Fine Structure of A and M Antigens from *Brucella* Biovars†

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Brucella **A and M epitopes were found on single O-polysaccharide chains of all biotype strains of this species. Lipopolysaccharides from the type and reference strains of five of the six *Brucella* species, *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, and *B. neotomae*, were extracted and purified. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, in conjunction with silver staining and immunoblotting developed by monoclonal antibodies, showed bands characteristic of A, M, or mixed A and M antigens. The A antigen previously described as an exclusively α1,2-linked homopolymer of 4,6-dideoxy-4-formamido-D-mannopyranose was shown by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy to possess a fine structure consistent with the low-frequency occurrence of α1,3-linked 4,6-dideoxy-4-formamido-D-mannopyranose residues. This feature was previously attributed only to the M antigen, which is also a homopolymer of the same sugar. *B. melitensis* biotype 3 and *B. suis* biotype 4 lipopolysaccharides showed characteristics of mixed A and M antigens. Immunoabsorption of these O polysaccharides on a column of immobilized A-antigen-specific monoclonal antibody enriched polymer chains with A-antigen characteristics but did not eliminate M epitopes. Composite A- and M-antigen characteristics resulted from O polysaccharides in which the frequency of α1,3 linkages, and hence, M-antigen characteristics, varied. All biotypes assigned as $A^+ M^-$ expressed one or two α1,3-linked residues per polysaccharide O chain. M antigens ($M^+ A^-$) also possessed a unique M epitope as well as a tetrasaccharide determinant common to A-antigen structures. *B. canis* and *B. abortus* 45/20, both rough strains, expressed low-molecular-weight A antigen.**

The structures of the *Brucella* A and M antigens have been elucidated by application of high-resolution ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques (6, 12). This approach was necessitated by the difficulty in isolating and identifying the component monosaccharide of the *Brucella* O-polysaccharide antigens, a 4-amino-4,6-dideoxy-D-mannose, which existed in both A and M antigens as the N-formyl derivative. This structural feature is important not only because it forms a crucial element of the A and M epitopes but also because formamido residues exhibit rotational isomerism, existing in two forms, E and Z (T. Peters, J.-R. Brisson, and D. R. Bundle, submitted for publication). The simplest case, the A antigen, a linear homopolymer of essentially α1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl residues (12), exhibits two sets of resonances in ^{1}H and ^{13}C NMR spectra (7, 10, 12), as each rotamer affects all other resonances of the repeating unit. In the case of the M antigen, a linear pentasaccharide repeating unit composed of four α1,2- and one α1,3-linked sugars, the E and Z rotamer distribution caused spectra to be so complex that structural elucidation by NMR spectroscopy was impossible without first removing the source of the isomeric microheterogeneity by N-deformylation and N-acetylation (6). NMR spectra of the native antigens thus possess an unusually large number of low base-line intensity resonances which could be ascribed to rotational isomerism. These resonances, which are partially obscured by base-line noise, could also result from

other fine-structural features. In studies of A and M antigens, the former alternative was assumed; however, a detailed survey of *Brucella* type strains revealed additional fine structures in the A and M antigens. This report provides results of complete structural studies done in this laboratory on *Brucella* A and M antigens and reports detailed structural investigations of lipopolysaccharides (LPSs) from all *Brucella* biotypes. In addition to physical methods such as NMR spectroscopic analysis, extensive analytical use was made of a panel of A- and M-antigen-specific monoclonal antibodies; these monoclonal antibodies are described in the accompanying paper (5).

Throughout this report reference is made to three prototype structures, the *Brucella* A and M antigens and a related O antigen from *Yersinia enterocolitica* O:9 (11). The latter antigen is related to the *Brucella* A antigen, as determined initially through cross-serological activity (1), which was shown to be based on the near identities of the O-polysaccharide structures (11, 12). The structures of A and M antigens determined for the polysaccharides extracted from *Brucella abortus* 1119-3 (A antigen) and *Brucella melitensis* 16M (M antigen) should be regarded as prototypes of a range of A- and M-antigen structures, the detailed elaboration of which forms the basis of this report.

MATERIALS AND METHODS

Preparation of LPS. Cells of 16 *Brucella* biotypes (wet weight, 1 to 6 g) were heat killed in 10 mM Tris hydrochloride (pH 7.0) containing 2% phenol and 1% (wt/vol) NaCl, made up to 20 ml with the same buffer, and stored at 4°C for 14 days. The cells were centrifuged (10,000 × g, 30 min), suspended in 10 ml of the same buffer (4°C, 16 h), and centrifuged again (10,000 × g, 30 min). The combined

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supernatants were extensively dialyzed and then lyophilized. These fractions were termed the Tris wash.

The cells washed in Tris hydrochloride were suspended in 50 ml of H_2O and extracted with 50 ml of 90% phenol (12). The aqueous and phenol phases were extensively dialyzed and then lyophilized.

Three lyophilized fractions (the Tris wash, aqueous, and phenol fractions) were each redissolved in 5 ml of H_2O and centrifuged (10,000 $\times$ g, 20 min) to remove insoluble material. The supernatants were ultracentrifuged (140,000 $\times$ g, 20 h), and the pellets were dissolved in water (1 ml) and lyophilized.

Large-scale extractions were carried out on *B. melitensis* 3, *Brucella suis* 4, and *Y. enterocolitica* O:9. Cells were digested enzymatically prior to phenol extraction (6, 14), and LPS was precipitated from the phenol phase with 8 volumes of methanol containing 1% (wt/vol) sodium acetate. The methanol-washed precipitates were dissolved in water and lyophilized. This crude LPS was dissolved in 1% (wt/vol) sodium chloride solution (10 to 20 mg/ml), and the solution was centrifuged (10,000 $\times$ g, 30 min) to remove insoluble material; this was followed by ultracentrifugation (4°C, 105,000 $\times$ g, 17 h). The pellets and the viscous layer immediately above the pellets were dissolved in water. The solutions of LPS were dialyzed and then lyophilized.

Pure LPS from *B. abortus* 1119-3 and *B. melitensis* 16M were available from previous studies (6, 12).

Preparation of O polysaccharide from LPS. LPS (5 mg/ml) was hydrolyzed in aqueous 4% (vol/vol) acetic acid at 100°C for 2 h. The solution volume was reduced to 5 ml by rotary evaporation and codistillation with toluene and centrifuged (10,000 $\times$ g, 20 min). The supernatant was applied to a column of Sephadex G-50 (2.6 by 95 cm) and eluted with pyridine-acetic acid-water buffer (5:3:992 [vol/vol]; pH 5.0) at a flow rate of 30 ml · h^{-1}. The fractions containing O polysaccharides, which were detected by monitoring the refractive index, were pooled and lyophilized. If necessary, the O polysaccharide was dissolved in water (10 to 20 mg/ml) and ultracentrifuged (105,000 $\times$ g, 17 h) to remove any unhydrolyzed LPS.

Sodium dodecyl sulfate-gel electrophoresis. LPS samples (1.2 μg) were run in 14% polyacrylamide gels by using a 5% stacking gel (16). Carbohydrate was detected by silver staining followed periodate oxidation (18).

Preparation of cells for O-polysaccharide quantitation. Cells were dialyzed against distilled water and then lyophilized. The dried cells (~0.1 g), which were suspended in 1.0 ml of 4% (vol/vol) acetic acid, were heated at 100°C for 2 h and then centrifuged (10,000 $\times$ g, 15 min). Supernatants (500 μl) were lyophilized and dissolved in phosphate-buffered saline (PBS; 2.5 ml).

Competitive enzyme immunoassay. The following monoclonal antibodies were used: YsT9-1 (an A-antigen-specific antibody), YsT9-2 (a cross-reactive A- and M-antigen-specific antibody) (4), and Bm-10 (an M-antigen-specific antibody) (5, 9). These monoclonal antibodies were used to coat plates for competitive inhibition assays with O-polysaccharide–biotin conjugates (P. J. Meikle and D. R. Bundle, submitted for publication) and streptavidin-horseradish peroxidase conjugates (Sigma Chemical Co., St. Louis Mo.). *Y. enterocolitica* O-polysaccharide–biotin conjugate was used with YsT9-1 and YsT9-2 antibodies, while *B. melitensis* O-polysaccharide–biotin conjugate was used with the Bm-10 antibody.

In a typical enzyme immunoassay (EIA), 96-well microtiter plates were coated with 100 μl of protein A-purified

antibody (5 to 10 μg/ml) in PBS for 3 h at 20°C. The plates were washed three times with PBS, and 50 μl of inhibitor (2 ng/ml to 2 mg/ml) and 50 μl of O-polysaccharide–biotin conjugate (2 to 8 ng/ml) solution were added to each well. Both solutions were prepared in PBS containing 1% (wt/vol) bovine serum albumin. The plates were incubated at 20°C for 3 h for quantitation of the O antigen or for 16 h for relative affinity measurements (Meikle and Bundle, submitted; E. Vorberg and D. R. Bundle, submitted for publication). After the wells were washed three times, wells with labeled antigens were incubated with streptavidin-horseradish peroxidase conjugate (25 ng/ml) in PBS (100 μl per well) at 20°C for 1 h. The plate was washed three times, the substrate 2,2-azido-di-(3-ethyl benzthiazoline sulfonic acid) (0.55 mg/ml) in 0.1 M citrate buffer (pH 4.0) with 0.003% H_2O_2 was added, and the A_{414} was read following 30 to 60 min of incubation at 20°C.

Quantitation of O-polysaccharide by EIA. To quantitate the amount of O polysaccharide present in cell hydrolysates, serial dilutions of the hydrolysates were prepared in PBS containing 0.1% bovine serum albumin. Inhibition by polysaccharide of O-polysaccharide–biotin conjugate binding to the solid-phase antibody was plotted against the inhibitor concentration, and the concentration of antigen required for 50% inhibition was determined. Concurrently, standard curves were prepared by using purified O polysaccharide in order to determine the actual amount of the polysaccharide O chain required for 50% inhibition.

Preparation of a monoclonal antibody affinity column. Sepharose 4B (10 ml) in 40 ml of 1 M Na_2CO_3 at 4°C was activated by the addition of cyanogen bromide solution (1 ml) in acetonitrile (1 g/ml). The gel was stirred vigorously for 2 min and then filtered, washed with 50 ml of 0.1 M $NaHCO_3$ buffer (pH 9.5), and dried on a sintered glass funnel. The activated gel was then added to 20 ml of 0.1 M $NaHCO_3$ buffer (pH 9.5) containing ~15 mg of protein A-purified monoclonal antibody YsT9-1. Coupling was allowed to proceed at 4°C for 18 h and then for a further 2 h at 20°C. The antibody-coupled gel was filtered and washed with 0.5 M sodium chloride in 0.1 M $NaHCO_3$ buffer (50 ml) and then in 50 mM glycine hydrochloride buffer (pH 2.5) containing 50 mM sodium chloride. From the protein content of the combined washings, which was determined by measuring the A_{280}, 12 mg of antibody was estimated to be coupled to the gel.

Affinity chromatography of polysaccharide antigens. The affinity column was equilibrated in PBS (pH 7.0), and polysaccharide samples (3 to 5 mg, 1 mg/ml) in PBS were applied to the column at a flow rate of 20 ml · h^{-1}. The column was washed with an additional 50 ml of PBS, and bound O polysaccharide was eluted with 50 mM glycine hydrochloride buffer (pH 2.5) containing 0.15 M sodium chloride. The unabsorbed and desorbed O polysaccharides were collected, extensively dialyzed, and then lyophilized.

NMR spectroscopy. ^1H and ^{13}C NMR spectra of O polysaccharides dissolved in D_2O (0.5 ml) were recorded with a spectrometer (AM-500; Bruker) at 500 and 125 MHz as described previously (6, 12).

Immunoblotting of sodium dodecyl sulfate-polyacrylamide gels. LPS samples (2 to 10 μg) were developed in 14% polyacrylamide gels (thickness, 0.75 mm; 15 by 15 cm) with a 5% stacking gel and with an applied current of 7.5 mA for stacking and 15 mA for separation (15). The gel was blotted onto nitrocellulose (60 V, 4 h), which was blocked by a 1% (wt/vol) bovine serum albumin solution in PBS and then probed with a solution of *B. melitensis*-specific monoclonal
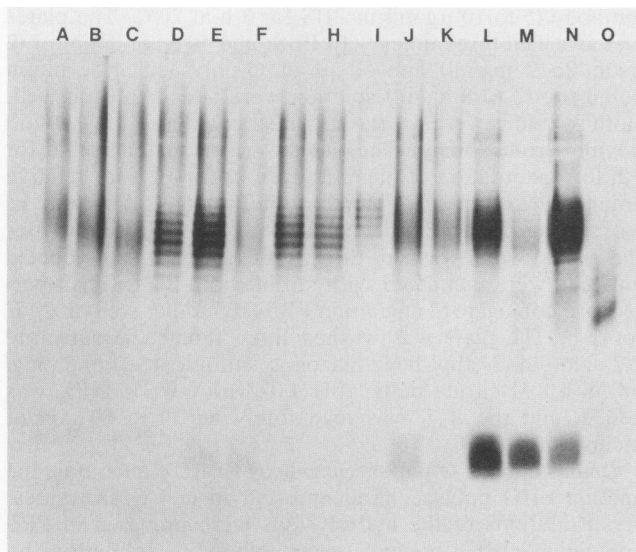
FIG. 1. SDS-PAGE of LPSs from *B. abortus* biotypes 1 to 6 (lanes A to F, respectively), *B. abortus* biotype 9 (lane H), *B. melitensis* biotypes 1 to 3 (lanes I to K, respectively), and *B. suis* biotypes 1 to 4 (lanes L to O, respectively). Discrete banding indicates an M-antigen-type pentasaccharide repeating unit. Unresolved banding suggests a monosaccharide repeating unit.

antibody Bm-10 (25 μg/ml in PBS containing 0.1% [wt/vol] bovine serum albumin for 18 h at 20°C). The sheet was washed in PBS (three times for 15 min each time) and developed by incubation for 2 h in a solution of goat anti-mouse immunoglobulin G-horseradish peroxidase conjugate (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:1,000 in PBS.

RESULTS

Prolonged incubation (14 days) of *Brucella* cells from all biotypes with Tris buffer, followed by phenol extraction (12, 14), gave three components after ultracentrifugation. The material leached from cells by Tris buffer contained substantial amounts of cyclic β1,2-D-glucans (7, 8), which was recovered in the unsedimented supernatant. Impure LPS was obtained from the pelleted material of the Tris wash after ultracentrifugation, while LPS in the purest form was obtained after ultracentrifugation of the phenol-phase extract of cells. In this case, LPS was present both as a pellet and as a viscous semi-gel layer above the pellet. The aqueous phase also yielded a pellet that was subsequently shown by colorimetric analysis and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to be devoid of carbohydrate. In agreement with earlier studies (6), *Brucella* LPS was found both in Tris buffer cell washings and in the phenol phase of a phenol-water cell extract. Often, the amount of LPS leached from the cell exceeded the amount of LPS extracted by phenol. Polysaccharide B, the mixture of cyclic β1,2-D-glucans, is also most effectively recovered by leaching cells in Tris buffer (8).

Analysis of the LPS samples from *B. abortus*, *B. melitensis*, and *B. suis* biotypes (insufficient LPS was isolated from *Brucella canis* or *Brucella neotome*) by SDS-PAGE followed by silver staining gave banding patterns consistent with the serological characterization of biotypes as either A antigen > M antigen or M antigen > A antigen (Fig. 1). Two

exceptions were *B. melitensis* biotype 3 and *B. suis* biotype 4, which were designated as $A^+ M^+$ serotypes. In these biotypes, the smeared staining characteristic of a monosaccharide repeating unit typical of the A-antigen LPS was observed without any evidence of the distinct M-antigen banding attributed to a pentasaccharide repeating unit (6). Thus, both of these biotypes appeared as A-antigen types based on SDS-PAGE banding patterns. With the exception of *B. suis* biotype 4, which exhibited a low molecular weight, the molecular weight distribution of the *Brucella* LPS chains was uniform, although the *B. suis* biotypes possessed a large proportion of R-type LPS core structures uncapped by O polysaccharide (18).

High-resolution ^{13}C NMR spectra of the unmodified *Brucella* A and M antigens have been reported previously (7), and these showed resonances that suggest the presence of α1,3-linked monosaccharide residues in O-polysaccharide preparations that are composed predominantly of α1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl units. These spectral features may be difficult to distinguish from background noise or possible contributions of core saccharides because of the deterioration of the signal-to-noise ratio caused by the E and Z rotational isomerism of the formamido residues and the low relative proportions of α1,3 to α1,2 linkages (Peters et al., submitted). The most suitable frequency range to distinguish and quantitate the two linkage types was that covering the region from 50 to 60 ppm, in which the resonance signal of C-4 was found. Whereas a C-2-substituted unit gave two signals at 57.7 and 52.7 ppm, C-3-substituted monosaccharide residues gave two resonances at 56.3 and 51.7 ppm. Integration and peak intensities of these C-4 resonances provide reliable estimates of the ratio of α1,2 to α1,3 linkages. A second set of characteristic resonance frequencies also well suited to assessment of the presence of the M-antigen character was the formamido carbonyl resonances that occurred at 168.8 and 165.9 ppm for an α1,2-linked monosaccharide, as opposed to a resonance at 165.3 ppm that distinguished the α1,3-substituted residue. The anomeric resonances at 102.4 and 101.6 ppm were also characteristic of the presence of an α1,3 linkage, while the C-1 of α1,2-linked polymers resonated at 101.4 ppm.

The ^{13}C NMR spectra of four *Brucella* O polysaccharides and that of *Y. enterocolitica* O:9 are shown in Fig. 2. The spectrum of the *B. melitensis* 16M polysaccharide (Fig. 2A) exhibited the two types of C-4 resonances, and integration of these signals indicated a 1:4 ratio of α1,3 to α1,2 linkages. This corresponded to the pentasaccharide repeating unit structure determined for the M polysaccharide by NMR studies of N-deformylated and N-acetylated polysaccharide derivatives (6). The *B. melitensis* biotype 3 O-polysaccharide, one of the two biotypes reported to contain both A and M antigens, exhibited A-antigen-type characteristics by SDS-PAGE analysis of the intact LPS and gave a ^{13}C NMR spectrum (Fig. 2B) resembling that of an A-antigen polysaccharide (Fig. 2C); but integration of its C-4 resonances indicated the ratio of α1,3 to α1,2 linkages was 1:12. *B. suis* biotype 4 O polysaccharide (Fig. 2D), the second $A^+ M^+$ biotype, gave an α1,3 to α1,2 ratio of 1:7. The ^{13}C NMR spectra of O polysaccharides from *B. abortus* 1119-3 (Fig. 2C) and *Y. enterocolitica* O:9 (Fig. 2E) were then reexamined for the presence of α1,3 linkages. *B. abortus* 1119-3 polysaccharide gave a ratio of α1,3 to α1,2 of 1:49, whereas no α1,3 linkages could be detected in the *Y. enterocolitica* O:9 polysaccharide (Fig. 2E).

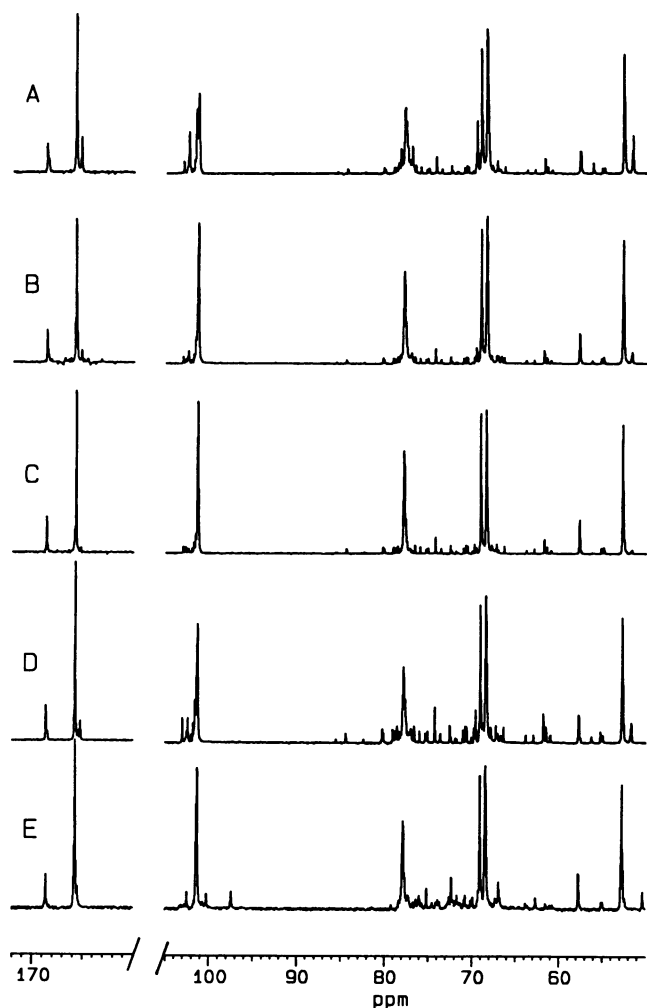Monoclonal antibodies with high specificities for the *Bru-*

FIG. 2. ^{13}C NMR spectra of *Brucella* O polysaccharides from the following representative biotype strains: *B. melitensis* 16M (A), *B. melitensis* biotype 3 (B), *B. abortus* 1119-3 (C), *B. suis* biotype 4 (D), *Y. enterocolitica* O:9 (E).

TABLE 1. Direct EIA to classify A- and M-antigen character by O-polysaccharide inhibition of antibody binding to O-antigen–biotin conjugates

O-polysaccharide inhibitor	Amt (ng/ml) required for 50% inhibition of the following antibody O-chain–biotin conjugates:		
	A-antigen-specific antibody YsT9-1 (YsT9-biotin)	A- and M-antigen-specific antibody YsT9-2 (YsT9-biotin)	M-antigen specific antibody Bm-10 (*B. melitensis*-biotin)
B. abortus 1119-3	3.5	8.4	14,125
B. melitensis 16M	4,841	8.4	41.4
B. suis 4	512	53	1,035

cella A and M antigens were used in combination with polysaccharide enzyme conjugates to accurately quantify O-polysaccharide (E. Vorberg and D. R. Bundle, submitted for publication). In the accompanying paper (5), the binding characteristics of three antibodies that were well suited to this purpose are defined, since detection of either the A or the M antigen in the presence of the other requires a highly specific binding profile. Two antibodies meeting these criteria were identified; and a third, which bound the A or the M antigen with equal affinity, served to quantitate both antigens (5). O-polysaccharide–biotin conjugates were prepared from the *Y. enterocolitica* O:9 (pure α1,2-linked homopolysaccharide) and *B. melitensis* 16M O-polysaccharides (Meikle and Bundle, submitted), and these were bound by solid-phase antibody. Quantitative inhibition of this binding was calibrated with each of the two unlabeled polysaccharides. The quantity of *Brucella* polysaccharide required for 50% inhibition with the three antibodies and two biotin-polysaccharide conjugates (Table 1) shows the high specificities of the monoclonal antibodies for the homologous antigen. Because of its relatively low molecular weight, the O antigen from *B. suis* biotype 4 was treated separately from the other A and M

antigens. Antibody YsT9-1 (A-antigen specific) was effectively inhibited by *B. abortus* 1119-3 polysaccharide but required 10^3 times as much M antigen from *B. melitensis* 16M to reach 50% inhibition (Table 1). The two antigens were equally effective as inhibitors of A- and M-antigen-specific antibody (YsT9-2). The M-antigen-specific antibody (Bm-10) showed a 350-fold difference in the binding of the prototype M and A antigens. These data are in contrast to the behavior of the *B. suis* biotype 4 and *B. melitensis* biotype 3 polysaccharides, which exhibited intermediate binding strengths with the A- or M-antigen-specific antibodies. The A- and M-antigen-specific antibody (YsT9-2), however, bound these antigens more effectively than did either the A- or M-antigen-specific antibodies. The competitive EIA with the three antibody-binding profiles provided not only a quantitation of the A or the M antigen but also a convenient qualitative screen for the A, M, or A^+ M^+ character of *Brucella* polysaccharides.

Extension of this EIA to the quantitation and screening of cell wall polysaccharides confirmed the established serotyping of *Brucella* biotypes (Table 2). It was also demonstrated that O polysaccharide accounted for between 1 and 9% of the cell dry weight and that even rough strains such as *B. canis* expressed small quantities of O polysaccharide. This was consistent with our unpublished observations, based on ^{1}H NMR spectra that other rough strains, e.g., *B. abortus* 45/20 and *B. abortus* S19, also express O polysaccharide. Of 16 biotypes examined, 14 were typed unambiguously as expressing the A or M antigen by EIA, and based on quantitation, each of these showed less than 1% (wt/wt) of the other antigen. *B. melitensis* and *B. suis* biotypes 3 and 4 were identified as expressing mixed A and M $(A^+ M^+)$ antigens.

Direct competitive EIA with the set of three defined monoclonal antibodies was applied to the elucidation of fine-structural features. The M-antigen-specific antibody Bm-10 has been shown to derive its specificity through recognition of α1,3 linkages as well as adjacent α1,2-linked residues (5), and it appeared to be well suited to an evaluation of the occurrence of this structural feature in *Brucella* O polysaccharides. The need for this arose from a comparison of the inhibitory powers of the three antigens, *Brucella* A antigen (*B. abortus* 1119-3), *Brucella* M antigen (*B. melitensis* 16M), and *Y. enterocolitica* O:9 antigen with antibody Bm-10 (Fig. 3). The A antigen was 10 times more active than the *Y. enterocolitica* O:9 antigen. This suggests that the A antigen possesses an important feature that is lacking in the *Yersinia* polysaccharide. Supported by NMR data (Fig. 2), this missing feature was seen to be the presence in *Brucella*

TABLE 2. Quantitation of O polysaccharide by direct competitive EIA and differentiation of A and M antigens

Biotype	Strain	Cell dry weight (mg)	Extracted O-polysaccharide (mg)[a]	% Polysaccharide/ cell dry weight	Estimated A-antigen polysaccharide (mg)[b]	Estimated M-antigen polysaccharide (mg)[c]	Serotype
A1	544	27.0	1.12	4.2	1.38	0.005	A
A2	86/8/59	23.9	1.05	4.4	1.27	0.004	A
A3	TULYA	23.6	1.12	4.7	1.19	0.007	A
A4	292	33.1	1.23	3.8	0.0004	0.98	M
A5	B1396	52.2	2.03	3.9	0.0005	1.60	M
A6	870	51.6	2.98	5.8	2.54	0.008	A
A9	C-68	41.0	1.97	4.8	0.012	1.44	M
M1	16M	24.7	2.29	9.2	0.005	2.08	M
M2	63/7	31.4	2.98	9.5	2.62	0.016	A
M3	Ether	55.9	4.12	7.5	2.39	0.38	A⁺ M⁺
S1	1330	48.2	1.41	2.9	1.44	0.006	A
S2	Thomsen	24.2	0.46	1.9	0.58	0.002	A
S3	BA136	23.7	0.96	4.0	1.13	0.002	A
S4		49.5	0.38[d]	0.8	0.31[d]	0.35[d]	A⁺ M⁺
N		8.3	0.058	0.7	0.057	ND[e]	A
C		14.2	0.00032	0.002	0.00026	ND	A

[a] Estimated with antibody YsT9-2.
[b] Estimated with antibody YsT9-1.
[c] Estimated with antibody Bm-10.
[d] Calculations based on standard curves obtained with the B. suis 4 O chain.
[e] ND, Not determined.

A polysaccharide of one or two α1,3-linked residues that were absent in the *Yersinia* antigen.

This EIA analysis for fine structure was extended to the O polysaccharide of *B. melitensis* biotype 3. The relative inhibitory powers of the A and M polysaccharides obtained from *B. abortus* 1119-3 and *B. melitensis* 16M were measured with the three defined antibodies YsT9-1 (A-antigen specific), YsT9-2 (A- and M-antigen specific), and Bm-10 (M-antigen specific). At the same time, an artificial mixture of 2 parts A polysaccharide and 1 part M polysaccharide was prepared, and its inhibitory power was compared with those

of the A antigen, *Y. enterocolitica* O:9 antigen, M antigen, and *B. melitensis* biotype 3 antigen. Whereas all four antigen preparations gave virtually superimposable binding curves with the YsT9-2 antibody (Fig. 4), the amount of antigen required for 50% inhibition of Bm-10 differed for the M antigen, *B. melitensis* biotype 3 antigen, and the 2:1 A- and
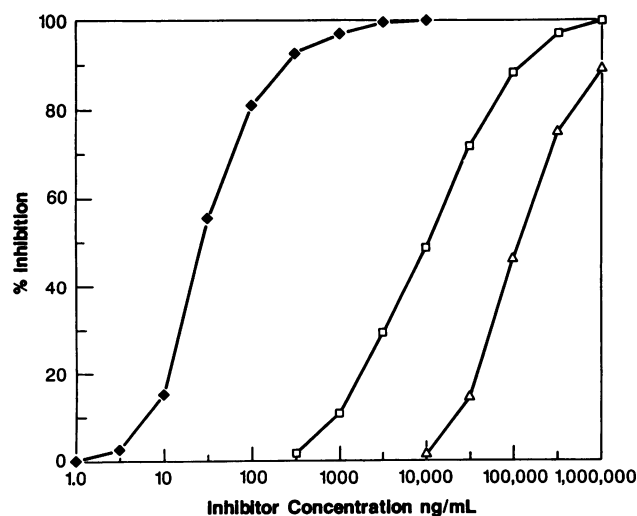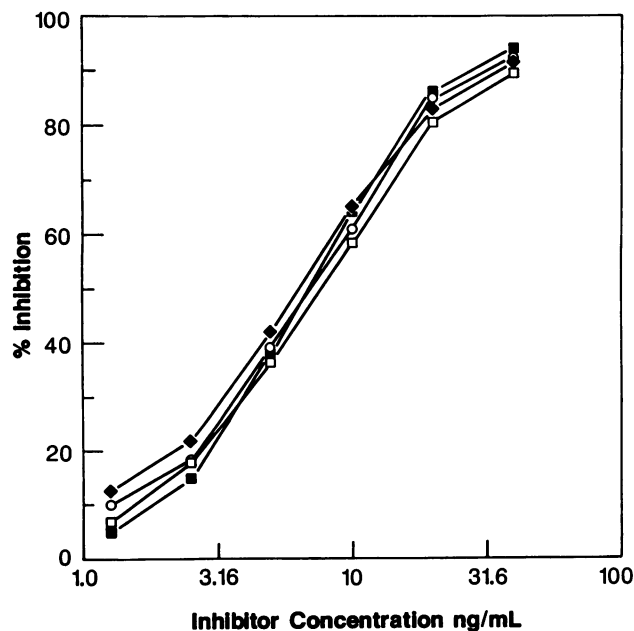

FIG. 3. Inhibition curves for binding of monoclonal antibody Bm-10 by O polysaccharides from *B. melitensis* 16m (◆), *B. abortus* 1119-3 (□), and *Y. enterocolitica* O:9 (△). Conditions were as follows: antibody coating, 5 μg/ml; *B. melitensis* O-polysaccharide–biotin conjugate, 1.5 ng/ml.

FIG 4. Inhibition curves for binding of monoclonal antibody YsT9-2 by O polysaccharides from *B. abortus* 1119-3 (□), *B. melitensis* 16M (◆), *B. melitensis* biotype 3 (■), and a 2:1 mixture of *B. abortus* 1119-3 and *B. melitensis* 16M (○). Conditions were as follows: antibody coating, 5 μg/ml; *Y. enterocolitica* O:9 O-polysaccharide–biotin conjugate, 2 ng/ml.
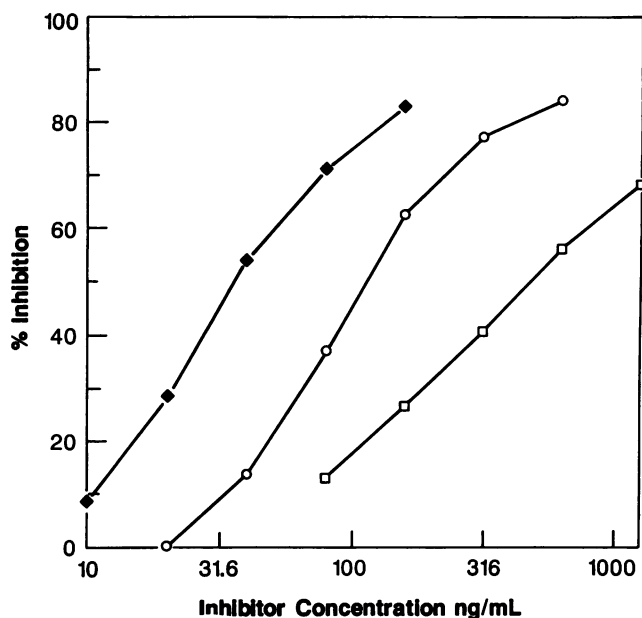
FIG. 5. Inhibition curves for binding of monoclonal antibody Bm-10 by O polysaccharides from *B. melitensis* 16M (◆), *B. melitensis* biotype 3 (□), and a 2:1 mixture of *B. abortus* 1119-3 and *B. melitensis* 16M (○). Conditions were as follows: antibody coating, 5 μg/ml; *B. melitensis* 16M O-polysaccharide–biotin conjugate, 1.5 ng/ml.
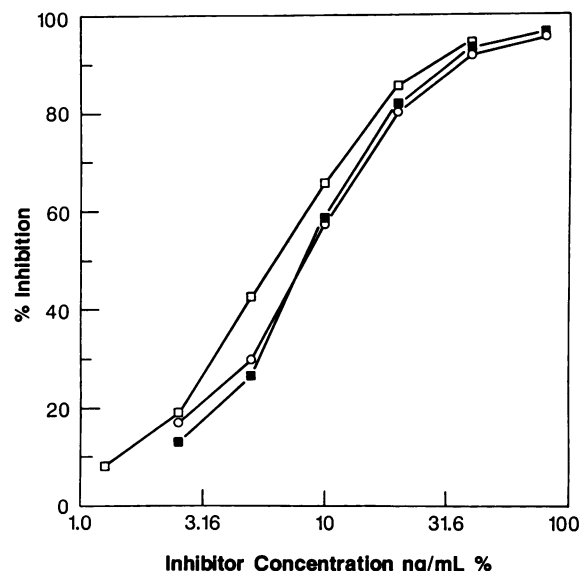

FIG. 6. Inhibition curves for binding of monoclonal antibody YsT9-1 by O polysaccharides from *B. abortus* 1119-3 (□), *B. melitensis* biotype 3 (■), and a 2:1 mixture of *B. abortus* 1119-3 and *B. melitensis* 16M (○). Conditions were as follows: antibody coating, 10 μg/ml; *Y. enterocolitica* O:9 O-polysaccharide–biotin conjugate, 4 ng/ml.

M-antigen mixtures (Fig. 5). By comparison, when the YsT9-1 antibody was used, the latter two antigens exhibited similar inhibitory powers (75% of that exhibited by pure A antigen) (Fig. 6). The EIA data suggest that the *B. melitensis* biotype 3 antigen, although of similar A-antigen content to the mixture (2:1) of A and M antigens, contains no more than 10% of M-antigen-like structure if these are present as unique M-antigen polysaccharides. However, from NMR measurements the content of α1,3 linkages in the biotype 3 polysaccharide was determined to be 8%, which was identical to that in the artificial mixture of A and M antigens (Table 3). The observed binding properties of the biotype 3 polymer were not consistent with those of the mixed A- and M-antigen chains but could result if M-antigen-like epitopes and A-antigen-like epitopes were expressed within one polysaccharide chain.

To test this possibility, an affinity column prepared by covalent attachment of YsT9-1 antibody to Sepharose 4B was used in attempts to fractionate either a 2:1 (wt/wt) mixture of pure A- and M-polysaccharide antigens or the *B. melitensis* biotype 3 polysaccharide antigen. Since the column was easily saturated, unretained material was not examined. Instead, the material that was retained and specifically desorbed from the column was analyzed by ¹H NMR spectroscopy (Fig. 7). The anomeric proton region of spectra of the absorbed and desorbed polysaccharides were examined, and for comparison, spectra of the A and M antigens are presented (Fig. 7A and B). The spectra for the two samples prior to chromatography are also shown (Fig. 7C and D). After affinity enrichment the desorbed fraction from the mixture of A and M antigens (Fig. 7E) showed a ¹H NMR spectrum resembling that of pure A antigen. The desorbed *B. melitensis* biotype 3 polysaccharide sample (Fig. 7F) showed very little difference in the content of α1,3 linkages compared with that present in material applied to

the column (Fig. 7C), indicating that pure A-antigen polysaccharide was either absent or present in only low quantities. This suggests that in such A^+ M^+ polysaccharides isolated chains with an exclusively A-antigen character are absent.

The possible presence of distinct M and A polysaccharide chains in the *B. melitensis* biotype 3 polysaccharide was investigated further at the LPS level by SDS-PAGE and immunoblotting. When the blot was probed with Bm-10 antibody, LPS from *B. abortus* 1119-3 failed to stain, while LPS from *B. melitensis* 16M as well as the 1:2 mixed M- and A-antigen LPS sample gave clear M-antigen-type banding, as was seen earlier by silver staining (Fig. 1). The LPS from *B. melitensis* biotype 3 stained when it was probed with Bm-10, but the staining pattern resembled that seen for A-antigen-type LPS with the silver reagent (smeared banding). Similar results were seen with *B. suis* biotype 4 LPS,

TABLE 3. Estimation of α1,3 linkages in O polysaccharides by ¹³C NMR spectroscopy and direct EIA analysis

Bacterial strain	Biotype	¹³C NMR estimation (%)[a]		Antigenic character by inhibition of EIA
		α1,2	α1,3	
B. melitensis 16M	M	79	21	M
B. abortus 1119-3	A	98	2	A
B. melitensis 3	$A^+ M^+$	92	8	$A > M^b$
B. suis 4	$A^+ M^+$	87	13	A M[c]
Mixture of (1:2) *B. melitensis* 16M and *B. abortus* 1119-3		92	8	

[a] Quantitation based on C-4 resonance signal intensities at δ_c 52.7 and 51.7 ppm.
[b] Based on inhibitory activities in Fig. 5 and 6.
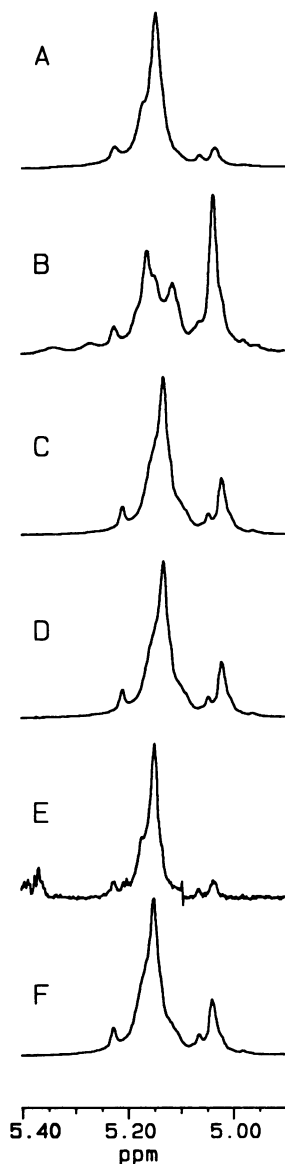[c] Low molecular weight prevented quantitation.

FIG. 7. ¹H NMR spectra of polysaccharide eluted from a YsT9-1 antibody affinity matrix. The anomeric regions at 5.4 to 5.0 ppm of the following O polysaccharides are shown: Brucella A polysaccharide (A), Brucella M polysaccharide (B), B. melitensis biotype 3 O polysaccharide prior to affinity separation (C), mixed (2:1 [wt/wt]) A and M polysaccharides (D), desorbed O polysaccharide from an artificial mixture of A and M polysaccharides (E) (cf. panel D), desorbed O polysaccharide from affinity absorption of B. melitensis biotype 3 antigen (F).

although in this instance faint banding was superimposed on the smeared A-antigen-type pattern. These data do not support the presence of discrete A and M antigens in the A^+ M^+ biotypes but, rather, suggest a range of O-polysaccharide structures with variable A- and M-epitope densities on a single polysaccharide chain.

DISCUSSION

New details of the fine structure of the Brucella A antigen were revealed by a detailed examination of the high-resolution ¹³C NMR spectra of the O polysaccharides extracted

from Brucella biovars and the corresponding published data for the Brucella A and M antigens (6, 7, 10, 12).

The polysaccharide antigens of Brucella are intrinsically difficult structures to analyze by either classical or modern methods, since acid hydrolysis leads to destruction of the single-component sugar 4,6-dideoxy-4-formamido-D-mannose; and because the A and M antigens are homopolymers, the poor dispersion of ¹H and ¹³C NMR resonances makes assignment of individual signals a particularly demanding task. This challenge is enhanced by the microheterogeneity that results from the rotational isomerism of the formamido moiety, which is an immunodominant feature of each monosaccharide residue (4) (Peters et al., submitted). The successful application of NMR spectroscopy to polysaccharide structure elucidation (3) depends on the stereoregularity of bacterial polymers. This feature simplifies spectral details, provided that the degree of polymerization is sufficiently high and that the effective concentration of internal repeating units exceeds those at either end of the polymer. When heterogeneity abrogates this condition or biosynthetic pathways no longer yield a stereoregular polymer, the degeneracy of resonances breaks down, and in the extreme cases, each monosaccharide residue may be unique. In the case of Brucella O polysaccharide, heterogeneity caused by both rotational isomerism (Peters et al., submitted) and repeating unit irregularity arises, possibly as the result of altered biosynthetic assembly (6, 15).

The A antigen, a simple homopolymer with a monomeric repeating unit of α1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl residues, exhibited microheterogeneity resulting from the E and Z isomers of the formate group; but the complexities of the resonances were confined to twinning of resonances. The M antigen, a linear pentasaccharide repeating unit of four α1,2- and one α1,3-linked monosaccharide residues, was immediately too complex to permit structural studies without first removing the source of heterogeneity (6). This was achieved by N-deformylation. In this way, the structure of the M antigen was successfully elucidated, and the two extremes of structure were regarded as the prototype A and M antigens. Based on results of these structural studies, it was appreciated that the C-4 resonance provided a window to assess quantitatively the number of α1,3-linked residues in a given polysaccharide preparation. This allowed the structures of key Brucella biovars to be studied without prior modification. Thus, reexamination of B. abortus 1119-3 O polysaccharide showed the presence of a small number (2%) of α1,3-linked 4,6-dideoxy-4-formamido-D-mannopyramosyl residues. The ratio of α1,3 to α1,2 linkages in the M antigen remained constant at 1:4. An intermediate situation arose for the B. melitensis biotype 3 and B. suis biotype 4 antigens, which are said to be A^+ M^+ based on established serology (20). The B. suis antigen, with a ratio of 1:7 for α1,3 to α1,2 linkages (Table 3), was not a typical M antigen and gave SDS-PAGE banding patterns more characteristic of an A antigen. Both A^+ M^+ antigens from B. melitensis biotype 3 and B. suis biotype 4 were investigated more thoroughly since the NMR data (Table 3) alone did not permit an unambiguous structural solution to the possibility that the antigens were either mixtures of appropriate ratios of pure A and M molecules or that the molecules were A-like antigens with a higher percentage of α1,3 linkages than was seen for the prototype A antigens. An intermediate solution, and the one that we favor, describes these antigens as a continuum of A-antigen-type structures with variable α1,3 linkage frequencies. The data leading to this conclusion came from serological studies with defined

monoclonal antibodies (Fig. 5 and 6) and SDS-PAGE data, in conjunction with immunoblotting, and finally, affinity enrichment of the A^+ M^+ antigen.

Competitive EIA firmly established the distinction between *B. abortus* A antigen and the A-antigen-like *Y. enterocolitica* O:9 antigen (Fig. 3 and 4 and Table 3). This distinction was confirmed by ^{13}C NMR spectroscopy (Fig. 2) and required that *Y. enterocolitica* be regarded as an exclusively α1,2-linked homopolymer devoid of α1,3 linkages. *Brucella* A antigen was bound 10 times more effectively by the M-antigen-specific antibody Bm-10 (Fig. 3), and from NMR data this could be attributed to the presence of α1,3 linkages. The ability of antibody YsT9-2 to bind all A, M, and *Y. enterocolitica* O:9 polysaccharide antigen structures with equal affinities (Fig. 4) results from its specificity and the size of its combining site (4). This site was fully satisfied by a tetrasaccharide determinant; and this structural element may be found in all A^+ M^-, A^- M^+, and A^+ M^+ structures. The A-antigen-specific antibody YsT9-1 was demonstrated to require at least an α1,2-linked pentasaccharide for the most effective binding, and this feature was only found in *Brucella* A antigen or *Y. enterocolitica* polysaccharides.

The absence of M-antigen-type banding in the immunoblots of *B. melitensis* biotype 3 and *B. suis* biotype 4 LPS antigens suggested that these were not mixtures of A and M antigens but, rather, a composite polysaccharide expressing both A and M epitopes on a single molecule. That the A-antigen character could not be clearly separated for M antigen but could only be enriched was established by immunoabsorption on an affinity column prepared from the A-antigen-specific monoclonal antibody YsT9-1.

There were two clearly identified *Brucella* polysaccharide antigens A and M, as typified by the strains *B. abortus* 1119-3 and *B. melitensis* 16M, respectively. Rough strains such as *B. abortus* 45/20 and S19 also carried the O polysaccharide, but in low amounts, and they had low molecular weights. This observation is consistent with evidence that immunization with R-type strains can induce circulating anti-A-antigen polysaccharide antibodies (2, 13). Of 16 *Brucella* strains examined, only 2, *B. melitensis* biotype 3 and *B. suis* biotype 4, exhibited A^+ M^+ characteristics. The weight of experimental evidence points toward a heterogeneous distribution of α1,3 linkages in these polysaccharide chains. The mean frequency of this distribution lies between the extremes represented by prototype A or M antigens. In this connection, it is tempting to suggest that *B. melitensis* is indeed the *Brucella* species type strain with a species O antigen (M antigen) and that the A antigen of *B. melitensis* biovar abortus (19) has arisen by alteration of the biosynthetic assembly of the O polysaccharide (6). In such a scheme, *B. melitensis* biovar 3 and *B. melitensis* biovar suis 4 represent strains in which the control of assembly as pentameric units has been lost but the frequency of α1,3 linkages has not fallen to that of typical A antigens. In earlier reports based on the structural features of A and M antigens, we argued in favor of two distinct modes of assembly for the M and A antigens (6, 15, 17). The M antigen conforms with the well-established biosynthetic scheme for O polysaccharides, which results in stereoregular antigens (17). The existence of random heterogeneity in the polysaccharides of A^+ M^- and A^+ M^+ *Brucella* strains is not readily reconciled with this biosynthetic scheme.

In concluding our structural studies on *Brucella* polysaccharide antigens, it is appropriate to comment on the validity of the paradigm proposed by Wilson and Miles (21) over 50 years ago. The fine structure of the A antigen confirms that M-antigen-type structural features, α1,3 linkages, are found within the A antigen. The presence of tetrasaccharide segments containing exclusively α1,2-linked 4,6-dideoxy-4-formamido-D-mannopyranosyl residues in the M antigen provides the structural basis for a common A-antigen-type determinant (6). In the subsequent report (5), we define in more precise immunochemical terms the extent and nature of the A and M epitopes, especially as defined by monoclonal antibody typing reagents.

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