Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and Immunoblotting Analysis of Smooth-Lipopolysaccharide Heterogeneity among Brucella Biovars Related to A and M Specificities

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Smooth (S)-lipopolysaccharide (LPS) preparations from reference and field strains of several biovars of Brucella abortus, B. melitensis, and B. suis were prepared by (i) the hot phenol-water method, (ii) hot sodium dodecyl sulfate extraction and protease K digestion, or (iii) dimethyl sulfoxide extraction. These S-LPS-enriched fractions were further analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining after periodate oxidation. Immunoblots were developed by using either monoclonal antibodies specific for Brucella A or M antigens or polyclonal polyspecific or monospecific sera from rabbits, cattle, and goats. The specificity of monoclonal antibodies reactive with Brucella unique (A or M) epitopes was demonstrated by enzyme-linked immunosorbent assay, LPS latex agglutination, or agglutination inhibition. The most-represented subunits of S-LPS ranged in M, from 30,000 to 70,000 relative to marker proteins. According to A or M immunodominance, two sodium dodecyl-sulfate-polyacrylamide gel electrophoresis blotting patterns were clearly distinguished among biovars, whatever the fraction tested: a close succession of regularly spaced narrow bands for A>M strains and regularly spaced triplets of bands including either (i) a first thick band followed by two thick bands for B. abortus M>A strains or (ii) one thick band between two thin bands for B. melitensis or B. suis M>A strains. Moreover, A and M specificities were reaffirmed by sandwich enzyme immunoassay and latex agglutination inhibition with monoclonal antibodies and polyclonal sera.

Lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria, is important for structure and function (13, 15). In Brucella spp., active immunization with LPS from smooth colony cells (S-LPS) and passive immunization with S-LPS-specific monoclonal antibodies (MAbs) have been reported to induce protective immunity in mice (12, 21). The serological response resulting from exposure to smooth Brucella spp. is largely against S-LPS (17). Moreover, antibodies to S-LPS form the basis of standard serological diagnostic tests (1). Wilson and Miles described two dominant antigens (A and M) in S-LPS (27). This description was reaffirmed recently on the basis of the reproducibility of the binding profile of absorbed rabbit sera (26) or MAbs (3, 16) and on the basis of DNA homology (25). Smooth Brucella strains carry both the A and M antigens in a ratio that defines the classification of strains in serovars (1). Three serovars can be classically distinguished by the slide agglutination test with polyclonal monospecific sera, A+M−, M+A−, and A+M+ (1). The differences in A and M distribution are related to the molecular structure of the S-LPS O-chain polysaccharide, and the structures of the Brucella A and M epitopes were recently postulated by using mouse MAbs and oligosaccharides (2, 3, 16). Analysis of Brucella LPS by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) has been carried out by several groups (16, 18-20). After periodic acid-Schiff staining, fraction 5 of the phenol-water extract (f5) gave a broad-smeared distribution pattern (18). After periodic acid silver staining, butanol-extracted S-LPS appeared as a diffuse band as well, but a slight indication of multimeric bands was claimed (20). After silver staining, S-LPS f5 and S-LPS prepared from a whole-cell lysate digested by proteinase K gave different patterns, a well-resolved banding pattern allowing distinction between A>M and M>A antigens (5), a broad diffuse pattern (19), and a broad diffuse pattern with some degree of better resolution for M>A S-LPS (16). This report describes the preparation of S-LPS-enriched fractions from several biovar strains of Brucella spp. by three different methods and analysis by SDS-PAGE. Immunoblotting analysis was performed by using MAbs and polyclonal mono- and polyspecific sera from rabbits, cattle, and goats. Two major well-resolved S-LPS patterns based on the immunodominance of A or M antigens (5) were confirmed and extended.

MATERIALS AND METHODS

Bacterial strains. Smooth biovar reference and field strains (Table 1) were kept freeze-dried in the Laboratoire Central de Recherches Vétérinaires (Maisons-Alfort, France) culture collection. Purity and colony type phase were checked by using standard procedures (1). Cells of a 48-h culture on 5% (vol/vol) equine serum (bio Mérieux)-supplemented blood agar base no. 2 (Oxoid) in Roux flasks were harvested and washed in distilled water, and except as stated otherwise, inactivated by being heated at 60°C for 1 h. The cells were then centrifuged at 12,000 × g for 30 min at 4°C. The pellet was then used for LPS antigen preparation or immunizations.

LPS antigens. Proteinase K-digested whole-cell lysates (LPS-PK) were prepared by the method of Hitchcock and

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Brown (5, 7) with some modifications. Briefly, cells were suspended in 0.0625 M Tris buffer (pH 6.8) containing 2% (wt/wt) SDS (0.5 g [wet weight] of cells per 10 ml). Samples were heated at 100°C for 10 min, and the lysates were cooled to 55°C. Proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany) was added at 0.15 mg/ml, and the samples were incubated at 55°C for 3 h and then kept overnight at 20°C. SDS (0.02 g/ml) was added before heating at 100°C for 10 min. The samples were centrifuged at 12,000 × g for 30 min at 20°C, and the LPS was precipitated by addition of 3 volumes of isopropanol at 4°C to the supernatant. After 30 min, the precipitate was harvested by centrifugation at 12,000 × g for 30 min at 4°C and dissolved in 10 ml of distilled water. After a second isopropanol precipitation, the pellets containing LPS were recovered in 1 ml of distilled water and lyophilized. The phenol-water fractions (LPS-fs) were isolated by the hot aqueous phenol procedure of Leong et al. (10). In this work, LPS-fs consisted of the first solubilization of the methanol precipitate without further purification. Dimethyl sulfoxide (DMSO) LPS-enriched fractions (LPS-DMSO) were prepared by the method of Hoffmann and Houle (8). Protein contamination of the LPS-enriched fractions was evaluated in samples by using a Micro BCA protein assay (Pierce Chemical Co., Rockford, Ill.) Rough LPS (R-LPS) crude extract of the rough strain of Brucella melitensis B115 was prepared as described previously (6).

**Rabbit, bovine, and goat polyclonal poly- and monospecific sera.** Anti-Brucella sp. polyclonal poly- and monospecific sera were prepared with either living or killed bacteria cultured and harvested as described above. Eight rabbits were initially injected intravenously with either living (5 × 10^9 cells) or killed (5 × 10^11 cells) bacteria (strain 544 or 16M), bled 7 days later, given five weekly intradermal booster injections of 5 × 10^11 killed cells of the respective organism, and then bled. Four goats and four cows were immunized in the same way, except that 5 × 10^12 living cells of either strain B19 or 53H38 were used for primary intravenous injection and the same but killed organisms were used for further intradermal injections for goats, while all injections were performed with either strain 544 or strain 16M (5 × 10^11 killed cells) in cattle. Two serum samples from B. abortus biovar 1 or B. melitensis biovar 1 culture-positive cows were also used. All sera were absorbed as described by Alton and al. (1), except that either strain 99 for M>A-specific antisera or strain 53H38 for A>M-specific ones were used as absorbing antigens. All sera were tested with a seroagglutination test as previously described (1) with either strain 99 or 53H38 as the antigen. After absorption, antibodies reacting with heterologous bacteria were dissociated from immune complexes by the method of Terminck and Avrameas (22) by using a buffer (pH 2.8) containing 0.2 M HCl and 0.2 M glycine.

**MAbs.** A and M antigen-specific MAbs (MAb-A and MAb-M) were produced as described previously (12). Iso-types were determined by enzyme-linked immunosorbent assay (ELISA) using S-LPS-coated plates and isotype-specific antisera (Bio-Rad Laboratories, Richmond, Calif.). SDS-PAGE and Western blots (immunoblots). Samples previously heated at 100°C for 10 min were run at 70 mA of constant current for 75 min (Protein II Slab Cell; Bio-Rad). The running (11% acrylamide) and stacking gels were prepared as described by Lugtenberg et al. (14) and Laemmli (9), respectively, and were 0.75 mm thick. Escherichia coli O55:B5 LPS W (Difco Laboratories, Detroit, Mich.) was used as the carbohydrate standard, and protein molecular weight markers (Rainbow; Amersham) were used to determine M, S. LPS molecules were revealed by silver staining after periodate oxidation as described previously (24). Protein contamination of the LPS-enriched fractions was visualized by the silver staining method of Damerval et al. (4). Electrophoresis of nitrocellulose sheets (0.45-μm pore size; Millipore, Molsheim, France) was performed as described by Towbin et al. (23) in a buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 20% (vol/vol) methanol at an constant voltage of 80 V for 2.5 h with a Trans-Blot Cell apparatus (Bio-Rad). After transfer, nitrocellulose sheets were first incubated overnight in Tris hydrochloride-buffered saline (20 mM Tris, 50 mM NaCl, pH 7.5) containing 1% (wt/vol) gelatin, and then with MAbS or polyclonal sera. Antigen-bound antibodies were visualized by incubation with sheep anti-mouse, donkey anti-rabbit or anti-goat (Amersham), or rabbit anti-bovine (Biosys) immunoglobulin G (IgG) biotinylated whole antibodies and then with streptavidin-horseradish peroxidase (Amersham). Immunological reactions were then revealed with the horseradish peroxidase color reagent (Bio-Rad).

**Immunooassays.** ELISAs were performed as previously described (11, 12), with either LPS or anti-Brucella sp. immunoglobulin coating for antibody or antigen titration, respectively. Immunoassays by latex agglutination or agglutination inhibition for antibody or antigen titration were performed as previously described (11, 12), with a computer-controlled automated analyzer, by which the degree of agglutination was determined by counting the nonagglutinated particles.
TABLE 2. Characteristics of MAbs

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<tr>
<th>MAb</th>
<th>Specificity</th>
<th>Isotype</th>
<th>A/M ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>LPS latex agglutination&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Agglutination inhibition&lt;sup&gt;b&lt;/sup&gt;</th>
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<tr>
<td>O4F9</td>
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<td>IgG2a</td>
<td>3 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<td>&lt;1 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>G2E11</td>
<td>M</td>
<td>IgG3</td>
<td>2.5 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>2.5 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>&gt;10&lt;sup&gt;-2&lt;/sup&gt;</td>
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<sup>a</sup> LPS-f5-A from B. abortus biovar 3 (A>M) and LPS-f5-M from B. melitensis biovar 1 strain 16M (M>A).

<sup>b</sup> Ratio of ELISA or agglutination titers of MAb-A and MAb-M.

FIG. 1. Sandwich ELISA titration curves for LPS-f5 measured with MAb-A (a) and MAb-M (b) coated plates. LPS-f5 samples were prepared from B. melitensis biovar 1 strain 53H38 (C) and 16M (b) and B. abortus biovar 1 strain 99 (C) and biovar 3 (b). O.D., Optical density.

FIG. 2. Sandwich ELISA titration curves for LPS-PK measured with MAb-A (a) and MAb-M (b)-coated plates. LPS-PK samples were prepared from B. melitensis biovar 1 strains 53H38 (P) and Rev.1 (A); B. melitensis biovar 3 (A); B. abortus biovar 1 strains 544 (C), 99 (C), and B19 (C); B. abortus biovar 5 (b); and B. suis biovar 4 (A). O.D., Optical density.

FIG. 3. B. abortus biovar 3 (a) or B. melitensis 16M (b) LPS-f5-coated latex agglutination inhibition curves for binding of MAb-A (a) and MAb-M (b) by LPS-f5 from B. melitensis biovar 1 strains 53H38 (P) and 16M (b) and B. abortus biovar 1 strain 99 (C) and biovar 3 (b).

RESULTS

Characteristics of MAb. The A or M specificities of the two Mabs used (MAb-A, O4F9; MAb-M, G2E11) were assessed by ELISA, LPS-coated latex agglutination, and agglutination inhibition (Table 2) and then confirmed in the experiment with LPS-PK. Isotypes were IgG3 for G2E11 and IgG2a for O4F9.

Characteristics of LPS-enriched fractions. Rates of Brucella sp. S-LPS-enriched fraction recovery expressed in dry weight units per unit of bacterial wet weight were higher for LPS-PK (1.5 to 3%) than for LPS-DMSO (1 to 1.5%) and LPS-f5 (0.8 to 1.5%). Silver staining of gels for proteins showed higher protein contamination in LPS-DMSO than in LPS-PK and LPS-f5 (data not shown). BCA protein assays revealed higher protein contamination for LPS-PK (10 to 20%) than for LPS-f5 (5 to 15%).

Comparative quantification of A and M antigens on LPS-PK or LPS-f5 fractions from various strains. Figures 1 to 4 show the results obtained by LPS titration with MAb-A or MAb-M with either sandwich ELISA or A- or M-dominant LPS-coated latex particle agglutination inhibition for LPS-PK or LPS-f5 from various Brucella strains. The A and M distribution observed with both assays was very similar to that classically obtained with slide agglutination of bacterial cells with monospecific polyclonal anti-A or anti-M rabbit serum (Table 1). The S-LPS of B. melitensis biovar 3 behaved like typical A>M S-LPS with MAb-A but reacted with MAb-M in a way intermediate between typical M>A and A>M S-LPS. The inverse was true of B. suis biovar 4 S-LPS (A+M+ with monospecific polyclonal sera), which appeared as an M>A antigen (Fig. 4A and B).

S-LPS SDS-PAGE and immunoblotting patterns based on A or M immunodominance and Brucella species. The silver-stained electrophoretic profiles of the LPS fractions were compared with that of S-LPS from Escherichia coli O55:B5 and crude R-LPS extract of B. melitensis B115 (Fig. 5). The migration pattern of LPS extracted from various Brucella strains by the hot phenol-water technique was identical to those of LPS-DMSO and LPS-PK (data not shown). The profiles of S-LPS preparations consisted of a fast-migrating low-M<sub>r</sub> band and an orderly series of high-M<sub>r</sub> bands which formed a characteristic ladderlike pattern. The fastest-migrating component is believed to represent the lipid A-core oligosaccharide portion of the LPS (R-LPS) and is identical to that obtained from the R-LPS extract of B. melitensis B115. The slower-migrating components represent S-LPS molecules with increasing numbers of O side chain repeated units. Moreover, two major electrophoretotypes were distin-
guished on the basis of A or M immunodominance: a close succession of regularly spaced narrow bands for A>M strains and regularly spaced triplets of bands including either (i) a first thin band followed by two thick bands for B. abortus M>A strains or (ii) one thick band between two thin bands for B. melitensis and B. suis M>A strains (Fig. 6). B. melitensis biovar 3 and B. suis biovar 4 LPS showed electrophoretic profiles consistent with the above-described considerations, but their patterns were fuzzier. The molecular weight distribution of the Brucella S-LPS chains was consistent from one strain to another, although the B. suis biovars exhibited a larger proportion of shorter chains. The $M_s$ of the most common S-LPS subunits ranged from 30,000 to 70,000 relative to those of marker proteins. The fast-migrating LPS (R-LPS) had an $M_s$ of approximately 5,000, identical to that of the R-LPS of the rough strain of B. melitensis B115. The S-LPS patterns were species independent and were confirmed whatever the fraction tested (LPS-PK, LPS-f5, or LPS-DMSO). Some contaminating (or LPS-linked) proteins were also observed, and the most common had $M_s$ of 25,000 to 27,000 relative to those of marker proteins.

Visualization of high-$M_s$ S-LPS molecules by immunoblotting. Figure 7 shows the results obtained after SDS-PAGE separation, gel blotting on nitrocellulose sheets, and development with either MAb-A or MAb-M. A- or M-dominant S-LPS was recognized only by the corresponding MAb. The patterns obtained were identical to those obtained as described above for SDS-PAGE coupled with silver staining. These electropherotypes were all confirmed by using polyclonal polyspecific sera whatever the species bled and the type of immunization (data not shown). Absorbed sera, like MAbs, recognized either A>M or M>A S-LPS electropherotypes, according to their respective specificities. Eluted antibodies recognized both A and M antigen-related electropherotypes (data not shown). In conclusion, all minor or major bands of electropherotypes were labeled by specific or common antibodies.

**DISCUSSION**

LPS-PK fractions are easier to obtain and less contaminated by proteins than are LPS-enriched fractions prepared by the DMSO method. While LPS-PK fractions are not as well purified as the classical f5 fraction, as previously reported in salmonellae (7), they allow, after easy and rapid preparation, precise immunochemical and electrophoretic studies of Brucella S-LPS.

A and M specificities assessed by sandwich ELISA and
LPS-coated latex agglutination inhibition are consistent with the early proposal of Wilson and Miles (27) and practical results currently observed in standard biotyping of brucellae (Table 1) (1). This description was recently confirmed with pure preparations of polysaccharides bearing A and M specificities in other immunological systems (3, 16). It is noteworthy that no difference was observed among different strains of the same biovar, i.e., for either B. abortus biovar 1 strain, 544 (reference strain) or 99 (strain commonly used for antigen preparations in Europe) and B19 (vaccinal strain) or for B. melitensis biovar 1 strains 16M (reference strain), 53H38, and Rev.1 (vaccinal strains).

Fine separation of the Brucella LPS molecules was dependent upon the concentration of the LPS-enriched fraction loaded on the gel (1 to 2 μg), and proper conditions for gel electrophoresis, including the resolving system of Lugtenberg et al. (14). Differences in the LPS banding patterns of smooth Brucella biotypes correlated positively with A and M antigens (polyclonal monospecific sera) or A and M epitopes (MAbs) when analyzed by SDS-PAGE and visualized by periodic acid and silver staining. For A>M strains, a close succession of regularly spaced narrow bands was observed. For M>A strains, a succession of regularly spaced triplets of bands was seen, including (i) a first thin band followed by two thick bands for B. abortus or (ii) one thick band between two thin bands for B. melitensis and B. suis.

MAbs of either A or M specificity and polyclonal monospecific sera revealed all of the S-LPS bands bearing the corresponding immunodominant A or M antigen and gave the same A- or M-dominant antigen-related patterns as those evidenced by the gel silver staining. The labeling of minor and major bands of the M S-LPS triplet banding pattern indicated that the M-type patterns were not a mixture of M and A bands but contained a unique type of molecules bearing both A and M specificities. Furthermore, the labeling of all of the S-LPS bands, whatever their A or M characteristics, by eluted antibodies, i.e., previously absorbed on a heterologous strain during the preparation of polyclonal monospecific anti-A or -M sera, may indicate the presence of a common epitope on the O chain. However, it is also conceivable that labeling could be due to homologous antibodies absorbed by the heterologous strain.

It is not possible to correlate these findings with biochemical data on Brucella S-LPS structure, but differences were recently evidenced in the S-LPS structure of A-dominant and M-dominant antigens of reference strains from five of the six Brucella species. The structure of the M-dominant O polysaccharide of S-LPS is known as a linear polymer of unbranched pentasaccharide repeating units consisting of four α1,2-linked and one α1,3-linked 4,6-dideoxy-4-formamido-d-mannopyranosyl residues, while the A-dominant O polysaccharide of S-LPS consists of an α1,2-linked homopoly saccharide of 4,6-dideoxy-4-formamido-d-mannopyranosyl units with a low-frequency occurrence of α1,3-linked 4,6-dideoxy-4-formamido-d-mannopyranosyl residues (3, 16). Composite A and M antigen characteristics result from O polysaccharides in which the frequencies of α1,3-linkages, and hence, O epitope characteristics, vary (16). All biovars assigned as A>M express 0 to 8% of α1,3-linked residues per polysaccharide O chain, while M>A biovars also possess a unique M epitope, with 13 to 21% of an α1,3-linked residue.
probably representing the major part of the M epitope (2). Tri- and tetrasaccharides containing exclusively α1,2-linked 4,6-dideoxy-4-formamido-3-n-mannopyranosyl residues were found to inhibit MAbs with equal A and M affinities (3). This is consistent with the results obtained here with antibodies eluted after heterologous absorption which recognize the A and M antigens. The peculiar aspect of smeared staining in SDS-PAGE or in immunoblotting obtained for B. melitensis biovar 3 and B. suis biovar 4 can also be correlated both with previous results showing structural characteristics of linkage in A-M antigens of mixed A and M specificities for these two strains (16) and with the results obtained here with immunoassays.

Our results confirm the reality of A and M epitopes and suggest that Brucella A-dominant S-LPS biosynthesis likely differs appreciably from that of M-dominant S-LPS. They also show that differences can be observed in M-dominant S-LPS, depending on the bacterial species (B. abortus or B. melitensis). The profiles of proteinase K-digested lysates were similar to the profiles of purified LPS and may be used to provide preliminary data concerning the LPS type without further purification. The two MAbs used proved to be well suited for use as standard Brucella typing or detection reagents. Furthermore, SDS-PAGE and silver staining, coupled with fast preparation of proteinase K LPS-enriched fractions, seem suitable as a complementary test in Brucella typing and diagnostic antigen verification.

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LITERATURE CITED