Characterization of O-polysaccharide specific monoclonal antibodies derived from mice infected with the rough *Brucella melitensis* strain B115

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Twenty-two monoclonal antibodies (mAbs) specific for smooth lipopolysaccharide (S-LPS) were generated by fusion of spleen cells from mice infected with the rough *Brucella melitensis* strain B115 with the NSO myeloma. According to reactivity in enzyme-linked immunosorbent assay (ELISA) with O-polysaccharide (O-PS) and absence of reactivity with rough lipopolysaccharide (R-LPS), it was postulated that these mAbs recognized epitopes present on the O-PS. Most of the mAbs reacted equally well in ELISA and immunoblotting with S-LPS types of *Brucella* A and M dominant strains and were designated as specific for common (C) epitopes. Three mAbs were highly specific for M dominant S-LPS. All these mAbs, in contrast to a mAb specific for the A epitope, showed little or no cross-reactivity with *Yersinia enterocolitica* O:9 S-LPS. S-LPS of *B. melitensis* B115 was extracted and analysed by immunoblotting and ELISA with mAbs specific for A, M and C epitopes. Reactivity of the mAbs with this S-LPS was compared to reactivity with S-LPS of A and M dominant smooth *Brucella* strains. The results suggest that S-LPS of *B. melitensis* B115 bears mainly C epitopes and a few M epitopes. The very weak reactivity of this S-LPS with the mAb specific for the A epitope and the fact that the mAbs specific for C and M epitopes showed little or no cross-reactivity with *Y. enterocolitica* O:9 S-LPS suggest that O-PS from this rough strain could be used to distinguish *Y. enterocolitica* O:9 infection from *Brucella* infection. The potential value of the rough *B. melitensis* strain B115 as a vaccine strain is also discussed.

Introduction

All smooth strains of *Brucella* bear on their smooth lipopolysaccharide (S-LPS) two distinct antigenic specificities defined either by polyclonal or monoclonal antibodies (mAbs) and designated A and M (Bundle *et al.*, 1989; Douglas & Palmer, 1988; Meikle *et al.*, 1989; Wilson & Miles, 1932). The relative amounts of the A and M specificities vary among smooth *Brucella* strains and they are absent on rough (R) strains lacking O side chain (Alton *et al.*, 1988). These smooth *Brucella* strains are classified into three serotypes, i.e. A⁺M⁻, A⁻M⁺ and

A⁺M⁺, according to slide agglutination using A and M monospecific polyclonal sera. These agglutination results correspond to strains expressing mainly the A or M antigens or both antigens in nearly equivalent amounts. These characteristics may be abbreviated as, respectively, A ≫ M, M ≫ A or A,M. mAbs to S-LPS of *Brucella* spp. have been reported with specificity for the A-LPS epitope, the M-LPS epitope and for common epitopes (C) cross-reacting or not with *Y. enterocolitica* O:9 S-LPS (Bundle *et al.*, 1989; Cloeckeaert *et al.*, 1992a; Douglas & Palmer, 1988; Garin-Bastuji *et al.*, 1990; Limet *et al.*, 1989; Palmer & Douglas, 1989).

The *B. melitensis* strain B115 is a rough strain by classical criteria, i.e. microscopic observation of colonies by obliquely reflected light, staining of R colonies with crystal violet and absence of agglutination by monospecific sera to the A and M antigens of *Brucella* (Alton *et al.*, 1988). Absence of reactivity of *B. melitensis* B115 bacterial cells with S-LPS specific mAbs has also been shown by colony-blot ELISA (Roop *et al.*, 1987),

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Abbreviations: mAb, monoclonal antibody; O-PS, O-polysaccharide; R-LPS, rough lipopolysaccharide; S-LPS, smooth lipopolysaccharide.

coagglutination (Vizcaino & Fernandez-Lago, 1992) and ELISA and immunoelectron microscopy (Cloeckaert *et al.*, 1992*b*). Jones *et al.* (1973) showed that injection of living or killed cells of *B. melitensis* strain B115 into rabbits produced low-titre agglutinins to smooth cells. However, they were unable to extract S-LPS from this strain by the phenol/water/chloroform/petroleum ether method of Galanos *et al.* (1969). mAbs specific for S-LPS were obtained by fusion of the NSO myeloma with spleen cells from mice infected with *B. melitensis* B115 (Cloeckaert *et al.*, 1990) or mice immunized with the cell wall fraction of *B. melitensis* B115 (unpublished data). By using one of these mAbs (12G12), of IgG1 isotype, which was specific for O-polysaccharide (O-PS) and reacted with S-LPS of both A and M dominant *Brucella* strains, we were able to demonstrate by immunoelectron microscopy the expression of O-PS in this rough bacterium (Cloeckaert *et al.*, 1992*b*). O-PS was localized intracellularly and not exposed at the cell surface as in the smooth *Brucella* strains. This mAb reacted in ELISA and immunoblotting with sonicated *B. melitensis* B115 bacteria or their cell wall fraction and was protective in mice against smooth virulent strains of *B. abortus* and *B. melitensis* (Cloeckaert *et al.*, 1992*a*). In the present study, we report the characterization of the mAbs derived from mice infected with *B. melitensis* B115, and the reactivity of S-LPS extracted from this rough strain with mAbs specific for A, M and C epitopes compared to S-LPS of A and M dominant smooth *Brucella* strains.

Methods

Bacterial strains. *B. melitensis* rough (R) strain B115 and *B. melitensis* 16M (A⁻M⁺), *B. melitensis* M3 (A⁺M⁺), *B. abortus* 99 (A⁺M⁻), *B. abortus* B3 (A⁺M⁻) smooth (S) strains were obtained from the Institut National de la Recherche Agronomique, Laboratoire de Pathologie Infectieuse et d'Immunologie, Nouzilly, Tours, France. Purity and R and S phenotypes were clearly established by standard procedures (Alton *et al.*, 1988). The R phenotype of *B. melitensis* B115 strain has been found to be stable over a period of 18 years (Alton *et al.*, 1988; Jones *et al.*, 1973). *Y. enterocolitica* O:9 was obtained from G. Wauters (Microbiology Department, Catholic University of Louvain).

LPS fractions. S-LPS fractions of *B. melitensis* 16M, *B. abortus* B3 and *Y. enterocolitica* O:9 were prepared by the phenol/water method (Leong *et al.*, 1970). R-LPS fraction of *B. melitensis* B115 was obtained by the phenol/water/chloroform/petroleum ether method of Galanos *et al.* (1969). Proteinase K-digested S-LPS fractions of *B. melitensis* 16M, *B. melitensis* M3, *B. abortus* 99 and *B. melitensis* B115 were prepared as described previously (Cloeckaert *et al.*, 1992*b*; Garin-Bastuji *et al.*, 1990). S-LPS fraction of *B. melitensis* B115 was isolated from cell walls. S-LPS fractions of *B. melitensis* 16M, *B. melitensis* M3 and *B. abortus* 99 were isolated from whole smooth bacterial cells. O polysaccharide (O-PS) of *B. abortus* 99 was prepared as described previously (Jacques *et al.*, 1991; Zygmunt *et al.*, 1988).

mAbs. Anti-S-LPS mAbs derived from mice infected with *B. melitensis* B115 were produced as described previously (Cloeckaert *et al.*, 1990, 1992*b*). Screening and specificity were determined by

immunoblot analysis and ELISA using cell walls and R-LPS of *B. melitensis* B115 and S-LPS of *B. melitensis* and *B. abortus* S strains. The mAbs specific for the A epitope (mAb 04F9) and M epitope (mAb 2E11) were produced as described previously (Limet *et al.*, 1989).

ELISA. ELISA was performed as described previously (Cloeckaert *et al.*, 1990, 1992*b*). S-LPS was coated on microtitre plates (Greiner Labortechnik, Stuttgart, Germany) by overnight incubation at a concentration of 4 µg ml⁻¹. Hybridoma supernatants or ascitic fluids were serially diluted (1 in 10, 1 in 30, 1 in 90, up to 1 in 21 870). Binding of antibodies to S-LPS was revealed by successive incubations with rabbit anti-mouse immunoglobulin serum and peroxidase-conjugated protein A (Cloeckaert *et al.*, 1990, 1992*b*) or with peroxidase-conjugated goat anti-mouse immunoglobulins (Kirkegaard & Perry Laboratories).

Immunoblot techniques. Immunoblotting was performed as described previously (Cloeckaert *et al.*, 1990, 1992*a, b*).

mAb isotypes. These were determined by direct latex agglutination immunoassay of the culture supernatants. Latex particles were coated with rat monoclonal anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgM and polyclonal anti-mouse IgA according to a protocol similar to that described by Limet *et al.* (1988).

Results

Monoclonal antibodies

S-LPS mAbs derived from mice infected with *B. melitensis* B115 were characterized by ELISA using two types of S-LPS, i.e. S-LPS of A dominant strain *B. abortus* B3 (A ≫ M) and M dominant strain *B. melitensis* 16M (M ≫ A). According to their reactivity in ELISA, these mAbs could be divided into three groups (Table 1). Typical binding of one mAb of each group is given in Fig. 1. Nine mAbs of IgG1 and IgM isotypes recognized epitopes expressed in equal amount on S-LPS of the A and M dominant *Brucella* serotypes and are designated as specific for a common epitope (C). These mAbs gave the same antibody titre and approximately the same maximal absorbance in ELISA with the two S-LPS types at the coating concentration used. Another group of ten mAbs of IgG3 and IgG2a isotypes reacted with both S-LPS types according to the maximal absorbance in ELISA but a higher titre was observed with S-LPS of the M dominant strain *B. melitensis* 16M. They are designated as specific for one or several epitopes common to both S-LPS types but more abundant or accessible to antibody in S-LPS of the M dominant strain (CM > A). The last group consisted of three mAbs which were highly specific for S-LPS of the M dominant strain (M) and these were of the IgG3 and IgM isotypes. Reactivity of the mAbs was confirmed by immunoblotting using the two S-LPS types (not shown).

All mAbs obtained except two specific for the M epitope (mAbs 18B04 and 04F03) reacted with O-PS of *B. abortus* 99. They did not bind to R-LPS of *B. melitensis* B115 and thus recognize epitopes of the O-PS.

Table 1. *ELISA binding of the anti-S-LPS MAbs to S-LPS of B. abortus B3 (A ≫ M), S-LPS of B. melitensis 16M (M ≫ A), R-LPS of B. melitensis B115 and O-PS of B. abortus 99*

mAbs (hybridoma supernatant)	Isotype	S-LPS B3		S-LPS 16M		R-LPS B115	O-PS 99
		Titre	Maximum absorbance*	Titre	Maximum absorbance	Absorbance‡	Absorbance‡
<i>S-LPS (C)†</i>							
05D01	IgG1	21870	3.138	21870	3.174	0.066	1.752
07F09	IgG1	7290	2.934	7290	3.213	0.108	1.731
12G12	IgG1	> 21870	3.802	> 21870	3.398	0.080	1.566
13A09	IgG1	21870	3.139	21870	3.287	0.157	1.790
14F12	IgG1	> 21870	3.128	> 21870	3.082	0.093	1.719
16F01	IgG1	21870	3.168	21870	3.239	0.103	1.708
13G11	IgM	> 21870	1.644	> 21870	1.874	0.024	1.124
O5E11	IgM	90	1.207	90	0.824	0.030	1.107
14H09	IgM	21870	2.056	21870	2.364	0.028	1.320
<i>S-LPS (C M > A)†</i>							
15B10	IgG2a	7290	3.023	> 21870	3.581	0.080	1.720
02B05	IgG3	810	2.882	> 21870	3.515	0.047	1.653
03D03	IgG3	810	3.536	21870	3.099	0.063	1.617
04A05	IgG3	270	2.933	21870	3.399	0.076	1.670
11H03	IgG3	270	2.963	21870	3.068	0.007	1.842
12B12	IgG3	2430	3.137	21870	3.135	0.080	1.589
17H02	IgG3	2430	3.215	> 21870	3.375	0.020	1.705
01F08	IgG3	810	2.808	21870	3.238	0.019	1.797
08C06	IgG3	810	2.825	7290	3.101	0.081	1.672
08B07	IgG3	2430	1.712	21870	2.109	0.099	1.423
<i>S-LPS (M)†</i>							
03E05	IgG3	30	1.377	> 21870	3.396	0.023	1.771
18B04	IgM	10	0.461	21870	3.325	0.039	0.032
04F03	IgM	10	0.592	> 21870	3.909	0.101	0.115

* Maximal absorbance after blank value subtraction.

† (C), (C M > A), (M): specific for a common epitope (C), an epitope common for both S-LPS types but more abundant on the M S-LPS type (C M > A), and for the M epitope (M), respectively.

‡ Absorbance value of a 1/2 hybridoma supernatant dilution.

Cross-reactivity with Y. enterocolitica O:9 S-LPS

In ELISA, in contrast to mAb 04F9, which is specific for the A epitope, the mAbs specific for common and M epitopes, which were derived from mice infected with *B. melitensis* B115, showed little or no reactivity with S-LPS extracted from the cross-reactive strain *Y. enterocolitica* O:9 (Fig. 2). This was also confirmed by immunoblot analysis (not shown).

Characterization of S-LPS of B. melitensis B115

(i) *Immunoblot analysis.* Proteinase K-digested S-LPS fraction of *B. melitensis* B115 was compared by immunoblotting using mAbs 12G12 (IgG1) specific for the C epitope (mAb-C), 04F9 (IgG2a) specific for the A epitope (mAb-A) and 2E11 (IgG3) specific for the M epitope (mAb-M), to Proteinase K-digested S-LPS extracted from *B. abortus* 99 (A⁺M⁻ serotype), *B. melitensis* 16M (A⁻M⁺ serotype) and *B. melitensis* M3 (A⁺M⁺ serotype) smooth strains (Fig. 3). mAb-C revealed all S-LPS types strongly. mAb-A revealed only S-LPS from the A

dominant strains *B. abortus* 99 (A⁺M⁻) and *B. melitensis* M3 (A⁺M⁺). mAb-M strongly revealed S-LPS of the M dominant strain *B. melitensis* 16M (A⁻M⁺), S-LPS of the *B. melitensis* strain M3 (A⁺M⁺) and, very weakly, S-LPS of the rough *B. melitensis* strain B115.

(ii) *ELISA.* Immunoblotting results were confirmed by ELISA using the same S-LPS fractions. mAb-C bound approximately equally well to the four strains tested (Fig. 4). The maximal absorbance observed with this mAb was always higher than or equal to that of the other mAbs. mAb-A bound to S-LPSs of the A dominant strains *B. abortus* 99 (A⁺M⁻) and *B. melitensis* M3 (A⁺M⁺), in agreement with serotyping results using A and M monospecific sera. mAb-A bound also to S-LPS of the M dominant strain *B. melitensis* 16M (A⁻M⁺) but the maximal absorbance observed was weaker than that observed for mAb-M. mAb-M bound to the S-LPSs of M dominant strain *B. melitensis* 16M (A⁻M⁺) and A dominant strain *B. melitensis* M3 (A⁺M⁺). In contrast to mAb-C, mAb-M and mAb-A bound very weakly to S-LPS of rough *B. melitensis* strain B115.

Fig. 1. Binding of anti-S-LPS mAbs (hybridoma supernatants) 12G12 (C epitope) (a), 12B12 (CM > A epitope) (b) and 04F03 (M epitope) (c) to S-LPS of *B. abortus* B3 (A⁺M⁻) (●) and *B. melitensis* 16M (A⁻M⁺) (○). Rabbit anti-mouse immunoglobulins and peroxidase-conjugated protein A were used to detect antibody reactivity.

Fig. 2. Binding of anti-S-LPS mAbs (hybridoma supernatants) 12G12 (C epitope) (□), 12B12 (CM > A epitope) (○), 04F03 (M epitope) (△) and 04F9 (A epitope) (●) to S-LPS of *Y. enterocolitica* O:9. Rabbit anti-mouse immunoglobulins and peroxidase-conjugated protein A were used to detect antibody reactivity.

Fig. 3. Immunoblot of proteinase K-treated S-LPS of *B. melitensis* B115 (R) (lane 1), *B. melitensis* M3 (A⁺M⁺) (lane 2), *B. abortus* 99 (A⁺M⁻) (lane 3) and *B. melitensis* 16M (A⁻M⁺) (lane 4) with mAb-A 04F9 (A), mAb-M 2E11 (M) and mAb-C 12G12 (C).

Fig. 4. Binding of mAb-A 04F9 (●), mAb-M 2E11 (○) and mAb-C 12G12 (□) (ascitic fluids) to S-LPS of *B. melitensis* B115 (R) (a), *B. abortus* 99 (A⁺M⁻) (b), *B. melitensis* 16M (A⁻M⁺) (c) and *B. melitensis* M3 (A⁺M⁺) (d). Peroxidase-conjugated goat anti-mouse immunoglobulins were used to detect antibody reactivity.

Discussion

mAbs specific for S-LPS were produced by fusion of spleen cells from mice infected with the rough *B. melitensis* B115 strain and the NSO myeloma (Cloeckaert *et al.*, 1990). The reactivity in ELISA with O-PS and absence of reactivity with R-LPS indicate that these mAbs were specific for O-PS epitopes. Immunoblotting

with R- (not shown) and S-LPS confirmed the O-PS specificity. Most of the mAbs produced were specific for common epitopes (C and C M > A) shared by the *Brucella* A and M dominant S-LPS types. The fact that the C epitope induced mainly IgG1 mAbs and the C M > A epitope mainly IgG3 mAbs remains to be explained. Two IgM mAbs highly specific for M LPS did not react with O-PS in ELISA since the latter was prepared from the A dominant strain *B. abortus* 99. All mAbs derived from mice infected with *B. melitensis* B115 showed little or no cross-reactivity in ELISA with *Y. enterocolitica* O:9 S-LPS. The weak or absent cross-reactivity of mAbs specific for common and M epitopes with *Y. enterocolitica* O:9 S-LPS was also shown by Palmer & Douglas (1989). mAbs showing a cross-reactivity with *Y. enterocolitica* O:9 generally recognize the A epitope (Douglas & Palmer, 1988) and this specificity is shown by mAb-A 04F9. Our results also suggest that these mAbs may be particularly useful for typing smooth *Brucella* strains.

The fact that mAb-C reacted with all *Brucella* S-LPS types (ELISA and immunoblotting) but that mAb-A did not react with *B. melitensis* B115 S-LPS while mAb-M reacted very weakly with this strain suggests that the O-PS expressed in rough *B. melitensis* B115 strain bears mainly C epitopes and very few M epitopes. mAbs were initially screened by ELISA using *B. melitensis* B115 antigen fractions, with rabbit anti-mouse immunoglobulins and peroxidase-conjugated protein A as secondary reagents (Cloeckaert *et al.*, 1990, 1992b). In these conditions mAb-M showed a better reactivity (higher absorbance value) against *B. melitensis* B115 than was obtained with peroxidase-conjugated goat anti-mouse immunoglobulins as shown in this study. Nevertheless, mAb-A still showed very weak reactivity against S-LPS of *B. melitensis* B115 when using rabbit anti-mouse immunoglobulins and conjugated protein A as secondary reagents (data not shown). Absence or very weak reactivity of this S-LPS with mAb-A and the fact that the mAbs specific for C and C M > A epitopes showed little or no cross-reactivity with *Y. enterocolitica* O:9 S-LPS suggest that O-PS from this rough strain could possibly be useful to distinguish *Y. enterocolitica* O:9 infection from *Brucella* infection. Sera from mice infected with *B. melitensis* B115 (five mice infected intraperitoneally with 10^7 c.f.u. were tested at 1 month post-challenge) also showed very weak antibody reactivity (not statistically different from negative control mice) or did not react with S-LPS of *Y. enterocolitica* O:9 in ELISA (data not shown) but showed high antibody reactivity against *B. melitensis* 16M S-LPS (titre between 1/500 and 1/1000 for an absorbance value of 1.0).

The *Brucella* O-PS structure has been defined as homopolymers of 4,6-dideoxy-4-formamido- α -D-manno-

pyranose residues. O-PS from A dominant strains is a linear α -1,2-linked polymer with about 2% of α -1,3-linkages, while O-PS from M dominant strains is a linear polymer of pentasaccharide repeating units containing one α -1,3-linked and four α -1,2-linked monosaccharide residues (Bundle *et al.*, 1989; Meikle *et al.*, 1989). Apparently the α -1,3-linkage is mainly involved in the structure recognized by M specific mAbs. This is reinforced by the fact that mAbs specific for M dominant S-LPS showed no reactivity against S-LPS of *Y. enterocolitica* O:9. The structure of the O-PS of *Y. enterocolitica* O:9 is identical to that of *Brucella* A dominant O-PS except that no α -1,3 linkages are detected (Meikle *et al.*, 1989). The α -1,2-linkage may be involved in the structure recognized by the A-specific mAb. This mAb also showed high reactivity against *Y. enterocolitica* O:9 S-LPS. Reactivity of mAbs specific for C epitopes is more difficult to explain according to the structures of the O-PS described in the literature since they showed high reactivity against S-LPS and O-PS of both A and M dominant *Brucella* strains but weak reactivity against *Y. enterocolitica* O:9 S-LPS. The lack of cross-reactivity with *Y. enterocolitica* O:9 also indicates that using serum antibody response to S-LPS of *Y. enterocolitica* O:9 as a criterion for detecting antibody response against *B. abortus* O-PS in infected animals (Schurig *et al.*, 1991) may not be sufficient. Since, according to ELISA and immunoblotting results, O-PS from the rough *B. melitensis* B115 strain seems to bear mainly common epitopes to A and M dominant smooth strains, it may be that its structure is different from the structures of the A and M S-LPS types described by Bundle *et al.* (1989) and Meikle *et al.* (1989). Determining the structure of this O-PS will help determine what kind of epitopes are recognized by mAb-C.

The fact that O-PS expressed in *B. melitensis* B115 strain bears mainly common epitopes and consequently the fact that it induces antibodies mainly against common epitopes is interesting when considering vaccine development. Vaccination actually performed in animals is based on live attenuated or killed smooth *Brucella* strains which induce an antibody response to S-LPS. Serodiagnosis by conventional tests does not permit distinction between vaccinated and infected animals because in these tests the S-LPS is the major immunodominant antigen. IgG and IgM mAbs specific for the C epitope derived from *B. melitensis* B115 infected mice were found to be protective in mice against challenge with virulent *B. abortus* and *B. melitensis* smooth strains (Cloeckaert *et al.*, 1992a). It was also shown by Dubray (1981) that the sodium dodecyl sulphate insoluble (SDS-I) cell wall fraction from *B. melitensis* B115 was the only SDS-I fraction among other *Brucella* rough strain SDS-I fractions to show a partial protective activity in mice

against *B. abortus*, probably mediated by anti-S-LPS antibodies induced by this fraction. This fraction has been shown by use of mAb-C to contain a small quantity of S-LPS or O-PS (unpublished result). Cell walls from *B. melitensis* B115 were also shown to induce antibodies specific for S-LPS (unpublished result). For these reasons, and because *B. melitensis* B115 (Jones *et al.*, 1973), like other rough *Brucella* strains (Alton, 1960; Harmon *et al.*, 1988; Kreutzer & Robertson, 1979; Schurig *et al.*, 1991; Tobias *et al.*, 1992), is much less virulent than smooth *Brucella* strains, *B. melitensis* B115 could possibly be used as a vaccine strain if distinction between vaccinated and infected animals could be based on specific detection in infected animals of antibodies against the A-LPS epitopes, as for example by use of competitive ELISA and/or O-PS of *Y. enterocolitica* O:9. High antibody reactivity in ELISA against *Y. enterocolitica* O:9 S-LPS has been observed in sheep and cattle experimentally infected with *B. melitensis* and *B. abortus* respectively (unpublished results).

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