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Use of Monoclonal Antibodies To Identify the Distribution of A and M Epitopes on Smooth *Brucella* Species†

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The smooth types of *Brucella* species express two lipopolysaccharides (LPSs) (A and M) which are antigenically distinct. Their existence as cross-reactive antigenic complexes makes definitive serology difficult. Murine hybridomas were produced and selected for their ability to produce monoclonal antibodies to the specific A- or M-LPS epitopes. The specificity of the monoclonal antibodies was determined by microplate enzyme-linked immunoassay, binding inhibition assay, microplate agglutination, and dot blot assay. Monoclonal antibody 12AE6 was specific for an epitope on the A LPS of *Brucella* spp., which was also expressed on *Yersinia enterocolitica* O:9. A unique epitope of M LPS was detected by monoclonal antibody 33.1.5. The two monoclonal antibodies did not exhibit cross-reactions when assayed with whole *Brucella* cells or purified M- and A-LPS preparations. Cross-reactive serology with polyvalent sera can be attributed to the presence of common antigenic determinants on both molecules. The use of A- and M-LPS-specific monoclonal antibodies has the potential to replace the more laborious methods involved in the production of monospecific sera.

The surface macromolecules of virulent and avirulent *Brucella* species have been examined with regard to total lipids, proteins, peptidoglycan content and composition, and lipopolysaccharides (LPSs) (8, 12, 15, 16, 21, 22). The major surface differences between the smooth species are found in the LPS fractions. The smooth-type *Brucella* spp. have been found to contain two distinct antigenic determinants designated A and M. The relative amounts of the two determinants vary among the smooth *Brucella* strains, and the determinants are absent on the rough strains (1). The A-LPS determinant is found on *Brucella abortus* 2308, 19, and 1119. The M-LPS determinant is found on *B. melitensis* 16M and 15M and *B. abortus* IV. *B. suis* IV is characterized as expressing nearly equivalent amounts of both A and M LPSs.

Historically, the A and M antigens of *Brucella* species were characterized by agglutination, cross agglutination, absorption, and cross absorption patterns obtained with heterologous and homologous *Brucella* antisera and strains. On the basis of the quantitative ability to make *B. abortus* antisera monospecific to the A antigen by absorption with *B. melitensis* 16M whole cells and vice versa, the two antigens were proposed to exist simultaneously in A/M ratios of 20:1 and 1:20 on the surfaces of smooth *B. abortus* and *B. melitensis*, respectively. The separation of the two antigens has been partially successful with absorbed, monospecific antisera and, in some cases, with cell extracts. Ether-water extracts of smooth *B. abortus* and *B. melitensis* show partial identity in immunoassays when run against unabsorbed sera. Most cross-reactions are eliminated when absorbed monospecific sera are used (8).

Monoclonal antibodies to *Brucella* spp. have been reported with specificity for common *Brucella* antigens (cross-reacting or not with *Yersinia enterocolitica* O:9 [23], the A antigen [11, 17], and antibodies that are not absorbable with the heterologous strain [19], that recognize group 3 outer membrane proteins [19], that compete with sera from infected animals in enzyme-linked immunosorbent inhibition assays [20], and that protect against infection in mice [14]). In this report, we present a description of the A- and M-LPS determinants of *Brucella* spp. as defined by monoclonal antibodies. The antibodies described are specific for unique epitopes of the A- and M-LPS determinants. Antibodies recognizing epitopes common to both smooth LPS types are also described.

MATERIALS AND METHODS

***Brucella* antigens.** Whole-cell bacterial antigens were acetone-killed preparations provided by B. L. Deyoe (U.S. Department of Agriculture, Ames, Iowa). The *Brucella* strains tested were smooth virulent *B. abortus* 2308, attenuated smooth vaccine *B. abortus* 19, rough vaccine *B. abortus* 45/20, A-LPS-deficient *B. abortus* IV, smooth *B. melitensis* 16M, smooth *B. suis* IV, rough *B. canis*, and rough *B. ovis*. *Y. enterocolitica* serotypes O:8 and O:9, *Escherichia coli* ATCC 25922, and *Salmonella typhimurium* ATCC 14028 were used in cross-reaction assays.

LPS antigens. Purified A-LPS extract from smooth *B. abortus* 1119.3 was prepared by K. Nielsen (Texas A & M University, College Station) and further treated with proteinase K (Sigma Chemical Co., St. Louis, Mo.). Purified M LPS was provided by E. C. Klaviter (Michigan State Department of Health). M LPS from *B. melitensis* 16M was prepared by lithium acetate extraction and precipitation with 2.3 volumes of isopropyl alcohol followed by proteinase K treatment. The purity of this preparation was confirmed by nuclear magnetic resonance analysis.

ELISA. Antibody-containing preparations were tested for activity in the enzyme-linked immunosorbent assay (ELISA) by using a modification of the microprocedure described by Douglas et al. (9). Briefly, whole-cell suspensions were used as antigens in 96-well Immulon 2 U plates (Dynatech Laboratories, Inc., Alexandria, Va.). LPS preparations adjusted to 5 μg/ml and dispensed at 0.050 ml per well were also used as antigens. Nonspecific protein binding was blocked with a solution of 10% normal goat serum and 10% powdered milk. The antibody preparations being tested were incubated for

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† This paper is dedicated to the memory of Lois M. Jones, who contributed so much to our knowledge of *Brucella* spp. and the A and M antigens.

TABLE 1. ELISA values of monoclonal antibodies

Antibody[a]	Reactivity (A_{492})[b] of antibody to:									
	Brucellae						Enteric bacteria			
	Purified LPS		*B. abortus* 2308[c]	*B. melitensis* 16M	*B. suis* IV	*B. ovis*	*Y. enterocolitica* O:9	*Y. enterocolitica* O:8	*E. coli* ATCC 25922	*S. typhimurium* ATCC 14028
	A[c]	M[d]								
A	1.60	0.00	1.52	0.00	0.58	0.00	1.01	0.00	0.00	0.00
M	0.00	0.72	0.00	0.70	0.19	0.00	0.00	0.00	0.00	0.00
C-1	0.40	0.84	0.35	0.91	0.37	0.00	0.00	0.05	0.06	0.00
C-2	0.20	0.82	0.30	0.69	0.42	0.07	0.00	0.00	0.00	0.07
C/Y-1	0.29	0.23	0.75	0.45	0.10	0.00	0.33	0.00	0.00	0.00
C/Y-2	0.65	0.66	0.85	1.21	0.50	0.06	0.22	0.00	0.05	0.00

[a] Ascites fluid diluted to 10^{-3}.
[b] Average A_{492}s of duplicate tests from at least three assays. Values of less than 0.05 were recorded as 0.00. Values of 0.00 were recorded for *B. abortus* 45/20 and *B. canis* for all antibodies tested.
[c] Purified LPS extract from *B. abortus* 1119.3 treated with proteinase K.
[d] Purified LPS from *B. melitensis* 16M prepared by lithium acetate extraction, isopropanol precipitation, and proteinase K treatment.
[e] Acetone-killed whole-cell preparations.

60 min at 37°C. Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Cooper Biomedical, Inc., West Chester, Pa.) was added to detect antigen-bound antibody and visualized by the addition of *o*-phenylenediamine substrate. A_{492}s of the microplate wells were read in a Titertek Multiscan ELISA plate reader.

Binding inhibition assay. Monoclonal antibodies were used to block rabbit monospecific anti-A-LPS and anti-M-LPS sera (F.A./W.H.O. Brucellosis Centre, Weybridge, England) in an ELISA. After the incubation of monoclonal antibodies in antigen-containing wells, 1 μg of goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.) per ml was used to block nonspecific goat anti-rabbit conjugate binding. Rabbit monospecific anti-A-LPS and anti-M-LPS sera were used at dilutions of 1:1,000. Goat anti-rabbit IgG (heavy and light chain specific) and goat anti-rabbit IgM (heavy chain specific) peroxidase conjugates (Cooper) were used to detect bound rabbit antibodies. Incubations and substrate development were performed as described above for the ELISA.

Monoclonal antibodies. BALB/c mice were immunized with whole *B. abortus* 2308 or *B. melitensis* 16M organisms. Spleen cells from immunized mice were fused with X63-Ag8.653 mouse myeloma cells by a modification of the method of Galfre et al. (10). The resulting hybridomas were screened for specific monoclonal antibody activity against whole-cell *Brucella* antigens by ELISA. The production of antibody-containing ascites fluid was accomplished by intraperitoneal injection of trypan blue-primed mice with hybridomas that had been cloned by limiting dilution.

Dot blot assay. Dot blots were performed with purified A- and M-LPS antigens and with whole-cell antigens of *B. abortus* 45/20, 2308, and 19, *B. melitensis* 16M, *B. abortus* IV, *B. suis* IV, and *Y. enterocolitica* O:9. Each antigen was spotted on nitrocellulose paper (Bio-Rad Laboratories, Richmond, Calif.) and allowed to dry. The antigen strips were incubated for 60 min in 1:10 dilutions of antibody. Unbound antibody was washed off, and the goat anti-mouse IgG conjugate was incubated on the strips. Unbound conjugate was then washed off, and the strips were immersed in a solution containing 4-chloro-1-naphthol (Bio-Rad) until a visible precipitate was formed.

RESULTS

ELISA and binding inhibition. The specific recognition of *B. abortus* A LPS was demonstrated by monoclonal antibody 12AE6 (monoclonal antibody A) (Table 1). Monoclonal antibody A produced in ascites exhibited substantial ELISA reactivity even when diluted at 1:100,000 and retained the highly specific A-LPS reactivity at the lowest dilution assayed (1:10) (Table 2).

The monoclonal antibody selected for specificity for *B. melitensis* 16M M LPS, 33.1.5 (monoclonal antibody M), lacked cross-reactivity, as indicated by ELISA values, to whole-cell preparations of smooth *B. abortus* strains and to purified A LPS (Table 1). Monoclonal antibody M recognized M-LPS-type whole cells and purified M-LPS preparations from *B. melitensis* 16M and 15M (Table 1).

B. suis IV was recognized by both monoclonal antibodies A and M, as expected for a strain expressing both A and M LPSs (Table 1). Neither monoclonal antibody A nor M recognized the rough *Brucella* strains *B. abortus* 45/20, *B. ovis*, and *B. canis*. *E. coli*, *S. typhimurium*, and *Y. enterocolitica* O:8 also gave negative reactions when run against monoclonal antibodies A and M.

When run in ELISA against purified A- and M-LPS preparations, monoclonal antibodies 9.1.2 and 29.1.2 (C-1 and C-2, respectively, in Table 1) showed cross-reactivity. Monoclonal antibodies 6.9D and 6.15A (C/Y-1 and C/Y-2, respectively, in Table 1) showed cross-reactivity with A and M LPSs and also with *Y. enterocolitica* O:9.

Monoclonal antibody A was able to inhibit the binding of monospecific A-LPS antiserum depending on the antibody subclass assayed. Monospecific rabbit IgG and IgM binding

TABLE 2. ELISA titers of ascites fluid monoclonal antibodies

Antibody	Titers ($\log_{10}$)[a]			
	B. abortus 2308[b]	A LPS[c]	*B. melitensis* 16M[b]	M LPS[d]
A	5	>5	0	0
M	0	0	4	4
C-1	5	>5	>5	>5
C-2	3	3	4	4
C/Y-1	4	3	4	4
C/Y-2	4	4	3	3

[a] Endpoint dilution was determined by an A_{492} of 0.10. The nearest $\log_{10}$ dilution is reported. Values of 0 were obtained for *E. coli* DH-1 for all antibodies tested.
[b] Acetone-killed whole-cell prepartions.
[c] Purified LPS extract from *B. abortus* 1119.3 treated with proteinase K.
[d] Purified LPS from *B. melitensis* 16M prepared by lithium acetate extraction, isopropanol precipitation, and proteinase K treatment.

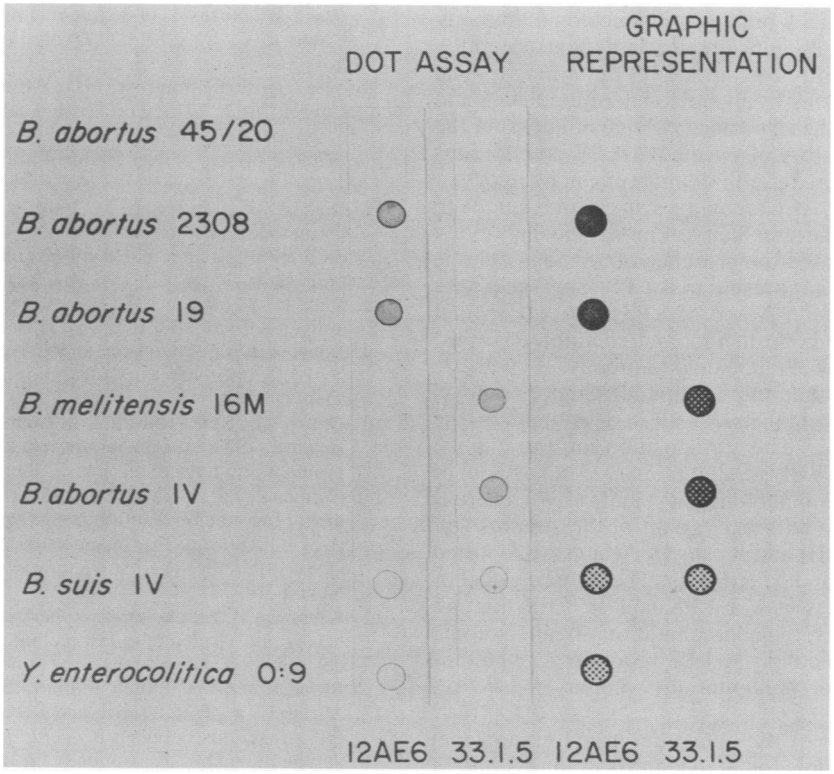

FIG. 1. Dot blot assay of monoclonal antibodies 12AE6 and 33.1.5. Whole-cell antigens (approximately 10^9 cells per ml) were spotted in 10-μl volumes on nitrocellulose. Immobilized antibodies were detected by conversion of 4-chloro-1-naphthol by peroxidase-conjugated anti-mouse antibodies. The graphic presentation is shown to clarify the differences in the intensity of reaction with the whole-cell antigens.

was inhibited by 36 and 66%, respectively. Monoclonal antibody M produced no demonstrable inhibition of monospecific M LPS.

Dot blot assay. Results of the dot blot assay of *Brucella* strains producing A or M LPS or both with monoclonal antibodies A and M are shown in Fig. 1. A-LPS-possessing *B. abortus* 2308 and 19 showed highly positive reactions when assayed with monoclonal antibody A. *B. suis* IV and *Y. enterocolitica* O:9 produced moderately positive reactions. No M-LPS cross-reaction was observed. *B. melitensis* 16M gave a highly positive reaction when assayed with monoclonal antibody M. *B. abortus* IV, which expresses M LPS, also gave a positive reaction. *B. suis* IV showed an intermediate positive reaction, demonstrating the presence of M and A LPSs on its surface.

DISCUSSION

Antibody response to *Brucella* surface molecules is perhaps the most important basis for differentiating the *Brucella* species (7, 18). Extracts of smooth *Brucella* strains have been found to be very similar and to induce similar responses. Cross-reactive antibodies are also elicited by other LPS-containing bacteria (5, 6, 13). *Y. enterocolitica* O:9, *S. typhimurium*, and *E. coli* are among the bacteria that induce cross-reacting antibodies that complicate the differential serological diagnosis of *Brucella* infection. The basis of the serological cross-reactivity of A LPS with the LPS of *Y. enterocolitica* O:9 has been related to the occurrence of 1,2-linked mannopyranosyl units in the O-chain polysaccharides of their LPSs (3, 4).

ELISA, dot blot, and immunoblot assays with monoclonal antibodies A and M showed that *B. abortus* 19 and 2308 possess distinct A-LPS epitopes, whereas *B. melitensis* and *B. abortus* IV possess distinct M-LPS epitopes. The presence of both LPS types is evident in the assay of *B. suis* IV. These observations are consistent with the classification scheme of *Brucella* biotypes developed with monospecific antisera (1). The data accumulated indicate that the A and M *Brucella* LPSs are distinct separate antigenic structures with unique and common epitopes. The cross-reactions and heterologous absorption data can be attributed to the presence of the common determinants of the A- and M-LPS molecules.

The structural difference between the two LPS types is partially defined. Studies of the A-LPS side chain by nuclear magnetic resonance have established that the cross-reactivity of smooth *B. abortus* and *Y. enterocolytica* is associated with the 1,2-linked N-acylated 4-amino-4,6-dideoxy-alpha-D-mannopyranosyl unit in the O-chain polysaccharide of their LPSs (2–4). Wilke et al. (23) described a monoclonal antibody to *B. melitensis* similar to C/Y-1 and C/Y-2 described here that cross-reacts with *Y. enterocolitica* O:9 and also reacts with A-LPS-antigen-possessing strains. The A-LPS-antigen-specific monoclonal antibody A described here strongly cross-reacted with *Y. enterocolitica* O:9, whereas the M-LPS-antigen-specific monoclonal antibody M failed to react with *Y. enterocolitica*. This result provides evidence for a common side chain or core for the two LPS types with a variation in linkage or branching in adjacent unique overlapping domains. The analysis of the LPS antigens by immunodiffusion indicates that the A LPS is a larger complex. The diffusion of A LPS in gel does not readily occur unless the molecule is partially hydrolyzed (8).

The inhibition of ELISA binding of monoclonal antibody A by monospecific A-LPS antisera clearly demonstrated the specificity of the antibody for an A-LPS determinant. The agglutination of organisms by monoclonal antibodies A and M further established their specificity. High affinity and the specific reaction in ELISA of purified A-LPS preparations but not of M-LPS preparations definitively indicated that monoclonal antibody 12AE6 recognizes an epitope unique to the A-LPS complex. The specificity of monoclonal antibody M in ELISA demonstrated the presence of a unique epitope in the M-LPS complex not present in A LPS. This specificity indicates that the *Brucella* A and M LPSs are two distinct molecular species with both distinct and common epitopes. The cross-reactivity of monoclonal antibody A with *Y. enterocolitica* implies that the 1,2-linked mannopyranosyl units of the LPS side chain make up at least part of the unique A-LPS epitope. The composition of the unique M-LPS epitope is reportedly related to one 1,3 linkage out of four 1,2 linkages of the mannopyranosyl units (2, 4). There is the possibility that an additional variant of the amino sugar exists for *B. melitensis* III and *B. suis* IV, which are reported to contain both the A and M antigens. The only physical or chemical difference between A LPS and M LPS that we demonstrated concerns their precipitation in an isotonic solution of isopropyl alcohol. M LPS required 2.3 volumes of isopropyl alcohol for precipitation, whereas A LPS required only 1 volume.

In summary, the use of the above-described monoclonal antibodies made it possible for us to identify unique A and M epitopes on smooth *Brucella* species and their LPS molecules. The distribution of the A and M epitopes among the smooth LPS-possessing *Brucella* strains established by agglutination schemes based on monospecific sera agrees with our analysis based on monoclonal antibodies in ELISA. The results with the monoclonal antibodies indicate that the A and M LPSs isolated from biotype I of either *B. melitensis* or *B. abortus* are unique molecules with A or M unique epitopes and shared epitopes. The hypothesis that biotype I brucellae possess differing ratios of A and M antigens was not supported by results of ELISA with monoclonal antibodies to the A and M epitopes.

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