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Characterization of Anti-Core Glycolipid Monoclonal Antibodies with Chemically Defined Lipopolysaccharides

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Five anti-core glycolipid monoclonal antibodies (MAb) (four against *Escherichia coli* J5 lipopolysaccharide [LPS] and one against the Re core glycolipid of *Salmonella typhimurium*) were characterized using LPS from several rough and smooth strains and derivatives of *E. coli* J5 LPS, obtained by N acetylation and hydrolysis. The MAb against *E. coli* J5 were not or only weakly cross-reactive with clinical isolates, whereas the anti-Re MAb was highly cross-reactive. The MAb differed in their reaction pattern with *E. coli* J5 LPS. MAb 4-7B5 (immunoglobulin M) and MAb 4-6A1 (immunoglobulin G1) cross-reacted with LPS of *Salmonella minnesota* R5 and *S. typhimurium* Ra and Rc and little with Re and lipid A. The dominant binding site of these MAb was located in the glucose-heptose-heptose region and was independent of phosphate substitution. The MAb 4-9A1 reacted with the terminal part of the core region (glucose-heptose) and was dependent on phosphate substitution of the LPS. The MAb BA7 (immunoglobulin G3) was *E. coli* J5 LPS specific and reacted with the glucosaminyl-heptose disaccharide. Antibody 8-2C1 was directed against the common parts of LPS, 3-deoxy-D-manno-octulosonic acid, and lipid A, which are not (or only weakly) recognized by the four anti-J5 LPS MAb. Thus, MAb that are not cross-reactive can be directed against at least three different antigenic determinants present on the core oligosaccharide of *E. coli* J5 LPS.

Antibodies raised against the rough mutant (R mutant) *Escherichia coli* J5 have been reported to protect against infections caused by gram-negative bacteria (19, 21, 24). Neutralization of the endotoxins (lipopolysaccharides [LPS]) by these antibodies has been postulated (23), but against which epitope of LPS these antibodies are directed is not known. *E. coli* J5 LPS is of chemotype RcP^+, and its inner core contains glucosamine, glucose, heptose, 3-deoxy-D-manno-octulosonic acid (KDO), phosphate, phosphoethanolamine, ethanolamine, and lipid A. Among members of the family *Enterobacteriaceae*, the structure of the inner core is highly conserved. However, *E. coli* J5 LPS is heterogenous and contains several molecular species (Fig. 1) (12). Additionally, a D-glucosaminyl-(α-1-7)-L-glycero-D-manno-heptose disaccharide is linked as a branch to the basal core structure (6, 12). Because *E. coli* J5 LPS is heterogenous, antibodies with different binding sites are induced after immunization. Some of these anti-*E. coli* J5 antibodies show cross-reactivity with other gram-negative clinical isolates, and some do not (9, 14–16). The most immunogenic part of the *E. coli* J5 LPS appears to be the nonreducing terminal sugar of the core region: glucose linked to heptose (6). It is not yet known to which epitope(s) the antibodies in cross-protective antisera are directed.

Only with chemically characterized *E. coli* J5 LPS is it possible to elucidate the antigenic binding site of antibodies on a molecular level. With the help of monoclonal antibodies (MAb) reactive with different antigenic determinants, it may then be possible to define the epitope that has the capacity to induce cross-protective antibodies in animals and humans. We have produced several MAb against the R mutants *E. coli* J5 and *Salmonella typhimurium* (5). These antibodies were characterized in this study and may, in the future, help to compare the reactivity and specificity of protective and nonprotective MAb.

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MATERIALS AND METHODS

Bacteria, LPS isolation, and chemical modifications of *E. coli* J5 LPS. *Serratia marcescens*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *E. coli* $O135K^+$, *Proteus mirabilis*, and *Morganella morganii* were isolated from the blood of patients with septic shock caused by gram-negative bacteria. Bacteria were grown on Isosensitest agar (Oxoid, United Kingdom) plates (18 h, 37°C). After harvesting, they were washed twice with phosphate-buffered saline (PBS) and heat killed in PBS for 1 h at 100°C.

LPS. LPS of the R strains *S. minnesota* R5 (RcP^-), *E. coli* J5 (RcP^+), and *S. typhimurium* Ra, Rc (RcP^+), and Re were isolated as described previously (7, 8). They were dissolved in distilled water (5 mg/ml), ultrasonicated (5 min), adjusted to pH 7 with triethylamine, and kept at −20°C. Lipid A, obtained from H. Brade, was isolated from the *E. coli* Re mutant strain F515 by acetate buffer hydrolysis (0.1 M, pH 4.4, 1 h, 100°C) (4).

Hydrolysis of *E. coli* J5 LPS. Sequential hydrolysis of LPS (20 mg/ml) was started with 20 mM acetate buffer (pH 4.4) for 3 h at 70°C. After dialysis against distilled water, dialysate 1 was concentrated by evaporation, and the remainder was further hydrolyzed with 0.1 M acetate buffer (pH 4.4) for 1 h at 100°C. After dialysis, dialysate 2 was obtained as described above. A part of the remainder (65%) was further hydrolyzed with 0.1 M HCl for 1 h at 100°C. The other 35% was termed J5 LPS-ACB (yield, 78%). Dialysate 3 was obtained as described above after 0.1 M HCl hydrolysis. This remainder was termed J5 LPS-HCl (yield, 31.4%).

After sequential hydrolysis with acetate buffer and HCl, samples of the dialysates were subjected to high-voltage paper electrophoresis as described before (4, 18) and stained with the Trevelyan reagent (22) for reducing sugars with ninhydrin for amino groups, with the Hanes-Isherwood reagent (11) for phosphate, and with thiobarbituric acid for KDO (3).

Dialysate 1, obtained after 20 mM acetate buffer treat-

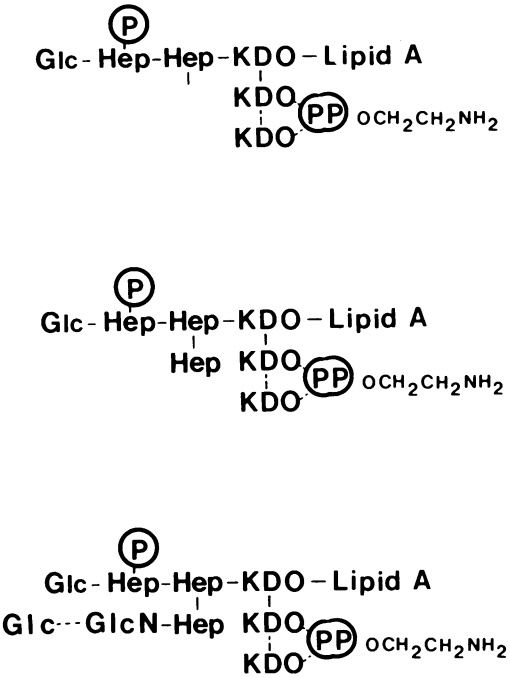


FIG. 1. Proposed structure of different molecular species of *E. coli* J5 LPS. Dotted lines indicate partial substitutions. Abbreviations: P, phosphate; PP-$CH_2CH_2NH_2$, pyrophosphorylethanolamine; Glc, Glucose; Hep, L-glycero-D-manno-heptose.

ment, contained only KDO monosaccharide. Dialysates obtained after 0.1 M acetate buffer and HCl (0.1 M) hydrolysis (dialysates 2 and 3, respectively) contained KDO oligosaccharides substituted with phosphate and KDO. After HCl hydrolysis, more monosaccharides were obtained.

By using gas-liquid chromatography–mass spectrometry, a glucose-heptose or heptose-glucose disaccharide could be detected in dialysates 2 and 3 after methylation by the method of Hakomori (10). Analysis (2, 12) of the preparations after 0.1 M acetate buffer and HCl hydrolysis showed loss of KDO, phosphate, and glucosamine, in agreement with the contents of the dialysates. The sediment after HCl treatment (J5 LPS-HCl) did not contain KDO but did have neutral sugars. Thus, even after HCl hydrolysis the lipid A part was not split off completely from the core, as was expected from results described before with LPS of other R strains (4, 8).

Kinetic hydrolysis. Samples of *E. coli* J5 LPS (2 mg/ml) in 0.1 M HCl at 100°C were taken at 0, 15, 30, and 60 min, neutralized with NaOH to pH 7.0, and kept at −20°C. The phosphate release was measured in the supernatant after centrifugation (10 min, 2,500 × *g*) by the method of Lowry et al. (13), in which almost all of the J5 LPS was sedimented.

LPS-HF. LPS were dephosphorylated twice with 48% hydrogen fluoride (LPS-HF) for 48 h at 4°C. After adjustment with NaOH (1 N) to pH 5 to 7, the solution was dialyzed and lyophilized (yield, 70%). LPS-HF was dephosphorylated for at least 95% as determined by phosphate analysis (12).

LPS-NAc. LPS (200 μl, 5 mg/ml) were N acetylated (LPS-NAc) with 50 μl of saturated $NaHCO_3$ and 2 μl of acetic acid anhydride, with continuous stirring for 30 min at room temperature. After dialysis against aquadest the solution was lyophilized. Analysis of the *E. coli* J5 LPS-NAc with gas-liquid chromatography–mass spectrometry showed that no detectable degradation products were formed.

Deamination of LPS. *E. coli* J5 LPS (200 μl, 5 mg/ml) was deaminated by treating with 2 ml of $NaNO_2$ (5%) and 2 ml of acetic acid (33%). This mixture was stirred for 1 h at room temperature, passed through an ion-exchange column (Amberlite H^+ IR 120), and washed three times with water. The eluate was dialyzed and lyophilized. The degree of deamination was tested by screening the dialysate after reduction for anhydromannitol (product of deaminated glucosamine, released after hydrolysis) by using gas-liquid chromatography–mass spectrometry. At least 25% (wt/wt) of original glucosamine was measured as anhydromannitol.

LPS-OH. *E. coli* J5 LPS (1 g) was de-O acetylated (LPS-OH) by drying under reduced pressure in the presence of P_2O_5 and stirred for 15 h at 37°C with 15 ml of anhydrous sodium methylate (0.25 M). After centrifugation the sediment was dissolved in 5 to 10 ml of water, and LPS-OH was precipitated with 60 to 80 ml of ethanol, centrifuged (10 min, 1,400 × *g*, 4°C), and dried (yield, 90%). LPS-OH was analyzed as described previously (12). It contained only half the original amount of 3-hydroxy-myristic acid. Nonhydroxylated fatty acids (12:0, 14:0) were absent, and there was no indication for changes in the amounts of other constituents. LPS-OH contained, as expected, two amide-linked 3-OH myristic acids, which were resistant to this alkali treatment.

MAb. MAb 4-7B5 (immunoglobulin M [IgM]), MAb 4-9A1 (IgG3), and MAb 4-6A1 (IgG1) against *E. coli* J5 LPS and anti-Re LPS MAb 8-2C1 (IgM) were obtained as described previously (5). The specific anti-J5 LPS MAb BA7 (IgG3) was a gift of B. J. Appelmelk (Free University of Amsterdam, The Netherlands).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed as previously described (5) on polyvinylchloride microdilution plates with horseradish peroxidase-conjugated anti-IgG and anti-IgM and detected with tetramethylbenzidine. Microdilution plates were coated with lipid A (0.1 ml per well, 5 μg/ml) in aquadest (adjusted to pH 7.0 with triethylamine) and cooled to 4°C. Cold HCl (25 μl, 0.4 N) was added, and the lipid A was precipitated for 15 to 30 min at 4°C. The plates were centrifuged (600 × *g*, 5 min, 4°C) and then washed with cold 0.01 N HCl for 15 min at 4°C. After centrifugation, the plates were washed with water and 0.05% Tween 20, and the ELISA procedure was followed as usual. Wells were coated with LPS (100 μl) diluted in PBS to 1 μg/ml or with bacteria (100 μl, adjusted to 2.5×10^8 bacteria per ml in PBS). Plates were incubated for 1 h at 37°C and overnight at 4°C.

Inhibition ELISA. Antibodies (diluted to a concentration that gave an optical density of 1.0 after reaction with the antigen used) were preincubated with decreasing amounts of antigen as serial twofold dilutions in microdilution plates (PBS–0.05% Tween 20) for 30 min at 37°C. The inhibited antibody solutions were tested in a normal ELISA and compared with uninhibited antibodies, except that the antigen-coated plates were first incubated with 4% bovine serum albumin, PBS, and 0.05% Tween 20 for 1 h at 37°C to block unbound plastic. The amount of antigen that gave an optical density of 50% compared with that of uninhibited antibodies was called the inhibitory amount, measured in nanograms.

Competition ELISA. *E. coli* J5 LPS (0.1 ml, 1 μg/ml)-coated microtiter plates were incubated after thoroughly washing with decreasing amounts of an IgG MAb for 1 h at 37°C in 4% bovine serum albumin–PBS–0.05% Tween 20. After washing, the plates were incubated with a competitive IgM MAb, adjusted to a dilution that produced an optical

density of about 1.0, for 1 h at 37°C. The bound IgM was detected with horseradish peroxidase-conjugated anti-IgM immunoglobulins by standard procedures. The IgG MAb was tested with anti-IgM as a control.

Passive hemolysis test (PHT). Sheep erythrocytes (SRBC), kept in Alsever solution, were washed three times with cold PBS. Cells (5% dilution in PBS) were coated with LPS (50 to 200 μg/0.2 ml of packed SRBC) for 30 min at 37°C. Coated SRBC were washed again three times with cold PBS and added to Veronal buffer (0.08 M sodium barbiturate [pH 8.6], 0.5 mM Ca^{2+}, and 0.5 mM Mg^{2+}) to a concentration of 0.5%. Antibodies were diluted twofold in Veronal buffer (50 μl) in microdilution plates (96 wells, V form; Greiner B.V., Alphen a/d Rijn, the Netherlands). Antigen-coated SRBC (50 μl) and complement (guinea pig serum [1:30] in 25 μl of Veronal buffer) were then added, and the plates were incubated for 1 h at 37°C. After incubation, the plates were centrifuged (3 min, 600 × *g*, 4°C), and the hemolytic titer could easily be read with the naked eye. The hemolytic titer was defined as the antibody dilution that gave 50% lysis. A hemolytic unit was defined as the dilution that was one twofold dilution step before the dilution with the hemolytic titer.

Inhibition PHT. MAb (25 μl) was added to the potential inhibitor (LPS antigens) (twofold serial dilutions, 25 μl) in microdilution plates. After gentle mixing the plates were incubated for 15 min at 37°C. Antigen coated on SRBC and complement was added, and the test was performed as described above. The inhibitory concentration was then read with the naked eye. The lowest amount of inhibitor that was just sufficient to give complete inhibition (no lysis) was expressed in nanograms.

TABLE 1. Characteristics of various LPS preparations and LPS derivatives

LPS or derivative	Chemotype or description
E. coli J5	RcP^+
S. typhimurium Ra	Ra, phosphorylated
S. typhimurium Rc	RcP^+
S. minnesota R5	RcP^-
S. typhimurium Re	(KDO)-KDO-KDO-lipid A
E. coli J5 LPS derivatives	
LPS-OH	After alkaline treatment only amide-linked 3-hydroxy myristic acids remain in the lipid A molecule; no changes in core region
Deaminated	Partially bound free amino group in core region has been specifically split off
LPS-NAc	Partially bound free amino group in core region has been acetylated
LPS-HF	By HF treatment all phosphate groups are cleaved off
J5 LPS-ACB	Hydrolysis with 0.1 M acetate buffer (pH 4.4) did not cleave J5 LPS in lipid A and core oligosaccharide; only some KDO, phosphate, and sugar residues were randomly split off
J5 LPS-HCl	Hydrolysis with 0.1 M HCl did cleave off some more phosphate, sugars, and all KDO, but the core region has not been split off from lipid A

RESULTS AND DISCUSSION

Reaction with bacteria, LPS, and lipid A. The reactivity of the MAb was tested against the clinical isolates in an ELISA; only the MAb raised against Re LPS (8-2C1) reacted with all clinical isolates tested. The anti-J5 LPS MAb 4-7B5 showed some weak reactions against *S. marcescens*, *E. cloacae*, *E. coli* O135 K^+, *P. mirabilis*, and *M. morganii*. The other anti-J5 LPS MAb did not react with any of the clinical isolates (data not shown). Also, the reactivity of the MAb against various LPS preparations, chemically modified *E. coli* J5 LPS, and the hydrolyzed fractions J5 LPS (Table 1) was tested in the ELISA (Table 2) and in the inhibition PHT (Table 3). In the inhibition assays MAb were first incubated with LPS or LPS derivatives and then added to SRBC (PHT) or the ELISA where SRBC or the polyvinylchloride wells were coated with *E. coli* J5 LPS (for anti-J5 MAb) or Re LPS (for 8-2C1). MAb 4-6A1 did not react in a PHT; therefore, ELISA inhibition experiments were conducted with this MAb instead.

MAb 8-2C1 reacted with all R LPS and lipid A. Reactions of MAb 8-2C1 could only strongly be inhibited by Re LPS and lipid A in the PHT. All anti-J5 LPS MAb reacted with RcP^+ LPS except MAb BA7, which did not react with RcP^+ derived from *S. typhimurium*. MAb 4-6A1 and MAb 4-7B5 showed high titers against *E. coli* J5 LPS and cross-reacted with LPS of the RcP^+, Ra, and R5 strains but only weakly with Re LPS and lipid A. However only MAb 4-7B5 cross-reacted strongly with R5 (RcP^-), and only the reaction with MAb 4-7B5 could be inhibited by lipid A and Re LPS in the PHT.

Reaction with chemically modified *E. coli* J5 LPS. All MAb (except 4-7B5) reacted better with alkali-treated *E. coli* J5 LPS (Tables 2 and 3). Antigenic determinants appeared to be better exposed after loss of the hydrophobic fatty acids. Dephosphorylation of *E. coli* J5 LPS resulted in a preparation (LPS-HF) that did not coat polyvinylchloride microdilution plates, probably due to a loss of charged phosphate groups. The reactivity of MAb 4-9A1 in the PHT could not be inhibited by LPS-HF. Only some inhibition by LPS-HF was observed in the PHT reaction with MAb 4-7B5. Also, MAb 4-6A1 did not react with dephosphorylated *E. coli* J5 LPS in the inhibition ELISA. Because MAb 4-7B5 also showed little inhibition with LPS-HF in the inhibition ELISA (data not shown), we concluded that the antigenic sites on LPS-HF were hindered under these conditions.

Only the reactivity between MAb BA7 and J5 LPS-coated SRBC was strongly decreased after deamination of *E. coli* J5 LPS (resulting in release of glucosamine). Therefore, it appeared that a glucosamine with a free amino group is essential for binding with MAb BA7. This conclusion is supported by the finding that *E. coli* LPS-NAc also failed to bind MAb BA7. These latter two derivatives of *E. coli* J5 LPS reacted in a similar fashion with the other three MAb. This means that glucosamine does not play an important role in their antigenic recognition.

Reaction with fractions of hydrolyzed J5 LPS. KDO monosaccharide present in dialysate 1 did not bind to the four MAb tested. The contents of dialysate 2, after 0.1 M acetate buffer hydrolysis, were able to inhibit the reaction with *E. coli* J5 LPS-coated SRBC of all four MAb (Table 3). Except for MAb 4-9A1, this was also seen with dialysate 3. The phosphate-substituted oligosaccharides that were released during acetate buffer hydrolysis contained epitopes recognized by all four MAb. After HCl hydrolysis, more monosaccharides and P_i were released, and the phosphorylated oligosaccharide recognized by MAb 4-9A1 may have been destroyed. Reactivity of all MAb with J5 LPS-ACB and J5 LPS-HCl showed that only with the last preparation did

TABLE 2. Reactivity of anti-core glycolipid MAb with various LPS and LPS derivatives in ELISA

LPS or derivative	ELISA titer[a]				
	Anti-Re LPS MAb 8-2C1	Anti-J5 LPS MAb			
		4-9A1	4-7B5	BA7	4-6A1
LPS					
E. coli J5	800	160	5,000	100	10,000
RcP$^+$ *S. typhimurium*	80	160	10,000	–	10,000
Ra	1,000	–	10,000	–	10,000
R5 (RcP$^-$)	800	–	50,000	–	20
Re	1,000	–	20	–	20
Lipid A	1,000	–	25.6	–	12.8
LPS derivatives					
LPS-OH	800	200	1,600	400	>3,200
Deaminated	800	200	1,600	–	>3,200
LPS-NAc	ND	200	ND	–	>3,200
Hydrolyzed J5 LPS					
J5 LPS-ACB	ND	80–320	80–320	640–6,400	>6,400
J5 LPS-HCl	ND	10–40	80–320	10–40	>6,400

[a] ELISA titer × 100; –, titer of <250. Polyvinylchloride microdilution plates were coated with 100 μg of antigen per well. Titer was defined as the reciprocal of the MAb dilution (ascites) at which the optical density was ≥0.2. ND, Not done.

reactivity decrease. It appears that hydrolysis in acetate buffer did not effectively cleave *E. coli* J5 LPS in core and lipid A. J5 LPS-ACB appeared to consist of *E. coli* J5 LPS in which some molecules were devoid of phosphorylated oligosaccharides. The four MAb could still bind to their antigenic determinants, even though the reaction of the four MAb with J5 LPS-HCL was diminished, because most sugars present in the core region and all KDO were cleaved.

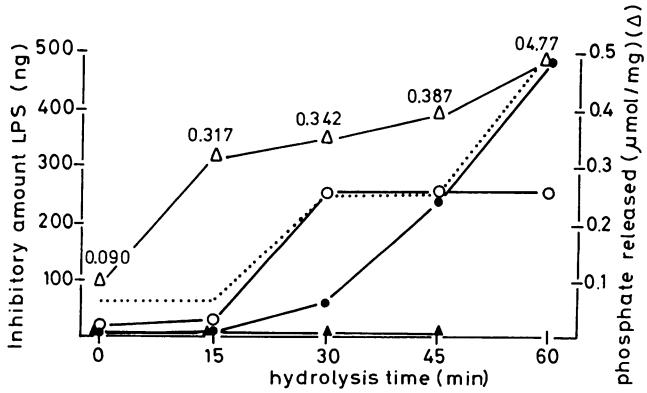
The amount of P_i released during kinetic hydrolysis of *E. coli* J5 LPS (0.1 N HCl, 100°C) (Fig. 2) was compared with the inhibition capacity of this hydrolyzed LPS of the PHT on MAb 4-9A1, 4-7B5, and BA7 and *E. coli* J5 LPS-coated SRBC. The total amount of phosphate was 1.250 μmol/mg of LPS (12). In *E. coli* J5 LPS approximately five phosphate residues are present. Thus, one phosphate residue refers to ±0.250 μmol/mg. One phosphate group was released after 15 min of hydrolysis, and almost all of the second phosphate group was released after 45 to 60 min. After 30 min of HCl hydrolysis, more *E. coli* J5 LPS (250 ng) was needed to achieve the same inhibition in the PHT with MAb 4-9A1 than with unhydrolyzed J5 LPS (31 ng). This was also observed with MAb BA7 after 30 to 45 min and with MAb 4-7B5 after 45 min of HCl hydrolysis. There was no loss of reactivity with MAb 4-6A1 and HCl-hydrolyzed *E. coli* J5 LPS. From these results one can conclude that the binding of MAb 4-9A1 and MAb BA7 with *E. coli* J5 LPS depends on an

TABLE 3. Inhibition by LPS and LPS derivatives of passive hemolysis with anti-core glycolipid MAb

LPS or derivative	PHT inhibition[a] (ng)				
	Anti-Re LPS MAb 8-2C1	Anti-J5 LPS MAb			
		4-9A1	4-7B5	BA7	4-6A1
LPS					
E. coli J5	125	31	1.3	31	12
RcP$^+$ *S. typhimurium*	–	–	125	10	125
Ra (RcP$^-$)	62.5	–	5	–	–
R5	125	–	10	–	–
Re	20	250	125	–	–
Lipid A	31	–	250	–	–
LPS derivatives					
LPS-OH	ND	10	5	10	–
Deaminated	ND	–	5–30	31	–
LPS-NAc	ND	40	1	500	5
LPS-HF[b]	ND	40	5	–	20
Hydrolyzed J5 LPS					
J5 LPS-ACB	ND	62.5	1	62.5	<20
J5 LPS-HCl	ND	500	20	125	80

[a] SRBC (0.2 ml of packed cells) were coated with 50 μg of J5 LPS (for J5 MAb) or 100 μg of Re LPS (for 8-2C1). The amount of antigen that inhibited lysis of SRBC is given. , Inhibition by >500 ng. The following dilutions of MAb were used: 4-7B5, 1:10,000; 4-9A1, 1:500; BA7, 1:500, 4-6A1, 1:40,000; 8-2C1, 1:10,000. ND, Not done.

[b] LPS-HF does not coat polyvinylchloride microdilution plates; therefore only the results in inhibition PHT were given.



(o 4-9A1, ... BA7, • 4-7B5, ▲ 4-6A1)

FIG. 2. Inhibition of the reaction between anti-*E. coli* J5 LPS MAb and J5 LPS with hydrolyzed (0.1 M HCl, 100°C) *E. coli* J5 LPS in the PHT. Dilution of MAb was 2 or 3 hemolytic units. SRBC (0.2 ml of packed cells) were coated with 50 μg of *E. coli* J5 LPS. P_i release during hydrolysis (0.1 M HCl, 100°C) of *E. coli* J5 LPS (△) was measured by the method of Lowry et al. (13). The standard deviation for all data points was 0.002 μmol.

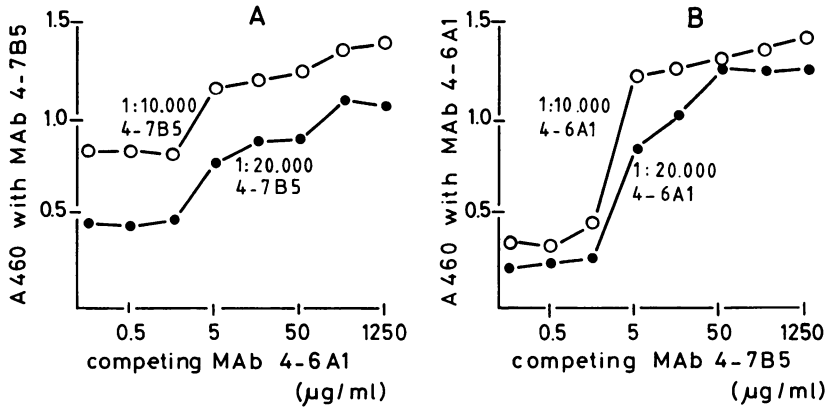


FIG. 3. Inhibition of reaction between MAb 4-7B5 (IgM) and MAb 4-6A1 (IgG) and J5 LPS in an ELISA. *E. coli* J5 LPS-coated microdilution plates were incubated (1 h) with twofold dilutions of MAb 4-6A1; after washing, one dilution (1:10,000 [○] or 1:20,000 [●]) of the competitive MAb 4-7B5 (ascites) was used. After 1 h of incubation, the amount of bound MAb 4-7B5 was detected with labeled anti-IgM antiserum (A). A second set of plates was incubated with MAb 4-7B5, and the amount of bound competitive MAb 4-6A1 was determined (B).

acid-labile phosphate substitution. It is possible that the glucosamine-heptose disaccharide is cleaved off during HCl hydrolysis and that therefore less reactivity with MAb BA7 could be seen in this experiment. For MAb 4-6A1 and MAb 4-7B5 the phosphate substitution is less important. Hydrolysis of sugar-sugar bonds, which form important antigenic binding sites, was responsible for the loss in their reactivity. MAb 4-6A1 probably binds to the heptose residues in the inner core, and these bonds may be more resistant to hydrolysis.

Competition experiment with MAb 4-7B5 and 4-6A1. *E. coli* J5 LPS-coated microdilution plates were incubated with twofold dilutions of MAb 4-6A1; after washing, the plates were incubated with the competitive MAb 4-7B5 (diluted 1:10,000 or 1:20,000) (Fig. 3A) and with MAb 4-6A1 as the competitive antibody (Fig. 3B). The amounts of MAb 4-7B5 (IgM; Fig. 3A) and of MAb 4-6A1 (IgG; Fig. 3B) were measured with labeled anti-IgM and anti-IgG, respectively. These competition experiments show that MAb 4-7B5 has more affinity to *E. coli* J5 LPS than does MAb 4-6A1. The proposed binding sites of the four MAb against *E. coli* J5 LPS are summarized in Fig. 4.

From the lack of activity of MAb 4-9A1 with LPS of the Ra, Re, and R5 types, dephosphorylated *E. coli* J5 LPS, and the products after kinetic hydrolysis of *E. coli* J5 LPS, we concluded that MAb 4-9A1 reacts with the terminal part of the core region, glucosyl-1,3-heptose, in which the heptose residue is probably phosphorylated. During hydrolysis of *E. coli* J5 LPS with HCl (0.1 N), the reactivity of MAb 4-9A1 is quickly lost. The glucose-1,3-heptose and the phosphate-heptose linkages are sensitive to HCl hydrolysis. Lack of binding of MAb 4-9A1 to Ra can be explained by the fact that the glucose-1,3-heptose is substituted on glucose and therefore sterically hindered in these LPS forms.

Because of lost activity after deamination and N acetylation of *E. coli* J5 LPS, we conclude that MAb BA7 reacts with glucosamine with a free amino group linked (α1-7) to heptose in the core region. The glucosaminyl-heptose has not been previously described as a constituent of LPS of *S. typhimurium* and *Salmonella minnesota* strains, and we therefore suggest that the antigenic binding site of BA7 is the glucosaminyl-heptose disaccharide. MAb BA7 showed no reactivity with LPS of *Vibrio cholerae*, which also contains this disaccharide (6, 12). This could be due to a different antigenic exposition.

Although, MAb 4-7B5 and MAb 4-6A1 react with the whole core structure of *E. coli* J5 LPS, only MAb 4-7B5 was active in a biological assay (PHT), probably because of an incomplete complement activation by the IgG1 isotype MAb 4-6A1. Phosphorylation does not influence the binding of both antibodies with oligosaccharides formed after hydrolysis with HCl (0.1 N). The moderate activity of both MAb with Re and Ra LPS indicates that L-glycero-D-manno-heptose and glucose are much more important for their antigenic recognition.

In contrast to anti-lipid A-KDO MAb 8-2C1, the anti-*E. coli* J5 LPS core oligosaccharide MAb (4-9A1, 4-6A1, and BA7) were not cross-reactive with gram-negative clinical isolates. This finding is supported by recent publications (1, 17, 21), which also suggest that reactivity of MAb with lipid A and/or KDO confers broad cross-reactivity and that reactivity of Rc/J5 specific MAb does not (20). The cross-

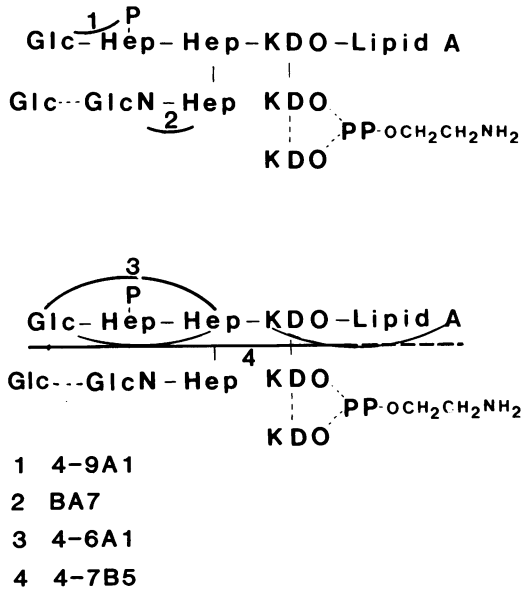


FIG. 4. Proposed binding sites of anti-*E. coli* J5 LPS MAb on a generalized structure of *E. coli* J5 LPS. Abbreviations are as described in the legend to Fig. 1.

reactivity of 4-7B5 may be explained by its additional moderate affinity for lipid A.

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