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Comparison of serological specificity of anti-endotoxin sera directed against whole bacterial cells and core oligosaccharide of *Escherichia coli* J5-tetanus toxoid conjugate★✪

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The rough mutants of Gram-negative bacteria are widely used to induce protective antisera but the nature of the target epitope for such antibodies is not precisely defined. Endotoxin is one of several antigens present on the surface of bacterial cells, which are able to elicit specific antibodies. We studied the specificity of antibodies produced against a conjugate of *E. coli* J5 endotoxin core oligosaccharide with tetanus toxoid. The use of chemically defined antigen for immunisation excludes the possibility of production of antibodies against other cell surface antigens. A comparison of this monospecific anti-endotoxin serum with antiserum against *E. coli* J5 whole cells was performed in order to distinguish the role that endotoxin core oligosaccharide plays in the interaction with humoral host defences from that of other potentially important Gram-negative bacterial surface antigens. The reactivity of both sera with smooth and rough lipopolysaccharides was determined in ELISA, immunoblotting

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Abbreviations: Ab, antibody; FAB-MS, fast atom bombardment mass spectrometry; FACS, fluorescence activated cell sorter; FCS, fetal calf serum; GLC-MS, gas liquid chromatography-mass spectrometry; IgG, immunoglobulin G; IL-6, interleukin 6; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; OMP, outer membrane protein; NO, nitric oxide; OS, oligosaccharide; OS J5-TT, *E. coli* J5 core oligosaccharide-tetanus toxoid conjugate; PBS, phosphate-buffered saline; TNFα, tumour necrosis factor.

and by flow cytometry. Both antisera reacted with similar specificity with most lipopolysaccharides of identical or related core type. Less distinct reactions with endotoxins of the antibacterial serum in comparison with the anti-conjugate serum were found in all serological tests. LPS of *E. coli* O100 that showed the strongest reactions with both sera was used to stimulate IL-6, TNFα and nitric oxide production by the J-774A.1 cell line. Both sera were used to inhibit that stimulation and no inhibitory effects of the examined sera in comparison with non-immune serum were observed.

Lipopolysaccharide (LPS) is the main component of the outer membrane of the cell envelope of Gram-negative bacteria. Lipopolysaccharide is responsible for the initiation of endotoxic shock, therefore this molecule is a target for new preventive and therapeutic strategies. It has been found that endotoxins derived from different bacterial species share common basic structure (Holst *et al.*, 1996). LPS consists of a polysaccharide part that includes an O-specific chain and core oligosaccharide (OS) covalently linked to lipid A. Most of the antibodies produced during immunisation with bacterial cells are directed against the O-specific part of endotoxin. The variability of O-serotypes among bacteria of one species excludes the use of anti-LPS antibodies as broad-protective candidates for therapeutic intervention in the case of infection. The core oligosaccharide is a conserved part of endotoxins. Structural analysis of lipopolysaccharides isolated from different strains of bacteria show lower variability among the core oligosaccharide parts in comparison with O-specific chains. These findings have prompted investigators to use rough mutants that lack the O-specific polysaccharide in an attempt to induce protective anti-core endotoxin antibodies. Such anti-core antibodies obtained as a result of immunisation with whole cells of rough mutants can recognise core oligosaccharide epitopes in the LPS structure on the surface of bacterial cells.

E. coli J5 is the most frequently studied rough mutant, isolated by Elbein and Heath (Elbein & Heath, 1965; Ziegler *et al.*, 1973) from a parent culture of *E. coli* serovar O111:B4. *E. coli* J5 produces LPS that lacks O-specific chain and possesses an incomplete core oligosaccharide of the Rc chemotype (Muller-Loennies *et al.*, 1994; Muller-Loennies *et al.,* 1999). Numerous experiments have been performed to examine therapeutic properties of antibodies directed against *E. coli* J5 whole cells during the last three decades. However, the results obtained by different groups were contradictory, regarding the existence of these broadly cross-reactive and cross-protective antibodies and the nature of the target epitope (Hustinx *et al.*, 1997). Several explanations of the lack of cross-protective activity of antibodies against whole cells of *E. coli* J5 were proposed. The heterogeneity of *E. coli* J5 vaccine and protection *via* anti-OMP (outer membrane proteins) antibodies are the main reasons of such a discrepancy in outcomes reported for cross-protection studies with *E. coli* J5 antisera. The antigenic and immunogenic differences in LPS of *E. coli* J5 vaccine strains depend on their origin and on the growth conditions. Some of the examined strains were identical with the original J5 strain and were unable to synthesise LPS with complete core structure substituted with O-specific polysaccharide. Several strains were found to contain LPS of the R3 chemotype while several others expressed complete R3 core structure and O-antigen, as a result of a reversion of the J5 mutant to the smooth form (Appelmelk *et al.*, 1986; Evans *et al.*, 1992; Appelmelk *et al.*, 1993). It was shown that antiserum against *E. coli* J5 contains antibodies reactive with OMP of heterogeneous Gram-negative bacteria. These results suggested that some of the *in vivo* protection described by earlier investigators could be conferred by anti-OMP IgG (Brauner *et al.*, 1986; Hellman *et al.*, 1997; Hellman & Warren, 1999; Hellman *et al.*, 2000). These findings prompted us to compare the specificity of

anti-*E. coli* J5 antiserum with antibodies obtained against chemically defined *E. coli* J5 LPS core oligosaccharide conjugated with tetanus toxoid (OS J5-TT).

Several antigens present on the surface of bacteria are able to elicit specific antibodies during immunisation with bacterial cells. We examined the immunogenicity and cross-reactivity of covalent conjugates of core oligosaccharides of *E. coli* R1, R2, R3, J5 and *Salmonella* Ra LPSs in our previous study (Lugowski *et al.*, 1996a; 1996b). The covalent conjugate of *E. coli* J5 core oligosaccharide with tetanus toxoid is an antigen with defined structure. The use of this conjugate to elicit anti-bacterial serum excludes the possibility of antibodies production against additional cell surface antigens such as OMP, *E. coli* R3 complete core structures and O-specific chains of *E. coli* O111:B4 LPS. We obtained and compared rabbit sera against *E. coli* J5 and the glycoconjugate (OS J5-TT).

This work was carried out in order to get insight into the role that endotoxin core oligosaccharide, a potentially important Gram-negative bacterial surface antigen, plays in the interaction with humoral host defences.

MATERIALS AND METHODS

Bacteria. The rough mutant strains of *E. coli* and *Salmonella typhimurium* were kindly provided by Prof. Helmut Brade (Research Center Borstel, Germany). The strains of *Bordetella pertussis* were obtained from the Sera and Vaccines Central Research Laboratory (Warsaw, Poland). *Plesiomonas shigelloides* O54 was obtained from the collection of the National Institute of Public Health (Prague, Czech Republic). Other strains of smooth bacteria were obtained from the collection of the Institute of Immunology and Experimental Therapy (Wrocław, Poland). Bacteria were grown in liquid medium, killed with 0.5% phenol and centrifuged using a CEPA flow laboratory centrifuge (Petersson *et al.*, 1997).

Tetanus toxoid. Tetanus toxoid preparation was obtained from Biomed (Kraków, Poland).

Cell lines. Cells of the mouse macrophage-like cell line J-774A.1 were obtained from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany), the TNFα-sensitive WEHI 164.13 mouse fibrosarcoma cells were kindly donated by Prof. Michał Zimecki (Institute of Immunology and Experimental Therapy, Wrocław, Poland). Murine macrophage cell line J-774A.1 cells were grown in Dulbecco's medium supplemented with 10% foetal calf serum (FCS) (Gibco, Biocult, Glasgow, U.K.). WEHI 164.13 cells were grown in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM pyruvic acid and 4 mM 2-mercaptoethanol.

Lipopolysaccharide and core oligosaccharides. LPS of *E. coli* J5 was extracted from bacterial cells by the phenol/chloroform/petroleum ether procedure (Galanos *et al.*, 1969). The yield of LPS was 2% of dry bacterial mass. LPS was degraded by treatment with 1.5% acetic acid at 100°C for 1 h. The reaction mixture was centrifuged to separate lipid A and supernatant was freeze-dried as described earlier (Niedziela *et al.*, 1996). The water-soluble mixture of oligosaccharides was fractionated on a Bio-Gel P-10 column (1.6 × 100 cm, equilibrated with 0.05 M pyridine/acetic acid buffer, pH 5.6) (Petersson *et al.*, 1997). Eluates were monitored with a Knauer differential refractometer. The mixture of oligosaccharides eluted from Bio-Gel P-10 was separated on a Bio-Gel P-2 column (1.8 × 90 cm, in the same buffer). Three fractions were eluted, freeze-dried and checked by ^{1}H NMR and matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF).

Analytical procedures. Sugars were analysed as their alditol acetates by GLC-MS (gas liquid chromatography-mass spectrometry). Methylations were performed according to the method of Hakomori (1964) and methylated sugars were analysed by GLC-MS as pre-

viously described (Petersson *et al.*, 1997). GLC-MS was carried out with a Hewlett-Packard 5971A system using the HP-1 fused-silica capillary column (0.2 mm × 12 m) and the temperature program 150– 270°C at 8°C min^{-1}. Dephosphorylation was carried out by treatment of core oligosaccharide sample (5 mg) with 48% HF (1 ml) at 4°C for 3 days.

Mass spectrometry. MALDI-TOF, in positive and negative ion mode, was run on a Kratos Kompact-SEQ instrument. 2,4,6-Trihydroxyacetophenone monohydrate (1% in acetonitrile/water, 1:1, v/v) was used as matrix for the analysis of the core oligosaccharide of *E. coli* J5 LPS. Fast atom bombardment (FAB) mass spectra were recorded on a JEOL JMS-SX/SX-102 A four sectors tandem mass spectrometer by bombardment of samples, dissolved in a glycerol matrix, with Xe atoms of the average translational energy of 6 keV.

NMR spectroscopy. NMR spectra of the oligosaccharides were obtained for ^2H_2O solutions at 35°C on a Bruker DRX 600 spectrometer. All spectra were obtained using acetone (δ_H 2.225 and δ_C 31.00) as internal reference. In the ^{31}P NMR experiments, 80% phosphoric acid was used as external reference. Oligosaccharides were repeatedly exchanged with ^2H_2O with intermediate lyophilization prior to analysis. The data were acquired and processed using standard Bruker software.

Preparation of core oligosaccharide conjugate with tetanus toxoid (TT). The conjugation was carried out according to the method of Jennings and Lugowski using reaction of reductive amination (Jennings & Lugowski, 1982). The core oligosaccharide was oxidised with 0.75% $NaIO_4$ (1 ml) in darkness at 4°C for 30–45 min. The excess of periodate was destroyed by adding ethylene glycol. After purification on a Sephadex G-25 (2.6 × 90 cm) column equilibrated with 0.02 M pyridine/CH_3COOH buffer, pH 5.4, the product was freeze-dried. The oxidised core oligosaccharide of *E. coli* J5 LPS was dissolved in 0.5 M K_2HPO_4, pH 9.0 (1 ml). Tetanus toxoid (3 mg), $NaBCNH_3$ (10 mg) and one drop of chloroform were added to the solution. The reaction mixture was kept in sealed vials for 12 days at 37°C and then applied to a Sephadex G-100 column (1.6 × 100 cm) equilibrated with phosphate-buffered saline (PBS), pH 7.5. Fractions containing the conjugate were concentrated by ultrafiltration.

Immunisation procedure. Rabbits were immunised with 50 μg of the conjugate suspended in Freund's complete adjuvant at days 0 and 21 as described (Lugowski *et al.*, 1996b). The animals were bled 14 days after the second injection. Polyclonal antibodies against the whole bacterial cells of *E. coli* J5 were obtained by intravenous immunisation of rabbits with freeze-dried bacteria suspended in PBS as described (Lugowski & Romanowska, 1978).

Microprecipitin test. Quantitative microprecipitin test was carried out by the method of Kabat & Mayer (1967). The reaction mixture contained in addition 2% polyethylene glycol 6000.

SDS/PAGE was carried out by the method of Laemmli (1970) with modifications (Romanowska *et al.,* 1988) and the LPS bands were visualised by silver staining (Tsai & Frasch, 1982).

ELISA and ELISA inhibition test. ELISA was performed by a modification of the method of Voller *et al.* (1975) as previously described (Lugowski *et al.*, 1996b).

In the inhibition studies the antiserum (100 μl) at concentration twice as high as that giving A_{405} in the range 0.8–1.1 was mixed with 100 μl of inhibitor solution and incubated for 1 h at 37°C. The mixture (100 μl) was transferred into the wells of a microtiter plate coated with *E. coli* J5 LPS and the reaction was carried out at room temperature for 15 min. The washing of the wells, incubation with second antibody conjugated with alkaline phosphatase and colour development were performed as described earlier (Lugowski *et al.*, 1996b).

Immunoblotting. Immunoblotting was performed on the SDS/PAGE separated LPS fractions as described earlier (Lugowski *et al.*, 1996a).

Immunofluorescence procedures. Bacteria were cultured in nutrient broth to logarithmic phase of growth, harvested, washed and suspended in PBS to an A_{600} of 0.6 (4×10^8 cfu ml^{-1}). Bacteria were mixed with antiserum (0.5 ml, 100 times diluted) and incubated at room temperature for 2 h. Non-immune sera were employed as negative controls. After three-times washing with PBS the bacteria were incubated for 3 h with FITC (fluorescein isothiocyanate)-labelled goat anti-rabbit IgG as a detecting antibody. The bacteria were washed with PBS and suspended in 0.5% paraformaldehyde (Lugowski *et al.*, 1996b).

Fluorescence activated cell sorter analysis. FACS analysis was performed using a FACSscan fluorescence-activated cell sorter (Becton Dickinson; argon-ion laser 488 nm at 14 mW). Twenty-thousand bacteria were evaluated in each analysis. Narrow angle forward light scatter and FITC (green fluorescence) emission signals were collected. Bacterial aggregates were electronically excluded on the basis of light scatter signals (Lugowski *et al.*, 1996b).

Inhibition of TNFα, IL-6 and NO release in J-774A.1 cells. J-774A.1 cells were plated in 24-well tissue culture plates (Nunc, Denmark) at 1×10^6 cells/well in 1 ml of Dulbecco's medium supplemented with 10% (v/v) FCS, incubated 16 h and washed twice with serum free medium. The lipopolysaccharide isolated from smooth strain of *E. coli* O100 (10 ng) was used as a stimulant. The cells were incubated in 1 ml of medium with: (A) LPS and anti-OS J5-TT conjugate rabbit serum (100 μl), (B) LPS and anti-*E. coli* J5 serum (100 μl), (C) LPS and non-immune serum (100 μl) and (D) PBS. The supernatants were collected to determine TNFα (after 4 h), IL-6 (after 24 h) and total concentration of nitrite and nitrate as stable metabolites of NO (after 48 h) and stored at –80°C until determination.

TNFa bioassay. TNFα concentration in the supernatants was determined in the cytotoxic assay using the WEHI 164.13 bioassay (Espevik *et al.*, 1986). Briefly, WEHI 164.13 cells were seeded at a concentration 2×10^4 cells/well in a 96-well plate (Nunc, Denmark). Decreasing dilutions of the assayed supernatants in RPMI 1640 medium (25 μl) were added to the target cells in the presence of actinomycin D (1 μg ml^{-1}). After incubation for 20 h, cell viability was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric assay. The concentration of TNFα was expressed as pg ml^{-1}, using mouse recombinant TNFα (Pharmingen, U.S.A.) as a standard. The detection limit of the WEHI 164.13 cell cytotoxicity assay was approximately 0.15 pg ml^{-1}.

IL-6 assay. IL-6 activity was determined using the OptEIA^{TM} kit (Pharmingen, U.S.A.). The test was performed using polystyrene microtiter plates (Maxisorb, Nunc, Denmark) according to the instructions provided. TMB (3,3′,5,5′-tetramethylbenzidine) reagent set provided by Pharmingen was used as a substrate for horseradish peroxidase. The absorbance was measured at 450 nm using a Behring EL311s microplate reader.

Nitric oxide determination. Total nitrite + nitrate (NO_2^- + NO_3^-) in the supernatants collected from stimulated J-774A.1 cells was determined by the Griess reaction in 100 μl of the test samples. Nitrate was reduced by nitrate reductase. Briefly, 50 mU (5 μl) of nitrate reductase (EC 1.6.6.2) and 1 μl of NADPH (stock, 0.65 mg in 100 μl) in Tris/HCl buffer, pH 7.5, were added to the tested supernatant (100 μl). The reaction was carried out at 25°C for 30 min, followed by the colorimetric test using Griess reagent. Samples were incubated for 10 min at room temperature with 100 μl of Griess reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl)-ethylene-diamine dihydrochloride in 5% H_3PO_4). Absorbance was measured at 540 nm using a

Behring EL311s microplate reader. NO_2^- concentrations were calculated by comparison with a standard curve prepared using $NaNO_2$ (Schmidt, 1995).

MTT colorimetric assay for cell growth / cell killing. The method was performed according to Mosmann (1983) with a modification (Hansen *et al.*, 1989). Briefly, MTT (5 mg ml^{-1} stock solution) was added (25 μl per well) at the end of the assay and after 3 h of incubation at 37°C in 100% humidity, 100 μl of lysis buffer (20% SDS, 50% dimethylformamide, pH 4.7) was added. After additional overnight incubation, absorbance was measured at 562 nm.

Statistical analysis. Assays were performed using triplicate cultures. Data are expressed as means ± standard deviation (± S.D.) and differences between them were analysed using Student's *t*-test. Differences at $P < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Structural analysis of the core oligosaccharide

Core oligosaccharides were purified by gel filtration on BioGel P-10 of the water soluble fraction obtained after mild acid hydrolysis of *E. coli* J5 lipopolysaccharide and further purified on a BioGel P-2 column. The results of methylation analysis of dephosphorylated oligosaccharides indicated the presence of five sugar residues: terminal Glc, 3-substituted heptose Hep, terminal GlcN, 7-substituted Hep and 3,7-substituted heptose. All of the ^1H and ^{13}C NMR spectra of the isolated core oligosaccharides contained main signals for five anomeric protons and carbons, and in addition a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) spin system confirming hexasaccharide structures (not shown). The ^{31}P NMR spectra indicated heterogeneity among the isolated core oligosaccharides. The observed heterogeneity concerned the substitutions of phosphate esters, diphosphomonoesters, diphosphodiesters and ethanolamine.

The positive mode FAB mass spectrum of the mixture of core oligosaccharides obtained after mild acid hydrolysis of LPS was similar to the spectrum obtained by Muller-Loennies *et al.* (1994; 1999) (Fig. 1A). Ions at *m/z* 1218.04 $(\mathbf{M}+P+H)^+$ and 1341.03 $(\mathbf{M}+P+PEtn+H)^+$ confirmed the calculated molecular mass of the core hexasaccharide (**M**) of LPS J5 (Fig. 1B) substituted with one phosphate group (P) and an additional 2-aminoethanol

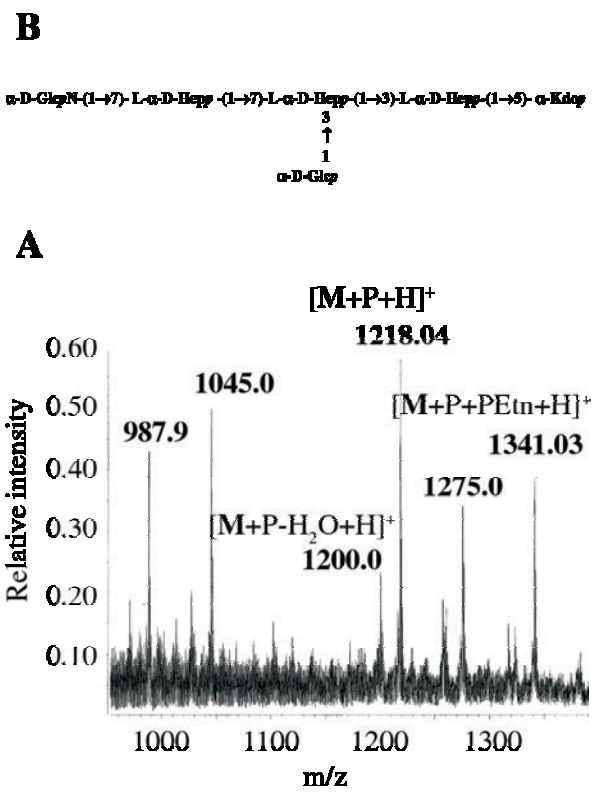

Figure 1. (A) Fragment of FAB-MS mass spectrum of the core oligosaccharide fraction obtained by mild acid hydrolysis of *E. coli* J5 LPS.

The core oligosaccharide fraction was obtained by mild acid hydrolysis of LPS with 1.5% acetic acid. The reaction mixture was centrifuged to separate lipid A and freeze-dried supernatant was fractionated by gel filtration. The spectrum was obtained in the positive mode using glycerol as a matrix. **(B)** Structure of the core hexasaccharide (**M,** molecular ion) of LPS *E. coli* J5 as reported (Muller-Loennies *et al.*, 1994; 1999). **α-D-Glc*p*N,** 2-amino-2-deoxy-D-glucopyranose (L-glucosamine); **α-D-Glc*p*,** α-D-glucopyranose; **L-α-D-Hep*p*,** L-*glycero*-α-D-*manno*-heptopyranose; **α-Kdo*p*,** 3-deoxy-α-D-*manno*-oct-2-ulopyranosonic acid.

phosphate (PEtn). The ion at *m/z* 987.9 represented an oligosaccharide composed of four sugar residues – two Hep, one Glc and one Kdo substituted with a phosphate group and an additional 2-aminoethanol phosphate.

The molecular ions at *m/z* 1045.0 and 1275.0 (Fig. 1B) corresponded to the glycoforms additionally substituted with a glycine residue as indicated by the mass difference of 57 Da in comparison with the ions at *m/z* 987.9 and 1218.04, respectively. Similar results were also obtained by MALDI-TOF mass spectrometry. Structural data confirmed the known structure of the core oligosaccharide isolated from *E. coli* J5 LPS and its heterogeneity that is a result of differences in the primary structure of the carbohydrate backbone and non-stoichiometric substitutions by phosphate residues and PEtn. The presence of glycine in the core region of LPS was observed previously (Li *et al.*, 2001; Niedziela *et al.*, 2002) and it contributes to the overall heterogeneity of the core oligosaccharides.

The *E. coli* J5 core oligosaccharide-tetanus toxoid conjugate

The core oligosaccharide was selectively oxidised in the Hep-Kdo region to achieve, on average, a single aldehyde group per molecule. The modified core oligosaccharide was used to obtain a covalent conjugate with tetanus toxoid using reductive amination. The calculated molar ratio of the core oligosaccharide to tetanus toxoid was 9:1.

The anti-conjugate sera

The conjugate and killed *E. coli* J5 bacteria were used to immunise rabbits in order to obtain anti-LPS antibodies. The antibody level in the immune sera was determined in a quantitative microprecipitin test with homologous lipopolysaccharide. *E. coli* J5 LPS precipitated in the equivalent point 1.6 mg of Ab ml^{-1} of anti-conjugate serum and 0.7 mg of Ab ml^{-1} of antiserum against whole bacterial cells. The conjugate of core oligosaccharide of *E. coli* J5 LPS was a strong immunogen yielding a high level of anti-OS core antibodies.

ELISA assay

The reaction of both sera with lipopolysaccharides isolated from rough and smooth strains of various bacteria were tested by ELISA. The reactions of the antisera with rough *E. coli* J5, R1, R2, R3, R4 and *Salmonella* Ra, Rb_1, Rb_2, Rc, Rd_1, Rd_2, Re lipopolysaccharides are shown on Fig. 2. The absorbance at 405 nm obtained with antisera 800-fold, 3200-fold and 25600-fold diluted

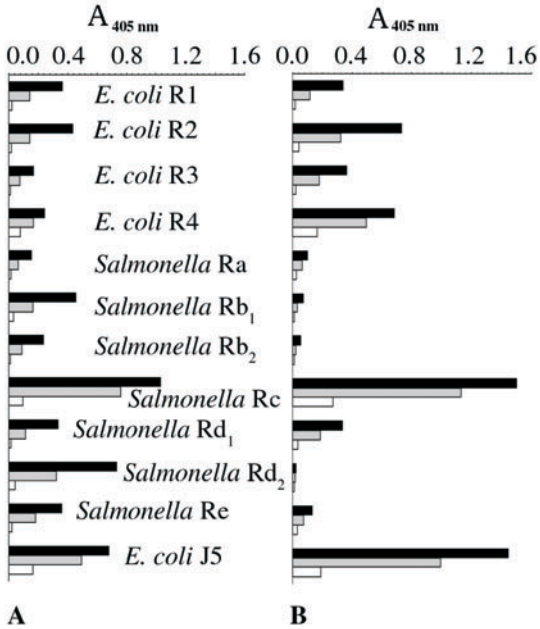

Figure 2. Comparison of the reaction of anti-*E. coli* J5 serum (A) and anti-*E. coli* OS J5-TT serum (B) with different lipooligosaccharides isolated from rough strains of bacteria in enzyme-linked immunosorbent assay (ELISA).

Wells were coated using 10 μg/ml solutions of lipooligosaccharides. Casein (2%) was used to block the surface of the plate and the wells were filled with serial two-fold dilutions of the sera (initial dilution: 1:100). Goat anti-rabbit IgG conjugated with alkaline phosphatase as a second antibody and *p*-nitrophenylphosphate as substrate were used for colour development. The figure shows A_{405} values for (■) 800-fold, (▩) 3200-fold, (❑) 25600-fold serum dilutions. Data represent the means of three replicates. Standard deviations did not exceed 5% and is not shown.

were compared. Both sera reacted strongly with homologous LPS of *E. coli* J5 and *Salmonella* Rc, but the cross-reaction profiles were different for each serum. Lipooligosaccharide of *Salmonella* Rd_2 reacted strongly with the serum against *E. coli* J5. The anti-endotoxin serum raised against the core oligosaccharide-tetanus toxoid conjugate showed broader specificity. This serum also reacted strongly at high concentration with LPS of *E. coli* representing R2 an R4 core type. Weak reactions were observed for the same LPSs with the anti-endotoxin serum obtained against *E. coli* J5. The strong reaction of the anti-*E. coli* J5 serum with LPS possessing Rd_2 core structure and even the Re type, suggested that deep core epitopes exposed on *E. coli* J5 bacterial cells are responsible for the induction of specific antibodies that are able to react with the Kdo-heptose region of LPS. The *E. coli* J5 core oligosaccharide present in the conjugate contained only one Kdo residue at the reducing end and was linked to the protein through a modified Kdo-heptose region. Such an immunogen exposes mainly epitopes of the terminal hexose region and induces antibodies against Rc and complete core structures.

Lipopolysaccharides isolated from various bacterial strains were used in ELISA to determine the ability of both antisera described above to recognise core epitopes in the structure of LPS of smooth bacteria (Fig. 3). LPSs of *E. coli*, *P. shigelloides*, *Klebsiella pneumoniae*, *Citrobacter freundii*, *S. typhimurium*, *Hafnia alvei*, *Shigella sonnei* Ph I and *B. pertussis* presenting different types of core oligosaccharides were used in ELISA. Both antisera showed similar specificity in the reaction with most of the lipopolysaccharides isolated from smooth and rough bacterial strains representing identical (*E. coli* O39, O64, *S. sonnei* Phase I and II, *S. flexneri* 2b) or related (*E. coli* O14, O100, *H. alvei* PCM 31 and 1207) inner core structure. The less distinct reactions with endotoxins of the antibacterial serum in comparison to the anti-conjugate serum were also found in this experiment.

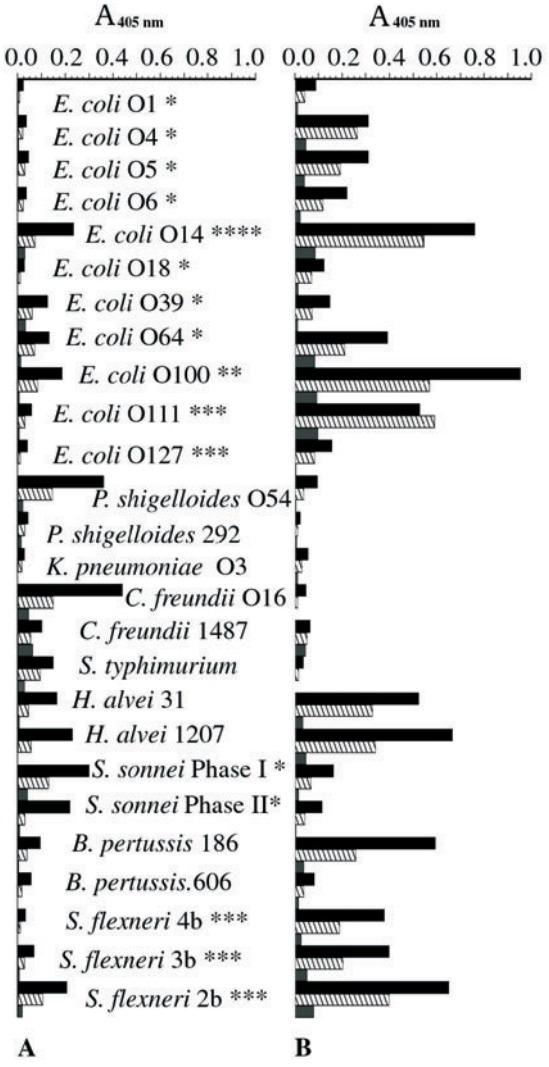

Figure 3. Comparison of the reaction of anti-*E. coli* J5 serum (A) and anti-*E. coli* OS J5-TT serum (B) with different LPSs of smooth and rough strains of bacteria in enzyme-linked immunosorbent assay (ELISA).

The symbols (*), (**) and (***) represent R1, R2 and R3 core types of LPS, respectively. The figure shows A_{405} values for (■) 800-fold, (▨) 3200-fold, (■) 25600-fold serum dilutions. Data represent the means of three replicates. Standard deviation did not exceed 5% and is not shown. Assay conditions as in Fig. 2.

The strongest reactions of both sera were observed with LPS of *E. coli* O14, O100, *S. flexneri* 2b and *H. alvei* 1207. The anti-conjugate serum showed additionally a distinct reaction with LPS of *B. pertussis* 186. In contrast to earlier studies (Lugowski *et al.*, 1996a) this antiserum reacted strongly even at low

concentration with *E. coli* O111 LPS. The antiserum directed against *E. coli* J5, in contrast to the anti-conjugate serum, reacted distinctly with LPS of *P. shigelloides* O54, *C. freundi* O16 and *S. sonnei* Ph I. Antibodies present in this serum did not recognize epitopes in the LPS of *E. coli* O111:B4. These results are in agreement with published data (Sakulramrung & Domingue, 1985; Appelmelk *et al.*, 1993).

Immunoblotting

In order to explain which region of lipopolysaccharides is involved in the reaction with specific sera the reactivity of the anti-conjugate and anti-*E. coli* J5 serum with lipopolysaccharides of various strains of smooth bacteria was compared in immunoblotting experiments. Lipopolysaccharides were separated by SDS/PAGE and transblotted from the gel onto nitrocellulose. All of the LPSs of smooth strains showed a high molecular mass ladder-like pattern of bands in SDS/PAGE analysis (Fig. 4A).

All the observed reactions with the anti-conjugate serum concerned mainly the fast migrating LPS fractions non-substituted with O-specific chains. Antibodies against OS J5-TT conjugate reacted with LPSs possessing identical (*E. coli* O1, O18, O64, O111, *S. sonnei* Phase I) and related (*E. coli* O10, O100, *H. alvei* PCM 31 and 1207, *B. pertussis* 186 and 606) inner core oligosaccharide. The lack of structural similarity between core oligosaccharide of *P. shigelloides* CNCTC 113/92 and *E. coli* J5 was responsible for the absence of reactions of that LPS with anti-conjugate serum. Both sera reacted strongly with core regions of LPS of *E. coli* O100, *H. alvei* 1207 and 31 (Fig. 4B and C). Lipopolysaccharides showing strong reactions in ELISA only with the anti-*E. coli* J5 serum, reacted in immunoblotting in the regions of slow migrating fractions. The immunoblotting pattern suggested that slight protein contaminants present in some LPS samples were involved in the observed reactions. LPS fractions obtained during phenol/water extraction contain only traces of proteins not detectable by standard analytical procedures such as SDS/PAGE silver-staining method, UV spectrophotometric assay and MALDI-TOF mass spectrometry. LPS of *C. freundii* was the only one that also reacted with the anti-*E. coli* J5 serum in the region of fast migrating fractions.

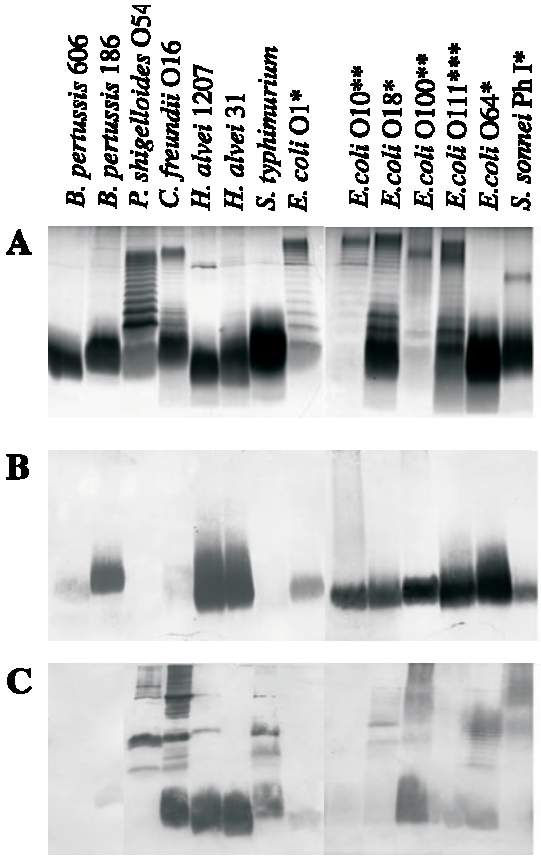

Figure 4. Silver-stained SDS/PAGE of various lipopolysaccharides (A). Immunoblots of the lipopolysaccharides with anti-OS J5-TT conjugate serum (B) and with anti-*E. coli* J5 serum (C).

LPSs were separated by SDS/PAGE. Suspensions (1 mg ml^{-1}) were mixed with sample buffer and 8 μl portions of LPSs were applied to a gel. Electrophoresis was performed in a 15% acrylamide slab gel. LPSs were detected by silver staining or were analysed by blotting with antibodies directed against anti-OS J5-TT conjugate (200-fold diluted).

Flow cytometry (FACS)

The ability of the sera examined to recognise core oligosaccharide epitopes of LPS on the surface of live bacteria were observed using

FACS analysis (Fig. 5). Three smooth strains were chosen for the experiment: *E. coli* O39, O100 and O111 representing LPS of the R1, R2 and R3 core types, respectively. The percentage of bacteria exhibiting fluorescence above background level was in excess of 95% for all tested strains and the ability of both sera to bind to bacterial cells was similar. The highest intensity of fluorescence was observed for *E. coli* O100. The similar reactivity of the anti *E. coli* J5 serum, containing lower level of anti-LPS antibodies than present in the anti-conjugate serum, is probably caused by the presence of antibodies against other bacterial surface antigens. This, being in agreement with earlier studies, suggests that antibodies directed against outer membrane proteins can play important role in the cross-reactivity of the anti-*E. coli* J5 serum.

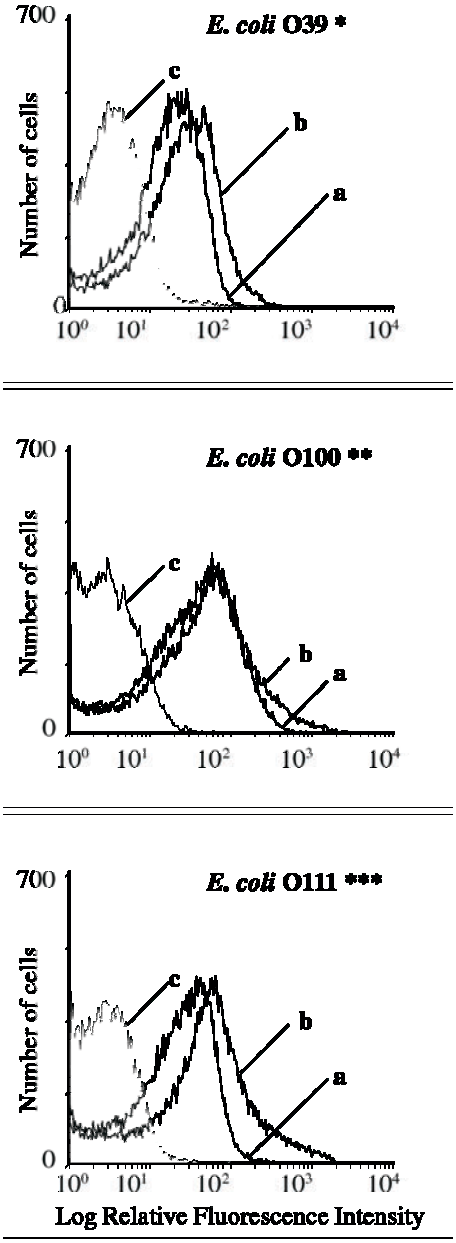

Figure 5. FACS analysis of the binding to smooth intact bacteria (*E. coli* O39, O100, O111) of (a) anti-*E. coli* J5 antibodies, (b) anti-OS J5-TT conjugate antibodies and (c) antibodies from non-immune rabbits.

Bacteria were suspended in PBS to an absorbance of 0.6 at 600 nm (4×10^8 cfu ml^{-1}) and mixed with antiserum. All antisera were 100-fold diluted. Non-immune sera were employed as negative controls. The symbols (*), (**) and (***) stand for R1, R2 and R3 core types of LPS, respectively.

ELISA inhibition assay

Core oligosaccharides of various core types were isolated from *E. coli* and *Salmonella* lipopolysaccharides (Fig. 6). The inhibitory ac-

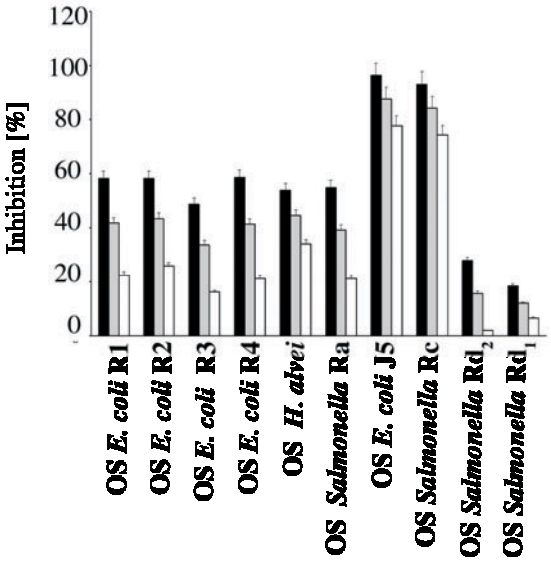

Figure 6. Inhibitory effect of different core oligosaccharides on the reaction of anti-OS J5-TT serum with *E. coli* J5 LPS in ELISA.

The antiserum (100 μl) at a concentration twice as high as that giving A_{405} in the range 0.8–1.1 was mixed with 100 μl of serial two-fold dilutions of inhibitor and transferred into wells coated with *E. coli* J5 LPS (10 μg/ml, 100 μl). The figure shows the results for concentrations of inhibitors: (■) 200 μg ml^{-1}, (▩) 50 μg ml^{-1}, (❑) 10 μg ml^{-1}. Data represent the means of three replicates. Standard deviation did not exceed 5% and is not shown.

tivity of isolated oligosaccharides in reactions between the anti-conjugate serum and *E. coli* J5 LPS was compared by ELISA. The reactions were inhibited by 77% to 100% by *E. coli* J5 and *Salmonella* Rc core oligosaccharide at 10–200 μg ml^{-1}. The oligosaccharides representing *E. coli* R1, R2, R3, R4, *Salmonella* Ra and *H. alvei* core types showed similar activity, i.e. they inhibited the binding of the anti-conjugate serum to *E. coli* J5 LPS. The inhibition for these oligosaccharides was in the range of 15–60% for the concentrations 10–200 μg ml^{-1}. The inhibitory activity of Rd core type oligosaccharides was significantly lower. These data suggested that immunisation with the OS J5-TT conjugate did not stimulate rabbits to produce antibodies directed against the heptose region of LPS which was probably not properly exposed in this immunogen.

Inhibition of cytokines and NO induction

LPS of *E. coli* O100 that showed the most distinct reactions with both sera in serological tests was used to stimulate IL-6, TNFα and NO production by the macrophage-like cell line J-774A.1. The anti-conjugate OS J5-TT and anti-*E. coli* J5 sera were used to inhibit this stimulation. The results are shown in Fig. 7. There were no inhibitory effects of the sera examined in comparison with non-immune serum.

It was shown in numerous previous studies (Dunn & Ferguson, 1982; Bhattacharjee *et al.*, 1994; 1996; Hogan *et al.*, 1999; Cross *et al.*, 2001) that anti-rough mutant *E. coli* J5 serum containing anti-LPS antibodies protected against challenge with heterologous Gram-negative bacteria or smooth LPS. We expected that anti-OS J5-TT serum possessing higher level of anti-J5 LPS core oligosaccharide antibodies than anti-*E. coli* J5 serum would show a better protective activity. Instead, we found that both sera were inactive even if the lipopolysaccharide of *E. coli* O100 was chosen for the challenge. Our earlier studies showed that antiserum obtained against a conjugate of the core oligosaccharide of LPS *E. coli* R1 with tetanus toxoid (OS R1-TT) attenuated the release of LPS-induced TNFα *in vitro* as well as *in vivo* (Lugowski *et al.*, 1996b).

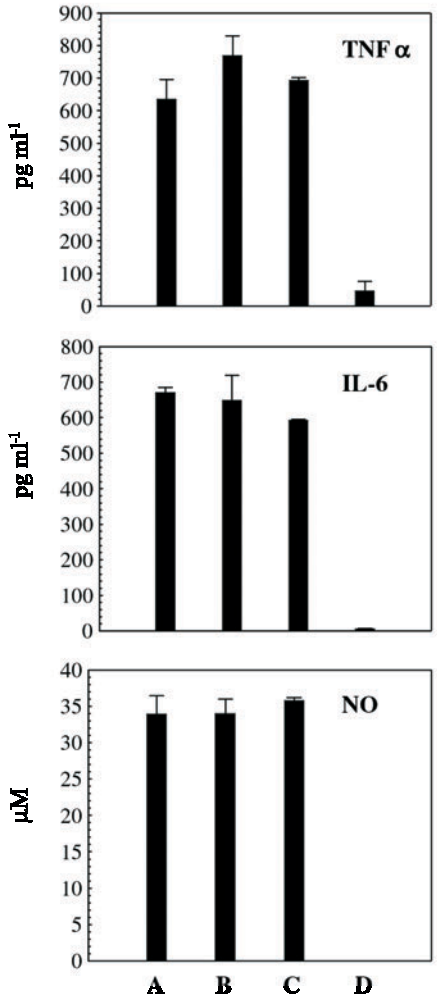

Figure 7. Effect of anti-OS J5-TT conjugate and anti-*E. coli* J5 serum on TNFα, IL-6 and NO production by J-774A.1 macrophages stimulated with LPS of *E. coli* O100.

Murine macrophage cell line J774A.1 (1×10^6 cells/well) was stimulated with 10 ng of LPS. Supernatants were collected after 4 h, 24 h and 48 h for TNFα, IL-6 and NO determination, respectively. TNFα concentration in the supernatants was determined in the cytotoxic assay using WEHI 164.13 bioassay. IL-6 activity was determined using the OptEIA^{TM} kit. Total nitrite (NO_2^-) concentration in the supernatants was determined by the Griess reaction in 100 μl of the test samples. Nitrate was reduced by nitrate reductase. **(A)** LPS + anti-OS J5-TT serum, **(B)** LPS + anti-*E. coli* J5 serum, **(C)** LPS + non-immune serum, **(D)** PBS + medium. Data are expressed as means ± S.D. of three replicates.

Lipooligosaccharide isolated from *E. coli* R1 possess complete structure of core OS containing both hexose and heptose regions. Antibodies against OS R1-TT serum recognise the complete core region present in LPSs of smooth bacterial strains. *E. coli* J5 is a rough mutant possessing non-complete core oligosaccharide of Rc type. This structure is an inner part of the complete core and is substituted with the sugars of the hexose region in the LPS of smooth bacterial strains. It may be assumed that antibodies directed against sugars present at the non-reducing end of complete core structure are responsible for the protective activity of the anti-conjugate serum. Antibodies of such specificity are not present in anti-OS J5-TT serum. This is in agreement with some earlier suggestions of other authors (Appelmelk *et al.*, 1993; Appelmelk *et al.*, 1986; Evans *et al.*, 1992) that the protective effects of anti-*E. coli* J5 serum were caused by the presence of bacterial revertants with complete R3 core type in the vaccine. The strain of *E. coli* J5 used in our experiments possesses only non-complete core region in LPS, which was confirmed in the structural analysis of isolated core oligosaccharide.

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