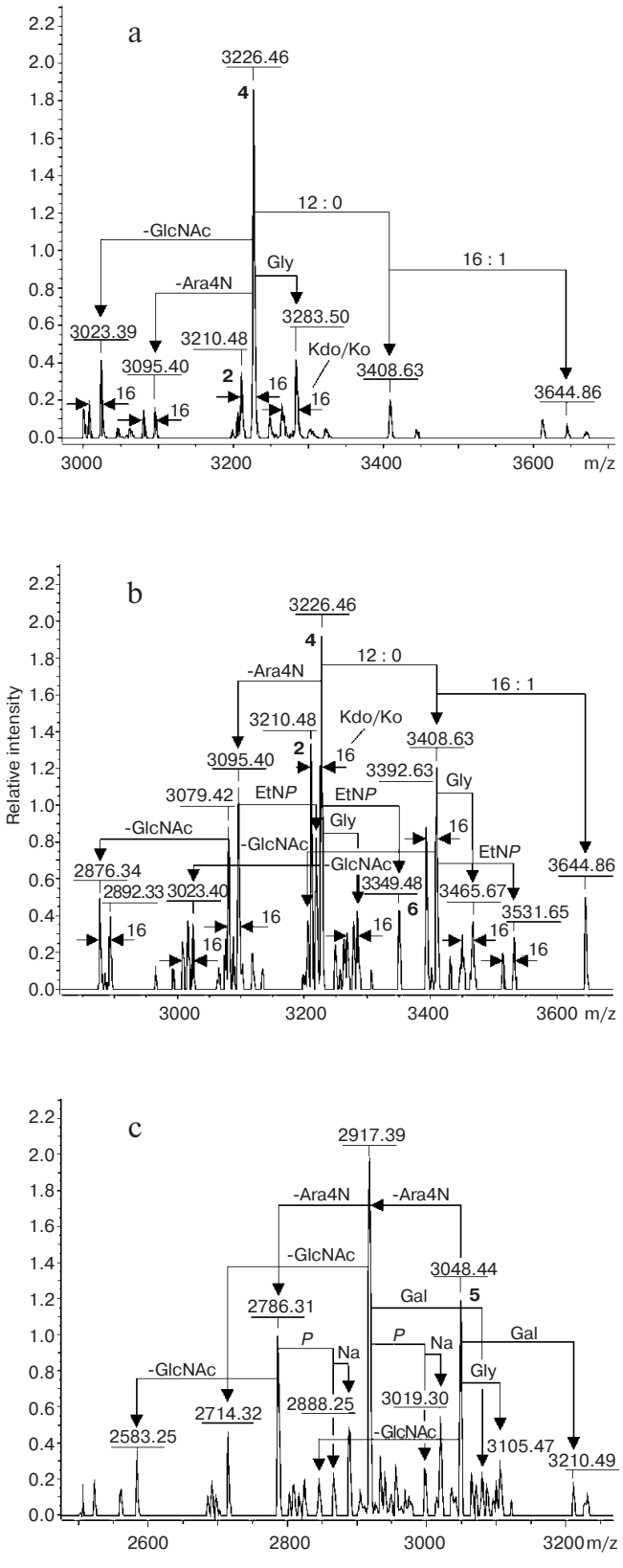

Fig. 2. Electrospray ionization mass spectra of the LPS of *Y. pestis* 1680p⁻ (a) and I-2377 (b, c) cultivated at 25°C (a, b) or 37°C (c).

strains of the main subspecies (ssp. *pestis*) (Fig. 2c and Table 1). Changes of the former type include a decrease in the content of galactose in the core from 100 to 42% for strain 1146 and to only 10% for strain I-2377-37. As the increase of the cultivation temperature caused also a replacement of Ko with Kdo, the core of I-2377-37 is mainly represented by glycoform 5 lacking one of the terminal monosaccharide residues (Fig. 1). As in the other *Y. pestis* "wild"-type strains studied, the increase of growth temperature resulted in a decrease of the degree of acylation in lipid A, which is present exclusively as the tetraacyl form in strain I-2377-37. At the same time, a marked decrease of the content of Ara4N in lipid A observed in strain I-2377-37 is typical of the main subspecies rather than of strain 1146 (Table 1). Therefore, the inability of strains 1146 and I-2377 to introduce DD-Hep into the core does not correlate with the ability to control by temperature the content of the Ara4N in lipid A, which is lost in 1146 but preserved in I-2377. The LPS of I-2377 contains no phosphoethanolamine, but some molecules (~25%) include additional phosphate and sodium phosphate groups. Lipid A of this higher phosphorylated structural variant contains no more than one Ara4N residue (Fig. 2c), which enables a suggestion that one of the Ara4N residues is replaced with the phosphate group to give a pyrophosphate group. Therefore, the elevation of the cultivation temperature of *Y. pestis* caused not only a decrease of the cell surface positive charge by a diminishing of the content of the cationic sugar Ara4N but also an increase of the negative charge by extensive phosphorylation.

The data obtained summarized in Table 1 expand the knowledge on the structural diversity and the character of temperature-dependent variations in the LPS of various subspecies of *Y. pestis*.

TNF-α secretion by LPS-stimulated mouse macrophage-like cells. Recent studies demonstrated the importance of pro-inflammatory cytokines, especially TNF-α, the main mediator of septic shock, in pathogenesis of infections caused by Gram-negative bacteria [19]. We studied the TNF-α-inducing activity of the LPS of *Y. pestis* using the mouse macrophages-like line J774A.1. The induction by LPS of the TNF-α production into the medium of J774A.1 cells was dose-dependent. The threshold LPS concentration, stimulating a detectable amount of the cytokine, was 3 ng/ml and the maximum induction was observed at 300 ng/ml.

Temperature-dependent changes in the TNF-α production were most pronounced for LPS concentrations between 10 and 300 ng/ml. The LPS preparations from the "wild"-type *Y. pestis* strains grown at 25°C showed a higher TNF-α-inducing activity than those from the high-temperature (37°C) cultures ($P < 0.05$) (Fig. 3). No correlation was observed between the growth temperature and TNF-α-inducing activity of the LPS from mutant strain *Y. pestis* ssp. *pestis* EV11M ($P > 0.05$).

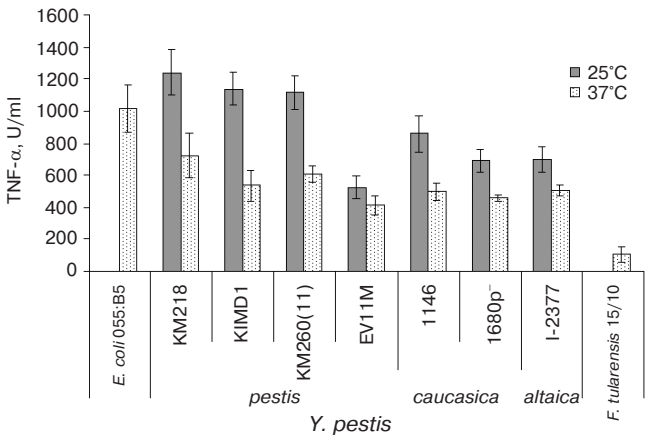

Fig. 3. Levels of TNF-α in the supernatant of mouse macrophages J774A.1 after stimulation by LPS preparations (100 ng/ml).

The cytokine-inducing activity of the low-temperature LPS preparations from three strains of *Y. pestis* ssp. *pestis* was comparable with that from *E. coli* O55:B5 ($P > 0.05$) and exceeded the activity of the LPS preparations from *Y. pestis* ssp. *caucasica* and ssp. *altaica* ($P < 0.05$) and mutant strain EV11M ($P < 0.05$) (Fig. 3). The TNF-α-inducing activity of all high-temperature LPS preparations was nearly identical ($P > 0.05$). The LPS of *F. tularensis* showed the lowest cytokine-inducing activity ($P < 0.05$).

LD_{50} level determination. Actinomycin D-sensitized mice are sensitive to toxic effect of the LPS [17]. We used this model to compare the toxicity of LPS from various *Y. pestis* strains. The LPS of the vaccine strain *F. tularensis* 15/10 was used as negative control as it showed no lethal toxicity for sensitized mice even at doses of up to 10 μg/mouse [13]. The LD_{50} values of the LPS preparations from all "wild"-type strains grown at 25°C were lower than those at 37°C (Table 2). The average LD_{50} values for strains KIMD1 and 1680p⁻ decreased nearly 60 and 26 times, respectively, for strain 1146 nearly 16 times, for strains KM218 and KM260(11) nearly six times, and for strain I-2377 nearly five times. However, taking into account the confidence intervals, the differences were reliable only for strains KIMD1 and 1680p⁻. The toxicity of LPS preparations from mutant strain EV11M-25 and EV11M-37 was nearly identical.

Table 2. LPS toxicity for actinomycin D-sensitized mice

LPS from strain	Growth temperature, °C	LD_{50}, μg*
Y. pestis KM218	25	0.79 (0.20-3.16)
	37	5.01 (1.26-19.95)
Y. pestis KIMD1	25	0.13 (0.03-0.50)
	37	7.94 (1.99-31.62)
Y. pestis KM260(11)	25	1.99 (0.50-7.90)
	37	12.59 (3.10-50.12)
Y. pestis 1146	25	0.079 (0.02-0.30)
	37	1.26 (0.30-5.00)
Y. pestis 1680p⁻	25	0.05 (0.01-0.21)
	37	1.30 (0.33-5.16)
Y. pestis I-2377	25	0.36 (0.09-1.42)
	37	1.79 (0.45-7.12)
Y. pestis EV11M	25	0.32 (0.08-1.25)
	37	0.50 (0.12-1.99)
F. tularensis 15/10	37	> 1000.00

* 95% confidence interval is given in parentheses.

DISCUSSION

Lipopolysaccharide is the main component of the outer layer of the outer membrane of Gram-negative bacteria and is capable of triggering the protective systemic pro-inflammatory response in macroorganisms. An excessive response to LPS may be harmful for the host organism owing to development of endotoxic shock. Studies of the influence of the chemical structure on the endotoxic activity of the LPS have shown that the degree of the monocyte/macrophages activation depends on structural features of lipid A [20-22]. Although *Y. pestis* belongs to the *Enterobacteriaceae* family, the chemical structure of its LPS differs from that of the other enterobacteria in the absence of any O-antigen [8, 10]. Another peculiar feature of *Y. pestis* is growth temperature-dependent variations in lipid A and core oligosaccharide structures [8-10, 23, 24].

Production by macrophages of several key cytokines plays a critical role in regulation of inflammation and in outcome of infections caused by yersiniae [25-27]. TNF-α has been recognized as the main mediator of septic shock induced by the Gram-negative microorganisms [19]. Therefore, the ability of various LPS preparations to induce the TNF-α production by mouse macrophage cell lines was used as a criterion of their *in vitro* inflammatory potential. The data obtained revealed a high TNF-α-inducing activity of the LPS from *Y. pestis* ssp. *pestis* strains KM218, KM260(11), and KIMD1 grown at 25°C. The increase of the activity correlates with a higher degree of acylation in the low-temperature LPS preparations, in particular, with the presence of the hexaacyl form of lipid A, which is absent from the high-temperature preparations [8, 10]. These findings are in agreement with published data [23, 24] obtained in mouse and human macrophage lines.

The cytokine-inducing ability of the LPS of *Y. pestis* ssp. *caucasica* 1146 and 1680p⁻ as well as *Y. pestis* ssp. *altaica* I-2377 was definitely lower than that of strains of the main subspecies. The difference in the levels of TNF-

α production between the low- and high-temperature LPS preparations was preserved, which is in accord with the chemical structure of their lipid A, namely the presence of tetraacyl and higher-acylated forms in the former and only the tetraacyl form in the latter. It could be suggested that the difference in the LPS core structure between the main and other subspecies, in particular, the lack of the terminal DD-Hep residue from the latter, results in different lipid A conformations, which ultimately predetermines the endotoxic activity of the LPS [21].

The deep R-mutant *Y. pestis* EV11M is characterized not only by the absence of the substantial part of the core but also by changes in the composition and content of fatty acids in lipid A as well as by the lack of temperature-dependent variations in the LPS structure [8]. The latter feature is in agreement with the same TNF-α-inducing activity of the LPS preparations from EV11M-25 and EV11M-37.

According to published data, sublethal quantities of some metabolic inhibitors, such as actinomycin D [17], can increase the sensitivity of mice to the toxic effect of the LPS more than 1000-fold. The actinomycin D-sensitized outbred Swiss Webster white mice were used to determine the difference in the toxicity between low- and high-temperature LPS preparations from various biovars and subspecies of *Y. pestis*. The general trend was a higher lethal toxicity of the low-temperature LPS preparations, though the experimental differences were reliable only for strains *Y. pestis* KIMD1 and 1680p$^-$.

There is no clear correlation between the *in vitro* induction of TNF-α by the LPS in the mouse macrophage-like cell line J774A.1 and the *in vivo* lethal toxicity of the LPS. The LPS preparations from two strains of *Y. pestis* ssp. *pestis* showing the highest TNF-α-inducing activity (KIMD1-25 and KM260(11)-25) substantially differed in the LD_{50} levels. Moreover, the *in vitro* TNF-α-inducing activity of the LPS of deep mutant EV11M was comparable to that of the non-toxic LPS of *F. tularensis*, while its lethal toxicity for mice was nearly identical to that of *Y. pestis* "wild"-type LPS, which demonstrated the highest TNF-α-inducing activity *in vitro*. These observations are in accordance with other findings [28, 29] for LPS preparations from other Gram-negative bacteria. Therefore, although the usage of isolated animal cells cultivated *in vitro* in a nutrient medium as a model of such complex processes as septic shock allows studies of fine mechanisms of interactions between endotoxins and isolated eukaryotic cells, it is a significant oversimplification of the process as a whole. Therefore, a comparison of results from *in vivo* experiments with data of isolated eukaryotic cells requires a serious critical appraisal.

As compared to published data, a relatively low decrease of the lethal toxicity of the high-temperature LPS preparations was observed on the actinomycin D-sensitized mouse model. Most likely, this is accounted for by a different content of the hexaacyl form of lipid A, which may result from differences in the nutrient medium used for cultivation of *Y. pestis* cells. Thus, the highest toxicity hexaacyl form was predominant in cells cultivated at 21°C in liquid Luria–Bertani medium [24] and at 27°C on brain-heart infusion agar [23]. At the same time, in our LPS preparations isolated from cell cultures grown at 25°C in liquid fish flour hydrolyzate medium, the content of the hexaacyl lipid A form did not exceed 15% (Table 1).

A relatively high toxicity of the high-temperature LPS forms may be beneficial for the microbe since it allows a permanent circulation of the infection in natural plague foci due to the quick death of warm-blooded hosts, which forces fleas to look for new hosts for themselves and *Y. pestis*. On the other hand, the ability of *Y. pestis* to produce enough LPS to cause death of the non-sensitized animal hosts appears inconceivable. The sensitization of the host organism may occur through the presence of a number of bacterial factors playing a role on different stages of the disease. Thus, the murine toxin that, like the LPS, is released upon death and lysis of *Y. pestis* cells [6], is lethal for mice and rats but is harmless for guinea pigs and apes, whereas the combined introduction of non-lethal doses of the murine toxin and LPS causes death of both mice and guinea pigs [30].

The main outcome of our investigation is the finding of a correlation between the temperature-dependent changes in the LPS structures of various *Y. pestis* subspecies and the toxicity of the corresponding LPS preparations. At the same time, the specific biological importance of the changes in the LPS structure remains unclear as the laboratory cultivation of bacteria cannot fully reproduce the *in vivo* conditions. Additional studies are necessary to identify the LPS structures synthesized by the bacteria in the flea and mammal hosts. This would allow selection of the *in vitro* conditions that would secure the production of the LPS forms typical of the particular animal for further studies of their biological properties.

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