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THE COMPARISON OF MONOCLONAL ANTIBODIES SPECIFIC FOR TWO GENERAL EPITOPES LOCATED ON *Citrobacter* 036 LIPOPOLYSACCHARIDE*

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ABSTRACT

Monoclonal antibodies have been generated to *Citrobacter* 036 by the hybridoma technique and selected for lipopolysaccharide binding. The quantitative specificity of four clonotypes were analyzed by precipitin-inhibition assay, immunoblotting, and affinity electrophoresis. The 036-specific polysaccharide and core oligosaccharide fractions were used as inhibitors of the precipitation of these antibodies by the homologous lipopolysaccharide. Association constants of monomers of the monoclonal antibodies were determined by affinity electrophoresis. Association constants measured with lipopolysaccharide ranged from 8.3×10^2 to 1.7×10^4, and with the O-specific polysaccharide from 8.7×10^2 to 3.7×10^3. The results obtained by these three methods are in full accordance with the hypothesis that the monoclonal antibodies examined were raised to two general epitopes of *Citrobacter* 036 lipopolysaccharide. These are the O-specific polysaccharide and an *O*-acetylated structure that includes the O-specific oligosaccharide-core region.

INTRODUCTION

Monoclonal antibodies to *Citrobacter* 036 have been already characterized immunochemically by enzyme-linked immunosorbent assay (ELISA), passive hemagglutination, passive hemolysis, and bacterial agglutination[1]. In ELISA, the monoclonal antibodies were active with the homologous lipopolysaccharide and were inhibited by the O-specific polysaccharide of *Citrobacter* 036. This suggested that they were directed to the polysaccharide structure, which is a homopolymer of

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(1→2)-linked 4-deoxy-β-D-*arabino*-hexopyranosyl residues[2]. However, a distinct difference was observed in ELISA inhibition between one (CB-8.1) of the four monoclonal antibodies examined and the remaining three (CB-2.1, CB-4.1, and CB-6.1). The inhibition attained by 25 μg of 036-specific polysaccharides was only 20%, whilst the remaining three antibodies were 40 to 55% inhibited by the same quantity of inhibitor (see Fig. 1, ref. 1). This observation aroused doubt that the specificity of CB-8.1 monoclonal antibody was confined to the O-chain determinants. In this work, more detailed studies based on quantitative microprecipitin inhibition, immunoblotting analysis, and affinity electrophoresis were used to assess the specificity and affinity of these antibodies.

EXPERIMENTAL

Bacterial products. — *Citrobacter* strain neg^{s}Tc^s/serotype 036 and its rough mutant Tc^r from the stock collection of the Institute of Microbiology of Wroclaw University were used. The carbohydrate portion of lipopolysaccharide, O-specific polysaccharide, and core oligosaccharide fractions obtained after mild acid hydrolysis of lipopolysaccharide, followed by fractionation on Sephadex G-50, were prepared as described earlier[2]. The core fractions were rechromatographed on a Bio-Gel P-10 column (400 mesh; 1.6 × 90 cm) equilibrated with pyridine–acetic acid buffer, pH 5.4. The three core fractions isolated from the smooth lipopolysaccharide had the following distribution coefficients: Fraction Ia K_D 0.23, Fraction II K_D 0.43, and Fraction III, K_D 0.59. From the rough lipopolysaccharide, no O-specific polysaccharide was isolated, and only two core fractions, II (K_D 0.43) and III (K_D 0.59), were obtained.

Anti-Citrobacter *036 ascitic monoclonal antibodies.* — Ascites fluids were developed in BALB/c mice by use of each hybridoma cell line as previously described[1].

Quantitative microprecipitin test. — Precipitin tests were carried out essentially by the method of Kabat and Mayer[3]. Ascitic fluid (100 μL) of a ten-fold dilution was incubated with the antigen solution (100 μL) at various concentrations. The protein in the precipitates was determined by the method of Lowry *et al.*[4].

Inhibition of precipitation was performed by incubation of antibody solutions with increasing concentrations of inhibitors for 1 h at 37° prior to antigen addition. The optimal concentration of antigen required for maximal antibody precipitation was determined from the precipitin curve. The test mixtures contained 50 μL of inhibitor solution of various concentrations, 100 μL of ten-fold diluted ascitic fluid, and 50 μL of lipopolysaccharide 036 solution of the optimal concentration.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. — Lipopolysaccharide was analyzed by SDS-PAGE in a 15% acrylamide slab gel[5]. The lipopolysaccharide in the gel was detected by the silver-staining method of Tsai and Frasch[6].

Immunoblotting. — After SDS-gel electrophoresis, the separated

lipopolysaccharide was transblotted from the gel onto nitrocellulose (pore size, 0.45 μm)[7]. Electrophoretic transfer was done in 25μM Tris–192mM glycine buffer containing 20% methanol, pH 8.3, at 6 mA/cm^2 for 10–12 h. After transfer, the nitrocellulose paper was blocked with 5% bovine serum albumin (BSA) in 10mM K_3PO_4 buffer (pH 7.3) containing 0.15M NaCl and 0.05% Tween 20 (PBST) for 1 h at 40°. The transblot was incubated for 7 h at 30° with the ascitic fluid diluted 1:500 in PBST containing 5% BSA. The nitrocellulose paper was washed for 75 min in five changes of PBST buffer, prior to incubation with horseradish peroxidase conjugate, rabbit anti-mouse IgG –, or anti-mouse IgM prepared at a concentration of 10 μg Ab/mL of PBST containing 1% BSA for 2 h at 30°. After five 20-min washes in PBST, the nitrocellulose paper was developed in 3,3′-diaminobenzidine·4M HCl solution in the presence of H_2O_2. The developed nitrocellulose paper was dried and photographed.

Conjugates of rabbit anti-mouse IgG or rabbit anti-mouse IgM with horseradish peroxidase, type VI Sigma, were prepared by the method of Nakane and Kawaoi[8].

Affinity electrophoresis. — This was performed in slab gels (10 × 15 × 0.1 cm) according to Takeo and Kabat[9], and Sharon *et al.*[10]. A 5% acrylamide gel in Tris–glycine buffer (pH 8.3) was used. Ascitic fluid containing 2 μg of monoclonal antibody, as determined from the quantitative precipitin test, was applied to each lane of a slab gel. Samples were reduced in 0.15M 2-mercaptoethanol in sample buffer for 1 h at room temperature.

In order to maintain the same conditions, the electrophoresis was carried out on one slab divided in its length into two equal parts. In one part, antigen (200 μg/mL) was added to the gel, and in the other no antigen was added. The electrophoresis was run at 30 mA for 1 h.

The equation of Takeo and Kabat[9] (*1*) was used to calculate the dissociation constants (K^d) for the monoclonal antibody antigen system, where c is the antigen concentration in the gel(g/mL), R_{mo} the relative mobility of the antibody in the absence of antigen in the gel, and R_{mi} the relative mobility of antibody in the presence of antigen in the gel. The relative mobility of antibody was recorded as the ratio of the migration of antibody monomer (immunoglobulin) to that of albumin present in ascitic fluid.

$$K^d = cR_{mi}/(R_{mo} - R_{mi}) \qquad (1)$$

RESULTS

Quantitative microprecipitin inhibition. — The results of quantitative microprecipitin assay of the ascitic anti-*Citrobacter* 036 monoclonal antibodies with the homologous lipopolysaccharide are shown in Fig. 1. None of the four monoclonal antibodies precipitated the rough lipopolysaccharide isolated from Tc^r mutant. For determination of specificity, the monoclonal antibodies were examined in the

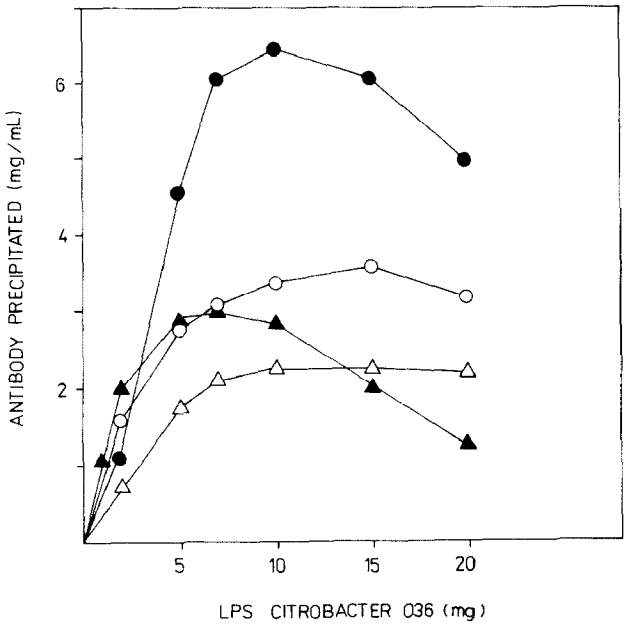

Fig. 1. Precipitation of anti-*Citrobacter* 036 monoclonal antibodies by the homologous lipopolysaccharide: (—●—●—) CB-2.1; (—○—○—) CB-4.1; (—△—△—) CB-6.1; and (—▲—▲—) CB-8.1.

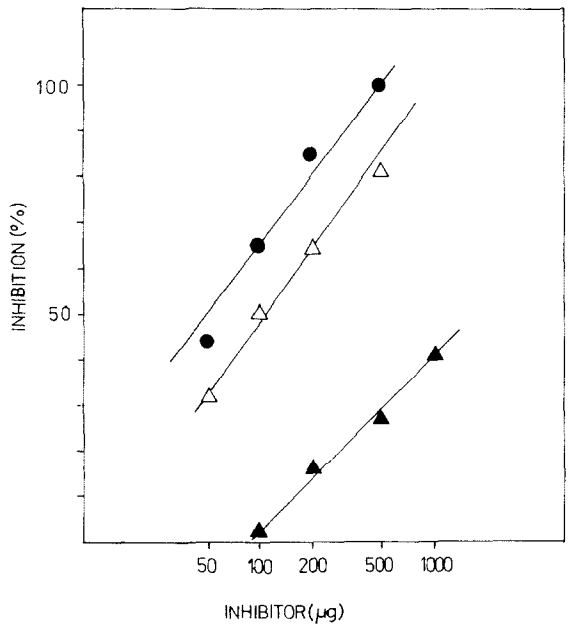

Fig. 2. Inhibition by 036-specific polysaccharide of precipitation of the anti-*Citrobacter* 036 monoclonal antibodies by the homologous lipopolysaccharide. Symbols as in Fig. 1.

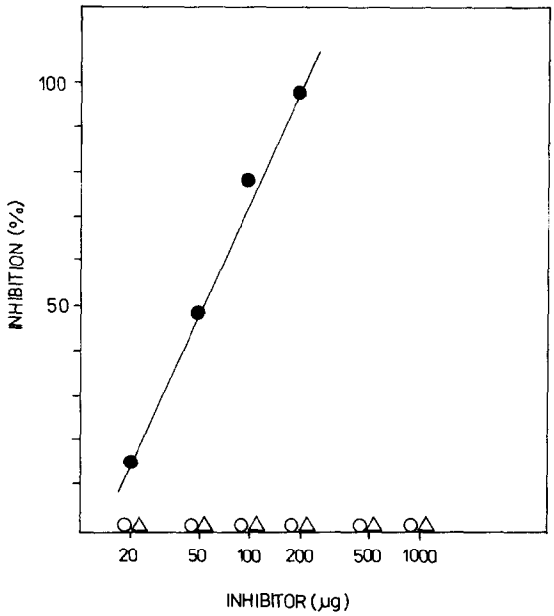

Fig. 3. Inhibition by the core oligosaccharide fractions of precipitation of CB-8.1 monoclonal antibody by *Citrobacter* 036 lipopolysaccharide: (—●—●—) Fraction Ia; (—○—○—) Fraction II; and (—△—△—) Fraction III.

precipitin inhibition assay with various fragments of the carbohydrate component of *Citrobacter* 036 lipopolysaccharide. As seen in Fig. 2, CB-2.1 and CB-6.1 antibodies were inhibited strongly by 036-specific polysaccharide (PS): 70–100 μg of 036 PS gave 50% inhibition, whereas antibody CB-8.1 was hardly inhibited by 036 PS, 50% inhibition requiring more than 1000 μg of inhibitor. The antibody CB-4.1, which is an immunoglobulin M, was not used in these experiments as it precipitated 036-specific polysaccharide itself. Maximal precipitation amounted to 2.15 mg of antibody/mg antigen.

Because antibody CB-8.1 did not show specificity towards the 036-specific polysaccharide, it was tested in precipitin-inhibition assays with three core fractions isolated from 036 lipopolysaccharide. As shown in Fig. 3, only fraction Ia exhibited marked inhibition of CB-8.1 antibody precipitation by 036 lipopolysaccharide antigen, and the weight required for 50% inhibition (50 μg of fraction Ia oligosaccharide) indicated a much higher specific activity than that of the 036-specific polysaccharide.

From structural studies of the core fractions of 036 serotype and its rough mutant[11], we established structure **1** for Fraction III (incomplete core), structure **2** for Fraction II (complete core), and structure **3** for Fraction Ia (complete core). After removal of the *O*-acetyl groups (0.1M NaOH, 16 h, room temperature), fraction Ia lost its inhibitory activity against CB-8.1 monoclonal antibody.

R-α-D-Glc*p*-(1→2)-α-D-Glc*p*-(1→3)-(Hep)$_3$-KDO
4
↑
1
β-D-Glc*p*
6
↑
1
α-D-Gal*p*

1 R = H

2 R = β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Glc*p*NAc)-(1→4)

3 R = (*O*-Ac-4-deoxy-β-D-*ara*-Hex*p*)$_3$-(1→3)-β-D-Gal*p*NAc-(1→4)-α-D-Gal*p*NAc-(1→3)-β-D-Glc*p*NAc)-(1→4)

Immunoblotting analysis of Citrobacter *036 lipopolysaccharide by monoclonal antibodies.* — Immunoblotting analysis was carried out to determine which epitopes located on *Citrobacter* 036 lipopolysaccharide are recognized by CB-2.1, CB-4.1, and CB-8.1 monoclonal antibodies. The silver-stained electrophoresis pattern of 036 lipopolysaccharide (Fig. 4A) showed at least three regions: (*a*) Four

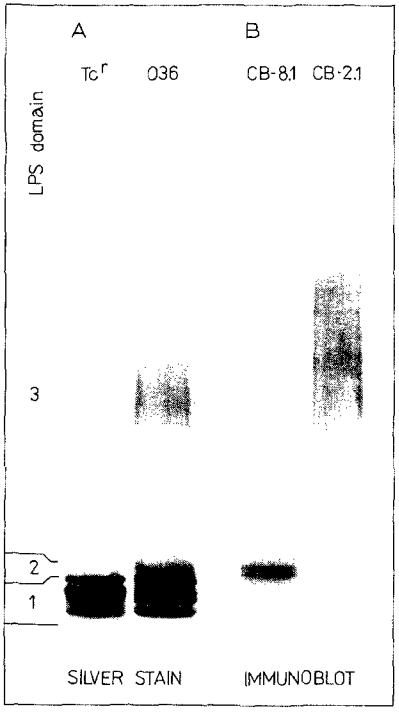

Fig. 4. (A) SDS-Polyacrylamide gel electrophoresis of *Citrobacter* 036 lipopolysaccharide (right lane, 1 μg) and of rough lipopolysaccharide of Tc^r mutant (left lane, 1 μg). (B) Immunoblot of *Citrobacter* 036 lipopolysaccharide with the monoclonal antibodies CB-8.1 and CB-2.1.

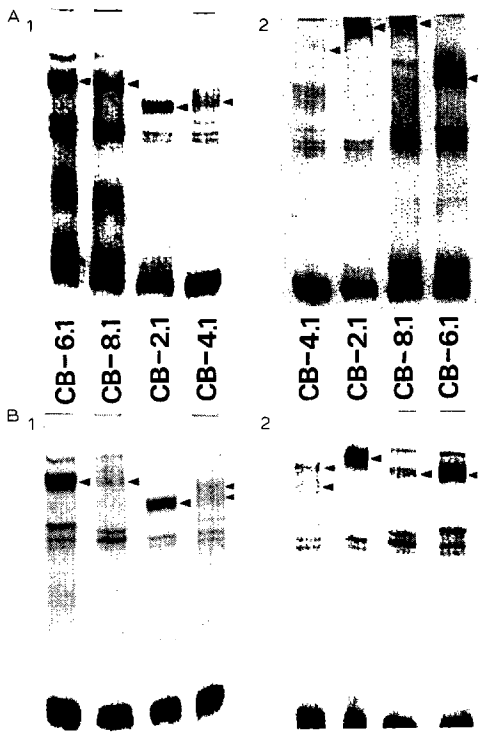

Fig. 5. Affinity electrophoresis of anti-*Citrobacter* 036 monoclonal antibodies in slab gels: (A1) No 036 lipopolysaccharide added to the gel; (A2) 036 lipopolysaccharide (200 μg/mL) added to the gel; (B1) no 036-specific polysaccharide added to the gel; and (B2) 036-specific polysaccharide (200 μg/mL) added to the gel. The arrows indicate bands of immunoglobulins. The gels were stained with Coomassie brilliant blue.

TABLE I

ASSOCIATION CONSTANTS K^a OF ANTI-*Citrobacter* 036 MONOCLONAL ANTIBODIES MEASURED WITH THE HOMOLOGOUS LIPOPOLYSACCHARIDE (LPS) AND 036-SPECIFIC POLYSACCHARIDE (PS)

Monoclonal antibody	$K^a_{LPS} \cdot mL^{-1} \cdot g^{-1}$	$K^a_{PS} \cdot mL^{-1} \cdot g^{-1}$
CB-2.1	1.7×10^4	3.7×10^3
CB-4.1	6.2×10^3	2.6×10^3
CB-6.1	8.3×10^2	1.1×10^3
CB-8.1	1.4×10^4	8.7×10^2

intensively stained fast-migrating bands which are related to the core lipopolysaccharide (region 1), (*b*) two bands of lower mobility, but in the vicinity of the core region (region 2), and (*c*) an unresolved continuous streak characteristic of O-specific lipopolysaccharide repeating-units (region 3). The rough lipopolysaccharide showed in SDS-gel electrophoresis only four fast-migrating bands

(region 1). As seen in Fig. 4B, CB-8.1 antibody reacted with region 2 of 036 lipopolysaccharide. On the other hand, CB-2.1, CB-4.1, and CB-6.1 antibodies reacted with 036 lipopolysaccharide containing O-specific chains region 3 (illustrated for CB-2.1). The results of the immunoblotting analysis are in full agreement with those obtained in the precipitin-inhibition assay. From precipitin-inhibition data, it may be concluded that the carbohydrate part of core lipopolysaccharide bands (region 1) has the composition of core Fractions II and III, whereas Fraction Ia represents the carbohydrate component of the lipopolysaccharide bands of region 2.

Determination of association constants of monoclonal antibodies specific for Citrobacter *036 lipopolysaccharide by affinity electrophoresis.* — Affinity electrophoresis was used for determining the association constants of anti-*Citrobacter* 036 monoclonal antibodies with the homologous lipopolysaccharide or with its O-specific polysaccharide. The relative mobilities of the antibody in SDS-gel electrophoresis were measured with and without the antigen. When 036 lipopolysaccharide or its O-specific polysaccharide was added to the gel, the migration of immunoglobulin monomers was retarded (Figs. 5 A and B), while the migration of other proteins of the ascitic fluids remained unchanged.

Association constants (reciprocal of K_d) calculated for anti-*Citrobacter* 036 ascitic monoclonal antibodies with the homologous lipopolysaccharide and 036-specific polysaccharide are listed in Table I. As seen by other methods, monoclonal antibody CB-8.1 has a relatively high K^a-value with the lipopolysaccharide, but the lowest K^a-value with 036-specific polysaccharide.

DISCUSSION

The results obtained from precipitin inhibition, immunoblotting analysis, and affinity electrophoresis confirmed that CB-2.1, CB-4.1, and CB-6.1 monoclonal antibodies are specific to linear homopolymer of (1→2)-linked 4-deoxy-β-D-*arabino*-hexose, although they show diversity in their affinity to specific antigen. The diversity is reflected in their association constants with homologous lipopolysaccharide, which range from 8.3×10^2 to 1.7×10^4, the highest being about 20 times greater than the lowest. The lack of identity among monoclonal antibodies of the same specificity has often been observed, *e.g.*, the anti-dextran hybridoma antibodies[10].

As proven in our experiments, CB-8.1 monoclonal antibody was directed neither to the homopolymer of 4-deoxy-D-*arabino*-hexose, nor to Fractions II and III complete and incomplete cores, respectively. It was, however, specific for Fraction Ia, which represents the *O*-acetylated oligosaccharide composed of the complete core and a 4-deoxy-D-*arabino*-hexopyranose trisaccharide side-chain (**3**). The *O*-acetyl group is immunologically important, because its selective removal destroyed the inhibitory activity of structure **3**. It appears that the epitope recognized by the antibody CB-8.1 includes the linkage between the core and the O-specific polysaccharide of *Citrobacter* 036 antigen. Actually, the position of the

specificity suggests that it is located in the vicinity of the linkage core–O-specific chain.

It should be emphasized that O-specific polysaccharide obtained after mild acid hydrolysis of lipopolysaccharide also contains the epitope that includes the O-specific chain-core region, but this epitope represents only a low percentage of the whole material. It is for this reason that the 036-specific polysaccharide exhibits some inhibitory activity against monoclonal antibody CB-8.1.

Citrobacter 036 lipopolysaccharide has shown great heterogeneity as far as its carbohydrate part is concerned. This was proven by gel filtration of the carbohydrate portion obtained from the lipopolysaccharide[2], as well as by SDS-gel electrophoresis of the lipopolysaccharide itself. O-Specific polysaccharide was eluted from a Sephadex G-50 column as a wide peak indicating various lengths of O-specific chains. This may also be seen in SDS gels of the smooth 036 lipopolysaccharide, region 3 (Fig. 4), which is observed as a long unresolved streak.

The results of the experiments reported herein have shown that a significant portion of lipopolysaccharide 036 molecules have core stubs unsubstituted by O-specific chains. Moreover, two kinds of cores were observed in this portion of 036 lipopolysaccharides, complete (Fraction II) and incomplete core (Fraction III), during gel filtration of the carbohydrate material. By comparison, SDS-electrophoresis of 036 lipopolysaccharide showed four fast-migrating bands for the core portion (Fig. 4A, region 1), which suggested further heterogeneity of the core region, probably caused by components such as ethanolamine diphosphate residues, which may be partly hydrolyzed to phosphate. Complete and incomplete cores were also observed in the rough lipopolysaccharide of Tc^r mutant.

In the population of 036 lipopolysaccharide molecules, a small portion of core stubs were substituted by an oligosaccharide composed of 4-deoxy-D-*arabino*-hexopyranosyl residues bearing an *O*-acetyl group (Fraction Ia), and these are identified as region 2 (Fig. 4A). Monoclonal antibodies selected from the murine response to *Citrobacter* 036 lipopolysaccharide are directed toward two epitopes, the O-specific polysaccharide and an *O*-acetylated structure that includes the region linking the O-specific oligosaccharide to the core.

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