ANALYSIS OF SPECIFICITY OF INTERACTION BETWEEN SYNTHETIC KEY SACCHARIDE COMPONENTS OF LIPOPOLYSACCHARIDES AND MONOCLONAL ANTIBODIES TO *COXIELLA BURNETII*

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Summary. – Two monoclonal antibodies (MAbs) against the lipopolysaccharides (LPSs) of *Coxiella burnetii* (*C.b.*) strains Priscilla and Nine Mile were prepared characterized by their interaction with synthetic glycoconjugates representing parts of LPSs of *C.b.* in virulent phase. Both MAbs were directed against immunodominant epitopes comprising core constituent of LPSs, Kdo (3-deoxy-α-D-*manno*-2-octulo-pyranosylonic acid). ELISA showed that the anti-Nine Mile MAb 4/11 bound preferably to disaccharides (α-Kdo (2 → 4) α-Kdo and α-Kdo (2→4) α-(5d) Kdo), while the anti-Priscilla MAb 1/4/H bound to all conjugates, though with various intensity. On the other hand, immunoelectron microscopy revealed a positive binding of only one glycoconjugate, namely the trisaccharide α-Kdo (2→4) α-Kdo (2→4) α-Kdo-BSA, to both MAbs. In competitive ELISA (cELISA), the anti-Priscilla MAb 1/4/H distinguished the strains Nine Mile and Priscilla, while the anti Nine Mile MAb 4/11 did not.

Key words: Coxiella burnetii; monoclonal antibodies; synthetic saccharides; lipopolysaccharides; ELISA; cELISA; immunoelectron microscopy

Introduction

C.b., the etiological agent of Q fever, is distributed worldwide. The disease occurs in animals and humans as subclinical, acute or chronic infection. Primary vectors are ticks that transmit the pathogen to domestic and wild animals, but the infection of humans spreads mainly via infectious aerosol, the respiratory route being a port of entry and the lungs a site of primary infectious focus.

This Gram-negative bacterium (Maurin and Raoult, 1999) is an obligate intracellular parasite of eukaryotic cells. Following endocytosis, replication of *C.b.* occurs exclusively in phagolysosomes. The infectious process and immune response have been studied in cell cultures and animal systems (Norlander, 2000).

Outer membrane protein antigens and LPS are the major surface antigens of *C.b.* Structural differences between various strains have been found by SDS-PAGE (Hackstadt *et al.,* 1985, 1986; To *et al.,* 1998), immunoblot analysis (Kováčová *et al.*, 1994), immunoelectron microscopy (McCaul *et al.*, 1991) and methods based on MAbs (Yu *et al.*, 1994).

C.b. LPS is chemically and serologically related to those of enterobacteria (Brade *et al.,* 1985, 1986) and is composed

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Abbreviations: BSA = bovine serum albumin; *C.b.* = *Coxiella burnetii*; cELISA = competitive ELISA; GlcN = 2-amino-2-deoxy-D-glucopyranose; GlcNAc = N-acetyl-D-glucosamine; Kdo = 3-deoxy-α-D-*manno*-2-octulopyranosylonic acid; (5d)Kdo = 5-deoxyderivative of Kdo; LPS = lipopolysaccharide; MAb = monoclonal antibody; PA = polyacrylamide; PBS = phosphate-buffered saline; PBST = PBS containing 0.5% Tween 20

of three parts: (i) lipid A, responsible for endotoxic activities (Galanos *et al*., 1985), (ii) a heteropolysaccharide, termed also core region, and (iii) a so-called O chain, composed of repeated oligosaccharide units (Amano *et al*., 1987). Each of these parts exhibits distinct immunoreactive properties, which are used for serotyping of Gram-negative bacteria.

It has been shown that the majority of antibodies in polyclonal rabbit antisera are directed against complex epitopes comprising parts of both the lipid A and the core region (Rozalski *et al*., 1989). A Kdo trisaccharide proximal to the lipid A appears to be the main component of the core region in native LPSs of *C.b.* Studies of these components of *C.b.* are substantial, as they harbor a genus-specific epitope consisting of virenose and dihydrohydroxystreptose (Schramek *et al.,* 1982, 1985; Škultéty *et al.,* 1998a). Immunodominancy of terminal sugars of LPSs has been described also for other bacteria (Brade *et al.,* 1985; Schramek *et al*., 1982; Mayer *et al.,* 1988).

In this study, we have attempted to characterize the factors determining the specificity of interaction of MAbs against *C.b.* and synthetic saccharide components of LPSs with immunoreactive properties. For this purpose, anti-Nine Mile (4/11) and anti-Priscilla (1/4H) MAbs were prepared. The study revealed certain extent of structural variability of saccharide components of LPSs that still allowed for the specificity of the interaction. Preliminary results of this study have been published earlier (Kováčová *et al*., 2004).

Table 1. Artificial glycoconjugate (GC) antigens

	Chemical structure
GC-1	α-Kdo-BSA
GC-2	α-Kdo (2 → 4) α-Kdo-BSA
GC-3	α-Kdo (2 → 4) α-Kdo (2 → 4) α-Kdo-BSA
GC-4	α-Kdo (2 → 4) α-Kdo (2 → 6) ß-GlcNAc (1 → 6) ß-GlcNAc-BSA
GC-5	α-Kdo (2 → 4) α-Kdo-copolymer (PA)
GC-6	α-Kdo (2 → 4) α-(5d) Kdo-copolymer (PA)
GC-7	α-Kdo (2 → 6) ß-GlcNAc-copolymer (PA)
GC-8	α-Kdo (2 → 4) α-Kdo (2 → 6) α-GlcNAc-copolymer (PA)

Materials and Methods

LPSs of phase I *C.b.* we purified according to Schramek *et al.* (1976, 1978).

Artificial antigens. Truncated ligosaccharides, cysteamine glycosides and Kdo oligosaccharides served as artificial antigens. Kdo oligosaccharides were prepared according to Kosma *et al*. (1987, 1988, 1990).

BSA-conjugates. α-Kdo-BSA (GC-1), α-Kdo (2 → 4) α-Kdo-BSA (GC-2), α-Kdo (2 → 4) α-Kdo (2 → 4) α-Kdo-BSA (GC-3) and α-Kdo (2 → 4) α-Kdo (2 → 6) ß-GlcNAc (1 → 6) ß-GlcNAc-BSA (GC-4) were used (Table 1). Kdo residues were covalently linked to bovine serum albumin (BSA) by the glutardialdehyde method (Holst *et al.,* 1991).

Polyacrylamide (PA) glycoconjugates. α-Kdo (2 → 4) α-Kdo-copolymer (PA) (GC-5), α-Kdo (2 → 4) α-(5d) Kdo-copolymer (PA) (GC-6), α-Kdo (2 → 6) ß-GlcNAc-copolymer (PA) (GC-7) and α-Kdo (2 → 4) α-Kdo (2 → 6) α-GlcNAc-copolymer (PA) (GC-8) were employed (Table 1).

MAbs. The anti-Priscilla MAb 1/4H was prepared in a standard way (Sekeyová *et al*., 1995).

The anti-Nine Mile MAb 4/11 was kindly provided by Prof. H. Krauss, Institut für Hygiene und Infektionskrankheiten der Tiere, Justus-Liebigs-Universität, Giessen,Germany.

Dot blot analysis was performed according to Cowley *et al.* (1992). Briefly, methanol-washed membranes (Immobilon P, Millipore) were cut into the circles of 1.5 cm in diameter, dried and used for applying 5 µg antigens in 1 µl of PBS per drop. Blots were incubated at +4°C for 24 hrs and saturated with a stop solution at room temperature for 15 mins. After washing, blocking were incubated with MAbs diluted 1/2000 in PBS at room temperature for 5 mins, washed 3 times and stained according to Willems *et al*. (1992).

Western blot analysis (WBA) was carried out according to Willems *et al.* (1992). Briefly, following SDS-PAGE gels were electroblotted to Immobilon P membranes (Millipore), which were washed with methanol and distilled water prior to use. Blots were thoroughly washed with PBS containing 0.5% Tween 20 (PBST), saturated with a blocking solution (3% fish gelatin in PBST), washed again and incubated with MAb diluted to 1/2000 in PBS. After the addition of anti-mouse IgG-alkaline phosphatase conjugate diluted to 1/20,000, blots were incubated at room temperature for another 45 mins. After washing 3 times with PBS, blots were stained with BCIP and Nitroblue tetrazolium salt. In each run, a mixture of prestained standards was included. Chemicals from Bio-Rad, Merck, Serva and Sigma were used.

Immunoelectron microscopy. Immunogold labeling and negative staining techniques in modifications according to Lin (1984) and Brenner *et al.* (1959) were used. Briefly, samples (20 µl) of stock suspensions of glycoconjugates (1 mg/ml) were layered on formvar carbon-coated grids (200 mesh gold grid, Polaron Equipment Ltd., England). After attachment (10 mins), the grids were rinsed with droplets of PBS (2 x 5 mins) and 1% BSA in PBS (30 mins). The immunolabeling reaction was carried out with purified MAbs (20 µl aliquots of 1 mg/ml stock solutions) for 30 mins. After washing with 1% BSA (3 x 5 mins), the grids were labeled with a goat anti-mouse IgG-gold (10 nm) conjugate (British BioCell International) diluted 1:20 in PBS/BSA (20 µl/grid) for 30 mins at room temperature. The grids were then washed with droplets of PBS (2 x 5 mins) and redistilled water (2 x 5 mins). After negative staining with 2% phosphotungstic acid pH 7.0 for 1 min electron microscopy was carried out with Philips EM 300 at 80 kV.

ELISA and cELISA. An indirect ELISA as described by Kováčová *et al*. (1994) and Thiele *et al*. (1992) was employed. cELISA was performed by preincubation of twofold serial dilutions of inhibitors with selected MAbs at 37°C for 1 hr. Ninety-six-well plates (Sarstadt, USA) were coated overnight at 4°C with LPSs (2.5 mg/ml), prepared from Nine Mile or Priscilla strain of *C.b.* in phase I (egg passage 3). After blocking, preincubated mixtures of inhibitors and MAbs (50 µl) were added to wells and the plates were

incubated at 37°C for 2.5 hrs. Then there followed additions of peroxidase-labeled swine anti mouse globuline (Sevapharma, Czech Republic) and substrate solution with DAB (Merck, Germany) (30 mins) and reaction stop. A_{450} was determined using a reader (Multiscan Photometer, Labsystems, Finland). The percentage of inhibition was calculated by comparison with a control without an inhibitor and plotted against the concentration of inhibitor. The concentration required for 50% inhibition was determined for each inhibitor used.

Results

Binding of MAbs to homologous and heterologous LPSs

Initially, a titration of each MAb against natural smooth LPSs of homologous and heterologous strains of *C.b.* in phase I was carried out by ELISA (Fig. 1). Both MAbs 1/4/H and 4/11 in higher concentrations (6.2–50 μg/ml) bound to their homologous LPSs by the same intensity. However, in lower concentrations (0.4–3.1 μg/ml), MAb 4/11 showed a lower binding compared with MAb 1/4H. The binding of both MAb to heterologous LPSs was clearly lower than that to homologous ones. This result indicates that heterologous LPSs had obviously a lower number of specific epitopes needed for a positive reaction with MAb as compared to homologous ones.

Immunoelectron microscopy of interactions of MAbs with glycoconjugates

In next step, attention was paid to an important part of LPSs, namely Kdo oligosaccharides. Exclusiveness of direct visualization of the reaction of a MAb with a Kdo oligosaccharide, using a relatively low amount of precisely defined and well-preserved Kdo oligosaccharide, was the main reason of using immunoelectron microscopy. The immunogold labeling allowed for the detection of saccharide conjugates. The method was able to show gold as small patches on the surface membrane resembling budding particles.

While MAb 1/4/H bound to all glycoconjugates, MAb 4/11 gave mostly negative results (Fig. 2). The only clearly positive reaction, comparable to those with MAb 1/4/H, was obtained with GC-5, while other glycoconjugates gave only a weak reaction, though stronger than that with negative control MAb. By reacting MAb 1/4/H with GC-2, GC-7 or GC-6 the gold was flocked into small spots and formed aggregates. With GC-4, more gold was dispersed over the background. An obviously specific binding of MAb 4/11 to GC-5 is demonstrated in Fig. 3 showing small patches of gold on the surface membrane of glycoconjugate particles.

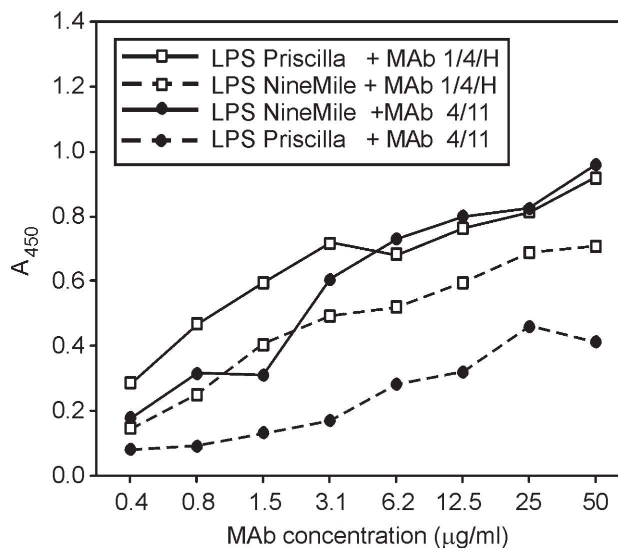

Fig. 1

Binding capacity of MAbs 1/4/H and 4/11 to LPSs of *C.b.* strains Priscilla and Nine Mile in phase I

ELISA. Solid lines: binding of MAb to homologous LPS; dashed lines: binding of MAb to heterologous LPS.

WBA shows binding of MAbs to single glycoconjugate only

In following the reaction of the MAbs with glycoconjugates GC-1 to GC-4 by WBA, a positive result, namely a 30 K band was obtained with GC-3, the trisaccharide conjugate α-Kdo (2 → 4) α-Kdo (2 → 4) α-Kdo -BSA (Fig. 4).

It is interresting to note that GC-2, GC-3, GC-4 and GC-7, when tested with the MAbs by a dot blot technique, gave positive results (data not shown).

cELISA reveals influence of structural variability of the glycoconjugates on the binding of the MAbs to C.b. LPSs

Since WBA does not give quantitative data, we employed cELISA, in which the binding specificity of the MAbs was investigated by using natural *C.b.* LPSs of strains Priscilla and Nine Mile as coating antigens, and artificial glycoconjugates as inhibitors (Fig. 5 and Table 2).

All the eight used glycoconjugates inhibited binding of MAb 1/4/H to both LPS-Priscilla and LPS-Nine Mile. Whereas GC-1, GC-2, GC-3 and GC-7 caused a 50% inhibition of the binding of MAb 1/4/H to LPS-Priscilla at a concentration range 12.8–19.8 μg/ml, GC-4, GC-5, GC-6 and GC-8 were stronger inhibitors; a 50% inhibition was achieved at a concentration below 7.8 μg/ml. In the case of LPS-Nine Mile, all the conjugates were fairly good inhibitors at a concentration below 7.8 μg/ml. Binding of MAb 4/11 to both homologous and heterologous LPSs was inhibited

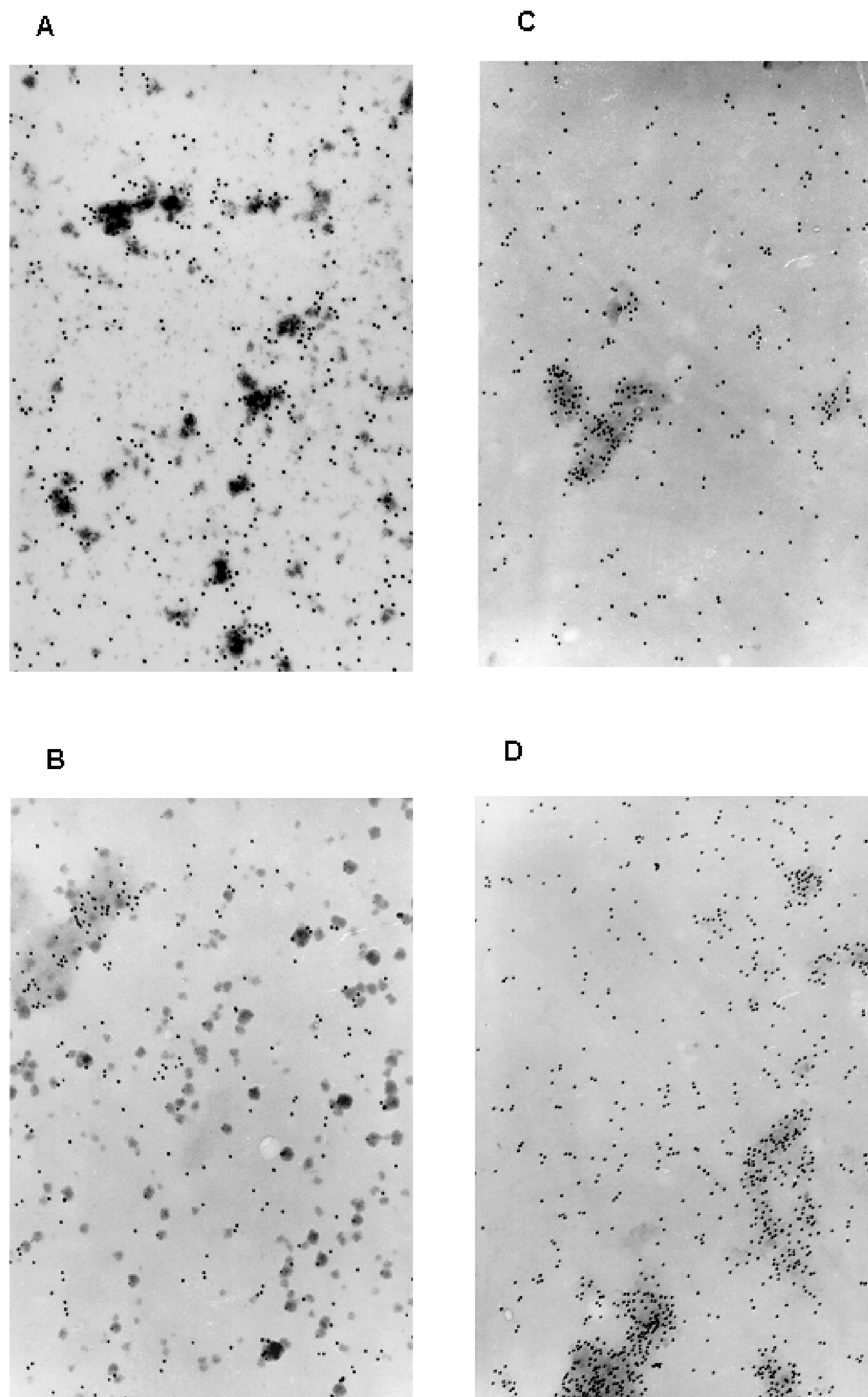

Fig. 2

Raction of the MAbs with the glycoconjugates as visualized by immunoelectron microscopy

A: MAb 1/4H + GC-2; **B**: MAb 1/4H + GC-7; **C**: MAb 1/4H + GC-6; **D**: MAb 1/4H + GC-4; **E**: MAb 4/11 + GC-2; **F**: MAb 4/11 + GC-7; **G**: MAb 4/11 + GC-6; **H**: MAb 4/11 + GC-4.

A highly dispersed immunoreactivity (A–D) contrasts with an immunoreactivity restricted to small areas (E–H). Magnification 42,000x.

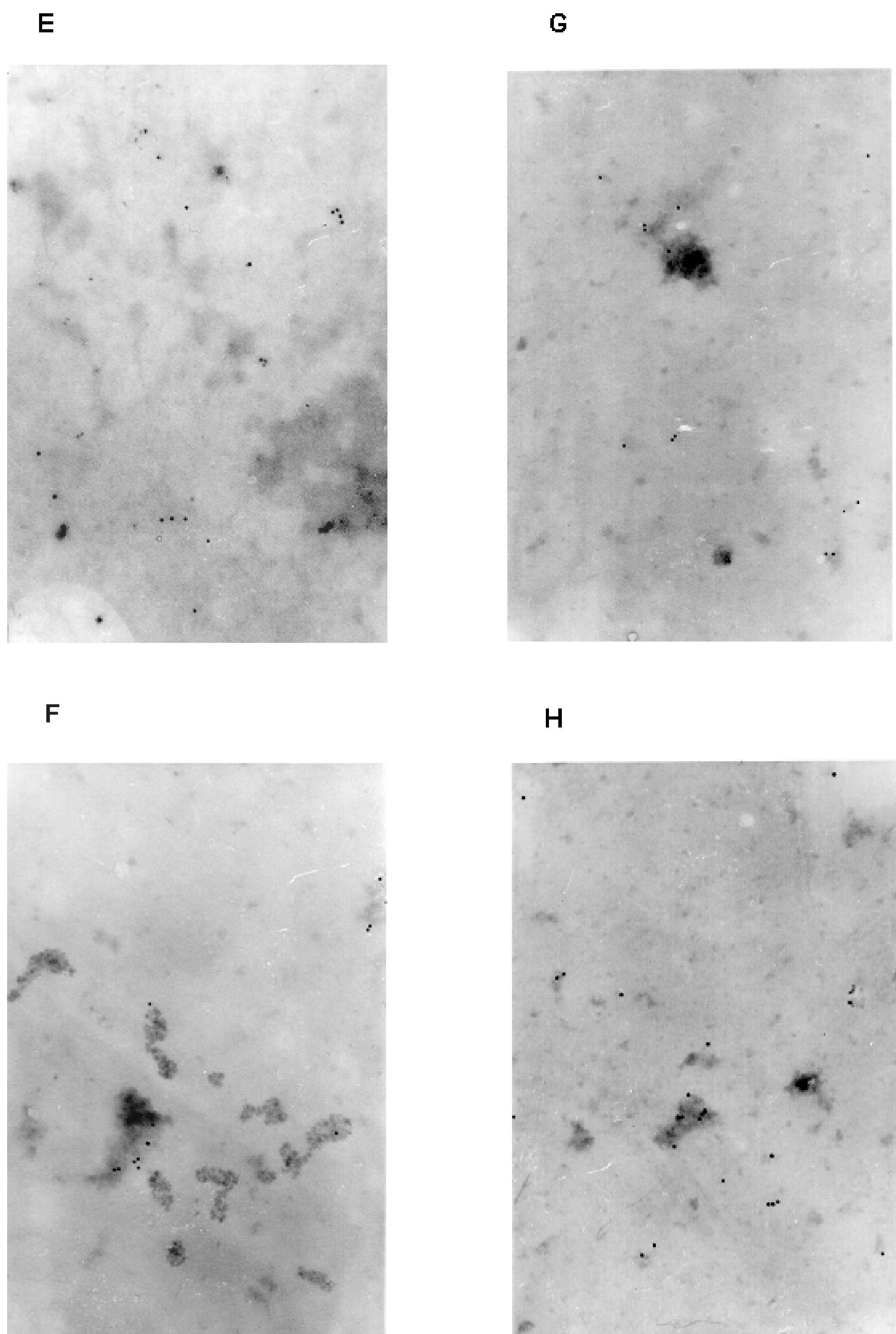

Fig. 2

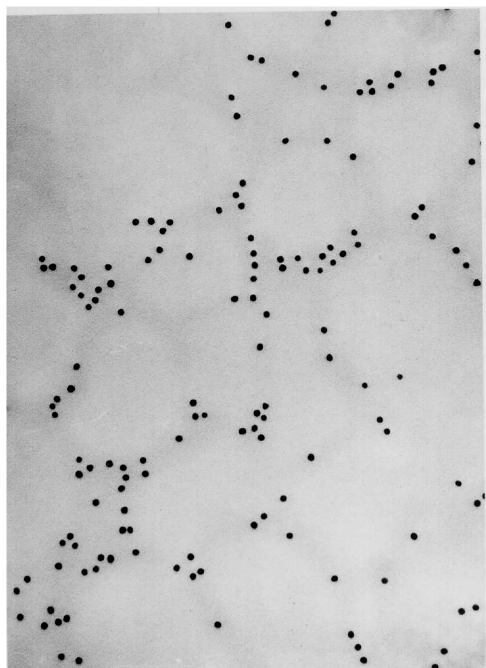

Fig. 3

Reaction of MAb 4/11 with glycoconjugate GC-5 – immunoelectron microscopy

A specific binding is demonstrated by small patches of gold on the surface membrane of glycoconjugate particles. Magnification 55,000x.

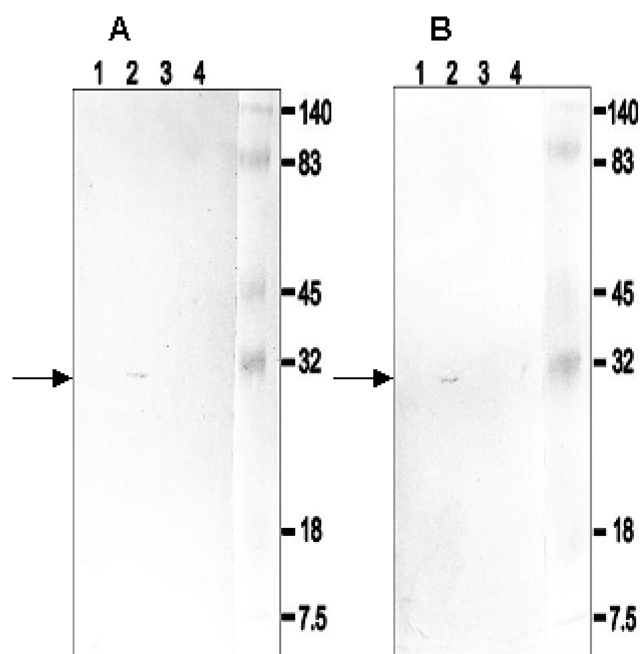

Fig. 4

Reaction of MAb 1/4/H (A) and MAb 4/11 (B) – Western blot analysis

GC-2 (lane 1); GC-3 (lane 2); GC-1 (lane 3); GC-4 (lane 4). Relative molecular mass markers in K values (the right end lane).

Table 2. IC_{50} values of glycoconjugates for the binding of the anti-Priscilla MAb 1/4/H and the anti-Nine Mile MAb 4/11 to *C.b.* LPSs of strains Priscilla and Nine Mile

Glyco-conju-gate	IC_{50} (μg/ml)			
	Anti-Priscilla MAb 1/4/H		Anti-Nine Mile MAb 4/11	
	LPS Priscilla	LPS Nine Mile	LPS Nine Mile	LPS Priscilla
GC-1	18.9	<7.8	>500	NI
GC-2	19.8	<7.8	7.1	6.7
GC-3	12.8	<7.8	NI	NI
GC-4	<7.8	<7.8	NI	NI
GC-5	<7.8	<7.8	23.5	21.6
GC-6	<7.8	<7.8	~500	~500
GC-7	14.8	<7.8	NI	NI
GC-8	<7.8	<7.8	NI	NI

NI = no inhibition at the highest inhibitor concentration.

over 50% by GC-2 and GC-5 in a concentration range 6.7–23.5 μg/ml (Table 2). A similarly high inhibition was obtained also with GC-6, but only at the highest concentration of the inhibitor (500 μg/ml). The remaining glycoconjugates gave a non-specific or no inhibition.

Discussion

MAbs 1/4/H and 4/11 are considered specific to LPSs, but the corresponding epitopes are apparently distributed on molecules of diverse molecular size. Irregularities in binding properties of the anti-Priscilla MAb 1/4/H to several *C.b.* isolates from France, Austria, Germany, Russia, Africa and Slovakia and varying WBA patterns have been observed in our previous studies (Sekeyová *et al.*, 1995, 1996a,b). *C.b.* isolates of three out of twenty established groups, distinguished according to *Not*I restriction analysis (Heinzen *et al.,* 1990; Thiele *et al.,* 1993), did not have any binding site for MAb 1/4/H on their surface (Sekeyová *et al.,* 1996a,b; Toman *et al.*, 1996). On the contrary, anti-Nine Mile MAb 4/11 reacted similarly with all the isolates tested. A single band corresponding to the same M_r was observed. Furthermore, a cross-reactivity of MAb 4/11 with chlamydial antigens in ELISA has been observed (Sekeyová *et al.*, 1996b).

Since a natural LPS is heterogenous even after extensive purification, it may lead to misinterpretation of the results. That's why MAbs are helpful in clarifying the nature of LPS structure, especially with regard to certain ambiguities on the level of strains (Brade *et al.*, 1987). To answer the

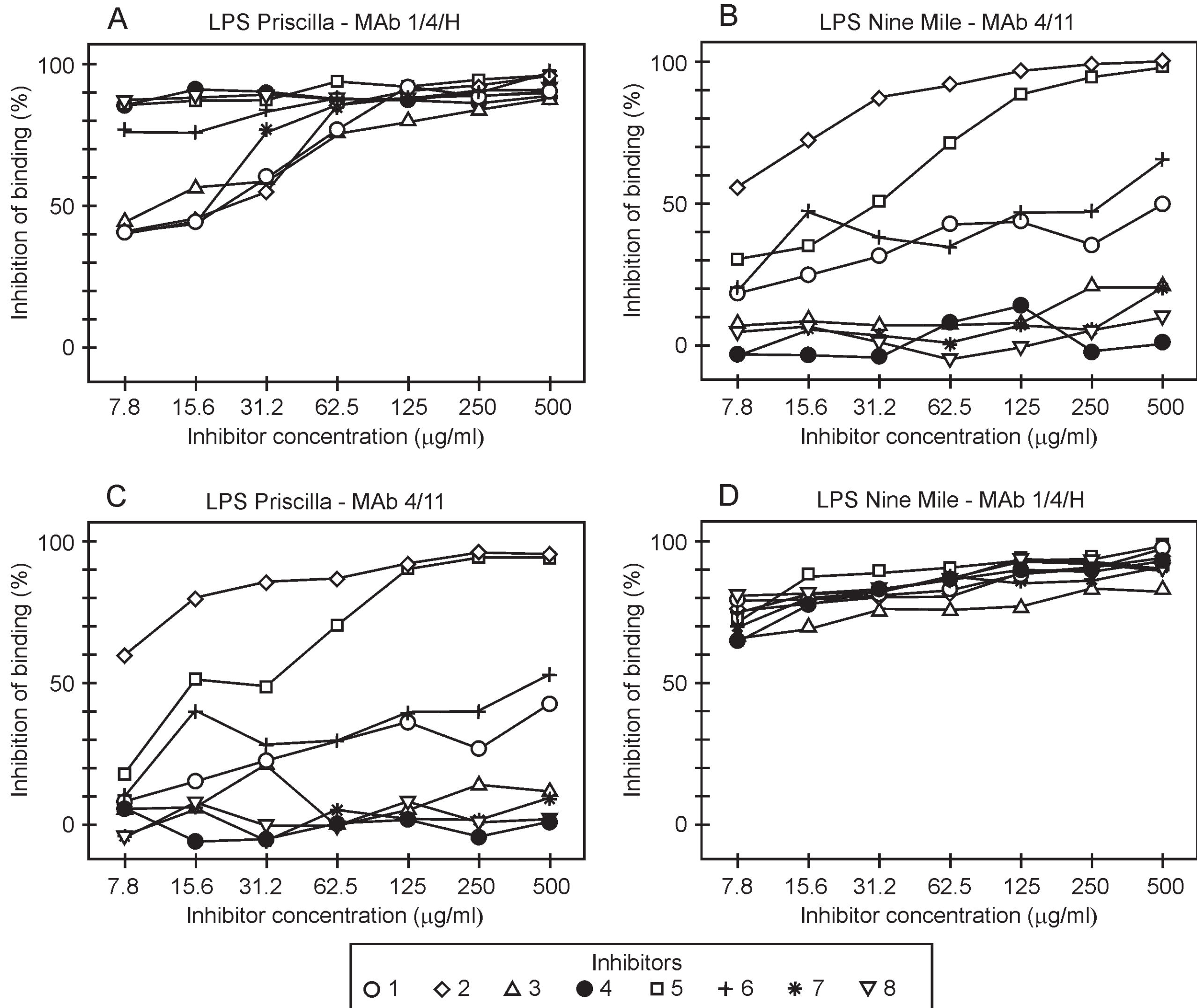

Fig. 5

Inhibition of binding of th MAbs to natural LPSs of *C.b.* strains Priscilla and Nine Mile by artificial glycoconjugates – cELISA

Inhibitors 1–8 correspond to glycoconjugates GC-1– GC-8.

questions concerning chemical and antigenic structures of LPSs, we decided to use artificial antigens (Kdo mono-di- or trisaccharides) in studying the ultimate goal, the interaction of *C.b.* with a host cell on molecular level. Thus we synthesized ourselves or obtained chemically homogenous saccharide conjugates that might exhibit *C. burnetii* strain specificity and be suitable for analysis by electrone microscopy, WBA and ELISA. Such an approach allows to disclose individual antigenic components in the humoral response.

Altogether eight synthetic glycoconjugates (Kdo oligosaccharides) were employed as artificial antigens. A precise immunolocalization of glycoconjugates by electron microscopy has been so far hampered by the fact that, in current methods, glycoconjugates are not immobilized by chemical fixation and thus get extracted during dehydration with organic solvents. Therefore we chose a unique approach that utilizes PA- and/or BSA-glycoconjugates, which form higher oligomeric structures and immunoaggregates detectable by electron microscopy and immunological cross-linking (Birkelund *et al.*, 1989; Ilk *et al.*, 1999).

In this study, immunogold labeling of the glycoconjugates tested clearly indicated binding of MAb 1/4/H to saccharide aggregates. Electron microscopy revealed that a KDO substituent influences the antigenicity of the inner core

region. The two MAbs behaved differently: whereas MAb 1/4/H reacted with all eight glycoconjugates, the reactions of MAb 4/11 were almost negative except for GC-5 (α-Kdo (2 → 4) α-Kdo-copolymer (PA)) This result suggests that MAb 4/11 is a specific antibody directed against an epitope containing a disaccharide.

This conclusion was supported by the results obtained with cELISA. This method also showed that the nature of the carrier of the disaccharide (copolymer or BSA) is not of primary importance. A significantly higher amount of the inhibitor was needed for 50% inhibition in the case of GC-6 compared to GC-2 or GC-5.

All eight glycoconjugates were able to inhibit the binding of MAb 1/4/H to LPSs of both *C.b.* strains, but the inhibition level depended on the strain, which from the LPS as coating antigen was prepared. With GC-1, GC-2, GC-3 and GC-7, the binding was lower for the Priscilla LPS. The anti-Priscilla MAb 1/4/H was able to distinguish the two strains in cELISA on the glycoconjugate level. This result and those from direct ELISA are in accord with our previous findings about the divergence of Kdo structures within LPS molecules of Nine Mile vs. Priscilla strain (Škultéty *et al.,* 1998a,b). In comparison to homologous LPSs, the presence of a lower number of specific epitopes on the surface of heterologous LPSs was detected. This might be due to configuration differences or absence of binding sites on the surface of the LPSs.

A different structural arrangement or conformation of the antigens in assays, as a result of their different treatments, resulted to certain discrepancies. Four glycoconjugates, GC-2, GC-3, GC-7 and GC-4 reacted positively in dot blot analysis.

In defining the MAb specificity, Pollack *et al*. (1989) have stressed the importance of using more than one assay due to assay-associated differences in antibody-binding patterns. In cELISA, a specific MAb directly binds to the a glycoconjugate inhibitor. On the other hand, the accessibility of a MAb-specific epitope on the surface of LPS may be affected by its association with the polystyrene matrix by electrostatic bonds which may influence the detection of binding. This may possibly account for the differences in epitope recognition when compared with other methods like dot blot hybridization and electron microscopy.

The WBA was restricted to four glycoconjugates contain KDO residues covalently linked to BSA, which enabled their migration in SDS-PAGE. Of these, only the trisaccharide glycoconjugate GC-3 (α-Kdo (2 → 4) α-Kdo (2 → 4) α-Kdo-BSA) reacted positively with the both MAbs with a single band of 30 K. An essential part of this molecule was the structure α Kdo2 → 4α Kdo2 → 4α Kdo, a basic component of *C.b.* LPS. It is possible that monosacharide or disaccharides molecules were not sufficiently large to be recognized by MAbs in this assay.

We may conclude, that within the *C.b.* antigen a partial binding components, required by the anti-Priscilla 1/4/H and anti-Nine Mile 4/11 MAbs, are structures of the Kdo region. The reactions with these saccharide conjugates were positive though not equally intensive in all the tests used. However, comparison of the results, especially those with natural LPSs, disclosed also involvement of multiple structures (sites), indispensable for specific recognition by the MAbs 1/4/H or 4/11 in the envelope of *C.b.*

These results extended previous knowledge of the phenomenon and provided clear evidence not only for the presence of saccharide conjugates on the surface of *C.b.* cells but also for substantial role they may play in differential diagnosis.

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