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Monoclonal Antibodies to Enterobacterial Common Antigen and to *Escherichia coli* Lipopolysaccharide Outer Core: Demonstration of an Antigenic Determinant Shared by Enterobacterial Common Antigen and *E. coli* K5 Capsular Polysaccharide

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We established hybridoma cell lines producing monoclonal antibodies against enterobacterial common antigen (ECA) and a substructure of the outer core of different *Escherichia coli* lipopolysaccharides (LPSs). Anti-ECA antibodies 865 and 898 reacted with ECA in extracts of heated *E. coli* and with ECA-bound R1 and R4 core-containing LPS preparations, as well as with a purified sample of ECA from *Salmonella montevideo*. Antibody 865, but not antibody 898, cross-reacted with K5 capsular polysaccharide, suggesting that 4-linked α-*N*-acetylglucosamine is part of an antigenic determinant shared by both K5 polysaccharide and ECA. Anti-LPS antibody 786 recognized an outer core structure common to *E. coli* K-12, B, R2, and R4 core type LPS, but not to R1 and R3 core type LPS. Its most probable target is the trisaccharide sequence Hexp(1→2)-α-D -Glcp(1→3)α-D-Glcp→(Hepp) (where Hex is hexose, p is phosphate, Glc is glucose, and Hep is heptose), the first glucose being the immunodominant moiety. These monoclonal antibodies may be used not only for the detection of ECA, K5, and LPS core structures but also for analysis of the molecular forms resolved on polyacrylamide gels (banding patterns) of both ECA and LPS, independently of one another.

Despite the availability of potent antibiotics, gram-negative bacteremia and sepsis is currently a frequent cause of deaths in hospitals. The development of vaccines against the most common etiologic agents of gram-negative sepsis (e.g., *Escherichia coli*) or of antibodies for passive immunization is therefore an important objective. An effective vaccine or protective antibody should be directed against antigenic determinants shared by many microorganisms. In *E. coli* the antigenic diversity expressed by the lipopolysaccharide (LPS) O-specific chains is substantial (23), whereas the number of different LPS core structures (14) and lipid A components (13) is small. On the other hand, these common determinants are often buried deeply within the cell envelope and are poorly accessible to antibodies. Although it has been reported that antibodies to lipid A or an inner core determinant of LPS protect against the lethal sequelae of gram-negative sepsis (34), it is difficult to imagine that these epitopes on whole living cells are accessible to antibodies; it is more probable that only the free endotoxin is neutralized (34).

In continuation of our previous studies to evaluate *E. coli* outer membrane antigens as potential factors of bacterial pathogenicity or as potential immunogens for vaccines (1, 5,15), we have produced monoclonal antibodies (mAbs) against the enterobacterial common antigen (ECA), an antigen present in the outer membrane of all members of the family *Enterobacteriaceae* (21), and against the hexose region of LPS outer cores that occur in a large number of *E. coli* strains. One of the anti-ECA mAbs was found to cross-react with K5 capsular polysaccharide, indicating the presence of a shared epitope between ECA and K5. The mAbs were found to react with cell extracts of a wide range of *E. coli* wild-type strains but not generally with whole bacteria, which indicates that ECA and the LPS outer core are not accessible to antibodies.

(Part of this work was conducted by M. Jürs in fulfillment of the requirements for the M.D. degree, University of Mainz, Mainz, Federal Republic of Germany.)

MATERIALS AND METHODS

Bacterial strains and cultivation. The strains used in this study are listed in Tables 1 and 2. *E. coli* K-12, C600 Rif^r(pKT146) (20), which produces the plasmid-specified outer membrane protein traTp, ECA (18), and LPS core, was used for immunization and for screening of hybridoma supernatant fluids. It was cultivated overnight at 37°C in tryptic soy broth containing tetracycline (10 μg/ml).

Salmonella typhimurium 4418 was a clinical isolate from a patient with sepsis at the Mainz University Hospitals. *E. coli* 2877/72, a galactose epimerase-deficient mutant (J5) of *E. coli* O111:B4, was a kind gift of the late A. I. Braude, University of California, San Diego. *E. coli* C375 (O132:K1) was kindly provided by F. Ørskov, Staatens Seruminstitut, Copenhagen, Denmark. R2 core strain 2657 and R4 core strain 2506, as well as all strains listed in Table 1, were from the strain collection of the Max Planck Institute, Freiburg, Federal Republic of Germany.

Capsular polysaccharide K1 antigen on *E. coli* clinical isolates was identified by enzyme-linked immunosorbent assay (ELISA) with anti-K1 mAbs (5). K1-negative derivatives were isolated from their parent strains as spontaneous (10) K1 phage-resistant mutants and shown to be K1 negative by a K1-specific ELISA (M. Frosch and D. Bitter-Suermann, manuscript in preparation). K5 capsular polysac-

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TABLE 1. Bacterial strains used for extraction of LPS, ECA, or capsular polysaccharides (K-PS)

Strain	Species and serotype	Material extracted	References (procedure/structural formula)
2847	*E. coli* K-12 wild type	LPS	6, 31/14, 22, 25
2661	*E. coli* K-12 mutant PL-2	LPS	6/22
2502	*E. coli* B	LPS	6/14, 24
F470	*E. coli* core type R1	LPS	6, 31/14
F1357	*E. coli* core type R1 mutant	LPS	6, 31/30
F539	*E. coli* core type R1 mutant	LPS	6, 31/B. Jann, unpublished data
F576	*E. coli* core type R2	LPS	6, 31/12
F653	*E. coli* core type R3	LPS	6, 31/14
2513	*E. coli* core type R4 (O14:K7)	LPS	6, 31/4
1827	*S. typhimurium* Ra	LPS	6, 31/14
2980	*E. coli* O18ac:K5	K-PS	9, 28/29
2164	*E. coli* O14:K7:H	K-PS	9, 28/27
2620	*E. coli* O2:K56:H	K-PS	9, 28/27
SH94	*S. montevideo*	ECA	21/Kuhn, thesis

charide-positive strains were identified with coliphage K5 (11). All wild-type strains were grown overnight on agar plates containing sheep erythrocytes. Strains used for the preparation of LPS and the K5, K7 and K56 capsular polysaccharides are listed in Table 1. For extraction of LPS, bacteria were cultivated in a laboratory fermentor (Eschweiler Co., Kiel, Federal Republic of Germany) in a medium which contained (per liter) casein peptone (Brunnengraeber C, Lübeck, Federal Republic of Germany), 15 g; glucose, 10 g; yeast extract (Difco Laboratories, Detroit, Mich.), 5 g; $Na_2HPO_4 \cdot 12H_2O$, 8 g; NaCl, 3 g; and $MgSO_4 \cdot 7H_2O$, 0.2 g (pH 7.2). After growth at 37°C under aerobic conditions for 6 to 8.5 h, bacteria were harvested with a Sharples centrifuge and washed once with water.

For the extraction of capsular polysaccharides, bacteria were cultivated in a laboratory fermentor (Eschweiler) in a dialysable medium which contained (per liter) Casamino Acids (Difco), 20 g; the dialysable portion of yeast extract (Difco), 100 ml from 100 g of yeast; glucose, 2 g; K_2HPO_4, 7 g; KH_2PO_4, 2 g; sodium citrate $\cdot 5H_2O$, 0.5 5 g; and $MgSO_4 \cdot 7H_2O$, 1 g (pH 7.2). After growth at 37°C for 5 to 6 h, an equal volume of 0.2% cetyltrimethylammonium bromide (Cetavlon; E. Merck A. G., Darmstadt, Federal Republic of Germany) was added. The mixture was kept overnight at 7 to 10°C and then centrifuged. The pellet was suspended in deionized water and worked up as described previously (9, 30).

Isolation of LPSs, capsular polysaccharides, and ECA. LPS was obtained by two extraction procedures. (i) The bacterial paste obtained from centrifugation of the liquid cultures was extracted with aqueous phenol (45% final concentration) at 65°C for 5 min. After dialysis of the aqueous phase, LPS was recovered by repeated centrifugation at 100,000 × *g* for 4 h, and the final pellet was freeze-dried (33). (ii) Acetone-dried bacteria were extracted with a mixture of 90% phenol, chloroform, and low-boiling petroleum ether (2:5:8) for 5 min with cooling in an ice bath. The bacteria were removed by centrifugation, and LPS was recovered from the supernatant fluid as described previously (6).

K5, K7, and K56 capsular polysaccharides were obtained from liquid cultures by precipitation with cetyltrimethylammonium bromide and purified by several cycles of precipitation with ethanol and extraction with cold 80% aqueous phenol (9, 30). ECA, extracted from *Salmonella montevideo* SH94 by a combined phenol-water–phenol-chloroform–petrol-ether procedure as described previously (19), was a generous gift of H. Mayer, Max Planck Institute, Freiburg.

Immunization, fusion, and cloning. The anti-ECA and anti-LPS hybridoma cell lines resulted from three fusion experiments followed by screening of hybridoma supernatant fluids for antibodies against *E. coli* K-12 outer membrane protein and carbohydrate antigens. For immunization, male BALB/c mice (6 weeks old; Zentralinstitut für Versuchstierkunde, Hannover, Federal Republic of Germany) were used. Mice were treated intraperitoneally with whole formalinized *E. coli* C600 Rif^r(pKT146) cells. The immunization-and-fusion protocol was exactly as described previously (1, 2). After fusion of the immune spleen lymphocytes with the hybridoma line X63-Ag 8.653 (16), the culture supernatant fluids of growing clones were screened by ELISA with whole glutaraldehyde-fixed bacteria or outer membranes as antigens (1).

Production and characterization of mAbs. Culture supernatant fluids were harvested from hybridoma clones isolated by limiting dilution. Ascites fluid was collected from Pristane (Sigma, Munich, Federal Republic of Germany)-primed BALB/c mice at about 2 weeks after intraperitoneal injection of hybridoma cells. Immunoglobulin class, subclass, and light chains were characterized in an ELISA with horseradish peroxidase-labeled goat anti-mouse immunoglobulin γ1, γ2a, γ2b, γ3, and μ heavy-chain antibodies and sheep anti-mouse κ and λ light-chain antibodies (Nordic, Tilburg, the Netherlands).

ELISA for determination of antibody specificity. ELISAs were performed in Costar (Cambridge, Mass.) 96-well V-shaped microtiter trays. Plates were coated with intact bacteria, supernatant fluid of heated bacteria, or purified LPS or ECA preparations. Before being coated with intact bacteria, microtiter plates were first activated with poly(D-lysine) (1 μg/50 μl per well; M_r 80,000; Sigma, Munich, Federal Republic of Germany). After the plates were washed twice with buffer A (0.01 M potassium phosphate buffer made isotonic with saline, pH 7.5), bacteria (5×10^8 cells per ml in physiological saline; 40 μl per well) were incubated for 1 h at 37°C. Subsequently, 100 μl of a 0.01% solution of glutaraldehyde was added and incubated for a further 10 min. Plates were washed twice with buffer A, and gelatin

TABLE 2. Bacterial strains used for ELISA and immunoblots

Strain	Species and serotype	Reference or source
C600 Rif^r(pKT146)	*E. coli* K-12	20
C375	*E. coli* O132:K1	22
2877/72 (J5)	*E. coli galE*$^-$ (mutant of *E. coli* O111:B4)	34
E412	*E. coli* ?:K1 (rough phenotype)	7
2657	*E. coli* O111, R2 core	Max Planck Institute
2506	*E. coli* O14:K7$^-$, R4 core	Max Planck Institute
4418	*S. typhimurium*	Mainz University Hospitals (clinical sepsis isolate)

(catalog no. 4078; Merck; 2% [wt/vol] in buffer A) was added to reduce nonspecific binding. Coating of microtiter plates with supernatant fluids of heated bacteria (5×10^8 cells per ml in physiological saline, heated for 10 min at 100°C; 40 μl per well) or purified LPS or ECA diluted in buffer A (1 μg/40 μl per well) was carried out [omitting the poly(D-lysine) and fixation steps] by overnight incubation at 37°C in a humid atmosphere. The further protocol for developing the ELISA with ascites fluid in dilutions of 1:1,000 to 1:2,000 as a source of mAbs, horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins (Dakopatts, Hamburg, Federal Republic of Germany), and peroxidase substrate [2,2'-azino-di(3-ethylbenzthiazolinsulfonate)] (6) (Boehringer GmbH, Mannheim, Federal Republic of Germany) has been described in detail elsewhere (1, 15).

The reactivity of mAbs with capsular polysaccharides K5, K7, and K56 (1 mg/ml in buffer A) was tested in an ELISA after untreated microtiter plates were coated with 20 μl of dilutions of the polysaccharides, air dried overnight at 37°C, and subsequently washed with buffer A containing 0.05% Tween 20.

SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Sodium dodecyl sulfate (SDS) slab gel electrophoresis (12%) was performed as described previously (15). Bacteria grown overnight on blood agar plates were washed twice with physiological saline and adjusted to a concentration of 5×10^8 cells per ml. After centrifugation, the pellet from 1 ml of the bacterial suspension was suspended in 100 μl of sample buffer (0.0625 M Tris-hydrochloride [pH 6.8], 5% 2-mercaptoethanol, 2% SDS, 12.5% glycerol) and heated for 10 min at 100°C. The equivalent of 10^8 bacteria (20 μl) was applied to the gel. Electrophoresis was carried out until the bromophenol blue tracking dye had reached the bottom of the gel. In every case in which a Western blot was performed, a second slab gel was run in parallel, and proteins and lipopolysaccharides were revealed by silver staining. Marker proteins (Sigma) having molecular weights of 92,000, 66,000, 45,000, 31,000, 21,000, and 14,000 were always run on the same slab gel (1, 15). Electrophoretic transfer of LPS, ECA, and proteins to nitrocellulose sheets (BA85; Scheicher & Schüll, Dassel, Federal Republic of Germany) was performed with a Transblot apparatus (Bio-Rad, Munich, Federal Republic of Germany) for 2 h at 25 V and 0.2 A, followed by 12 h at 50 V and 0.4 A. Subsequent steps for blocking unspecific binding of antibodies with gelatin and for staining were described in detail previously (15).

RESULTS

mAb isolation and characterization and evaluation of reactivity with *E. coli* wild-type strains. After ELISA screening of hybridoma culture supernatant fluids with bacterial outer membranes, cells which showed consistent reactivity after cloning by limiting dilution were maintained, and ascites fluids were produced. mAb 786 was an immunoglobulin G3 κ chain, mAb 898 was an immunoglobulin G2a κ chain, and mAb 865 was an immunoglobulin M κ chain. For determination of antibody specificity, hybridoma culture supernatants or ascites fluids were reacted with either intact bacteria or supernatant fluids of heat-treated bacteria. The reactivities of mAbs 865, 898, and 786 with whole, glutaraldehyde–fixed encapsulated and unencapsulated bacteria are shown in Table 3.

In general, the antibodies reacted only weakly with capsulated or smooth whole, glutaraldehyde-fixed bacteria, although they reacted strongly with extracts obtained by heat treatment of bacteria and with rough bacteria. mAb 786, especially, showed only marginal or no reactivity with whole bacteria, except with those of the rough K-12 strain G600 Rif^r(pKT146). Improvement of ELISA reactivity of mAb 786 was achieved in the case of *E. coli* C375 by heating the bacteria. mAb 865 reacted in an ELISA only with whole bacteria of rough strains, such as *E. coli* K-12 and unencapsulated E412 (K^-), but reacted with all supernatant fluids of heated *E. coli* strains. In addition, mAb 865 strongly reacted with whole *E. coli* cells carrying the K5 capsular polysaccharide. mAb 898 qualitatively showed the same reactivity pattern as mAb 865 with all strains tested but did not react with whole cells of K5 capsule-bearing *E. coli* 2980. Heating of the K5 capsule-bearing or capsuleless bacteria allowed antigen detection by both mAb 865 and mAb 898.

TABLE 3. ELISA reactivity pattern of anti-ECA mAbs 865 and 898 and of anticore mAb 786

E. coli strain	OD_{414}[a]					
	Whole bacteria with mAb:			Extracts of heated bacteria with mAb:		
	865	898	786	865	898	786
E412 $K1^+$	0.16	0.17	0.02	0.67	0.96	0.13
E412 $K1^-$	0.60	0.52	0.03	0.69	0.89	0.17
C375 $K1^+$	0.02	0.03	0.03	0.66	0.93	0.31
C375 $K1^-$	0.17	0.22	0.19	0.69	1.06	0.48
2980 $K5^+$	0.64	0.03	0.03	0.43	0.49	0.02
2980 $K5^-$	0.09	0.16	0.08	0.65	0.89	0.18
C600(pKT146)	1.23	1.32	0.77	0.71	1.10	0.92

[a] ELISA optical density reading at 414 nm after 1 h. For the ELISA protocol, see Materials and Methods. The dilution of mAbs (ascites) was 1:1,000.

ELISA with purified antigens. The epitope specificity of the three mAbs was analyzed with purified LPS and ECA as antigens. Anti-ECA mAb 865 bound to purified ECA from *S. montevideo* and to *E. coli* R1 and R4 LPS (Fig. 1). The ELISA reactivity with R4 LPS extracted by the phenol–chloroform–petrol-ether method (6) was about 60% of that obtained with the phenol-hot water procedure (33). The R4 LPS used in these studies is derived from *E. coli* O14:K7 (strain 2513), the classical ECA immunogenic strain. In both the R1 and R4 LPS preparations, some of the LPS molecules are covalently linked to ECA (21). In contrast, there is only marginal or no reactivity of mAb 865 with LPS preparations bearing the *E. coli* K-12, B, R2, and R3 core LPS and with an LPS preparation of ECA-free *E. coli rfe* mutant F1357 (32).

Since ECA contains *N*-acetylmannosaminuronic acid (19), a possible cross-reactivity of mAbs 865 and 898 with the mannosaminuronic acid-containing capsular polysaccharides K7 and K56 was studied in an ELISA. The capsular polysaccharides K7 and K56 showed no ELISA reactivity at loadings of 20 μg per well with either anti-ECA mAb, whereas mAb 865, but not mAb 898, reacted well with K5 polysaccharide at the nanogram level (Table 4).

Table 5 shows the structures of various *E. coli* LPS outer core types isolated from rough mutants lacking O-specific side chains and their relative ELISA reactivities with mAb 786. Binding of antibody to solid-phase LPS was optimal in the case of K-12 (100%) and R2, followed by *E. coli* B and R4 core. LPS preparations of the deep rough mutant strain PL-2

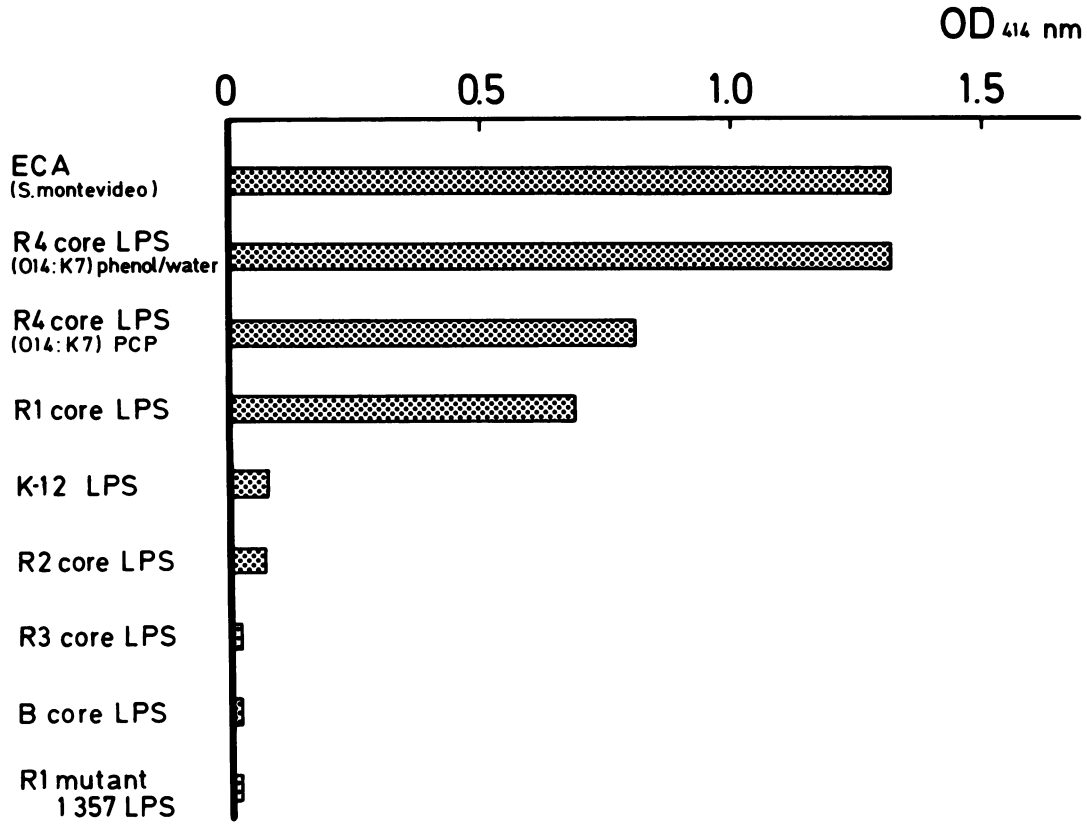

FIG. 1. ELISA reactivity patterns (optical density [OD] at 414 nm after 1 h) of mAb 865 with purified ECA and *E. coli* LPSs. The antigen concentration used for coating the microtiter trays was 25 μg/ml. The dilution of mAb 865 (ascites fluid) was 1:1,000.

and of an R1 mutant lacking the β-D-glucose phosphate substituent showed 63.8 and 90.8% reactivity, respectively, whereas native R1 LPS, R3 LPS, and an R1 mutant LPS with only one α-D-glucose phosphate in the outer core (and thus identical to the K-12 LPS inner core structure) failed to react with mAb 786.

Immunoblot analysis of established *E. coli* wild-type and mutant strains. Figure 2A shows an immunoblot of SDS-solubilized *E. coli* and *S. typhimurium* of different core types developed with mAb 865, which demonstrates the ladderlike banding pattern of ECA molecules. The molecular weights of the ECA species ranged from about 10,000 to 35,000 in all strains except for the R4 strain, which showed a markedly broader distribution of ECA molecules with a molecular weight range between about 10,000 and 80,000. mAb 898 yielded a nearly identical staining pattern with the same strains (Fig. 2B).

mAb 786 reacted in immunoblots only with the LPS of *E. coli* strains bearing the LPS core types K-12, R4, and R2 (Fig. 2C) and B (data not shown). This is in accordance with the data shown in Table 5. In the rough K-12 strain, as well as in R2 and R4 core strains, mAb 786 stained a fast-moving, diffuse band. In a smooth strain with the R2 core, mAb 786 also detected a complex pattern of slower-moving bands of higher molecular weight (Fig. 2C). Strain C375, not defined with regard to its core type, reacts with mAb 786 in a fashion similar to that of the smooth R2 core strain 2657.

The J5 mutant derived from *E. coli* O111:B4 bearing only one glucose molecule in the outer core (34), *S. typhimurium* (core structure shown in Table 5) and *E. coli* strains with R1 and R3 core types (data not shown) failed to react with anticore mAb 786 in immunoblots, but all reacted with anti-ECA mAbs 865 and 898 and exhibited ladderlike ECA patterns after polyacrylamide gel electrophoresis.

Anti-ECA mAb 865 detected in *E. coli* O18ac:K5, in addition to ECA, a high-molecular-weight antigen (ca. 80,000) which could not be detected in O18ac:K5$^-$ *E. coli* (Fig. 3A). mAb 898 failed to stain this high-molecular-weight band in either O18ac:K5 or O18ac:K5$^-$ *E. coli* (Fig. 3B). The same observation was made with four other sepsis isolates of K5 strains and with their isogenic unencapsulated mutant strains (data not shown).

TABLE 4. ELISA reactivity pattern of anti-ECA mAbs with purified K5 polysaccharide

mAb	OD_{414}[a] with K5 antigen at (ng/well):					
	10	3.3	1.1	0.37	0.12	O (Buffer)
865	0.801	0.568	0.357	0.130	0.058	0.017
898	0.061	0.036	—	—	—	0.037
Buffer	0.012	—	—	—	—	0.0

[a] ELISA optical density at 414 nm after 2 h. The dilution of mABs (ascites) was 1:1,000.

DISCUSSION

The evaluation of the epitope specificity of the three mAbs described in this paper is based on the availability of purified antigens with a known structure (17, 19, 25–27, 32), as well as on well-characterized strains and mutants.

The specificity of mAbs 865 and 898 for ECA is demon-

TABLE 5. ELISA reactivity pattern of mAb 786 with LPS purified from rough mutants of *E. coli* and *S. typhimurium*

Core type	LPS core structure[a]	ELISA reactivity[b]
E. coli K-12	α-D-GlcpNAc(1→6)-α-D-Glcp(1→2)-α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp 6 ↑ 1 α-D-Galp	100
E. coli K-12 mutant PL-2	α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp	63.8
E. coli B	α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp	61.4
E. coli R2	α-D-GlcpNAc(1→2)-α-D-Glcp(1→2)-α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp 6 ↑ 1 α-D-Galp	98.2
E. coli R4	α-D-Galp(1→2)-α-D-Galp(1→2)-α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp 4 ↑ 1 β-D-Galp	38.1
E. coli R1 mutant F1357	α-D-Galp(1→2)-α-D-Galp(1→2)-α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp	90.8
E. coli R1	α-D-Galp(1→2)-α-D-Galp(1→2)-α-D-Glcp(1→3)-α-D-Glcp(1→3)Hepp 3 ↑ 1 β-D-Glcp	5.2
E. coli R1 mutant F539	α-D-Glcp(1→3)Hepp	1.6
S. typhimurium Ra	α-D-GlcpNAc(1→2)-α-D-Glcp(1→2)-α-D-Galp(1→3)-α-D-Glcp(1→3)Hepp 6 ↑ 1 α-D-Galp	1.0
E. coli R3	α-D-Glcp(1→2)-α-D-Glcp(1→2)-α-D-Galp(1→3)-α-D-Glcp(1→3)Hepp 4 ↑ 1 α-D-GlcpNAc	1.2

[a] Abbreviations: Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Hep, heptose; p, phosphate.

[b] Given as the percentage of the optical density at 414 nm obtained with a reference *E. coli* K-12 LPS preparation. The antigen concentration used for coating the wells was 25 μg/ml. Data are means of duplicate determinations from a representative experiment.

strated by their reactivity with authentic ECA from *S. montevideo* and with R1 and R4 LPS preparations and by their nonreactivity with LPS from *rfe* mutant 1357, which lacks ECA covalently bound to the core (32). The fact that mAb 865 does not react with the *N*-acetylmannosaminuronic acid-containing K7 and K56 polysaccharides shows that the presence of this ECA constituent alone is not sufficient for antibody binding. The results described here do, however, indicate the presence of an antigenic determinant common to ECA and the K5 polysaccharide but absent from the K7 and K56 polysaccharides. The K5 polysaccharide has the disaccharide repeating unit 4-β-GlcUA(1→4)-α-GlcNAc-1 (31), and ECA has the trisaccharide repeating unit 4-β-ManNAcUA(1→4)-α-GlcNAc(1→3)-α-Fuc-4-NAc-1 (H.-M. Kuhn, Ph.D. thesis, University of Freiburg, Freiburg, Federal Republic of Germany, 1981). (In these structures, GlcUA is glucuronic acid, GlcNAc is *N*-acetylglucosamine, ManNAcUA is *N*-acetylmannosaminuronic acid, Fuc is fucose, and NAc is *N*-acetyl.) The K7 polysaccharide, which does not react with mAb 865, consists of the disaccaride repeating unit 3-β-ManNAcUA(1→4)β-GlcNAc-1 (29). On the basis of the data available at present, the chemical structure of the epitope recognized by mAb 865 cannot be defined with certainty. It is likely, however, that this epitope is the α-*N*-acetylglucosamine residue, which is substituted at C-4 by glucouronic acid in the K5 polysaccharide and by *N*-acetylmannosaminuronic acid in ECA. This substructure is not present in the K7 polysaccharide. It is possible that an adjacent charge is an important part of the epitope. This could be the carboxyl group of glucuronic acid in the K5 polysaccharide and of *N*-acetylmannosaminuronic acid in ECA. It cannot be decided at present whether the acidic constituent rather than α-*N*-acetylglucosamine is the dominant part of the epitope. Since glucuronic acid (K5) and *N*-acetylmannosaminuronic acid (ECA) residues differ in substitution and configuration at C-2, their participation, if any, in antibody binding is probably restricted to the C-3–C-6 regions.

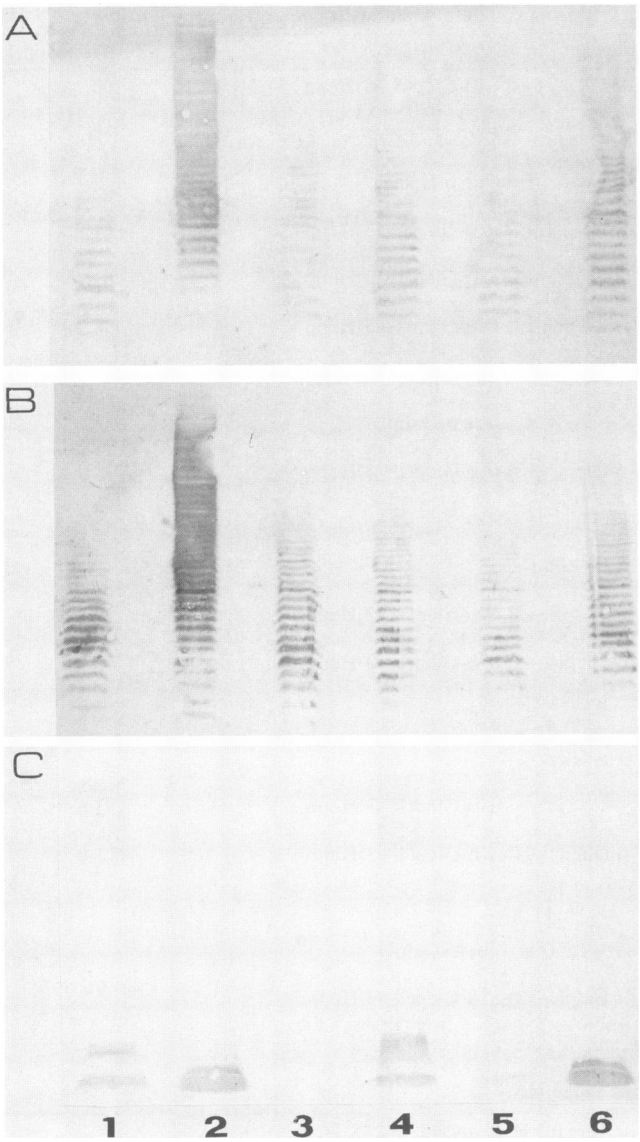

FIG. 2. Immunoblots of *E. coli* strains and an *S. typhimurium* strain, developed with ECA-reactive mAb 865 (A), ECA-reactive mAb 898 (B), and core-reactive mAb 786 (C). Lanes: 1, *E. coli* C375; 2, *E. coli* 2506; 3, *S. typhimurium* 4418; 4, *E. coli* 2657; 5, *E. coli* 2877/72 (J5); 6, *E. coli* C600 Rif^r(pKT146). The dilution of mAbs (ascites fluid) was 1:1,000. Note the lack of reactivity of *E. coli* 2877/72 (J5) and of *S. typhimurium* 4418 with mAb 786.

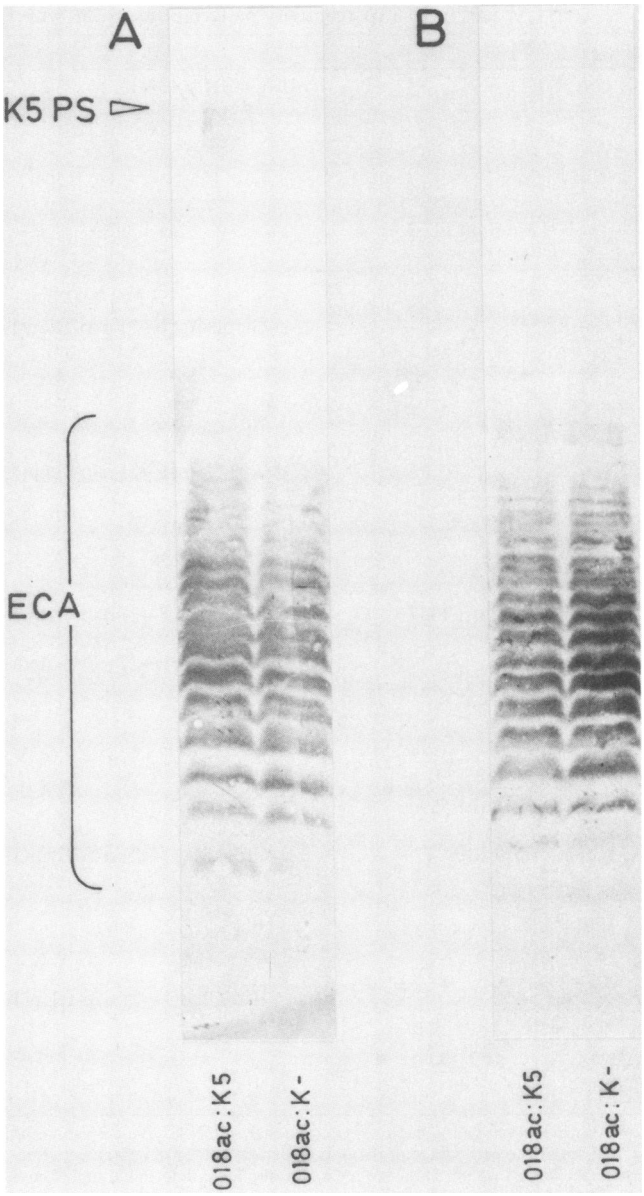

FIG. 3. Immunoblots of the isogenic pair *E. coli* O18ac:K5 and O18ac:K developed with mAb 865 (A) and with mAb 898 (B). The dilution of mAbs (ascites fluid) was 1:1,000. PS, Polysaccharide.

In immunoblots, SDS-solubilized, ECA-containing bacteria show a ladderlike pattern when incubated with mAb 865 or mAb 898 (Fig. 2 and 3). This could indicate a size heterogeneity of ECA, as demonstrated for LPS (24). However, a recent report of cyclic forms of ECA (3) does not agree with such an interpretation. It is also possible that in these bacteria the LPS core is substituted not only with the O-specific polysaccharide, but also with one or several ECA repeating units. In such a case the heterogeneity detected with an ECA-specific antibody would be due to LPS heterogeneity. This is obviously not the case for the rough *E. coli* strains whose LPS lacks O side chains (Fig. 2C). In addition, our finding that in an immunoblot of extracts of *E. coli* 2506 and C600, mAb 786 detected only one diffuse band of high electrophoretic mobility (Fig. 2C), in contrast to the complex ladderlike pattern revealed by mAb 865 or 898 (Fig. 2A and B), argues against an LPS dependency of ECA ladders. In this context it should be mentioned that ECA is substituted at its reducing end with a phosphatidic acid (Kuhn, thesis), as has been reported for capsular polysaccharides of invasive *E. coli* (8, 28). It is conceivable that ECA molecules of different sizes, each substituted with phosphatidic acid, form associations with SDS that give rise to the ladderlike pattern in SDS-polyacrylamide gel electrophoresis.

In a preliminary survey of strains with the ECA mAbs described here, ECA was detected in supernatant fluids of heated bacteria of clinical isolates of members of the family *Enterobacteriaceae* (*E. coli* and *Enterobacter*, *Klebsiella*, *Proteus*, *Salmonella*, *Shigella*, and *Yersinia* spp.) but not in *Acinetobacter*, *Pseudomonas*, *Campylobacter*, *Vibrio*, *Haemophilus*, *Bordetella*, *Neisseria*, or *Bacteroides* strains.

The epitope recognized by mAb 786 was tentatively defined as the sugar sequence Hexp(1→2)-α-D-Glcp(1→3)-α-D-Glcp→(Hepp) at the inner region of the outer core, in which the heptose-distal glucose is immunodominant. (In this structure, Hex is hexose, p is phosphate, Glc is glucose, and Hep is heptose.) Substitution of this glucose at C-3 (as in the R1 core), its lack (as in mutant 539), or its replacement by α-galactose (as in *S. typhimurium* Ra core) almost completely prevents antibody binding. Substitution of the immunodominant glucose at C-4 (as in the R4 core) has less influence on the epitope specificity.

The reaction patterns of mAbs 865, 898, and 786 with whole bacteria (Table 3) indicate that ECA and the outer core in smooth or encapsulated (or both) bacteria are not readily accessible to antibodies. This suggests that these common envelope structures may not be useful antigens for vaccines or for producing antibodies for therapy. On the other hand, the anti-ECA and anticore mAbs efficiently detected their corresponding antigens in extracts of heated cells and hence will be useful for chemical and antigenic analysis of isolates for diagnosis and taxonomic studies.

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