

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

BRUNO GARIN-BASTUJI,^{1*} RAUL A. BOWDEN,² GERARD DUBRAY,² AND JOSEPH N. LIMET³

Laboratoire Central de Recherches Vétérinaires, Centre National d'Etudes Vétérinaires et Alimentaires, 94703 Maisons-Alfort,¹ and Station de Pathologie de la Reproduction, Institut National de la Recherche Agronomique, 37380 Nouzilly,² France, and Unité de Médecine Expérimentale, Université Catholique de Louvain, 1200 Brussels, Belgium³

Received 22 February 1990/Accepted 16 July 1990

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 proteinase 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_r from 30,000 to 70,000 relative to marker proteins. According to A or M immunodominance, two sodium dodecyl sulfate-polyacrylamide gel electrophoresis banding 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 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* 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-smear distribution pattern (18). After periodic acid silver staining, butanol-extracted S-LPS appeared as a dif-

fuse 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

* Corresponding author.

TABLE 1. A and M immunodominance of the *Brucella* strains used

A and M antigen distribution and species	Biovar	Strain(s)	Agglutination with mono-specific serum	
			A	M
A>M				
<i>B. abortus</i>	1	544, 99, B19	+	-
	2	86/8/59	+	-
	3	Field strain	+	-
	6	870	+	-
<i>B. melitensis</i>	2	63/9	+	-
	3	Ether	+	+
<i>B. suis</i>	1	1330	+	-
	2	Field strain	+	-
	3	Thomsen	+	-
M>A				
<i>B. abortus</i>	4	292	-	+
	5	B3196	-	+
	9	C68	-	+
<i>B. melitensis</i>	1	16M, Rev.1, 53H38	-	+
<i>B. suis</i>	4	40	+	+
	5	513	-	+

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 were treated with RNase and DNase I (Boehringer), both at 0.01 mg/ml, at 37°C for 30 min and then by proteinase K at 0.01 mg/ml at 55°C for 3 h and then at 20°C overnight. After a third isopropanol precipitation, the pellets containing LPS were recovered in 1 ml of distilled water and lyophilized. The phenol-water fractions (LPS-f5) were isolated by the hot aqueous phenol procedure of Leong et al. (10). In this work, LPS-f5 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 5×10^9 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 Terninck 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). Isotypes 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 (Protean 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_r*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). Electrotransfer to nitrocellulose sheets (0.45- μ m 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 a 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).

Immunoassays. 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

MAb	Specificity	Isotype	A/M ratio ^a		
			ELISA ^b	LPS latex agglutination ^b	Agglutination inhibition ^c
O4F9	A	IgG2a	3×10^3	3×10^3	$<10^{-2}$
G2E11	M	IgG3	2.5×10^{-3}	2.5×10^{-3}	$>10^2$

^a LPS-f5-A from *B. abortus* biovar 3 (A>M) and LPS-f5-M from *B. melitensis* biovar 1 strain 16M (M>A).

^b Ratio of ELISA or agglutination titers of MAbs whose titers were determined on LPS-A and LPS-M.

^c Ratio of LPS-A/LPS-M concentrations giving 50% inhibition of agglutination in a homogeneous MAb-LPS-coated latex system, i.e., LPS-A with MAb O4F9 and LPS-M with MAb G2E11.

RESULTS

Characteristics of MAbs. 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

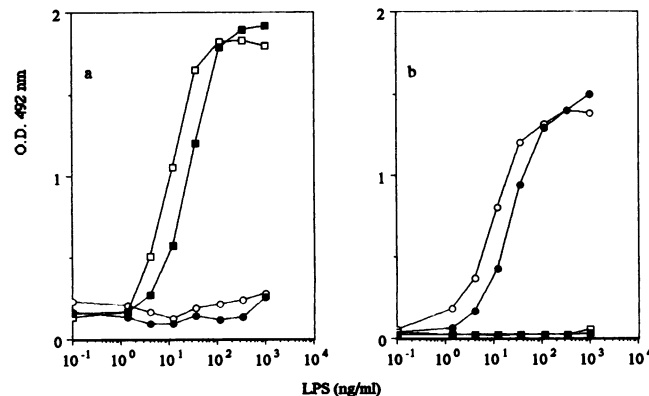


FIG. 1. Sandwich ELISA titration curves for LPS-f5 measured with MAb-A (a)- and MAb-M (b)-coated plates. LPS-f5 samples were prepared from *B. melitensis* biovar 1 strains 53H38 (○) and 16M (●) and *B. abortus* biovar 1 strain 99 (□) and biovar 3 (■). O.D., Optical density.

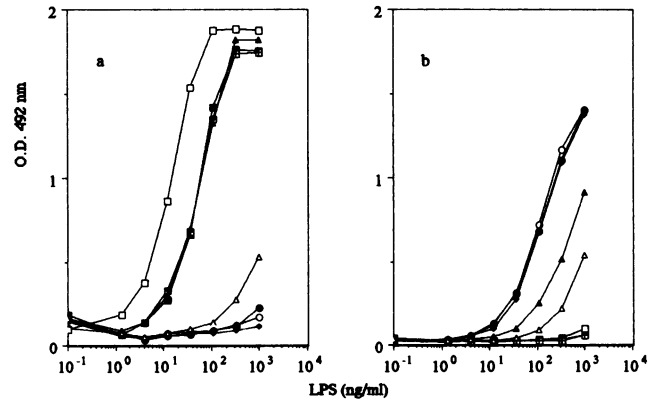


FIG. 2. Sandwich ELISA titration curves for LPS-PK measured with MAb-A (a)- and MAb-M (b)-coated plates. LPS-PK samples were prepared from *B. melitensis* biovar 1 strains 53H38 (○) and Rev.1 (●); *B. melitensis* biovar 3 (▲); *B. abortus* biovar 1 strains 544 (□), 99 (■), and B19 (⊞); *B. abortus* biovar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

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_r* band and an orderly series of high-*M_r* 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-

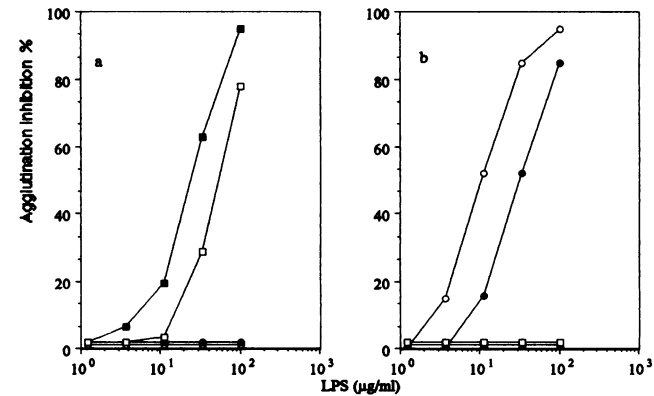


FIG. 3. *B. abortus* biovar 3 (a) or *B. melitensis* 16M (b) LPS-f5-coated latex agglutination inhibition curves for binding of MAb-A (a) and MAb-M (b) by LPS-f5 from *B. melitensis* biovar 1 strains 53H38 (○) and 16M (●) and *B. abortus* biovar 1 strain 99 (□) and biovar 3 (■).

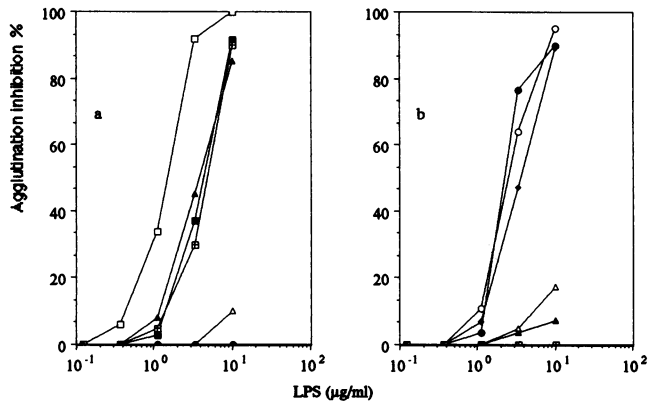


FIG. 4. *B. abortus* biovar 3 (a) or *B. melitensis* 16M (b) LPS-f5-coated latex agglutination inhibition curves for binding of MAb-A (a) and MAb-M (b) by LPS-PK from *B. melitensis* biovar 1 strains 53H38 (○) and Rev.1 (●); *B. melitensis* biovar 3 (▲); *B. abortus* biovar 1 strains 544 (□), 99 (■), and B19 (⊞); *B. abortus* biovar 5 (◆); and *B. suis* biovar 4 (△).

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_r 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_r 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_r s of 25,000 to 27,000 relative to those of marker proteins.

Visualization of high- M_r 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 electrophoretotypes 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 electrophoretotypes, 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

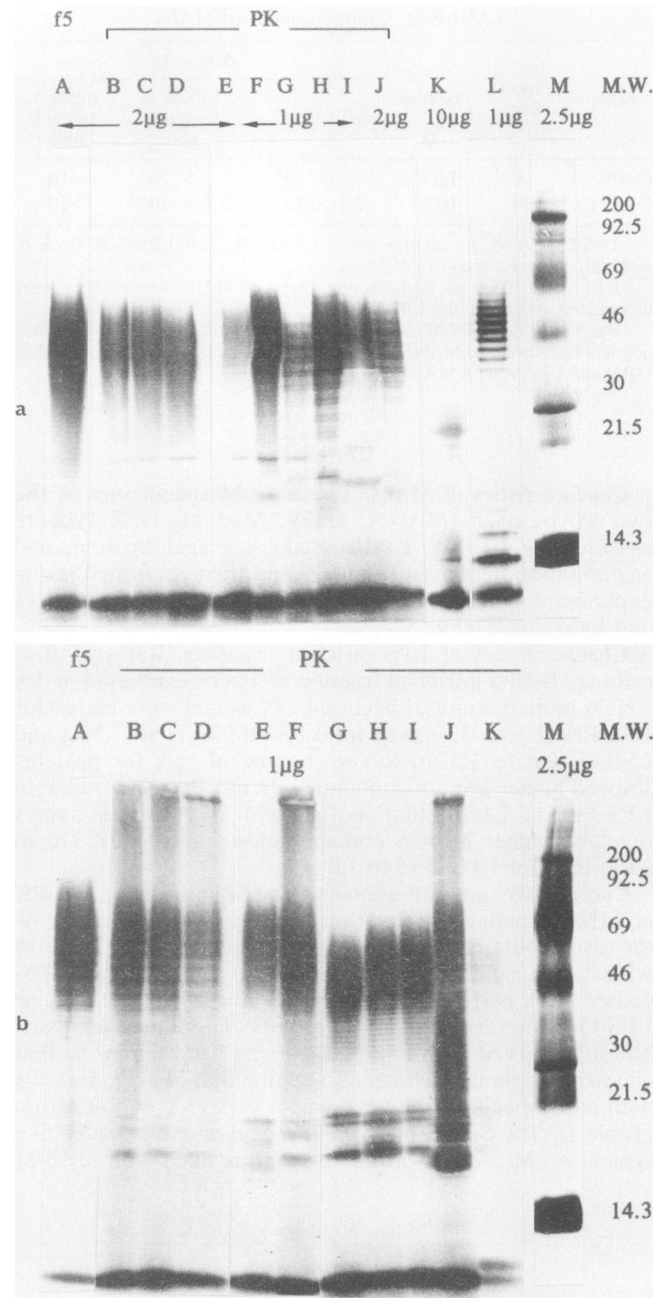


FIG. 5. Silver-stained SDS-polyacrylamide gels of LPS-f5 (lane A) and LPS-PK (other lanes) from *B. abortus* (a) and *B. melitensis* and *B. suis* (b). (a) *B. abortus* biovar 1 strains 99 (lanes A and B), 544 (lane C), and B19 (lane D) and biovars 2 to 6 (lanes E to I, respectively) and 9 (lane J); *B. melitensis* B115 crude extract (lane K); *E. coli* O55:B5 LPS (lane L); and marker proteins (lane M) (10^3). (b) *B. melitensis* biovar 1 strains H38 (lanes A and B), 16M (lane C), and Rev.1 (lane D) and biovars 2 and 3 (lanes E and F, respectively); *B. suis* biovars 1 to 5 (lanes G to K, respectively); and marker proteins (lane M) (10^3). M.W., Molecular weight.

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



FIG. 6. SDS-PAGE banding patterns of *Brucella* S-LPS according to species and serological specificity. Lanes, a, results; b, schematic representations of bands.

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* biovars 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

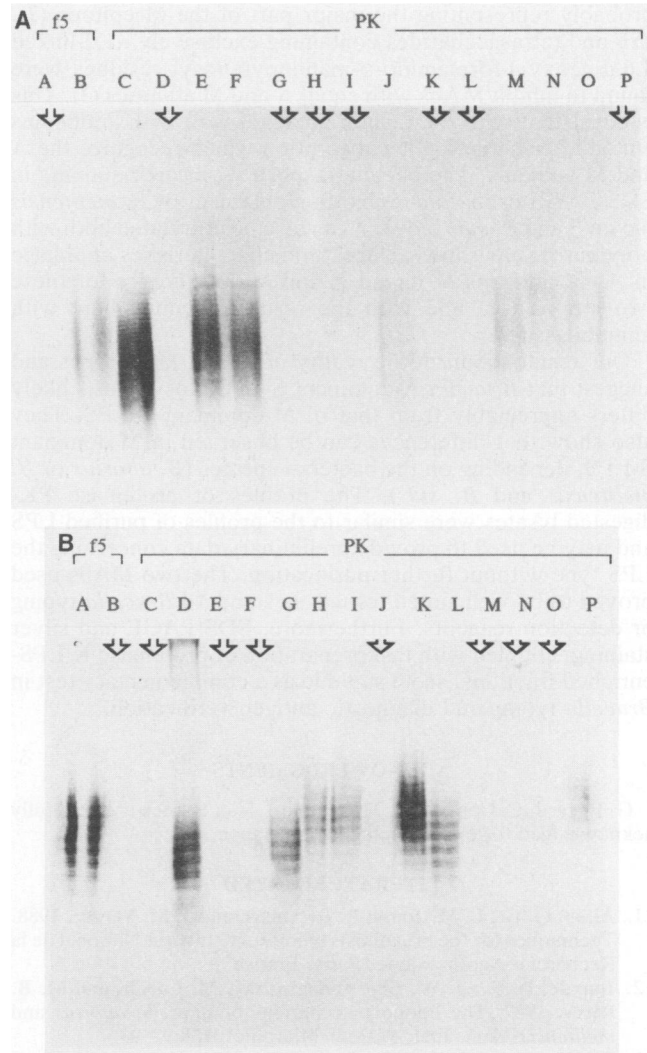


FIG. 7. (A) Western blot of LPS-f5 (lanes A and B) and LPS-PK (lanes C to P) (1 μg) of *Brucella* strains developed with MAb-A. LPS-f5 from *B. melitensis* biovar 1 strain H38 (lane A) and *B. abortus* biovar 1 strain 99 (lane B); LPS-PK from *B. melitensis* biovar 2 (lane C); *B. abortus* biovars 4, 3, 2, and 9 (lanes D to G, respectively); *B. suis* biovar 4 (lane H); *B. melitensis* biovar 1 strains Rev.1 and H38 (lanes I and K, respectively) and biovar 3 (lane J); *B. abortus* biovar 5 and biovar 1 strains 99, B19, and 544 (lanes L to O, respectively); and *B. melitensis* biovar 1 strain 16M (lane P). (B) Same gel developed with MAb-M. Arrowheads indicate the LPS-enriched fractions not revealed by MAb-A or MAb-M.

polysaccharide of S-LPS is known as a linear polymer of unbranched pentasaccharide repeating units consisting of four α 1,2-linked and one α 1,3-linked 4,6-dideoxy-4-formamido-D-mannopyranose residues, while the A-dominant O polysaccharide of S-LPS consists of an α 1,2-linked homopolysaccharide of 4,6-dideoxy-4-formamido-D-mannopyranosyl units with a low-frequency occurrence of α 1,3-linked 4,6-dideoxy-4-formamido-D-mannopyranose residues (3, 16). Composite A and M antigen characteristics result from O polysaccharides in which the frequencies of α 1,3-linkages, and hence, M epitope characteristics, vary (16). All biovars assigned as A>M express 0 to 8% of α 1,3-linked residues per polysaccharide O chain, while M>A biovars also possess a unique M epitope, with 13 to 21% of an α 1,3-linked residue

probably representing the major part of the M epitope (2). Tri- and tetrasaccharides containing exclusively α 1,2-linked 4,6-dideoxy-4-formamido-D-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* and *B. suis*). The profiles of proteinase PK-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.

ACKNOWLEDGMENTS

G. Bézard, C. Cau, A. M. Mahé, and J. Van Broeck are gratefully acknowledged for expert technical assistance.

LITERATURE CITED

- Alton, G. G., L. M. Jones, R. D. Angus, and J. M. Verger. 1988. Techniques for the brucellosis laboratory. Institut National de la Recherche Agronomique, Paris, France.
- Bundle, D. R., J. W. Cherwonogrodzky, M. Caroff, and M. B. Perry. 1987. The lipopolysaccharides of *Brucella abortus* and *melitensis*. *Ann. Inst. Pasteur Microbiol.* **138**:92-98.
- Bundle, D. R., J. W. Cherwonogrodzky, M. A. J. Gidney, P. J. Meikle, M. B. Perry, and T. Peters. 1989. Definition of *Brucella* A and M epitopes by monoclonal typing reagents and synthetic oligosaccharides. *Infect. Immun.* **57**:2829-2836.
- Damerval, C., M. le Guilloux, J. Blaisonneau, and D. de Vienne. 1987. A simplification of Heukeshoven and Dernick's silver staining of proteins. *Electrophoresis* **8**:158-159.
- Dubray, G., and J. Limet. 1987. Evidence of heterogeneity of lipopolysaccharides among *Brucella* biovars in relation of A and M specificities. *Ann. Inst. Pasteur Microbiol.* **138**:27-37.
- Galanos, C., O. Lüderitz, and O. Westphal. 1969. A new method for the extraction of R lipopolysaccharides. *Eur. J. Biochem.* **9**:245-249.
- Hitchcock, P. J., and T. M. Brown. 1983. Morphological heterogeneity among *Salmonella* lipopolysaccharide chemotypes in silver-stained polyacrylamide gels. *J. Bacteriol.* **154**:269-277.
- Hoffmann, E. M., and J. J. Houle. 1986. Purification of nonlipopolysaccharide antigen from *Brucella abortus* during preparation of antigen used for indirect hemolysis test. *J. Clin. Microbiol.* **24**:779-784.
- Laemmlis, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
- Leong, D., R. Djaz, K. Milner, J. Rudbach, and J. B. Wilson. 1970. Some structural and biological properties of *Brucella* endotoxin. *Infect. Immun.* **1**:174-182.
- Limet, J. N., A. C. Berbinschi, A. Cloeckaert, C. L. Cambiaso, and P. L. Masson. 1988. Longitudinal study of brucellosis in mice by immunoassay of lipopolysaccharide-related antigens in blood and urine. *J. Med. Microbiol.* **26**:37-45.
- Limet, J. N., N. Bosseray, B. Garin-Bastuji, G. Dubray, and M. Plommet. 1989. Humoral immunity in mice mediated by monoclonal antibodies against A and M antigens of *Brucella*. *J. Med. Microbiol.* **30**:37-43.
- Lüderitz, O., M. A. Freudenberg, C. Galanos, V. Lehmann, E. T. Rietschel, and D. H. Shaw. 1982. Lipopolysaccharides of gram-negative bacteria. *Curr. Top. Membr. Transp.* **17**:79-151.
- Lugtenberg, B., J. Meyers, R. Peters, P. van der Hoeck, and L. van Alphen. 1975. Electrophoretic resolution of the major outer membrane protein of *Escherichia coli* K12 into four bands. *FEBS Lett.* **58**:254-258.
- Mäkelä, P. H., and B. A. D. Stocker. 1984. Genetics of lipopolysaccharide, p. 59-137. In E. T. Rietschel (ed.), *Handbook of endotoxin*, vol. 1. Chemistry of endotoxin. Elsevier Science Publishing, Inc., Amsterdam.
- Meikle, P. J., M. B. Perry, J. W. Cherwonogrodzky, and D. R. Bundle. 1989. Fine structure of A and M antigens from *Brucella* biovars. *Infect. Immun.* **57**:2820-2828.
- Moreno, E., D. Borowiak, and H. Mayer. 1987. *Brucella* lipopolysaccharides and polysaccharides. *Ann. Inst. Pasteur Microbiol.* **138B**:102-105.
- Moreno, E., M. W. Pitt, L. M. Jones, G. G. Schurig, and D. T. Berman. 1979. Purification and characterization of smooth and rough lipopolysaccharides from *Brucella abortus*. *J. Bacteriol.* **138**:361-369.
- Palmer, D. A., and J. T. Douglas. 1989. Analysis of *Brucella* lipopolysaccharide with specific cross-reacting monoclonal antibodies. *J. Clin. Microbiol.* **27**:2331-2337.
- Phillips, M., G. W. Pugh, and B. L. Deyoe. 1989. Chemical and protective properties of *Brucella* lipopolysaccharide obtained by butanol extraction. *Am. J. Vet. Res.* **50**:311-317.
- Plommet, M. 1987. Brucellosis and immunity: humoral and cellular components in mice. *Ann. Inst. Pasteur Microbiol.* **138**:105-110.
- Terninck, T., and S. Avrameas. 1972. Polyacrylamide-protein immunoabsorbents prepared with glutaraldehyde. *FEBS Lett.* **23**:1574.
- Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350-4354.
- Tsai, C. M., and C. E. Frasch. 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. *Anal. Biochem.* **119**:115-119.
- Verger, J. M., F. Grimont, P. A. D. Grimont, and M. Grayon. 1985. *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int. J. Syst. Bacteriol.* **35**:292-295.
- Verger, J. M., F. Grimont, P. A. D. Grimont, and M. Grayon. 1987. Taxonomy of the genus *Brucella*. *Ann. Inst. Pasteur Microbiol.* **138**:235-238.
- Wilson, G. S., and A. A. Miles. 1932. The serological differentiation of smooth strains of the *Brucella* group. *Br. J. Exp. Pathol.* **13**:1-13.