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Monoclonal Antibody-Defined Specific C Epitope of *Brucella* O-Polysaccharide Revisited

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The C epitope of *Brucella* O-polysaccharide (O-PS) has so far lacked definitive structural identity. Revised structures for this antigen revealed a unique capping perosamine tetrasaccharide consisting of a sequence of 1,2:1,3:1,2 interresidue linkages. Here, using synthetic oligosaccharide glycoconjugates, the α-1,3 linkage of the O-PS is shown to be an integral structural requirement of this epitope. Although A-dominant strains possess only one or two copies of the capping tetrasaccharide, this creates a unique pentasaccharide antigenic determinant with the linkage sequence 1,2:1,3:1,2:1,2 that is always present in major pathogenic *Brucella* species.

Brucellae are Gram-negative, facultative, intracellular bacteria that can infect humans and many species of animals. The main animal- or human-pathogenic species of the genus *Brucella*, i.e., *Brucella melitensis*, *Brucella suis*, and *Brucella abortus*, carry a smooth (S) lipopolysaccharide (LPS) (S-LPS), a surface molecule that is a major virulence factor and the most important serodiagnostic antigen (1, 2). Its O-polysaccharide (O-PS) moiety represents the most exposed antigenic structure of the *Brucella* cell surface, and it carries the antigenic determinants involved in serotyping with polyclonal sera. At present, *Brucella* S strains are classified into three serotypes, i.e., A^+M^-, A^-M^+, and A^+M^+, according to slide agglutination with A and M monospecific polyclonal sera (3). Additionally, *Brucella* S strains share common epitopes on the O-PS with cross-reacting strains, of which the most important is *Yersinia enterocolitica* O:9 (1, 4, 5). By using monoclonal antibodies (MAbs), a number of epitope specificities on the O-PS have been reported: A, M, and epitopes shared by both A-dominant and M-dominant strains, which have been named common (C) epitopes (2, 5–12). The latter have been fur-

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TABLE 1 Binding of the anti-O-PS MAbs in ELISA to *Brucella* strains and synthetic PSs[a]

Antigen	Agglutination with monospecific serum: A	M	Binding titer (max. abs.) by ELISA of the following MAb[b]: 2E11 (M)	12G12 (C [A=M])	07F09 (C [A=M])	12B12 (C [M>A])	18H08 (C/Y [A=M])	04F9 (C/Y [A>M])	05D4 (C/Y [A>M])
Killed cells									
B. melitensis 16M (bv. 1)	−	+	90 (2.506)	7,290 (2.701)	2,430 (2.791)	90 (2.907)	90 (2.858)	30 (2.420)	810 (2.432)
B. suis 1330 (bv. 1)	+	−	<10	7,290 (5.272)	2,430 (4.573)	30 (3.234)	270 (4.368)	7,290 (4.669)	21,870 (4.317)
B. suis Thomsen (bv. 2)	+	−	<10	10 (2.422)	10 (1.249)	<10	90 (3.356)	7,290 (3.109)	7,290 (3.211)
Synthetic PSs[c] (structural feature)									
NVG-125 (Di, α-1,3)			<10	<10	<10	<10	<10	<10	<10
NVG-129 (Tri, α-1,3:1,2)			<10	<10	<10	<10	<10	<10	<10
NVG-133 (Tri, α-1,2:1,3)			<10	<10	<10	<10	<10	<10	<10
NVG-137 (Tetra, α-1,2:1,3:1,2)			<10	<10	<10	<10	<10	<10	<10
JG5 (Penta, α-1,2:1,3:1,2:1,2)			<10	810 (4.278)	270 (3.967)	<10	10 (1.486)	10 (1.451)	30 (1.859)
NVG-224 (Hexa, α-1,2:1,3:1,2:1,2:1,2)			<10	810 (4.559)	270 (4.337)	<10	<10	270 (3.405)	90 (1.875)
NVG-220 [Hexa, α-$(1,2)_6$]			<10	<10	<10	<10	10 (1.661)	2,430 (5.058)	7,290 (5.062)
JG9 [Nona, α-$(1,2)_4$:1,3:$(1,2)_3$]			<10	810 (4.575)	270 (3.724)	<10	10 (1.518)	2,430 (4.927)	7,290 (5.100)

[a] PSs, polysaccharides.
[b] Results are expressed as titers of the MAbs, i.e., the highest dilutions of the MAbs giving an absorbance value above 1.0. Maximal absorbance (max. abs.) was observed mostly at a 1/10 dilution of the MAb.
[c] Values inside the parentheses are the size and linkages of the PS structure.

FIG 1 Structural view of the C (A=M) and C/Y (A>M) epitopes. The C/Y (A>M) epitope is composed of ≥5 α-1,2-linked D-Rha4NFo residues (yellow circle). The C (A=M) epitope is composed of the disaccharide D-Rha4NFo (1,3) linked to α-1,2-linked D-Rha4NFo residues on each side (blue circle). All linkages are α-1,2 except the α-1,3 linkage indicated by the black arrows. +++, +, and − correspond to reactivity with MAbs.

ther subdivided according to relative preferential MAb binding in enzyme-linked immunosorbent assays (ELISA) into five epitopic specificities: C (M>A), C (A=M), C/Y (M>A), C/Y (A=M), and C/Y (A>M) (12). MAbs indicated with C are specific for *Brucella*, while those indicated with C/Y cross-react with *Y. enterocolitica* O:9. The preferential binding to A-dominant or M-dominant strains and equal binding to both strains are indicated by A>M or M>A and A=M, respectively.

The *Brucella* O-PS structure has been described as being constituted by homopolymers of 4,6-dideoxy-4-formamido-α-D-mannopyranose (*N*-formylperosamine) residues. In 1989, O-PS from A-dominant strains was reported as being a linear α-1,2-linked polymer, with about 2% α-1,3 linkages, while that from M-dominant strains constituted a linear polymer of pentasaccharide repeating units containing one α-1,3-linked and four α-1,2-linked monosaccharide residues (13). More recently, reinvestigation of the structure of *Brucella* O-antigens has defined the M epitope as a tetrasaccharide, missing one of the α-1,2-linked monosaccharide residues compared to the previously proposed structure (14), and this epitope always occurred as a capping element on the α-1,2-linked A-type polysaccharide. In addition, that study showed that M-dominant *Brucella* strain 16M contains several copies of the capping tetrasaccharide, thereby potentially creating additional unique sequences that are absent in those *Brucella* O-PSs with a single M capping tetrasaccharide. The differences between A-dominant and M-dominant strains were shown to be quantitative and depended on the length of α-1-2-linked polymer and the number of repeating M tetrasaccharides. In *B. melitensis* strain 16M, the α-1,2-linked chain was short, but the M-type polymer was long. In other strains, one or two M-type tetrasaccharides were present at the nonreducing end of the O-antigen.

Prior to the 2013 revision of the *Brucella* O-PS structure, MAbs specific for the C/Y epitopes were suggested to recognize α-1,2-linked tri- or tetrasaccharides of the O-PS (6). The α-1,3 linkage was suggested to be mainly involved in the structure recognized by MAbs specific for the M epitope, since such MAbs failed to react with *Y. enterocolitica* O:9, lacking the α-1,3 linkages, and their preferential binding to M-dominant O-PS correlated with an increased number of α-1,3-linked monosaccharide residues. However, in a more recent study, *B. suis* bv. 2 previously shown to lack the C epitope(s) (2) was also shown to lack α-1,3-linked monosaccharide residues in its O chain; it was thus suggested that the α-1,3 linkage would also be involved in the C epitope(s) MAb recognition. However, direct evidence for this has not been provided yet, and the role of the α-1,3 linkage in the recognition of the C epitope(s) remains questionable as, by definition, MAbs to such epitopes show equal binding to A-dominant, M-dominant or A+M+ *Brucella* strains, although they differ significantly in the levels of α-1,3 linkage expression in their O chain, from 2% to 20% (13). In light of the recent results of structural reinvestigation of the structure of *Brucella* O-antigens (14), in this study we reinvestigated the MAb specificities, in particular those previously defined as specific for the C and C/Y epitopes, using recently published synthetic oligosaccharide glycoconjugates (15, 16). These consisted of a series of nine oligosaccharides from di- to nonasaccharides, without or with α-1,3 linked *N*-formylperosamine situated in different locations within the oligosaccharide sequence.

Binding of the MAbs to these glycoconjugates was assessed by indirect ELISA using conditions previously published (2, 15, 16).

Results are shown in Table 1 and compared to binding data observed on whole bacterial cells of M-dominant *B. melitensis* strain 16M, A-dominant *B. suis* strain 1330, and A-dominant *B. suis* strain Thomsen (bv. 2) lacking the C epitope(s) (2). MAbs previously defined as specific for the M and C (M>A) epitopes did not bind at all to any oligosaccharide used in this study (Table 1). Comparatively, their binding titers on bacterial cells were also significantly lower than for the other MAbs. Their actual epitope specificity, thus, may be questionable. For those binding to the oligosaccharides, a first observation is that the minimal polymeric structure recognized by any of the MAbs is the pentasaccharide JG5 (Table 1). MAbs specific for the C/Y epitopes essentially recognize α-1,2-linked oligosaccharides, according to the high binding observed to NVG-220, consisting of an α-1,2-linked hexasaccharide without any α-1,3 linkage (Table 1). Both MAbs 12G12 and 07F09 specific for the C (A=M) epitope showed binding at high titers only to the penta-, hexa-, and nonasaccharides containing the α-1,3 linkage, i.e., JG5, NVG-224, and JG9, respectively (Table 1). These MAbs did not bind at all to the α-1,2-linked hexasaccharide NVG-220. Binding titers observed on the JG5, NVG-224, and JG9 oligosaccharides were identical regardless of the position of the α-1,3 linkage, indicating that the α-1,3 linkage is an essential feature in the recognition by the C (A=M)-specific MAbs. In agreement with this observation, a recent structural study on the O chain of *B. suis* bv. 2, which lacks this epitope (2), revealed the absence of the α-1,3-linkage in this particular O chain (17). The present study provides direct evidence that this *Brucella*-specific O chain linkage is essential to recognition by such antibodies.

Figure 1 summarizes the ELISA data in accordance with the oligosaccharide structures. From this figure, it becomes obvious that the C (A=M) epitope consists of a pentasaccharide where the α-1,3-linkage is exposed and located in the middle of the structure. However, it raises the question of why equal bindings, for MAbs recognizing this epitope, are observed on whole bacterial cells regardless of whether they are A dominant or M dominant, with the proportion of α-1,3 linkages of 2% and 20%, respectively. A possible explanation is that only the tip of the capped M epitope would be accessible and uniformly distributed on the bacterial surface from an antigenic point of view.

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