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Highly homogenous tri-acylated S-LPS acts as a novel clinically applicable vaccine against *Shigella flexneri* 2a infection

Vladimir A. Ledov [a], Marina E. Golovina [a], Anna A. Markina [a], Yuriy A. Knirel [b], Vyacheslav L. L'vov [c], Alexander L. Kovalchuk [d], Petr G. Aparin [a,*]

[a] *Laboratory of Carbohydrate Vaccines, National Research Center-Institute of Immunology, Federal Medical Biological Agency of Russia, 24, Kashirskoe Shosse, Moscow 115478, Russia*
[b] *N. D. Zelinsky Institute of Organic Chemistry, Russian Academy of Sciences, 47, Leninsky Prospect, Moscow 117913, Russia*
[c] *Laboratory of Preparative Biochemistry, National Research Center-Institute of Immunology, Federal Medical Biological Agency of Russia, 24, Kashirskoe Shosse, Moscow 115478, Russia*
[d] *The Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, NIH, Rockville, MD 20852, United States*

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ABSTRACT

Shigellosis, a major cause of diarrhea worldwide, exhibits high morbidity and mortality in children. Specificity of *Shigella* immunity is determined by the structure of the main protective O-antigen polysaccharide component incorporated into the lipopolysaccharide (LPS) molecule. Endotoxicity, however, precludes LPS clinical use. Thus, there is still no vaccine against the most prevalent shigellosis species (serotype *S. flexneri* 2a), despite ongoing efforts focused on inducing serotype-specific immunity. As LPS is highly heterogenous, we hypothesized that more homogenous pools of LPS might be less toxic. We developed a method to generate a homogenous *S. flexneri* 2a LPS subfraction, Ac_3-S-LPS, containing long chain O-specific polysaccharide (S-LPS) and mainly tri-acylated lipid A, with no penta- and hexa-acylated, and rare tetra-acylated lipid A. Ac_3-S-LPS had dramatically reduced pyrogenicity and protected guinea pigs from shigellosis. In volunteers, 50 μg of injected Ac_3-S-LPS vaccine was safe, with low pyrogenicity, no severe and few minor adverse events, and did not induce pro-inflammatory cytokines. In spite of the profound lipid A modification, the vaccine induced a prevalence of IgG and IgA antibodies. Thus, we have developed the first safe immunogenic LPS-based vaccine candidate for human administration. Homogenous underacetylated LPSs may also be useful for treating other LPS-driven human diseases.

Clinical trial registry: http://grls.rosminzdrav.ru/

1. Introduction

Shigellosis *S. flexneri* is still a poorly controllable enteric infection marked by high annual global morbidity and mortality due, among other factors, to the absence of vaccines with even moderate efficacy. A vaccine for prophylaxis of shigellosis is one of the major tasks identified by the WHO [1].

Development of vaccines against *Shigella* infections, including *S. flexneri,* is based on the generally accepted role of lipopolysaccharide (LPS) as the molecule possessing key protective serotype-specific O-antigen [2,3]. LPS is also a well-known *Shigella* virulence factor, as LPS molecular size influences bacterial invasion [4,5]. Discovered more than a century ago, LPS, a member of the family of amphiphilic glycolipids, is localized on the outer layer of the outer membrane of Gram-negative bacteria [6,7]. The so-called S-LPS that is usually produced by 'smooth' colony-forming bacteria, includes an O-specific polysaccharide (OPS), which determines the immunospecificity of the S-LPS and, as a consequence, the whole microorganism. S-LPS-specific antibodies play a key role in resistance against many bacterial infections and exhibit a highly protective capacity [7,8]. Even low doses of LPS are endotoxic, and the appearance of relatively small amounts of LPS in the circulation of an infected host causes a state of endotoxic shock that precludes any use of the intact LPS as a vaccine component.

Lipid A of some highly virulent (*Yersinia pestis*) or weakly virulent *(Helicobacter pylori, Porphyromonas gingivalis, Treponema pallidum)* microorganisms differ significantly from the classical endotoxic lipid A by a lower degree of acylation, variations in fatty acid chain length, the presence of multiple bonds in fatty acids, cyclic structures, and/or oxygen-containing groups as well as by modifications of the phosphate groups [9–11]. Although it is not

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* Corresponding author at: Laboratory of Carbohydrate Vaccines, National Research Center-Institute of Immunology, 24, Kashirskoe Shosse, Moscow 115478, Russia.

E-mail address: pg.aparin@nrcii.ru (P.G. Aparin).

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currently known whether the endotoxic and immunostimulatory properties of lipid A are due to the same or different components of the molecule, a hint has come from studies showing that a decrease in the degree of acylation of the 'classical' lipid A results in a noticeable reduction in TLR-4 stimulation and, consequently, of the pyrogenicity and endotoxicity of the LPS [9,12], with some of these LPSs being TLR-4 antagonists rather than agonists [13]. The finding that a dramatic decrease in LPS endotoxicity can be achieved by a reduction in the number of fatty acid residues encouraged studies directed at conversion of the endotoxic LPS to lower-acylated derivatives by chemical, enzymatic, and genetic manipulations. However, modified LPS preparations from endotoxic bacteria, which contain penta-acylated species or represent mixtures of tetra-, penta-, and hexa-acylated species, have not met clinical safety criteria [14–16].

The humoral immune response against carbohydrate antigenic determinants of the whole LPS is important for the development of adaptive immunity against Gram-negative bacteria [8,17]. This has led to another approach for construction of vaccines through utilization of LPS-associated antigenic determinants. Notably, LPS cleavage products were used as specific antigens for conjugation with protein carriers to afford neo-glycoconjugate vaccines. These were an OPS-core prepared by mild acid degradation of LPS or an OPS-core-di-N-acyl lipid A obtained by alkaline O-deacylation of LPS [18,19]. Despite this progress, no clinically applicable vaccine based on a complete LPS immunogen has yet been developed.

Here we show the potential utility of a vaccine comprising a highly homogenous LPS subfraction from the enteric bacterium *S. flexneri* 2a, the most prevalent species and serotype causing shigellosis in humans [20]. In this work, we delineate the preparation and testing of Ac_3-S-LPS derived from *S. flexneri* 2a for direct use as a vaccine in humans. We achieved an initial significant reduction in endotoxicity by gel chromatography fractionation of the intact LPS from *S. flexneri* 2a, enabling separation of S-LPS from the highly endotoxic R-LPS. We further removed penta- and hexa-acylated contaminants from the Ac_3-S-LPS preparation by partial alkaline deacylation of S-LPS under de-micellization conditions. The resulting Ac_3-S-LPS was immunogenic while showing low endotoxicity and pyrogenicity in laboratory animals as well as in human volunteers, and is thus promising as a new *Shigella* candidate vaccine.

2. Results

2.1. Preparation and chemical characterization of homogenous modified S-LPS of S. flexneri 2a

S. flexneri 2a 1605 were propagated in liquid medium, dried bacterial cells were extracted by the Westphal method [21]. A crude LPS preparation was freed from nucleic acids and proteins by treatments with RNAse, DNAse and proteinase K.

S-LPS, relatively free of R-LPS and containing <1% protein and <1% nucleic acids, was obtained by fractionation of the isolated LPS using gel-permeation chromatography on Sephadex G-150 in the presence of Na-deoxycholate as detergent (Supplemental Fig. 1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.12.067.

Partial deacylation of the isolated S-LPS was performed with aqueous ammonia, and modified Ac_2-S-LPS, Ac_3-S-LPS or Ac_4-S-LPS species were obtained by varying temperature and time of reaction and concentration of aqueous ammonia. SDS-PAGE confirmed the presence of high molecular mass species of Ac_3-S-LPS (Fig. 1, right lane).

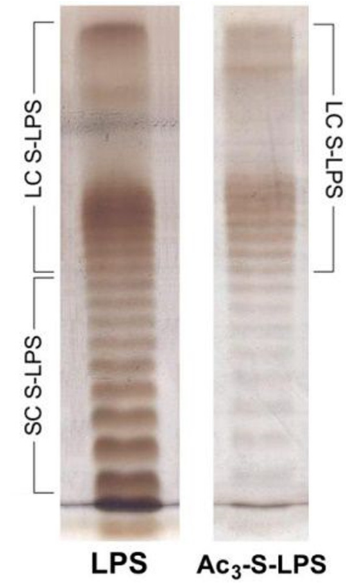

Fig. 1. SDS-PAGE analysis of Ac_3-S-LPS from *S. flexneri* 2a in comparison with the intact LPS. LC S-LPS and SC S-LPS indicate S-LPS with long-chain and short-chain OPS, respectively. Ac_3-S-LPS contains mainly long-chain OPS species.

A comparison of the ^{13}C NMR spectra of the non-modified S-LPS and Ac_3-S-LPS (Fig. 2A and B, respectively) showed that treatment with 8.3% aqueous ammonia eliminated O-acetyl groups from the OPS (a signal at δ 21.3 for methyl groups of OAc disappeared) and reduced the content of fatty acids in lipid A (the intensity of signals at δ 31–32 for methylene groups was significantly decreased). The spectra were essentially identical with respect to signals for the OPS [22], indicating that deacylation did not alter the OPS repeating unit structure.

Lipid A (LA) was released from the modified S-LPS by mild acid hydrolysis. The negative ion mode electrospray ionization mass spectrum of the released LA showed peaks for $[M-H]^-$ ions of various acyl variants (Fig. 3). The peak at m/z 871.50 belongs to an Ac_2-LA containing two N-linked 3-hydroxymyristoyl groups (Fig. 3A). The peaks at m/z 1053.67 and 1279.85 belong to Ac_3-LA and Ac_4-LA containing one lauroyl group and two or three 3-hydroxymyristoyl groups, respectively (Fig. 3B and C). Notably, no peaks for penta- and hexa-acyl derivatives were present in the mass spectra of the modified LA (Fig. 3A–C), whereas they were readily evident in the mass spectrum of LA from the intact S-LPS with clear hexa-acyl domination (Fig. 3D).

Importantly, deacylation caused no significant changes to the serological activity of Ac_2-S-LPS and Ac_3-S-LPS in reactions with homologous *S. flexneri* group-specific 3,4 and type-specific II antibodies as compared with the non-modified S-LPS. In contrast, the intact S-LPS that contained short OPS chains, had reduced serological activity due to a lower percentage of the species that carry group- and type-specific antigenic determinants (Fig. 4A and B).

2.2. Structural requirements for apyrogenicity of the modified S. flexneri 2a S-LPS

Pyrogenicity of the modified *S. flexneri* 2a S-LPS with long OPS chain preparations was examined in rabbits by comparing it to the intact S-LPS and the commercial Vi-antigen typhoid vaccine. Similar to the Vi-antigen vaccine, both Ac_2-S-LPS and Ac_3-S-LPS were apyrogenic ($\sum\Delta$ t °C = 0.9 °C) when injected intravenously (i.v.) into rabbits at a WHO approved Vi-vaccine pyrogenicity test dose of 0.025 $\mu g\ kg^{-1}$ (Fig. 4C), whereas the intact S-LPS was highly pyrogenic ($\sum\Delta$ t °C > 4 °C) regardless of the OPS chain length.

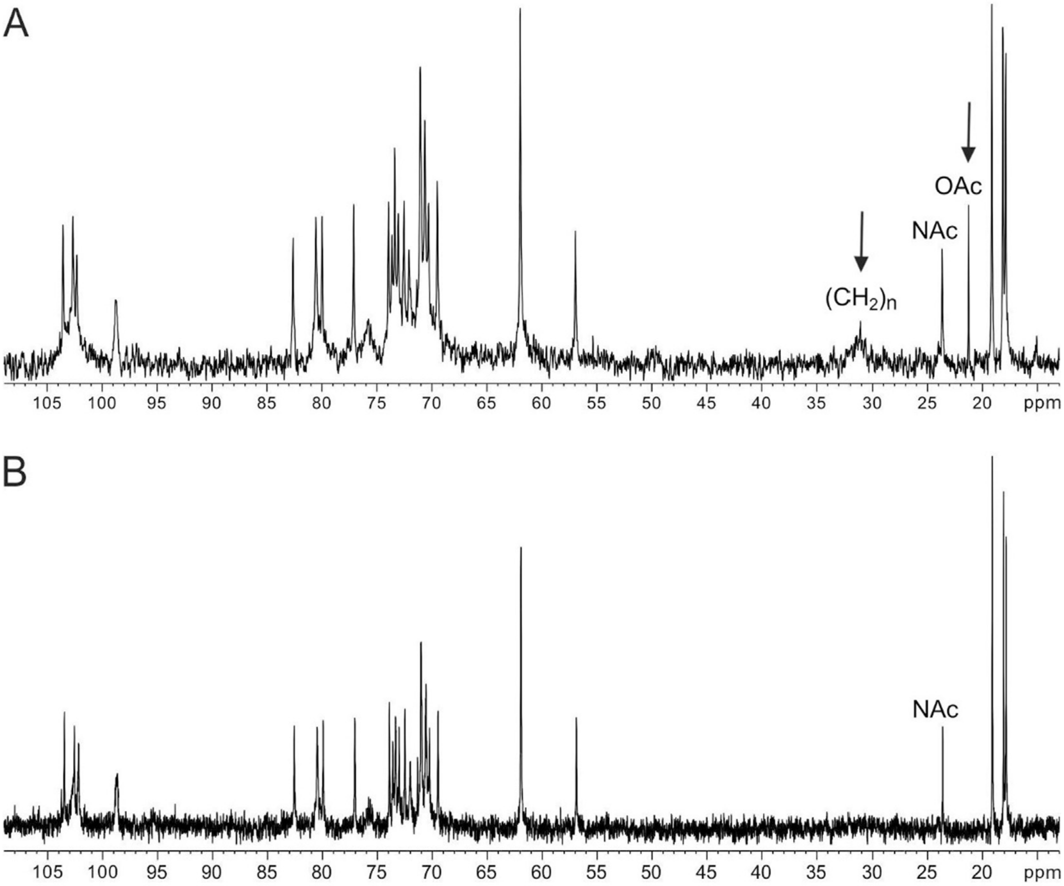

Fig. 2. ^{13}C NMR spectra of the long-chain S-LPS (A) and Ac_3-S-LPS (B) from *S. flexneri* 2a. NAc and OAc indicate N-acetyl and O-acetyl groups on monosaccharides, $(CH_2)_n$ indicates methylene groups of fatty acids. The spectra are similar to each other except for the absence from the Ac_3-S-LPS spectrum of signals for OAc at δ 21.3 and $(CH_2)_n$ at δ 31–32 (arrows).

Therefore, these modified S-LPS variants meet pyrogenicity requirements for a capsular polysaccharide vaccine [23].

Endotoxicity of Ac_3-S-LPS was also evaluated in the *Limulus* amoebocyte lysate (LAL) assay and compared with the intact S-LPS. The clotting activity in LAL assay of the Ac_3-S-LPS preparation was 8 Eu μg^{-1}, whereas the LAL-activity of the intact S-LPS was 1649 Eu μg^{-1} (Fig. 4D). These findings indicate that LAL clotting potency of the Ac_3-S-LPS was significantly reduced by partial deacylation.

Effect of the modified *S. flexneri* 2a S-LPS on production of pro-inflammatory cytokines IL-6, IL-8 and TNFα was assessed on the human pro-monocytic cell line U937, a widely used model of monocyte/macrophage functions as well as on PBMCs from healthy donors. Ac_3-S-LPS demonstrated significantly decreased capacity to stimulate pro-inflammatory cytokine production compared to intact S-LPS and commercially available LPS (Table 1). In these tests Ac_3-S-LPS did not differ significantly from monophosphoryl lipid A.

2.3. Immunogenicity of apyrogenic and pyrogenic variants of S. flexneri 2a S-LPS

The level of serum antibody response in mice immunized with intact S-LPS with a long OPS chain was higher than for those given short-chain S-LPS according to unpublished observations. This prompted us to study the antibody responses of mice to various preparations of modified S-LPS with only a long OPS chain (Fig. 5A).

Except for those given Ac_2-S-LPS, the levels of IgG and IgM in mice after intraperitoneal (i.p.) immunization with intact S-LPS, Ac_3-S-LPS or Ac_3-Ac_4-S-LPS were significantly higher than in the control group of animals ($p \leq 0.05$). The immunogenicity of low-endotoxic Ac_3-S-LPS was high and almost equal to that of the endotoxic and highly pyrogenic intact S-LPS.

The apyrogenic Ac_3-S-LPS immunogen was selected for further systemic immune response characterization and examination of the mouse serum IgG antibody profile. IgG_3 was dominant among other IgG subclasses. Either s.c. or i.p. immunization of mice with a dose of 12.5 μg induced the same 12.7-fold increase in IgG_3 (Fig. 5B). The observed mouse serum IgG antibody profile was typical for carbohydrate antigens, which are Type 2 thymus-independent antigens [24].

We next studied the mucosal immune response, as it is the basic mechanism of immunity against *Shigella*. A prime-boost intranasal immunization of mice with Ac_3-S-LPS at a dose of 50 μg induced significant increases in specific IgA and IgG titers in lung lavages (Fig. 5C). The mucosal IgA and IgG titers were 5- and 4-fold higher than those in the control group, respectively. IgM antibody titers were similar in groups of immunized and control mice.

Therefore, experimental immunization of mice with the *S. flexneri* 2a Ac_3-S-LPS resulted in significant increases in LPS-specific serum IgG and IgM, and in IgA and IgG antibodies on mucosal surfaces of mouse lungs.

2.4. Ac_3-S-LPS vaccine protects against S. flexneri 2a keratoconjunctivitis in a guinea pig model

We assessed protective *Shigella*-specific mucosal immunity after s.c. immunization with this Ac_3-S-LPS vaccine in a guinea

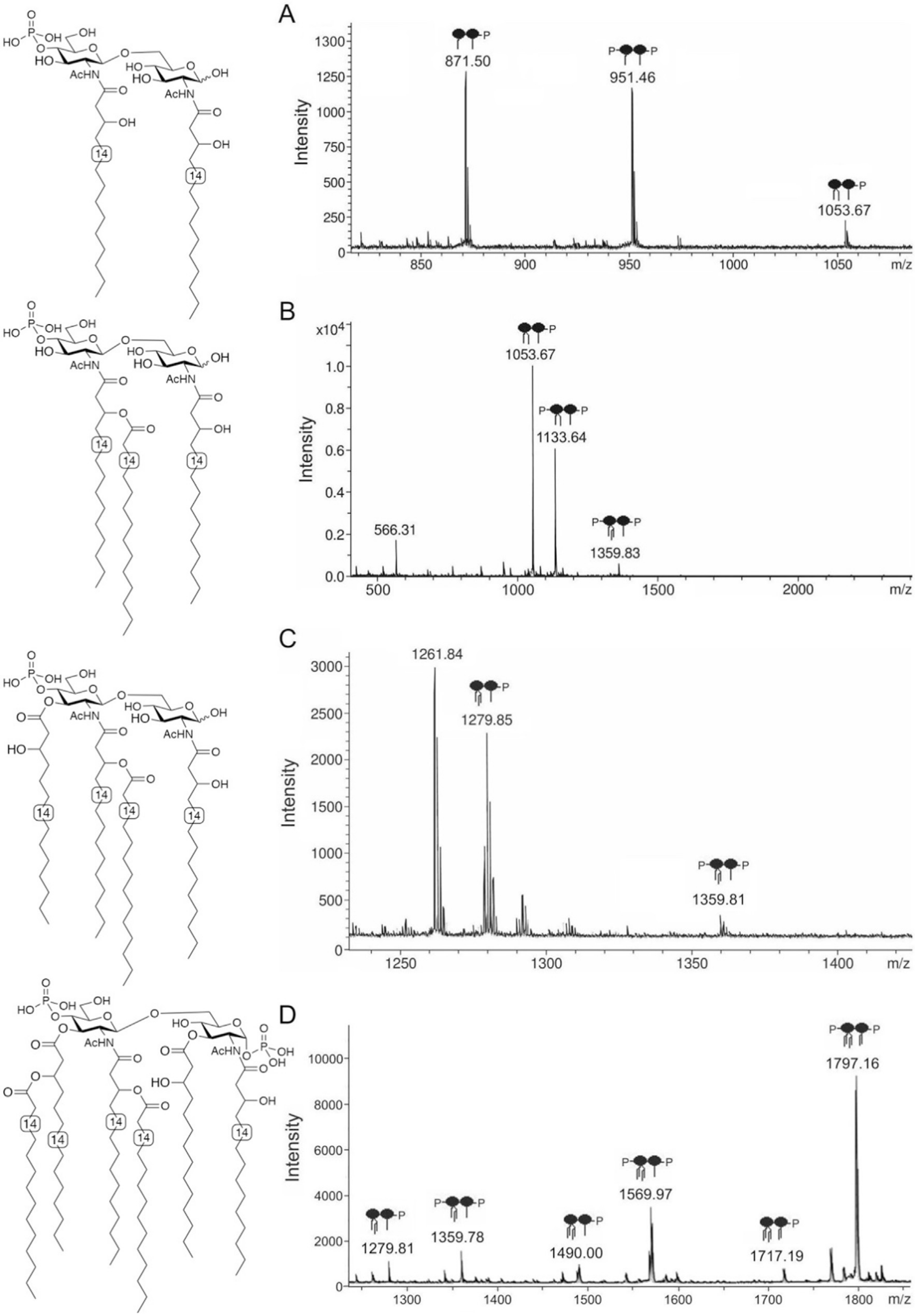

Fig. 3. Mass spectrometry analysis of lipid A species. LA samples were released from the modified S-LPS of *S. flexneri* 2a by mild acid hydrolysis (2% AcOH, 100 °C, 1 h). Negative ion mode electrospray ionization mass spectra of LA show $[M-H]^-$ ions of various acyl variants, Ac_2-S-LPS (A), Ac_3-S-LPS (B) and Ac_4-S-LPS (C) compared to the intact LPS (D). Major peaks at *m/z* 871.50, 1053.67 and 1279.85 are for monophosphoryl Ac_2-LA (A), Ac_3-LA (B) and Ac_4-LA (C), respectively, and minor peaks at *m/z* 951.46 (A), 1133.64 (B) and 1359.82 (C) for the corresponding bisphosphoryl LA species.

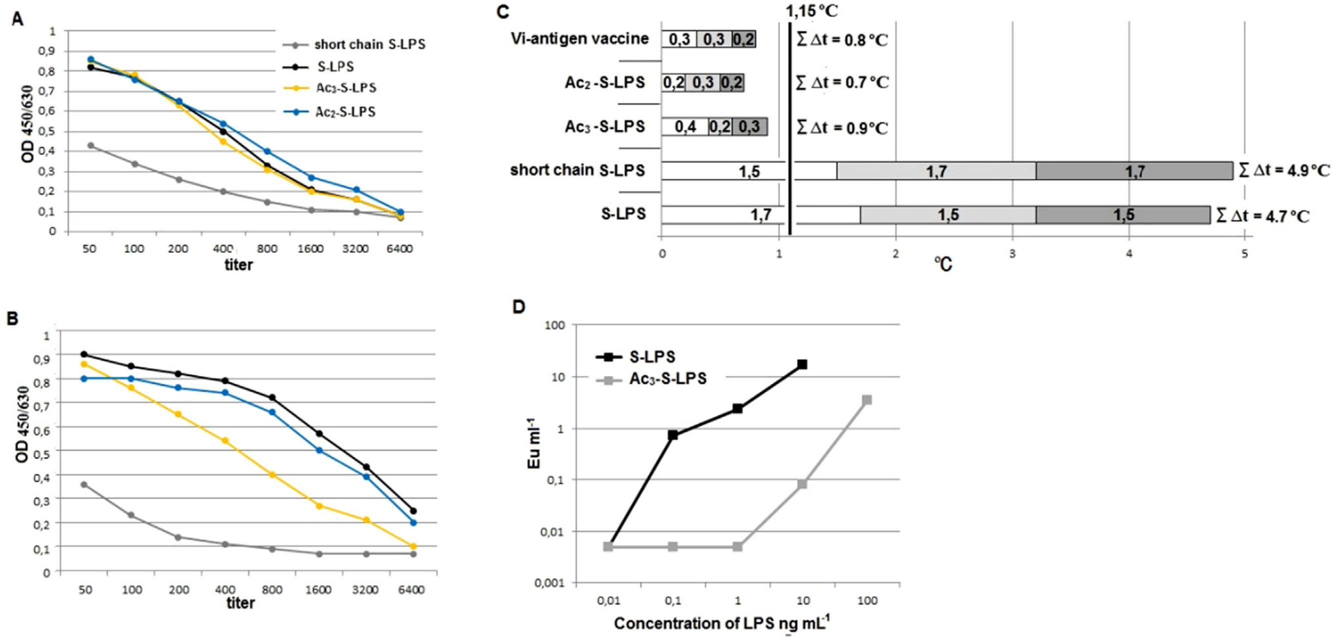

Fig. 4. Immunobiological characteristics of intact and modified S-LPS of *S. flexneri* 2a. (A). Binding of monoreceptor type II-specific and (B) binding of group 3,4-specific anti-*S. flexneri* rabbit antibodies to intact or modified S-LPS of *S. flexneri* 2a was studied by ELISA.Microwells were coated with short-chain intact S-LPS (—●—), long-chain intact S-LPS (—●—) or its long-chain O-deacetylated derivatives, Ac_3-S-LPS (—●—) and Ac_2-S-LPS (—●—). Type II- and group 3,4-specific antibodies bound poorly to short-chain intact S-LPS. (C) Rabbit pyrogen test data measured after i.v. injection of 0.025 µg per kg of intact or modified S-LPS of *S. flexneri* 2a in apyrogenic saline. Summed responses of three rabbits to Ac_3-S-LPS, Ac_2-S-LPS and Vi-antigen vaccine did not exceed the European Pharmacopeia pyrogenic threshold ($\sum\Delta$ t °C < 1.15 °C). After injection of intact S-LPS, all rabbits had Δt higher than 1.5 °C resulting in summed responses significantly exceeding the 1.15 °C pyrogenic threshold. (D) *S. flexneri* 2a Ac_3-S-LPS (—■—) exhibits decreased LAL clotting activity in comparison with intact *S. flexneri* 2a S-LPS (—■—).

Table 1
Pro-inflammatory cytokine concentrations (in pg mL^{-1}) in supernatants of LPS-stimulated human pro-monocytic cell line U937 or PBMC isolated from healthy volunteers.

	Ac_3-S-LPS *S. flexneri* 2a	LPS *E.coli* O55	Pyrogenic S-LPS of *S. flexneri* 2a	Monophosphoryl lipid A	Not stimulated cells
U937					
IL-6	20,0 ± 1,7	213 ± 21	132 ± 8	124 ± 3	4,0 ± 1
IL-8	22,3 ± 6,8	397 ± 21	353 ± 15	210 ± 44	8,3 ± 2,1
TNFα	38,0 ± 12,2	188 ± 8	235 ± 13	90,3 ± 4,5	5,0 ± 2,6
PBMC[a]					
IL-6	1965 ± 1796	5670 ± 368	7293 ± 327	688 ± 357	6,7 ± 3,2
IL-8	12841 ± 13524	46236 ± 1751	43883 ± 3981	6548 ± 4852	266 ± 167

Data are means ± SD.

[a] PBMC were isolated from three donors.

pig model of *Shigella* keratoconjunctivitis (Sereny test) [25], comparing it to immunization with the intact S-LPS. In the control group immunized with vaccine diluent all animals challenged with 2×10^9 cells of *S. flexneri* 2a (ID_{100}) developed keratoconjunctivitis (Fig. 5D), whereas double s.c. immunization of guinea pigs with the Ac_3-S-LPS vaccine at doses of 25 or 50 µg protected 40% and 60% of challenged eyes, respectively compared to 20% and 70% eye protection rates in guinea pigs, that were immunized with similar doses of the intact S-LPS. Therefore, the levels of the mucosal protection induced by the modified and intact S-LPS were similar, and mucosal anti-*Shigella* immunity can be induced by s.c. immunization of guinea pigs with the Ac_3-S-LPS vaccine.

Protection against keratoconjunctivitis was also associated with the development of systemic immunity: immunization of guinea pigs with Ac3-S-LPS and intact S-LPS at a dose of 50 µg elicited 4.1- and 6.9-fold increases of serum IgG titers, respectively, compared to control group (Fig. 5E).

2.5. Clinical safety study of the Ac_3-S-LPS vaccine in human volunteers

22 adult volunteers were injected s.c.with the Ac_3-S-LPS candidate vaccine at doses of 25 µg (10 persons) or 50 µg (12 persons). The parenteral route of vaccination can be an effective instrument of inducing protective mucosal responses [26]. Despite the vaccine dose exceeding 0.7 µg kg^{-1}, no serious adverse events (AE), including severe febrile responses or endotoxic shock, were registered, and the body temperature of the volunteers did not exceed 37.1 °C. Thus, Ac_3-S-LPS was as apyrogenic in humans as it had been in experimental animals. No systemic endotoxic manifestations, such as chills, tachycardia >90 bpm, systolic blood pressure increases of >140/100 mm Hg, headache, malaise, abdominal pain or nausea, were observed among the 22 adults (Table 2). Local AE including two cases of redness at the site of injection, were registered 2 h after injection of the Ac_3-S-LPS vaccine at a dose of 50 µg.

Hematological studies did not reveal any significant changes in blood cell counts, including erythrocytes, total white blood cell (WBC) count and differential 6 h and 30 days after vaccination with the Ac_3-S-LPS vaccine. Levels of the main blood biochemical parameters, including urea, uric acid, creatinine, ALT, AST, and glucose, were also in the normal ranges (Table 3).

We assessed attenuation of the LPS endotoxicity by direct measurement of cytokine responses 2, 4 and 6 h after s.c. immunization of volunteers with the Ac_3-S-LPS vaccine. At doses of 25 or 50 µg,

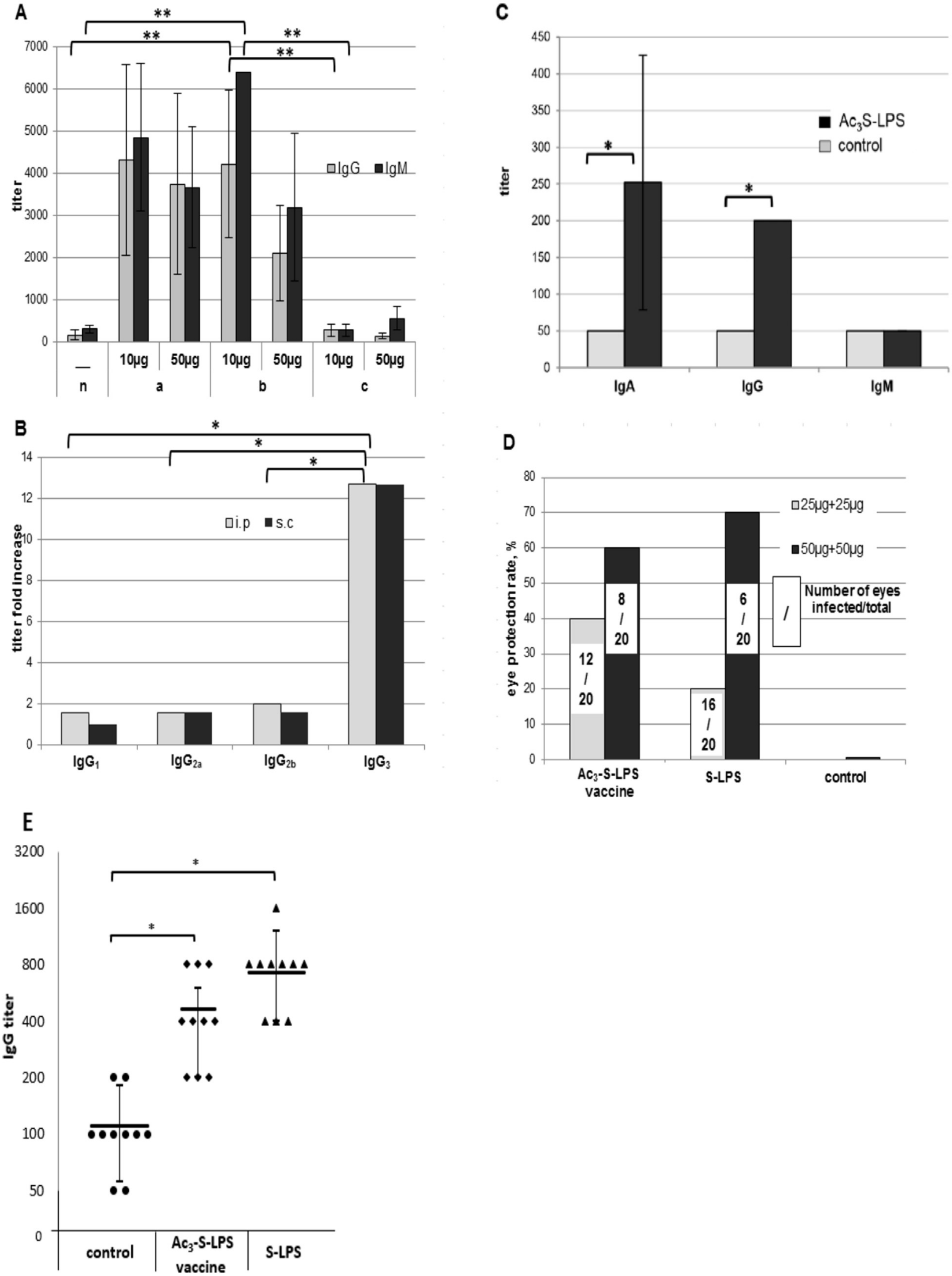

Fig. 5. Antibody responses and mucosal immunity induced by Ac_3-S-LPS *S. flexneri* 2a vaccine. (A) IgG and IgM serum antibody titers in mice immunized and boosted with intact or modified S-LPS *S. flexneri* 2a preparations. (n) control; (a) intact S-LPS; (b) Ac_3-S-LPS; (c) Ac_2-S-LPS. (B) Titers of specific IgG subclasses increased in mice immunized with Ac_3-S-LPS. Fold increase of IgG subclasses titers was calculated. Differences between IgG_3 and IgG_1, IgG_{2a} or IgG_{2b} titer increases were significant. (C) IgA, IgG, IgM end point anti-S-LPS antibody titers were detected in mouse lung lavages. The titers of mucosal IgA and IgG after Ac_3-S-LPS immunization were 1:252 and 1:200 respectively and were significantly higher than in the control group. (D) Protective mucosal anti-*Shigella* immunity was induced in guinea pigs by systemic immunization with intact pyrogenic S-LPS of *S. flexneri* 2a or Ac_3-S-LPS. Protection rate was estimated as percentage of non-infected eyes. (E) LPS-specific IgG titers were quantified by ELISA in individual sera of intact (control) and immunized guinea pigs. The results shown are means ± SD. Significant differences between values are indicated. (* $p < 0.05$, **$p < 0.01$).

Table 2
Local and systemic AE frequency after s.c. immunization of volunteers with *S. flexneri* 2a Ac$_3$-S-LPS vaccine.

	Adverse events	Dose 25 μg (n = 10)	Dose 50 μg (n = 12)
Local	Injection site pain	0	0
	Redness	0	2
	Swelling	0	0
	Regional lymph node enlargement	0	0
Systemic	Body temperature > 37.1 °C	0	0
	Arterial pressure > 140/100 mmHg	0	0
	Heart rate > 90 bpm	0	0
	Nausea	0	0
	Diarrhea	0	0
	Headache	0	0
	Abdominal pain	0	0
	Chills	0	0
	Malaise	0	0

serum concentrations of pro-inflammatory cytokines TNFα, IL-1β, IL-6 were low and did not significantly exceed those in serum taken from the volunteers before vaccination (Table 4). Concentration of TNFα increased very slightly at 2 h and that of IL-6 at 4 h after the vaccine administration, whereas the IL-1β concentration remained at basal levels.

This clinical study demonstrates a sharp decrease in the endotoxicity of the modified S-LPS as well as the safety of parenteral administration of the *S. flexneri* 2a Ac$_3$-S-LPS candidate vaccine to adults.

2.6. Human immune responses after immunization with the S. flexneri 2a Ac$_3$-S-LPS vaccine

Antibodies specific for LPS mediate the major protection after vaccination against shigellosis [27]. We therefore measured anti-*S. flexneri* 2a S-LPS-specific serum antibody titers after s.c. injection in healthy volunteers. The vaccine dose of 25 μg was less immunogenic than the 50 μg dose (Fig. 6A and B). The most prominent increases were in the titers of IgA and IgG, with approximately 5-fold increases on day 30 and 7–10 fold increases at day 60 (Fig. 6A). Agglutinating titers also increased, with 8.75- and 6.0-fold increases on days 30 and 60, respectively (Fig. 6B), while production of IgM was low.

2- and 4-fold seroconversion of anti-*S. flexneri* 2a LPS antibodies was observed in both groups of volunteers. 4-fold seroconversion rates for IgG (75%), IgA (50%) and agglutinating anti-LPS antibodies (100%) were highest on day 60 after vaccination with a dose 50 μg. 4-fold seroconversion rates on day 30 were lower and comprised 75% (IgG), 42% (IgA), 92% (LPS-specific agglutinating antibodies) and 25% (IgM) (Fig. 6C and D).

Therefore, homogenous Ac$_3$-S-LPS is safely apyrogenic and also immunogenic in adults, and thus represents a safe human immunogen capable of inducing IgA and IgG immune responses in spite of the profound lipid A modification. Importantly, the humoral immune response was induced by injection of Ac$_3$-S-LPS without the addition of any other adjuvant compound.

3. Discussion

Lipopolysaccharides are among the most heterogeneous biomolecules in nature [28].

The occurrence of multiple structural variants that differ in OPS chain length and the degree of acylation and phosphorylation of lipid A induce a wide variety of immunological and endotoxic responses to LPS. The novel vaccine reported here is based on highly homogenous Ac$_3$-S-LPS, a modification of the S-LPS in which we produced a tri-acylated form from the original LPS, which also includes hexa- and penta-acylated forms.

The ability of various structural variants of *S. flexneri* 2a S-LPS to induce pyrogenic reactions in rabbits is defined mainly by the degree of acylation of lipid A and, to a lesser extent, by OPS chain

Table 3
Blood count and biochemical data of volunteers immunized s.c. with a 50 μg dose of *S. flexneri* 2a Ac$_3$-S-LPS vaccine.

Test	Before vaccination	6 h after vaccination	On day 30 after vaccination	Normal range
RBC (×10^{12} L^{-1})	5.0 ± 0.6	4.9 ± 0.4	4.7 ± 0.7	4.5–5.0
Hb (g L^{-1})	159 ± 24	154 ± 28	142 ± 33	130–160
WBC (×10^{9} L^{-1})	5.6 ± 1.2	5.8 ± 1.4	9.0 ± 3.2	4.5–9.5
Neutrophils (%)	69 ± 11	71 ± 3	70 ± 5	50–72
Lymphocytes (%)	26 ± 14	23 ± 15	25 ± 17	18–38
Monocytes (%)	5 ± 2	5 ± 2	7 ± 2	2–10
ESR (mm h^{-1})	4 ± 2	3 ± 2	3 ± 2	1–5
ALT (IU L^{-1})	21 ± 12	29 ± 17	17 ± 14	5–40
AST (IU L^{-1})	24 ± 14	31 ± 16	19 ± 12	5–40
Creatinine (μmol L^{-1})	108 ± 22	119 ± 20	113 ± 17	70–130
Urea (μmol L^{-1})	4.6 ± 3.1	4.7 ± 4.1	3.9 ± 2.8	3.38–8.32
Uric acid (μmol L^{-1})	325 ± 128	340 ± 142	290 ± 118	240–500
Glucose (μmol L^{-1})	4.6 ± 3.5	5.1 ± 4.2	4.1 ± 3.8	3.5–6.1

Data are means ± SD.

Table 4
Pro-inflammatory cytokine concentrations in volunteer's serum before and after immunization with *S. flexneri* 2a Ac$_3$-S-LPS vaccine.

Vaccine dose (μg)	Time (h)	Cytokine concentration[a] (pg mL^{-1})		
		TNFα	IL-1β	IL-6
25	0	12.7 ± 1.4	0	1.18 ± 0.63
	2	17.7 ± 2.3	0	0.74 ± 0.49
	4	13.5 ± 0.1	0	1.78 ± 0.74
	6	19.9 ± 1.7	0	2.83 ± 1.32
50	0	11.2 ± 2.2	0	1.26 ± 0.91
	2	18.0 ± 4.3	0	1.96 ± 1.53
	4	13.5 ± 4.2	1.49 ± 0.51	6.06 ± 2.1
	6	11.8 ± 0.1	0	2.59 ± 0.1

[a] Data are means ± SD of blood samples of 10 volunteers immunized with a dose of 25 μg and 12 volunteers with a dose of 50 μg.

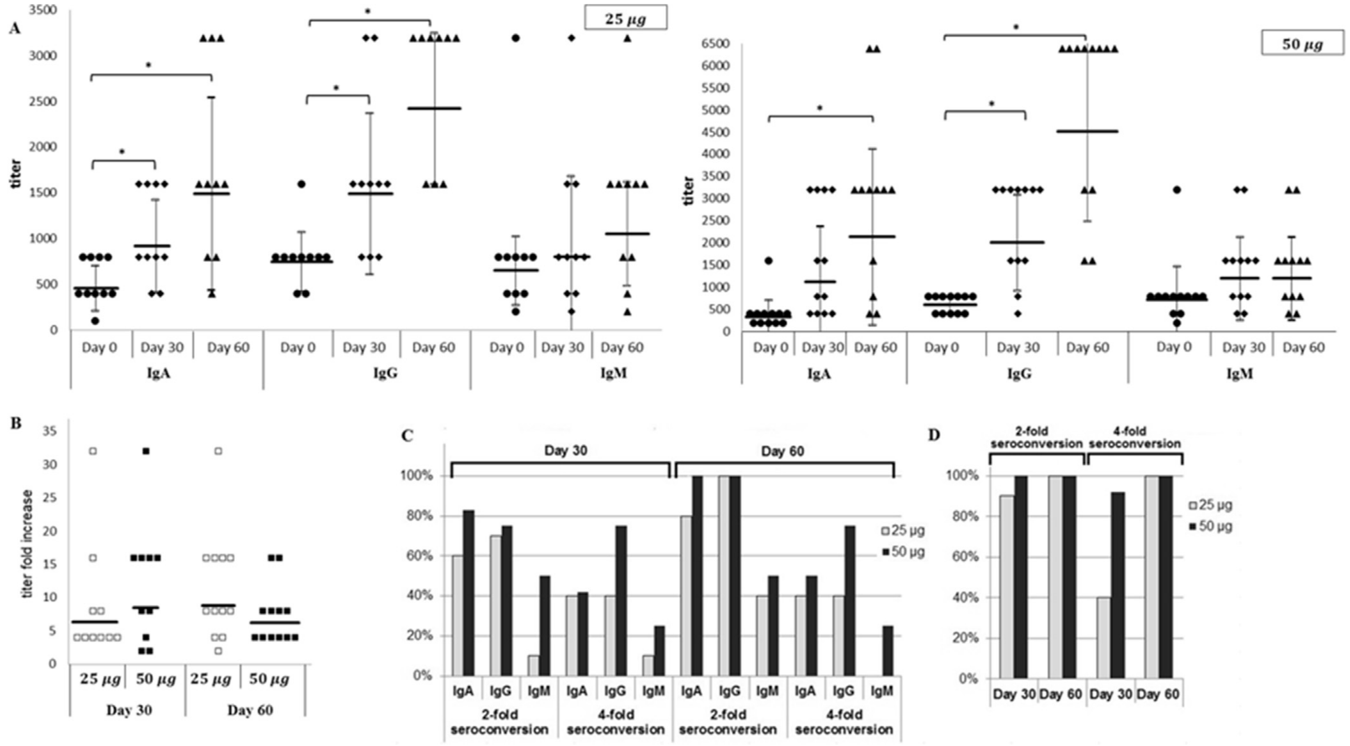

Fig. 6. S.c. immunization of volunteers with Ac_3-S-LPS *S. flexneri* 2a vaccine led to development of specific humoral immune responses. (A) Specific IgA, IgG and IgM serum antibody titers were quantified by ELISA in individual sera of immunized volunteers. Data are means ± SD of samples of 10 volunteers immunized with a dose of 25 µg and 12 volunteers given a dose of 50 µg. Significant differences between pre- and post- vaccination levels of IgA and IgG antibodies are indicated (*$p < 0.05$). (B) Serum LPS-specific agglutinating antibody titers were increased in volunteers immunized with the Ac_3-S-LPS vaccine. Titer increases were significant at any combination of dose and time compared with the corresponding pre-vaccination levels. (C, D) 4-fold and 2-fold seroconversion rates of serum anti-LPS IgA, IgG and IgM (C) and agglutinating antibodies (D) were observed in groups of volunteers immunized with the Ac_3-S-LPS vaccine. 4-fold seroconversion rate for agglutinating antibodies reached 100% on day 60 in both groups of volunteers. Paired *t*-test was used to compare postvaccination responses. Differences were considered significant at $p < 0.05$.

length. Using the rabbit pyrogen test, the transition from apyrogenic to pyrogenic modified *S. flexneri* 2a S-LPS samples was determined showing that the pyrogenicity greatly decreased upon conversion to the tri-acylated form.

Dependence of the magnitude of antibody responses on the lipid A domain acylation pattern is a characteristic feature of the immunogenicity of *S. flexneri* 2a S-LPS derivatives in mice. Experimental samples of intact S-LPS, which contains tri-, tetra-, penta-, and hexa-acylated variants of lipid A, and purified samples containing the underacylated and apyrogenic form Ac_3-LPS, induced high levels of serum LPS-specific IgG and IgM in mice, whereas Ac_2-S-LPS, the other apyrogenic homogenous form, was non-immunogenic. Thus, modified *S. flexneri* 2a Ac_3-S-LPS was highly immunogenic and its lipid A domain structure is sufficient for activation of the immune response. Ac_3-S-LPS was also effective as an experimental mucosal immunogen, inducing antigen-specific IgA and IgG in mouse lung lavages.

The Sereny test is an experimental model for assessing *Shigella* protective immunity in guinea pigs. Instead of the intranasal route of guinea pig vaccination used by Noriega et al. for *Shigella* vaccine strains [29], we utilized parenteral double injections of LPS immunogen s.c. taking into consideration, that this route of immunization can be used for volunteer's immunization. Activation of mucosal immunity in response to experimental *S. flexneri* 2a infection provides a direct demonstration of Ac_3-S-LPS usefulness as a parenteral vaccine immunogen.

The most important aim of these studies was to conduct clinical trials designed to confirm or disprove the concept of the highly homogenous Ac_3-S-LPS *S. flexneri* 2a as a vaccine preparation suitable for clinical application. After s.c. immunization of adult volunteers with a candidate *S. flexneri* 2a Ac_3-S-LPS vaccine at doses up to 0.7 µg kg^{-1}, no serious AE were observed: i.e. no endotoxicosis, pyrogenic reactions, changes in hematological profiles, blood biochemical parameters or elevations in pro-inflammatory cytokines.

In addition, administration of Ac_3-S-LPS *S. flexneri* 2a induced practically no adverse events, compared to administration of *E. coli* LPS. For example, i.v. administration to humans of United States Pharmacopoeia LPS standard *E. coli* O113 lot 5 at dose of 2 ng kg^{-1} was found to be unsafe, as it induced temperature elevations to 38–39 °C, tachycardia, and increases in systolic blood pressure, time-dependent increases in serum concentrations of TNFα and IL-6 that peaked at >500 pg mL^{-1} $\sim$ 2 h after injection, and significant alterations in differential white blood cell counts (neutrophilia, lymphopenia) during the first 12 h after injection [30]. In contrast, the complete absence of temperature increases after s.c. immunization with Ac_3-S-LPS, and low cytokine responses, indicate that S-LPS deprived of hexa- and penta-acylated derivatives represents an apyrogenic, clinically applicable form of S-LPS.

The modified *S. flexneri* 2a S-LPS with tri-acyl LA has low LAL clotting activity, similar to modified lipid A molecules studied earlier [31]. These results indicate that the low LAL activity of the modified Ac_3-S-LPS correlates well with the safety characteristics of the vaccine. Similarly to monophosphoryl lipid A (a clinically applicable heterogeneous modified lipid A preparation) Ac_3-S-LPS was also a much less potent inducer of TNFα, IL-6 and IL-8 production by cells of the human pre-monocytic leukemia line U937 or PBMCs from healthy donors, when compared to intact S-LPS of *S. flexneri* 2a or LPS of *E. coli.*

LPS strongly influences the immunopathogenesis of shigellosis [32–34]. Moreover, structural remodeling of LPS is likely associated with the initial epithelial invasion stage of infection. The length of

OPS chains was shortened and the lipid A of intracellular LPS became highly heterogeneous, consisting of tri-, tetra-, penta-, and hexa-acylated derivatives [35,36]. Therefore, protection against infection is targeted to neutralization of *Shigella* cells producing different LPS molecules during invasion. The common antigenic determinant, O-antigen, expressed on all LPS structural variants of invasive *Shigella* determined the serotype-specific nature of immunity [27,37].

Human immune protection against *S. flexneri* infection, similar to infection with *S. sonnei*, correlates with significant quantities of circulating LPS-specific antibodies, especially of IgA [37,38]. The immune response profile upon immunization of volunteers with the *S. flexneri* 2a Ac_3-S-LPS experimental vaccine was characterized by a prevalence of IgG and IgA antibodies and relatively unchanged levels of IgM (IgG > IgA>>IgM). This is uncommon for a response to the 'classical' LPS antigen marked by high IgM production. Induction of an IgA response after vaccination is associated with protection of volunteers against challenge with *S. flexneri* 2a [38].

Production of specific IgG and IgA antibodies after just a single injection of Ac_3-S-LPS may indicate reactivation of memory B-cells specific to polysaccharide antigens. Similar findings have been reported for several bacterial antigens [39]. For example, carbohydrate-specific pre-existing IgG- and IgA-switched human B-memory cells were detected in primary adult responses to *Haemophilis influenzae* type b conjugated polysaccharide vaccine [40]. Detection of pre-existing antibodies to *S. flexneri* 2a and *Shigella dysenteriae* type 1 LPS in healthy adults that have not had a history of shigellosis, may be a manifestation of the formation of *Shigella*-specific B-memory cells [34,37,41], and may also be due to cross reactions with the antigens of other microbes. In our hands, the volunteers had pre-existing titers of antibodies, and activation of IgA and IgG antibody-producing cells in response to *S. flexneri* 2a Ac_3-S-LPS occurred without increased expression of pro-inflammatory cytokines.

Our highly homogenous LPS represents the first standardized type of LPS preparation with a fully defined structure. Such preparations can be used for fine agonistic or antagonistic targeting of the TLR-4 receptor, that is, for example, important for studying microglia activation in Alzheimer's disease [42,43].

In summary, the present study identifies highly homogenous LPS preparations that are clinically safe when injected s.c. to humans at immunogenic doses similar to doses of licensed polysaccharide vaccines. Taking into account that humans are constantly colonized by a vast number of species of microorganisms or are invaded by LPS-expressing pathogens, the proposed original method for preparation of LPS-based vaccines provides a new approach for immunoprophylaxis of various bacterial infections.

4. Materials and methods

4.1. Bacterial strain and growth conditions

Biomass of *Shigella flexneri* 2a strain 1605 was obtained through fermentation using a Hottinger liquid culture medium (NutriMedia) in a 250 L fermenter (BioR 250, Prointech). Bacterial cells were separated from the liquid phase by flow centrifugation (Z-41, Cepa).

4.2. Isolation and degradation of S-LPS

20 g of dried bacterial cells were extracted with 45% aqueous phenol (Sigma-Aldrich) at 68–70 °C. The aqueous phase was separated, dialysed against distilled water, and lyophilized to give a crude LPS preparation (960 mg). The preparation was dissolved in 0.05 M TRIS-buffer, pH 7.2, containing 0.01% (w/w) $CaCl_2$ and $MgCl_2$ solution. RNAse (Sigma-Aldrich) (100 $\mu g\ mL^{-1}$) and DNAse (Sigma-Aldrich) (10 $\mu g\ mL^{-1}$) were added and the solution was stirred at 37 °C for 16 h. The reaction mixture was treated with proteinase K (Sigma-Aldrich) (20 $\mu g\ mL^{-1}$) for 2 h at 55 °C and then dialyzed using ultrafiltration with a 50 kDa passage limit membrane (Vladisart), concentrated, and lyophilized to give a purified LPS preparation (530 mg). S-LPS was obtained by gel-permeation chromatography on Sephadex G-150 (Sigma-Aldrich). A LPS sample was applied to a column (100 × 5 cm) of Sephadex G-150 in 0.2 M NaCl containing 0.001 M EDTA, 0.01 M TRIS, 0.01% NaN_3 (Sigma-Aldrich), and 2% Na-deoxycholate (Sigma-Aldrich), and then eluted with the same buffer containing 0.25% Na-deoxycholate [44], at a flow rate of 1 mL min^{-1}. Monitoring was performed by a UV detector at 226 nm (ÄKTApurifier 10). Fractions that contained S-LPS were combined and freeze-dried. The residue was treated with 1% AcOH in ethanol, the precipitate was separated (20 min, 4200 g on a Rotina 420R centrifuge from Hettich), vacuum-dried, dissolved in water and freeze-dried.

After removal of lipid A (2% AcOH, 100 °C, 1 h), the carbohydrate portions of the native LPS and S-LPS were profiled by an Agilent 1260 HPLC system with UV- and RI-detection on a TSK gel G3000PW (Toyopearl) (7.8 mm I.D. × 30 cm) column, by using 0.2 M NaCl elution solution and flow rate of 0.5 mL min^{-1}. A *S. flexneri* 2a S-LPS preparation containing short OPS chains was also isolated. The ^1H and ^{13}C NMR spectra showed that the isolated S-LPS preparations were free from Na-deoxycholate [45].

4.3. Partial de-acetylation of S-LPS

A solution of S-LPS (300 mg) was heated with stirring in 8.3% aqueous ammonia and water (100 mL) containing 100 mg of Na-deoxycholate at 30 °C for 8 h, then cooled to 5–10 °C, diluted with 200 mL of water, neutralized with AcOH, and freeze-dried. The product was treated with 100% ethanol (200 mL), the precipitate was separated by centrifugation, washed with 100% ethanol (2 × 200 mL), vacuum-dried, dissolved in water and freeze-dried to give Ac_3-S-LPS (213 mg). Similar treatment of S-LPS with 8.3% aqueous ammonia at 50 °C for 18 h afforded Ac_2-S-LPS. Treatment of S-LPS with 6.25% aqueous ammonia at 30 °C for 8 h gave Ac_4-S-LPS.

4.4. Chemical and physical analyses

Protein and nucleic acid contents in the LPS preparations were determined by the methods of Bradford [46] and Spirin [47], respectively. SDS-PAGE was performed on a 12% acrylamide gel according to the Laemmli [48] method using a Bio-Rad Mini-Protean electrophoresis system. Gels were stained with silver nitrate reagent [49].

Electrospray ionization high-resolution mass spectra of lipid A samples were measured in the negative ion mode using a micrOTOF II instrument (Bruker Daltonics).

^1H and ^{13}C NMR spectroscopy was performed for solutions in 99.95% D_2O at 323 K on a Bruker DRX-500 spectrometer using sodium 3-trimethylsilylpropanoate-2,2,3,3-d_4 (δ_H 0) and acetone (δ_C 31.45) as references for calibration.

4.5. Preparation of the S. flexneri 2a Ac_3-S-LPS vaccine

The candidate vaccine was dispensed in ampoules by Chumakov Institute of Poliomyelitis and Viral Encephalitides. The preparation was manufactured based on the *S. flexneri* 2a Ac_3-S-LPS as the active substance and excipients were: phenol 0.75 mg, NaCl 4.15 mg, Na_2HPO_4 0.052 mg, NaH_2PO_4 0.017 mg (all Sigma-Aldrich), and sterile pyrogen-free water for injection 0.5 mL. Commercial Vi-antigen vaccine VIANVAC was used as a heterologous vaccine control.

4.6. Pyrogenicity and Limulus amoebocyte lysate activity

All animal studies on guinea pigs, mice and rabbits were conducted in accordance with protocol 5512 approved by the Institutional Animal Care and Use Committee at the NRC-Institute of Immunology, Moscow. This protocol is in compliance with the National Institutes of Health guidelines for the humane use and care of laboratory animals.

Pyrogen tests were conducted on Chinchilla rabbits weighing 2.8–3.05 kg in accordance with European Pharmacopoeia requirements [50] and WHO requirements for the Vi-polysaccharide vaccine. A substance was considered to be apyrogenic if the cumulative temperature rise of three rabbits did not exceed 1.15 °C.

Doses of 0.01, 0.1, 1, 10 ng mL^{-1} of the Ac3-S-LPS vaccine or intact S-LPS were mixed with LAL reagent (Endosafe KTA, Charles River), and LAL activity was determined with a quantitative chromogenic assay. Data were processed using the specialized software, EndoScan-V.

4.7. Immunogenicity in mice

Groups of mice (CBA × C57BL/6) F_1 were immunized i.p. with doses of 10, 12.5 or 50 µg of the intact or modified S-LPS. One month after primary injection, the mice were boosted i.p. with the same doses. At day 15 after the primary and the secondary immunizations, serum samples were collected, and levels of S-LPS specific IgM, total IgG, IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3 were evaluated by ELISA using intact S-LPS adsorbed on microplates (Greiner). For evaluation of the mucosal immune response, Ac_3-S-LPS was introduced intranasally to mice at a dose of 50 µg twice with a 14-day interval. Fourteen days after the last immunization, mice were sacrificed and bronchoalveolar lavage was performed according to a standard technique. Specific IgG, IgA, and IgM antibodies were detected in lavage fluid by ELISA.

4.8. Efficacy evaluation of the S. flexneri 2a Ac_3-S-LPS vaccine

To examine the Ac_3-S-LPS vaccine for induction of *S. flexneri* 2a-specific mucosal immunity, groups of 10 guinea pigs (200–250 g) were twice dorsally immunized s.c. with doses of 25 or 50 µg of vaccine or intact S-LPS at an interval of 10 days. Control animals were given saline. Ten days after the last immunization, keratoconjunctivitis was induced by inoculation into the mucosal surface of the conjunctiva of both eyes with a suspension of virulent strain *S. flexneri* 2a 1605 at a dose of 2×10^9 cells in 30 µL of sterile saline. Keratoconjunctivitis development was assessed 7 days after challenge by visual inspection. The efficacy of the Ac_3-S-LPS vaccine and S-LPS was evaluated by protection rate, percent of non-infected eyes in all groups of animals. Additional animals (10 per group) were immunized according to the same protocol with 50 µg dose of Ac_3-S-LPS or intact S-LPS to monitor immune response and bled ten days after the last immunization for evaluation of specific IgG by ELISA.

4.9. Safety monitoring of the S. flexneri 2a Ac_3-S-LPS vaccine

The protocol of the Phase I proof-of-concept double-blind randomized clinical trial of the Ac_3-S-LPS vaccine was approved by the Ethics Committee of the Ministry of Health of the Russian Federation (Approval №04-16507/09) and the adult volunteers (28–46 years old without history of shigellosis) gave informed consent. 0.5 mL of the vaccine was injected s.c. in the upper third of a shoulder to two randomized groups of 10 and 12 adult volunteers at a single dose of 25 or 50 µg, respectively. During the trial, the volunteers were under close clinical supervision and were monitored for possible minor and serious AEs, including endotoxic shock. The occurrence and types of AEs was documented in diary cards.

4.10. S. flexneri 2a Ac_3-S-LPS vaccine immunogenicity study in adult volunteers

Antibody responses were examined on day 30 and 60 after immunization with the Ac_3-S-LPS vaccine. Sera from the volunteers were separated and stored at −70 °C until assayed. Titers of *S. flexneri* 2a LPS-specific serum IgG, IgA, and IgM were determined by ELISA. Indirect haemagglutination test was performed using a commercial *S. flexneri* diagnostic kit (Microgen). The end-point titers or titer fold increase of S-LPS-specific antibodies were calculated for comparing immunogenicity of the preparations. The immunogenicity of the vaccine was also estimated by calculating the rates of 2- and 4-fold seroconversions, i.e. the frequencies of subjects with 2- or 4-fold or greater rise of LPS-specific antibodies in immune sera above the pre-vaccination level. Paired *t*-test was used to compare postvaccination responses. Differences were considered significant at $p < 0.05$.

On day 30 after vaccination with a dose of 50 µg Ac_3-S-LPS, antibody titers to the heterologous Vi-antigen composed of α1 → 4-linked 2,3-di-*N*,*O*-acetyl-D-galactosaminuronic acid did not change significantly, therefore the humoral immune response elicited by the vaccine is carbohydrate-specific and is targeted to LPS antigen.

4.11. Detection of pro-inflammatory cytokines in sera of volunteers

Serum levels of pro-inflammatory cytokines TNFα, IL-1, and IL-6 were determined after s.c. immunization with Ac_3-S-LPS vaccine at doses of 25 or 50 µg. Serum samples were taken prior to immunization and 2, 4, and 6 h after vaccine administration. Concentration of cytokines (pg mL^{-1}) was determined using ELISA test systems (BioSource).

4.12. Cell culture and in vitro stimulation

Normal human PBMC were isolated using gradient centrifugation in Ficoll-Paque solution (PanEco). PBMC and the human premonocytic leukemia cell line U937 were cultured in RPMI 1640 (Gibco) supplemented with heat inactivated 10% fetal bovine serum (Sigma), 2 mM L-glutamine (Sigma) and 25 µg mL^{-1} gentamicin (Gibco) in a humidified atmosphere (5% CO_2) at 37 °C.

200 µL aliquots containing 2×10^4 U937 cells or 2×10^5 PBMC were put in 96-well plates (Nunc) and incubated for 24 h in the presence of various types of LPS: Ac_3-S-LPS *S. flexneri* 2a, intact S-LPS of *S. flexneri* 2a, LPS *E.coli* O55 (Sigma-Aldrich), monophosphoryl lipid A (Sigma-Aldrich). Final concentration of LPS preparations was 1 µg mL^{-1} in U937 and 100 ng mL^{-1} in PBMC culture.

Cell-free supernatants were collected and TNFα, IL-6 and IL-8 levels were measured by commercially available ELISA kits (Vector-Best) according to the manufacturer's instructions.

4.13. Hematological and biochemical studies

Venous blood samples were collected from volunteers before, 6 h and 30 days after vaccination. Automated complete blood count, differential white blood cell counts (WBC) and biochemical analyses were performed in a centralized laboratory.

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Conflict of interest

The authors declared that there is no conflict of interest.

Author contributions

P.G.A. and V.L.L. designed research; V.A.L., V.L.L., M.E.G., A.A.M., P.G.A. performed research; P.G.A., A.L.K., V.L.L.,Y.A.K.,V.A.L., M.E.G., A.A.M analysed data and P.G.A., A.L.K., Y.A.K., V.A.L., V.L.L., M.E.G., A. A.M wrote the paper.

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