Immunological Detection of Heterogeneous O-antigen Containing Lipopolysaccharides in *Escherichia coli*

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The components of the cell envelopes of *Escherichia coli* O1:K1, O7:K1, O18:K1 and O83:K1 strains were separated on SDS–polyacrylamide gels. Longitudinal slices (50 µm thick) of the gel were incubated with typing sera for *E. coli* O1, O7, O18 and O83, followed by detection of the bound antibodies with ¹²⁵I-labelled protein A and autoradiography. The antisera reacted with many cell envelope components of strains both with the homologous O-serotype and heterologous O-serotypes. With O-typing sera cross-reactions with heterologous cells and cells boiled for 2 h were found. Up to 40 serotype-specific bands at regular positions with molecular weights between 12000 and 100000 were demonstrated. Since these bands were also observed when purified lipopolysaccharide and unabsorbed homologous O-typing sera were used, it was concluded that these bands represented lipopolysaccharide molecules with increasing molecular weight, all of which contained O-antigen specific immunodeterminants. The band patterns were not influenced by the growth conditions of the cells or the various isolation procedures for the cell envelopes. Comparison of various strains serotyped as O18 revealed strain differences with respect to their lipopolysaccharide band patterns. In the case of O21- and O83-serotyped strains lipopolysaccharide cross-reactions, which were detected by agglutination, were analysed in detail using the gel immunoradioassay method. These cross-reactions appeared to be caused by the presence of common determinants on their lipopolysaccharides and polysaccharide-like material. The cross-reacting antibodies could be removed by cross-absorption. It is concluded that the immunological detection of lipopolysaccharides and other components of *E. coli* in gels is an important tool in (1) the control of the specificity of typing antisera, (2) the study of the nature of cross-reacting antigens and (3) the study of the nature and uniformity of the various O- and K-serotypes.

INTRODUCTION

The outer membrane of Gram-negative bacteria contains protein, phospholipid and lipopolysaccharide. Lipopolysaccharide is exclusively located in the outer monolayer of the outer membrane, directing its sugar chains into the medium (Mühlradt & Golecki, 1975). It is an important component for the structure and function of the outer membrane (Alphen *et al.*, 1976; Burnell *et al.*, 1980; Osborn, 1979).

Lipopolysaccharide molecules are composed of a lipid A part, carrying fatty acids necessary for the embedding of lipopolysaccharide in the membrane, and of a sugar chain which is hydrophilic. In many strains from natural sources this sugar chain contains the O-antigen in addition to the core part (Galanos *et al.*, 1977). *Enterobacteriaceae* containing lipopolysaccharide with O-antigen demonstrate a smooth colony morphology. In contrast, colonies of bacteria lacking this part of the lipopolysaccharide molecule have a rough appearance. The O-antigen is strongly immunogenic and forms the basis of serological typing schemes. For *Escherichia coli*, 164 O-serotypes have been described (Ørskov *et al.*, 1977), indicating that the composition of the

O-antigen is widely variable in nature. The structures of the core and lipid A part of lipopolysaccharide are much more conserved (Galanos *et al.*, 1977).

Heterogeneity in the molecular size of lipopolysaccharide within a strain has frequently been observed, but only recently has detailed information become available from the analysis of radiolabelled lipopolysaccharide in SDS-polyacrylamide gels. *Escherichia coli* O111 (Galanos *et al.*, 1977), *Salmonella typhimurium* LT2 (Goldman & Leive, 1980; Palva & Mäkelä, 1980) and *Chromatium vinosum* (Hurlbert & Hurlbert, 1977) lipopolysaccharides consist of at least 40 different molecular species with increasing numbers of repeating O-antigen subunits (Goldman & Leive, 1980). Molecules with short or long side chains are predominant. The length distribution of the lipopolysaccharide molecules is probably caused by transfer of incomplete O-antigen from the polyisoprenoid intermediate to the core-lipid A backbone during the biosynthesis of lipopolysaccharide (Goldman & Leive, 1980; Robbins & Wright, 1971).

In this paper we describe the heterogeneity of lipopolysaccharides from several invasive *E. coli* strains which were isolated from neonates with meningitis and from standard strains which are in use for O-serotyping of *E. coli*. Analysis of lipopolysaccharides was performed immunochemically on slices of SDS-polyacrylamide gels with antisera specific for lipopolysaccharide (Poolman, *et al.*, 1978; Poolman & Zanen, 1980). The implications of these results for O-serotyping are discussed.

METHODS

Strains and growth conditions. *Escherichia coli* was isolated from the cerebrospinal fluid or blood from neonates with meningitis living in The Netherlands. Most of these strains are encapsulated (K1-serotype); OK-serotypes were determined at the Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands. The reference strains *E. coli* O83:K⁻, O21:K⁻, O7:K⁻, O21:K20, O18:K76 and O4:K3 were obtained from Dr P. A. M. Guinée, Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands. *Escherichia coli* K12 strain PC1349 (Phabagen Collection, from Miss H. S. Felix, Padualaan 8, Utrecht) was used as a rough non-pathogenic strain. Cells were grown overnight in Brain Heart Infusion broth (Difco) at 37 °C under aeration unless otherwise indicated.

Isolation and characterization of cell envelopes. The procedure for the isolation of cell envelopes described by Lugtenberg *et al.* (1975) was followed. Cells were harvested by centrifugation (10 min at 10000 g) washed with 0.9% (w/v) NaCl and disrupted by ultrasonic treatment in 50 mM-Tris/HCl pH 8.0 using a Branson sonifier at 70 W. In some experiments cells were either disrupted with a French pressure cell (Burnell *et al.*, 1980) or by osmotic lysis (Heerikhuisen *et al.*, 1975). Whole cells were removed from the lysate by low speed centrifugation (20 min at 3000 g) and the cell envelopes obtained from the supernatant by ultracentrifugation (45 min at 225000 g). The cell envelopes were washed once with 2 mM-Tris/HCl pH 7.8 and the ultracentrifugation step was repeated. The pellet was solubilized in a mixture containing 62.5 mM-Tris/HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue, pH 6.8; after boiling for 5 min the solution was applied to 11% (w/v) SDS-polyacrylamide gels (Lugtenberg *et al.*, 1975) in a slab gel apparatus (Pharmacia). Proteins were stained with Coomassie brilliant blue (Fairbanks *et al.*, 1971) and (lipo)polysaccharides by employing periodic acid-Schiff staining (Segrest & Jackson, 1972).

For the immunological detection of lipopolysaccharides, unstained gels were sliced longitudinally into 50 µm slices at -30 °C in a Bright 5030 cryostat microtome. Slices were incubated with type-specific antisera and bound antibodies were visualized by (1) incubation with goat anti-rabbit IgG-peroxidase followed by enzymic staining with 3-amino-9-ethylcarbazole [the SDS-gel immunoperoxidase technique (SGIP; Poolman *et al.*, 1978)] or (2) by incubation with ¹²⁵I-labelled protein A, followed by autoradiography [the gel immunoradioassay technique (GIRA; Poolman & Zanen, 1980)].

Antisera and O-agglutination. Antisera for typing of O1, O7, O18, O21 and O83 lipopolysaccharides were obtained from the *E. coli* typing centres in The Netherlands (Rijks Instituut voor de Volksgezondheid) and in Denmark (International *E. coli* and *Klebsiella* Center, Copenhagen). OK-sera and O-sera were raised in rabbits against cells of strains 790163I (O83:K1), 780401I and 780699 (O18:K1), 800014 (O7:K1), 770706 (O1:K1) and 781123 (O21:K1) as described (Ørskov *et al.*, 1977). Sera were absorbed with equal amounts of fresh and boiled (2 h) cells of strains with an O-serotype different from that of the homologous strain. Strain O4:K3 was used for absorption of O1- and O7-antiserum; similarly strains O4:K3 and 790163I (O83:K1) were used to absorb O18-antiserum, and strains O4:K3 and 780401I (O18:K1) for O83-antiserum. Boiled cells were washed twice with 10 mM-sodium phosphate/0.1 M-NaCl buffer pH 7.4. For absorption, packed cells were suspended in serum in a 1:1 (v/v) ratio. Serum was recovered from the incubation mixture as the supernatant obtained after ultracentrifugation (30 min at 100000 g). The specificity of the antisera was analysed by means of the SGIP and/or GIRA

techniques using cell envelopes of *E. coli* O1:K1, O7:K1, O18:K1 and O83:K1 strains as antigens. This treatment was apparently sufficient to remove most of the cross-reacting antibodies. In some cases additional absorptions were required with cells (fresh or boiled) or with lysates of cells obtained by ultrasonic treatment (Lugtenberg *et al.*, 1975) in order to remove particular protein reactions.

Agglutination was performed in V-shaped microtitre trays with serial dilutions of anti-O sera, using cells boiled for 2 h as antigen. The results were read after incubation for 16 h at 50 °C (Ørskov *et al.*, 1977).

Isolation of lipopolysaccharide. Lipopolysaccharide was isolated from cell envelopes by use of the phenol/water method (Westphal & Jann, 1965) as modified by Lindberg & Holme (1972). The isolated lipopolysaccharide contained less than 1% protein based on protein staining of an SDS-polyacrylamide gel loaded with 100 µg lipopolysaccharide. It was negative in the coagglutination test for K1 polysaccharide at a concentration of 10 mg ml⁻¹ and contained less than 1% nucleic acid, based on measurements of the absorbance at 254 and 280 nm.

RESULTS

Immunological detection of O1, O7, O18 and O83 lipopolysaccharide in SDS gels

Escherichia coli strains with serotypes O1, O7, O18 and O83 are frequently isolated from patients with neonatal meningitis in The Netherlands (Alphen *et al.*, 1982). Four representative strains from our collection were chosen for this study. The protein pattern of the cell envelopes of these four strains is shown in Fig. 1*a*. Periodic Schiff staining of SDS gels resulted in a colour reaction at the front of the gel (Fig. 1*b*), where rough lipopolysaccharide is expected (Goldman & Leive, 1980). Longitudinal slices of the gel were incubated with antisera prepared for O-serotyping by agglutination. The results of the analysis with anti-O83, anti-O18, anti-O7 and anti-O1 sera followed by subsequent incubation with ¹²⁵I-labelled protein A and autoradiography are shown in Fig. 1*c*, *d*, *e* and *f*, respectively. Many cell envelope components were recognized by the antisera and extensive cross-reactions were observed with strains belonging to heterologous serotypes. This phenomenon appeared to be general for many agglutinating typing antisera which are used for O (and K) serotyping. Further absorption of anti-O1, anti-O7, anti-O18 and anti-O83 sera rendered them monospecific for the homologous strains as tested by GIRA. This coincided with a two to fourfold reduction in O-agglutination titres, mainly due to the disappearance of cross-reacting antibodies against cell envelope proteins and rough lipopolysaccharide. The GIRA results observed for the absorbed anti-O83, anti-O18, anti-O7 and anti-O1 sera are shown in Fig. 1*g*, *h*, *i* and *j*, respectively. Repeating band patterns were observed in the cell envelopes of the homologous strains. These bands have apparent molecular weights from about 12000 up to 100000 as assessed by the use of protein markers. The various serotypes were associated with different patterns; the distances between the bands and the relative intensities of the bands varied by serotype. However, most bands were found in the low (10000–25000) and high (40000–80000) molecular weight regions.

Since O-serotyping depends on determinants on lipopolysaccharide molecules we supposed that the bands probably represent a set of lipopolysaccharide molecules, each of which carries serotype-specific immunodeterminants. Therefore, the nature of the bands was further investigated by analysing purified lipopolysaccharide immunochemically. The results for O83 lipopolysaccharide are summarized in Fig. 2. A band pattern identical to the pattern obtained with cell envelopes was observed with purified lipopolysaccharide using specific anti-O83 serum (lanes 4 and 5). Moreover the same pattern was seen with crude OK-serum which contained antibodies against lipopolysaccharide and many outer membrane proteins. Similar results were obtained for O1, O7 and O18 lipopolysaccharide and the corresponding antisera. In addition, various antisera obtained from different rabbits gave identical results for each of these lipopolysaccharides. The results obtained using the GIRA technique agreed with those obtained with SGIP analyses.

The influence on the band pattern of various cell growth conditions and different procedures for the isolation of cell envelopes was analysed for O83 and O18 lipopolysaccharides. *Escherichia coli* O83:K1 strain 790163I and O18:K1 strain 780401I was harvested at (1) stationary phase (16 h after growth at 22 °C without shaking), (2) late-exponential phase ($A_{660} = 0.9$; after growth at 37 °C with vigorous agitation), and (3) stationary phase (16 h after growth at 37 °C with vigorous agitation). These variations did not result in differences in the band patterns of the

Fig. 1. Immunodetection of O1, O7, O18 and O83 lipopolysaccharides in slices of SDS-polyacrylamide gels. A gel with cell envelopes of representative *E. coli* strains from patients with meningitis was sliced and the slices were incubated with O-typing antisera. In each case cell envelopes were obtained from: lane 1, strain 790163I (O83:K1); lane 2, strain 780401I (O18:K1); lane 3, strain 780755 (O7:K1); lane 4, strain 770706 (O1:K1). The reactions between antibodies and antigens were visualized by autoradiography after incubating with ^{125}I -labelled protein A. (a) Polypeptide pattern shown by Coomassie brilliant blue staining, (b) periodic Schiff staining. (c-j) Autoradiograms obtained after incubation with the following typing sera: (c) anti-O83, (d) anti-O18, (e) anti-O7, (f) anti-O1, (g) absorbed anti-O83, (h) absorbed anti-O18, (i) absorbed anti-O7, (j) absorbed anti-O1. The positions of molecular weight marker bands are indicated.

Fig. 2. Analysis of the molecular nature of O83 band patterns. Lane 1, molecular weight markers; lane 2, protein staining of cell envelopes of strain 790163I (O83:K1); lane 3, protein staining of lipopolysaccharide isolated from strain 790163I (O83:K1); lane 4, GIRA pattern of cell envelopes and lane 5, GIRA pattern of isolated lipopolysaccharide of strain 790163I (O83:K1), both with specific O83 antiserum.

cell envelopes after reaction with specific O18 and O83 antisera. Moreover, the band patterns were not dependent on the way in which the cell envelopes were isolated. The bands were also observed when intact cells were applied to the gel, although the results were difficult to interpret because of the relatively low amount of lipopolysaccharide which is present in whole cell lysates.

Strain specificity

Several reference O18-serotype strains were analysed with anti-O18 serum by GIRA. The results for five such strains are shown in Fig. 3. In cell envelopes of four out of five strains (lanes 1, 3, 4 and 5) band patterns became visible after incubation with anti-O18 serum. The differences observed in the relative positions of the bands in the gel slices could not be ascribed to changes in the lipopolysaccharides resulting from storage (Goldman & Leive 1980) since only freshly isolated material was used. Since strain H882-79 still agglutinated with anti-O18 serum in high titres and showed a polysaccharide-like reaction (Poolman *et al.*, 1978) at the top of the gel (lane 2), the results indicate that the O18 serotype is heterogeneous.

Fig. 3. (a) Polypeptide patterns of cell envelopes shown by Coomassie brilliant blue staining. (b) Immunochemical detection by GIRA of O18 lipopolysaccharide in the cell envelopes of serotype O18 strains. In each case cell envelopes were obtained from: lane 1, strain 780401I (O18:K1); lane 2, strain H882-79 (O18:K1); lane 3, reference strain O18:K⁻; lane 4, reference strain O18:K76; lane 5, strain H755-79 (O18:K76).

Cross-reactions between strains typed as O83 and O21

Cross-reactions between O-serotypes are well known (Ørskov *et al.*, 1977). The nature of the cross-reactions between serotype O83 and O21 strains was investigated. The O83 strains were agglutinated by anti-O21 serum and vice versa (Table 1). As shown in Fig. 4*b* antiserum specific for O83-lipopolysaccharide cross-reacted with lipopolysaccharide of strain 781123 typed as O21:K1. Anti-O83 serum absorbed with boiled cells of the O21 strain 781123 lost its reactivity both in GIRA and in agglutination towards O21-lipopolysaccharide (Fig. 4*d* and Table 1). Similarly, anti-O21 serum absorbed with boiled cells of strain 790163I, typed as O83, lost its reactivity towards cell envelopes of strain 790163I in GIRA (compare Fig. 4*c* and *e*) and did not agglutinate O83 cells (Table 1). The nature of the cross-reacting antigens was different in both cases from type-specific lipopolysaccharides (compare Fig. 4*b* and *c* with *d* and *e*) since the band patterns for O83 and O21 were different. In the case of strain 790163I (O83:K1) the cross-reacting antigen is also lipopolysaccharide, since the cross-reaction was also observed with isolated lipopolysaccharide. The cross-reacting antibodies in anti-O21 serum reacted with polysaccharide-like antigens, which migrate in the top part of the gel (Poolman *et al.*, 1978).

Table 1. *Cross-reactions between O21 and O83 antisera measured by micro-agglutination*

Agglutinating strain	Agglutination titre with:			
	Anti-O21 serum*		Anti-O83 serum†	
	Unabsorbed	Absorbed	Unabsorbed	Absorbed
O21 st	≥10000	≥10000	5000	—
781123 (O21:K1)	≥10000	≥10000	2560	—
O21:K20 st	≥10000	ND	5000	—
790163I (O83:K1)	≥10000	80	5000	5000
H1137-79 (O83:K1)	≥10000	160	10000	10000
H908-78 (O83:K1)	≥10000	ND	10000	5000
O4:K3	—	—	—	—

ND, Not determined; —, no agglutination.

* Antiserum found to be specific by SGIP was used as 'unabsorbed' serum. This was further absorbed with boiled cells of strain 790163I (O83:K1).

† Antiserum found to be specific by SGIP (cf. Fig. 1) was absorbed with boiled cells of strain 781123 (O21:K1) ('absorbed').

Fig. 4. Cross-reactions observed with anti-O83 and anti-O21 sera. In each case lane 1 contains cell envelopes of strain 790163I (O83:K1) and lane 2 those of strain 781123 (O21:K1). (a) Polypeptide pattern shown by Coomassie brilliant blue staining. (b-e) Immunochemical profile, after incubation with the following typing sera: (b) anti-O83, (c) anti-O21, (d) anti-O83 absorbed with O21 cells, (e) anti-O21 absorbed with O83 cells. The positions of molecular weight marker bands are indicated.

DISCUSSION

Lipopolysaccharides from several invasive *E. coli* strains, which contain O-antigenic side chains appeared heterogeneous, since multiple bands could be visualized in gels after reaction with specific antisera (Figs 1 and 2). Each serotype had a type-specific band pattern. These band patterns resemble the multiple band patterns observed for *E. coli* O111, *Salmonella typhimurium* LT2 and *Chromatium vinosum* lipopolysaccharides, for which it was shown that these bands represent lipopolysaccharide molecules with different degrees of polymerization of the O-antigen subunits (Goldman & Leive, 1980; Palva & Mäkelä, 1980; Hurlbert & Hurlbert, 1977). This heterogeneity is probably caused by coupling of O-antigen polysaccharide precursors (with a heterogeneous length distribution) to the core-lipid A region of lipopolysaccharide during its synthesis (Robbins & Wright, 1971; Palva & Mäkelä, 1980). In addition, post-translocational modification of the side chains, which sometimes results in form variation (Yuasa *et al.*, 1969), might contribute to the heterogeneity of lipopolysaccharide. Finally, heterogeneity of the core-lipid A part of lipopolysaccharide has been described for *E. coli* and *S. typhimurium* (Alphen *et al.*, 1980; Mühlradt *et al.*, 1977) with respect to the amount of phosphate and substituents, which might influence the charge and thereby the electrophoretic mobility of the molecule. The slight differences in band patterns observed in various strains serotyped as O18 might be explained by the latter kind of heterogeneity (Fig. 3). It is very unlikely that the band patterns resulted from various degrees of aggregation of one defined lipopolysaccharide molecule since (1) in the case of *E. coli* O111 the amount of O-antigenic subunit sugars increases relative to the amount of lipid A phosphate with increasing molecular weight of the bands (Goldman & Leive, 1980), (2) the band patterns were similar in cell envelopes, cells and isolated lipopolysaccharides and did not depend on boiling in SDS (results not shown).

Not all the lipopolysaccharide molecules of strains with O18-serotypes appeared as band patterns with type-specific antiserum in GIRA or SGIP analyses (Fig. 3). In some strains only specific immunodeterminants were present on polysaccharide-like components which hardly entered the gel (Fig. 3). This was not due to loss of the O-antigen of the lipopolysaccharide since boiled cells of these strains strongly agglutinated in anti-O18 serum, indicating that specific determinants were present on the cells. Summarizing, in O18-serotyped strains the serotype-specific determinants are associated with various molecular structures.

Another exception to the regular banding pattern was found for O21 lipopolysaccharide (Fig. 4). The broad smear in the gel in addition to the band at the front is indicative of polysaccharide structures (Poolman & Zanen, 1980). It might be that O21 represents a heat-stable polysaccharide not exposed on the cell surface in untreated cells or that the linkage between O-antigen and core-lipid A is weak, resulting in polysaccharide and rough lipopolysaccharide. Since lipopolysaccharides can be discriminated from polysaccharides by the GIRA method (Poolman & Zanen, 1980) it would be useful to apply this technique to the analysis of the polysaccharide or lipopolysaccharide nature of the known O- and K-serotypes in *E. coli*.

The main purpose of this paper was to combine the separating capacity of SDS-polyacrylamide gels with an immunological detection of the O-antigens in order to get a better understanding of the molecular nature of the serotypes and the specificity of the typing antisera. The GIRA method appeared to be more sensitive than the SGIP method, a fact of special importance when weak antisera are analysed. However, the SGIP technique does have the advantage that the results are obtained faster and that radiolabelling is not required.

Absorption of typing sera to remove cross-reactivity usually coincided with a two- to fourfold reduction in agglutination titre, indicating that a considerable portion (50–75%) of the antibodies were directed against cross-reactive antigens. This may have considerable implications as regards the typing of strains which appear to agglutinate with low titres. The nature of these cross-reactions appeared complex. Antibodies against several proteins (Fig. 1) occurred next to antibodies against other lipopolysaccharides (Fig. 4b) and (possibly) polysaccharide (Fig. 4c).

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