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Characterization of Smooth Lipopolysaccharides and O Polysaccharides of *Brucella* Species by Competition Binding Assays with Monoclonal Antibodies

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Previously, four epitope specificities on the O chain of *Brucella* species were reported: M, A, C, and C/Y. In this work, according to monoclonal antibody binding to smooth lipopolysaccharides of *Yersinia enterocolitica* O:9, *Brucella abortus* W99 (A-dominant strain), and *B. melitensis* Rev1 (M-dominant strain), seven O-chain epitope specificities were defined: M, A, C (M > A), C (M = A), C/Y (M > A), C/Y (M = A) and C/Y (A > M). Competitive binding assays between these monoclonal antibodies suggested that these different epitopes are probably overlapping structures.

Brucellae are facultative intracellular bacteria responsible for infections in animals and humans. *Brucella abortus* 19 (S19), an attenuated smooth strain, is currently used to vaccinate cattle against *Brucella* infections (33). Nevertheless, both the current vaccine and virulent field strains elicit a sustained antibody response mainly directed against the O chain of the smooth lipopolysaccharide (S-LPS). These serological responses prevent discrimination between vaccinated and infected animals by the currently used serological tests (4, 35). Attempts to develop new serological techniques able to discriminate persistently infected S19-vaccinated cattle from those that eliminate S19 after vaccination also failed (1, 50). The development of new vaccines that stimulate a protective immune response but do not cross-react on standard serological tests is therefore an important issue in bovine brucellosis (11–13, 24, 25, 43–48). To be protective, these new vaccines must contain relevant proteins to induce a cellular immune response (2), but also O chain determinants of the S-LPS. Indeed, a major part of the protective response depends on the presence of antibodies raised against the O chain as demonstrated by passive protection experiments (28, 29, 31) and by vaccinations with rough strains (13, 24, 25, 39, 45).

The S-LPS is also the source of antigenic cross-reaction between smooth *Brucella* spp. and other bacteria which possess an O chain similar to that of *Brucella* (18, 49, 52). Among these bacteria, *Yersinia enterocolitica* O:9 is the major cause of confusion in the serological diagnosis of brucellosis (17, 30) because its S-LPS O chain is almost identical to that of *B. abortus* (9).

To develop new vaccines and to understand the molecular basis of serological cross-reactions, the chemical and the immunological characteristics of the O chain of *Brucella* have been extensively studied. This O chain is an unbranched polysaccharide of 4,6-dideoxy-4-formamido-D-mannopyranosyl (D-Rha4NFo) residues (6, 10). Up to now, four types of epitopes have been described, each characterized by specific monoclonal antibodies (MAbs): the M and A epitopes, present on M and A dominant *Brucella* strains, respectively; the common (C) epitope, strictly specific for smooth *Brucella* spp., either A or M dominant; and the C/Y epitope, which is common to smooth *Brucella* spp. and *Y. enterocolitica* O:9 (5, 8, 19, 36).

It has been proposed that the structure of the M epitope resides in a linear pentasaccharide unit consisting of an α1,3-linked D-Rha4NFo disaccharide with adjacent α1,2-linked residues (5). This is in agreement with the absence of this epitope on the O chain of *Y. enterocolitica* O:9, which is devoid of α1,3 bonds (9). It was also established that the A epitope is formed by four or five α1,2-linked D-Rha4NFo residues (5). This structure, shared by A-dominant *Brucella* strains and *Y. enterocolitica* O:9, is at the origin of the serological cross-reactivity observed between these strains. The C/Y epitope appears to be a sequence of two to four units of α1,2-linked D-Rha4NFo residues (5). The C epitope is still to be unambiguously defined.

In this study, the characterization of some MAbs by indirect enzyme-linked immunosorbent assay (ELISA) shows that the diversity of the epitopes localized on the O chain is more complex than previously described, and competition binding assays between these MAbs suggest that these different epitopes are probably overlapping structures.

Epitope specificity of anti-O-PS MAbs determined by using S-LPSs. To study epitope specificity, seven anti-O polysaccharide (O-PS) MAbs derived from mice infected with different *Brucella* species (14, 16, 28, 29) were characterized by using S-LPSs of *Brucella* species expressing the main O-PS epitopes in different amounts: S-LPS of an A-dominant strain (*B. abortus* W99) and S-LPS of a M-dominant strain (*B. melitensis* Rev1). The extent of serological cross-reactions between *Brucella* spp. and other gram-negative bacteria was also analyzed by testing these MAbs in indirect ELISA with S-LPSs of *Y. enterocolitica* O:9, *Salmonella urbana*, *Escherichia coli* O:157, and *Vibrio cholerae.*

The *Y. enterocolitica* O:9 strain was isolated from a stool specimen by B. Limbourg, Centre de Dépistage des Maladies Animales, Erpent, Belgium. *B. abortus* W99, *S. urbana*, *E. coli* O:157, and *V. cholerae* strains were obtained from the Institut

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TABLE 1. Indirect ELISA results for hybridoma supernatants

MAb	Ig class	S-LPS Rev1		S-LPS W99		S-LPS 09		Epitope recognized	PS[a] Rev1		PS W99	
		Titer[b]	OD[c]	Titer	OD	Titer	OD		Titer	OD	Titer	OD
16C10	IgG3	1,500	2,598	59	1,773	40	910	C/Y (M > A)	340	1,235		43
16A4E7	IgG3	530	2,595	250	2,545	44	910	C/Y (M = A)	200	766		71
4F9	IgG3	10^4	2,451	10^5	2,676	10^4	2,582	C/Y (M < A)		50	$>10^4$	2,880
15B1G10	IgG3	1,700	2,516	55	960		4	C (M > A)	250	1,335		17
12G12	IgG1	9,800	2,828	8,900	2,748		108	C (M = A)	10^4	>3,000	5,600	>3,000
6B3B2	IgG3	260	1,810		16		3	M		142		32
2C8C4	IgG3		19	1,700	2,536		91	A		23		21

[a] PS, polysaccharide.
[b] Reciprocal of the highest dilution reaching an optical density of 0.2 after 20 min of substrate incubation.
[c] Optical density (OD) of 1/9 hybridoma supernatant dilution.

National de Recherches Vétérinaires, Brussels, Belgium. The M-dominant *Brucella* strain was obtained from the Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et d'Immunologie, Nouzilly, France. S-LPS fractions were prepared by the phenol-water method (27), and O-PS was prepared as described by Jacques et al. (23).

Microtiter plates (2-69620; Nunc) were coated with 1 μg of S-LPS per ml. Serial dilutions of MAbs were added, and bound antibodies were detected with a peroxydase conjugated sheep anti-mouse immunoglobulin G followed by *o*-phenylenediamine and H_2O_2 substrate. The results were expressed as $A_{490-630}$.

The reactivities of the MAbs in indirect ELISA are shown in Table 1. None of the MAbs, even those that reacted with S-LPS of *Y. enterocolitica* O:9, reacted with S-LPSs of *S. urbana* and *E. coli* O:157 (data not shown). For these two species, known to be involved in cross-reactions in classical serological tests, their O-PS structures are more divergent from those of *Brucella* spp. and *Y. enterocolitica* O:9 because the α-D-mannopyranosyl residue is only one of the four sugars of the repeating O unit (7, 37, 38). Nevertheless, these MAbs failed also to react with the S-LPS of *V. cholerae* (data not shown), for which the O-PS is an unbranched polysaccharide of N-acylated 4-amino-4,6-dideoxy-D-mannose residues where the acylating acid is 3-deoxy-L-glycerotetronic acid (26, 41). Thus, this O-PS differs from that of *Brucella* and *Y. enterocolitica* O:9 only in the *N*-acyl grouping. Consequently, the nature of the *N*-acyl group seems to be crucial in the binding of MAbs specific for C/Y epitopes.

Of the seven MAbs tested, only three (16C10, 16A4E7, and 4F9) cross-reacted with S-LPS of *Y. enterocolitica* O:9. As they reacted also against both *Brucella* A- and M-dominant strains, they could be referred to as specific for the C/Y epitope. Interestingly, they showed different types of reactivities toward the S-LPS of the A- and M-dominant *Brucella* strains. The first (MAb 16C10) bound to both A- and M-dominant S-LPS but showed a better binding (higher titer and optical density [OD]) to M-dominant S-LPS and was thus referred to as being specific for the C/Y (M > A) epitope. The second (MAb 16A4E7) bound equally well in indirect ELISA to S-LPS of the A-dominant and M-dominant *Brucella* strain. This MAb was thus referred to as being specific for the C/Y (M = A) epitope. These two MAbs presented the same level of reactivity against the S-LPS of *Y. enterocolitica* O:9. The last (MAb 4F9) presented a higher reactivity against the S-LPS of *Y. enterocolitica* O:9 than the first two. It bound also to both A- and M-dominant S-LPS but showed a better binding to A-dominant S-LPS and was referred to as being specific for the C/Y (A > M) epitope. The MAb 4F9 was previously described as an anti-A MAb (15, 29). In this study, it appears that MAb 4F9 is an anti-C/Y (A > M) MAb. This might explain why this MAb is able, following passive transfer, to protect against infection by M-dominant strain whereas anti-M MAbs are not able to protect against infection by an A-dominant *Brucella* strain (15). The structure of this epitope and other C/Y epitopes could depend on the number of α1,2-linked D-Rha4NFo residues. Indeed, in an O chain where the percentage of α1-3 links is important (e.g., O-PS of *B. melitensis* 16M) (32), a structure formed of around four residues with α1,2 links will be rarely present and thus it would be defined a C/Y (A > M) epitope. On the other hand, two or three α1,2-linked D-Rha4NFo residues could define the C/Y (A = M) epitope since this structure is common to A-dominant *Brucella*, M-dominant *Brucella*, and *Y. enterocolitica* O:9 O chains (9, 32). However, the presence of an epitope recognized by the MAb with C/Y (M > A) specificity also suggests that α1,2 links are probably not the sole structural feature for MAb recognition, as this MAb bound better on M-dominant *Brucella* S-LPS than on A-dominant *Brucella* and *Y. enterocolitica* O:9 S-LPS.

The four remaining MAbs were specific for *Brucella* species. MAbs 15B1G10 and 12G12 bound to both A- and M-dominant S-LPS and could be referred to as being specific for the C epitope. Nevertheless, MAb 15B1G10 bound better to M-dominant S-LPS than to A-dominant S-LPS, and MAbs 12G12 bound equally well to A- and M-dominant S-LPS. Consequently, these two MAbs were referred to as being specific for the C (M > A) and C (M = A) epitopes, respectively. MAb 6B3B2 bound at high titer to S-LPS of the M-dominant *Brucella* strain and not at all to S-LPS of the A-dominant *Brucella* strain and thus was shown to be highly specific for M-dominant S-LPS. MAb 2C8C4, in contrast, bound only to A-dominant S-LPS and was referred to as being specific for the A epitope. Surprisingly, it presented no reactivity against the S-LPS of *Y. enterocolitica* O:9. The specificities of these four MAbs can still not be explained by the known O-PS structure of *Brucella* or *Y. enterocolitica* O:9. (i) It was demonstrated that A-dominant O-PS possess α1,3 links, but anti-M MAbs are not able to react against this antigen. The low percentage of these links (2%) (32) indicates that either they are not readily accessible to anti-M MAbs or M epitopes are present on A-dominant O-PS but in insignificant quantities. However, their absence on O-PS of *Y. enterocolitica* O:9 (9) may explain the high specificity of anti-M MAbs for M-dominant S-LPS. (ii) It was also established that the structure of the A epitope was a pentasaccharide or larger oligosaccharide composed of α1,2-linked D-Rha4NFo. Nevertheless, the structure of the epitope recognized by some MAbs, such as 2C1 (40) and 2C8C4, is still not known. As these MAbs react only against A-dominant O chain,

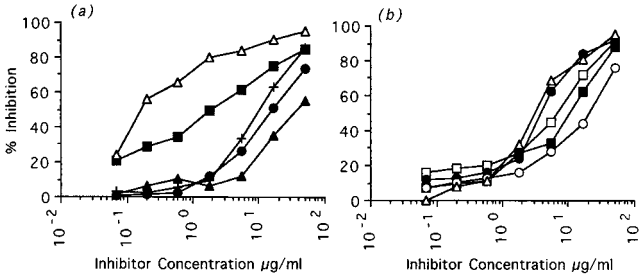

FIG. 1. Measurement of relative inhibitory abilities of O-PS of *B. melitensis* Rev1 (a) and O-PS of *B. abortus* W99 (b). Increasing amounts of O-PS inhibitors were added together with MAb 16C10 (●), MAb 16A4E7 (□), MAb 4F9 (▲), MAb 15B1G10 (■), MAb 12G12 (△), MAb 6B3B2 (+), and MAb 2C8C4 (○) to wells of ELISA plates that were coated with S-LPS of *B. melitensis* Rev1 and S-LPS of *B. abortus* W99.

the structure of this epitope would not be shared by both *Brucella* A-dominant strains and *Y. enterocolitica* O:9. (iii) Finally, the structure of C epitope remains unknown. In conclusion, the classification of the epitopes along the O chain of *Brucella* species appears to be more complex than previously described and their structures are not totally resolved.

Characterization of O-PS of A- and M-dominant *Brucella* spp. by MAbs. (i) Epitope specificity of anti-O-PS MAbs determined by using O-PSs. The reactivities of the seven anti-O-PS MAbs were also studied by using microtiter plates coated with 5 μg of O-PS of M-dominant *B. melitensis* Rev1 or O-PS of A-dominant *B. abortus* W99 per ml (Table 1). Generally, on the O-PS of *B. melitensis* Rev1, the reactivities of MAbs were weaker than that observed on the S-LPS. Surprisingly, MAb 6B3B2, referred to as specific for the M epitope, failed to bind to O-PS. This lack of reactivity was also observed for MAb 4F9 and as expected for MAb 2C8C4. Only the MAb specific for the C (A = M) epitope presented similar reactivities on both O-PS and S-LPS of *B. melitensis* Rev1. Of the six MAbs which bound to the S-LPS of *B. abortus* W99, two bound to the O-PS of this A-dominant strain. The first was MAb 4F9, for which the reactivities were nearly the same for both O-PS and S-LPS. The second was MAb 12G12. Its reactivities were similar on the four antigens tested.

(ii) Competition binding assays between O-PS and S-LPS. Some anti-O-PS MAbs showed absence or limited reactivity to purified O-PS. These MAbs also did not react with purified rough LPS in indirect ELISA (data not shown) and must therefore logically be specific for O-PS epitopes. The discrepancy between O-PS and S-LPS indirect ELISA results could be due to a difference in antigen attachment to the polystyrene matrix as suggested by Nielsen et al. (34). To verify this hypothesis, competition binding assays were performed between O-PS and S-LPS. MAb hybridoma supernatants (at the appropriate dilution) were incubated with various amounts of O-PS on S-LPS-coated microtiter plates. Under these conditions, the free antigen (O-PS) competed in the binding of the MAb with the adsorbed antigen (S-LPS). Binding of MAbs was visualized by using a peroxidase-conjugated sheep anti-mouse immunoglobulin. With this assay, all MAbs which reacted on the S-LPSs but not on the O-PSs in indirect ELISA showed reactivity against the competitive antigen (Fig. 1). Thus, these results confirmed that these MAbs are directed to epitopes of the O-PS moiety of S-LPS.

Competition binding assays between MAbs. To map the different epitopes previously described on S-LPS and O-PS, antigenic sites were defined by competition binding assays. MAbs were labelled with horseradish peroxidase (20) and incubated on S-LPS-coated microwell plates, at the optimal dilution, simultaneously with decreasing amounts of unlabelled MAbs. To present the results obtained with the panel of MAbs, three degrees of competition were established: total (more than 50%), partial (25 to 50%), and minimal (less than 25%) when the hybridoma supernatants (unlabelled MAbs) were used at a 1/4 dilution. The percentage of competition was compared to that on a control assay using either an anti-A MAb (MAb 2C8C4) when S-LPS of the M-dominant strain was coated or an anti-M MAb (MAb 6B3B2) when S-LPS of the A-dominant strain was used. Table 2 presents the results of the competitions according to these criteria.

When microtiter plates were coated with a M-dominant S-LPS, binding of the peroxidase-conjugated MAbs 12G12 and 6B3B2 was totally blocked by the other MAbs (M, all C, and C/Y epitopes) except for MAb 4F9 and, as expected, by the control MAb 2C8C4. However, the binding of peroxidase-conjugated MAb 4F9 was totally inhibited by all MAbs which were reactive against M-dominant S-LPS.

With regard to the A-dominant *Brucella* S-LPS, binding of peroxidase-conjugated MAb 12G12 was partially inhibited by the MAbs specific for the C (M > A), C/Y (M > A), C/Y (M = A), and C/Y (A > M) epitopes but not at all by MAb 2C8C4 and by the control MAb 6B3B2. Binding of peroxidase-conjugated MAb 2C8C4 was totally inhibited by MAb 4F9 and partially inhibited by MAb 16C10. Binding of peroxidase-conjugated MAb 4F9 was not at all inhibited by the MAbs specific for the other epitopes. As expected, these two latter peroxidase-conjugated MAbs were not inhibited by the control MAb 6B3B2.

With regard to data for competitions between MAbs obtained with A- and M-dominant S-LPS, it appears that the

TABLE 2. Competition binding assays among anti-O-PS MAbs[a]

Competitor MAb	Epitope recognized	Binding to indicated coated antigens					
		S-LPS Rev1			S-LPS W99		
		12G12	6B3B2	4F9	12G12	2C8C4	4F9
6B3B2	M	++	++	++	−	−	−
15B1G10	C (M > A)	++	++	++	+	−	−
12G12	C (M = A)	++	++	++	++	−	−
16C10	C/Y (M > A)	++	++	++	+	+	−
16A4E7	C/Y (M = A)	++	++	++	+	−	−
4F9	C/Y (A > M)	−	−	++	−	++	++
2C8C4	A	−	−	−	−	++	−

[a] MAbs were conjugated to horseradish peroxidase and tested in competition binding assays against the panel of competitor unlabelled MAbs. Levels of competition are defined as follows: −, less than 25%; +, 25 to 50%; and ++, more than 50%.

different O-PS epitopes are probably overlapping structures. The fact that anti-C/Y MAbs inhibit the fixation of anti-C MAbs may explain why sera from animals infected with *Y. enterocolitica* O:9 (53) or after a vaccination with *B. abortus* S19 (unpublished data) were found positive in competitive ELISA using M-dominant S-LPS and a peroxidase-conjugated MAb directed to the C epitope like MAb 12G12 (53). The competition binding observed between anti-C/Y and anti-C MAbs seems to be more pronounced on M-dominant O chain than on A-dominant O-chain, which suggests that competitive ELISA using S-LPS of A-dominant strains and anti-C MAbs might be a better way to improve the specificity of serological diagnosis of bovine brucellosis. Nevertheless, these observations contradict the proposal of Vizcaino et al., who suggested the use of M-dominant S-LPS (51).

These data indicate that the development of new vaccines and serological tests (which fail to detect vaccinated animal and animal infected with cross-reacting bacteria) still requires first the determination of the exact nature of the C epitope and second its isolation. These two goals could be approached either by the use of synthetic oligosaccharides as described by Bundle et al. (5) or, as currently under way in our laboratory, by the screening of synthetic random peptide libraries expressed on phages with MAb 12G12. Such an approach has been used successfully to identify peptides which mimic carbohydrate substances like the Lewis^y antigen (22) or an epitope of the S-LPS of *Shigella flexneri* (21, 42).

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