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Analysis of *Brucella* Lipopolysaccharide with Specific and Cross-Reacting Monoclonal Antibodies

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Monoclonal antibodies which bind *Brucella* A lipopolysaccharide (LPS)-specific, M LPS-specific, or cross-reactive epitopes were used as reagents in quantitative dot blot, Western blot (immunoblot), and immunoprecipitation analysis of *Brucella* whole cells, whole-cell extracts, and purified LPS preparations. This set of monoclonal antibodies detected four unique epitopes on *Brucella* LPS. The specificity of monoclonal antibodies reactive with *Brucella* unique (A and M) and common (C and C/Y) LPS epitopes was demonstrated by blot analysis. The serotype specificity of monoclonal antibodies for A LPS of *B. abortus* 1119.3 or M LPS of *Brucella melitensis* 16M was confirmed. Type C monoclonal antibodies recognized epitopes on *Brucella* A and M LPS and did not cross-react with *Yersinia enterocolitica* O:9. In Western blots, type C monoclonal antibodies were bound by epitopes on *Brucella* A and M LPSs ranging in M_rs from 30,000 to 70,000, relative to marker proteins. Type C/Y monoclonal antibodies were cross-reactive with *Y. enterocolitica* O:9 and recognized *Brucella* A LPS epitopes with a restricted M_r ranging only from 40,000 to 50,000, relative to marker proteins. Type C/Y monoclonal antibodies also displayed a more restricted pattern of binding to *Brucella* M LPS. The monoclonal antibodies were able to detect 5 to 50 pg of a purified A LPS preparation in dot blots. The limits of detection by the monoclonal antibodies of a purified M LPS preparation ranged from 0.05 to 50 pg. Monoclonal antibody analysis of whole-cell preparations also demonstrated quantitative differences in the presence of the respective epitopes. The binding profiles of the monoclonal antibodies to *Brucella* whole cells varied between acetone- and chloroform-killed organisms as well as between species and strains. The lower limit of detection of any whole-cell preparation by the dot blot technique was 10^5 CFU. Binding profiles in Western blots and endotoxin activity of immunoprecipitates obtained with these monoclonal antibodies further defined the *Brucella* LPS antigens. These monoclonal antibodies and the techniques described may be useful in monitoring the antigenic content of *Brucella* vaccines and diagnostics.

Diagnosis of brucellosis depends mainly on the serological response of the infected host. The serological responses resulting from exposure to smooth *Brucella* spp. are predominantly against the outer membrane lipopolysaccharide (LPS) and polysaccharide (PS) complexes. Protective, passive, and active immunity have been reported in mice with anti-LPS antibody and with vaccination with LPS complexes (2, 3, 23, 25, 31). The epitopes associated with this protection have not been adequately defined. The induction of protective immunity through vaccination produces serological reactions to the immunodominant LPS-PS complexes of smooth *Brucella* spp. which complicate distinguishing between vaccinated and infected hosts.

Brucella LPS-PS complexes consist of the *Brucella* O chains, native hapten, and polysaccharide B (6, 8, 10). The existence of this complex has confounded efforts to dissect the epitopes important in *Brucella* serology (17, 29). This complex possesses LPS type-specific O-antigen epitopes (A or M) and recently described epitopes which are common to both LPS types (C and C/Y) (13). The contributions of native hapten, polysaccharide B, and LPS to these common epitopes have not been established. Better characterization of these LPS-PS complexes is necessary for understanding their role in inducing serological reactions and immunity.

Preparations of supposedly purified *Brucella* LPS, native hapten, and polysaccharide B antigens possess determinants that can partially cross-absorb LPS-specific polyclonal antibodies (18, 26, 30, 34). This suggests that *Brucella* PS antigens copurify with LPS. These PS antigens must, therefore, be considered when describing the immunochemistry of such preparations. Monitoring the content of *Brucella* carbohydrate antigens for different types of PS ratios may be important in the preparation of *Brucella* vaccines and diagnostic reagents (17, 27, 35).

In this report, immunoblotting techniques, in conjunction with monoclonal antibodies specific for smooth *Brucella* LPS epitopes, are shown to provide a means with which to further our understanding of these smooth *Brucella* antigens. These well-characterized monoclonal antibodies are also used to determine the ratios of specific and shared LPS epitopes on smooth *Brucella* species.

MATERIALS AND METHODS

***Brucella* antigens.** Whole-cell bacterial antigens were acetone- or chloroform-killed preparations provided by W. Deyoe (U.S. Department of Agriculture, Ames, Iowa). The *Brucella* strains tested were smooth virulent biotype 1 *Brucella abortus* 2308, avirulent biotype 1 smooth vaccine *B. abortus* 19, A-LPS-deficient *B. abortus* biotype 4, and smooth *Brucella melitensis* 16M.

LPS antigens. Extracts of chloroform-killed *Brucella* spp. were prepared as described by Dubray and Limet (15). Briefly, concentrated cell solutions were pelleted by centrifugation and lysed with 1% sodium dodecyl sulfate (SDS) in 0.0625 M Tris (pH 6.8) for 10 min at 100°C. Proteinase K (12.5 U) (Sigma Chemical Co., St. Louis, Mo.) was added, and the suspension was incubated for 2 h at 50°C. The suspensions were then incubated overnight at 20°C. Particulate cell debris was removed by centrifugation, and the

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supernatant was saved. The concentrations of LPS in the extracts were determined by the quantitative dot blot.

Purified A LPS from *B. abortus* 1119.3 was an extract prepared by K. Nielsen (Animal Disease Research Institute, Ontario, Canada). Phenol-extracted, purified M LPS from *B. melitensis* M15 was provided by E. C. Klaviter (Michigan State Department of Health). Purified A and M LPS were treated with proteinase K (Sigma). Proteinase K and LPS at a 1:1 (wt/wt) ratio were incubated as described for whole-cell extraction. Samples were then centrifuged 200,000 × *g* for 4 h. The resulting pellets were briefly rinsed twice and suspended in 0.9% saline at a concentration equivalent to 10 mg of the starting LPS per ml. Less than 1% protein was detectable by the Bradford technique (5) in the treated LPS preparations.

ELISA. Antibody-containing preparations were tested for activity by enzyme-linked immunosorbent assay (ELISA) by a modification of the micro procedure described by Douglas et al. (12). Briefly, whole-cell suspensions were used as antigens in 96-well Immulon 2 U plates (Dynatech Laboratories, Inc., Alexandria, Va.). LPS preparations adjusted to 5 μg/ml and dispensed 0.05 ml per well were also used as antigens. Nonspecific protein binding was blocked with a solution of 10% normal goat serum and 10% powdered milk. The antibody preparations tested were incubated for 60 min at 37°C. Peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Cooper Biomedical, Inc., Malvern, Pa.) was added to detect antigen-bound antibody and visualized by the addition of *o*-phenylenediamine substrate. The A_{492}s of the microplate wells were read in a Titertek Multiscan ELISA plate reader.

Monoclonal antibodies. Monoclonal antibodies were produced as described previously (21). Briefly, BALB/c mice were immunized with whole *B. abortus* 2308 or *B. melitensis* 16M organisms. Spleen cells from immunized mice were fused with X63-Ag8.653 mouse myeloma cells in a modified method of Galfre et al. (19). The resulting hybridomas were screened for specific monoclonal antibody activity against whole-cell *Brucella* antigens by ELISA. The production of antibody-containing ascites fluids was accomplished by intraperitoneal injection of trypan blue-primed mice with hybridomas that had been cloned by limiting dilution (16).

PAGE and Western blots. Samples containing the equivalent of 5 to 10 μg of purified LPS per lane were run in 10 or 12% SDS-polyacrylamide gel electrophoresis (PAGE) at 30 mA constant current for 75 min. Proteinase K-treated *Escherichia coli* O55:B5 LPS W and *Salmonella typhimurium* LPS W (Difco Laboratories, Detroit, Mich.) were used as carbohydrate standards. Low-molecular-weight protein standards (Bio-Rad Laboratories, Richmond, Calif.) were used to determine M_r. Migration patterns were visualized by silver staining (20, 32).

Samples separated by PAGE were electrophoretically transferred (Western blotted) to nitrocellulose paper (Bio-Rad Laboratories) in a buffer (pH 8.3) containing 0.025 M Tris, 0.192 M glycine, and 20% methanol (vol/vol) at 150 mA for 2 h. After transfer, the nitrocellulose was cut into strips and incubated in ELISA blocking buffer without the goat serum. Monoclonal antibodies in excess concentrations were used to detect specific epitopes present in the blots. The strips were incubated for 1 h at room temperature with equivalent excess monoclonal antibodies, as determined in ELISA, diluted in blocking buffer. Antigen-bound antibody was visualized by incubation with rabbit anti-mouse IgG (Cappel Laboratories, Malvern, Pa.) followed by incubation with ^{125}I-labeled protein A (Dupont, NEN Research Products, Boston, Mass.) or protein A-gold (Bio-Rad Laboratories). Three 5-min washes were performed after each incubation. Blots incubated with radiolabel were autoradiographed. Exposure times were maximized to ensure detection of all bound antibody. Protein A-gold-stained strips were washed for 10 min in phosphate buffer plus 1% Triton X-100 and twice in buffer alone and were silver stain enhanced (4).

Immunoprecipitation. Purified *Brucella* LPS preparations were immunoprecipitated by overnight incubation at 4°C with excess monoclonal antibodies. Bound and unbound mouse IgG was then precipitated by incubation at 4°C overnight with rabbit anti-mouse IgG at an optimum relative concentration for precipitation of the mouse IgG. The precipitates were pelleted by centrifugation. The supernatants from the precipitates were removed and saved. The pelleted precipitates were centrifuged and washed three times with ice-cold saline. The precipitates and supernatants were then proteinase K treated to digest the immunoglobulins away from the precipitated LPS. The concentration of LPS in the supernatants and precipitates was determined by the quantitative dot blot assay described below.

Immunoaffinity chromatography. Monoclonal antibody C-1 was coupled to an Affi-Gel 10 (Bio-Rad Laboratories) slurry in 0.1 M MOPS (morpholinepropanesulfonic acid) buffer, pH 7.5, at 4°C for 4 h. Unbound antibody was washed from the gel matrix with three bed volumes of 0.01 M $NaPO_4$ plus 0.9% NaCl (wt/vol) and followed by three bed volumes of the same buffer containing 4 M urea. The column was then equilibrated in buffer without urea (24).

Purified proteinase K-treated *Brucella* strain 1119.3 A LPS was allowed to absorb to the column bed at 4°C overnight. Unbound LPS was washed through the column and collected. Antibody-bound LPS was eluted from the column with buffer containing 4 M urea and was collected. Fractions were screened by PAGE for LPS content. Fractions containing LPS were pooled, proteinase K treated to remove residual antibody protein, washed by pelleting at 200,000 × *g*, and suspended in 0.9% NaCl. The immunoreactivity of the pools was determined in blot assays.

Quantitative dot blots. The concentration of LPS antigens detectable, relative to the purified LPS preparations, by each monoclonal antibody was determined by serial dilution of antigen preparations. The dilutions were spotted in 10-μl volumes on nitrocellulose strips and allowed to dry. The strips were blocked at room temperature for 30 min as by the Western blot procedure. Monoclonal antibodies were diluted in blocking buffer and incubated with the strips overnight at 4°C. The antigen-bound antibody was then visualized as described for protein A-gold staining above. The last dilution of antigen showing visible staining after silver-stain enhancement was recorded as the minimum detectable concentration of antigen. The concentration of LPS in whole-cell extracts was determined by comparison of the dilution of extracts showing endpoint detection to the known endpoint concentration of purified LPS preparations.

The ability of the monoclonal antibodies to detect acetone- and chloroform-killed bacteria was determined by spotting 10 μl of bacterial suspensions in 0.9% saline on nitrocellulose strips. The bacteria were then reacted with the monoclonal antibodies and visualized as described for the LPS preparations.

***Limulus* lysate gelation assay.** The endotoxin content of the monoclonal antibody-precipitated LPS preparations was determined by the *Limulus* lysate gelation assay, Pyrotell (Associates of Cape Cod, Woods Hole, Mass.). The assay

TABLE 1. ELISA reactivities of monoclonal antibodies

Antibody	Presence (+) or absence (−) of reactivity in:					
	Purified LPS[a]		Whole-cell preparations[b]			
	A	M	*B. abortus* 2308	*B. melitensis* 16M	*B. suis* IV	*Y. enterocolitica* O:9
A	+ (5)	−	+	−	+	−
M	−	+ (4)	−	+	+	−
C-1	+ (5)	+ (5)	+	+	+	−
C-2	+ (3)	+ (4)	+	+	+	−
C/Y-1	+ (3)	+ (4)	+	+	+	+
C/Y-2	+ (4)	+ (3)	+	+	+	+

[a] Purified A LPS was obtained from *B. abortus* 1119.3 treated with proteinase K. Purified M LPS was obtained from *B. melitensis* 16M treated with proteinase K. Data represent the results of duplicate ELISAs from at least three assays. Endpoint dilution values of less than 0.10 (A_{492}) were recorded as negative. The numbers in parentheses represent the nearest positive $\log_{10}$ dilution of ascites fluids.

[b] Acetone-killed whole-cell preparations were used. Data represent the results of duplicate ELISAs from at least three assays on ascites fluids diluted 10^{-3}. Values of less than 0.05 (A_{492}) were recorded as negative. Negative values were recorded for *B. abortus* 45/20, *B. canis*, *B. ovis*, *Y. enterocolitica* O:8, *E. coli* (ATCC 25922), and *S. typhimurium* (ATCC 14028) for all antibodies tested.

was performed according to the instructions of the manufacturers.

RESULTS

ELISA. We have previously described monoclonal antibodies specific for *Brucella* A LPS or M LPS, cross-reactive common monoclonal antibodies (C-1 and C-2), and common monoclonal antibodies, which also cross-react with *Yersinia enterocolitica* O:9 (C/Y-1 and C/Y-2) (13). None of these monoclonal antibodies recognize rough *Brucella* strains *B. abortus* 45/20, *Brucella ovis* or *Brucella canis*, *E. coli* (ATCC 25922), *S. typhimurium* (ATCC 14028) or *Y. enterocolitica* O:8. The reactivities of these monoclonal antibodies are summarized in Table 1.

PAGE and Western blots. Unlike proteins, the true molecular weights of carbohydrates cannot be determined by their M_rs in PAGE; however, the distribution of oligosaccharides in a sample can be visualized. Compared with those of the *E. coli* and *S. typhimurium* standards, whose large O-chain subunits produce distinct ladder-banding patterns, *Brucella* A LPS and M LPS O-chain subunits consisting of one and five monosaccharides, respectively, produce broad smear PAGE distribution patterns (6, 15, 28).

In Western blots (Fig. 1), type C monoclonal antibodies were bound by epitopes on purified A and M LPSs ranging from 30,000 to 70,000 relative to marker proteins. The type C/Y monoclonal antibodies recognized A LPS epitopes ranging only from 40,000 to 50,000, relative to marker proteins. The type M and C/Y monoclonal antibodies were also more restricted in binding to M LPS than were the type C monoclonal antibodies.

Western blots of crude, whole-cell extracts also displayed heterogeneous binding patterns (Fig. 2). The C/Y-1 monoclonal antibody recognized a distinct low-molecular-weight band at 20,000, relative to marker proteins in extracts of *B. abortus* 19 not detected by the other monoclonal antibodies. This band was not detected in extracts of *B. abortus* 2308. The C-2 and C/Y-2 monoclonal antibodies lacked reactivity with the faster-migrating epitopes in extracts of *B. abortus* 19 and 2308 which were recognized by monoclonal antibodies A and C-1.

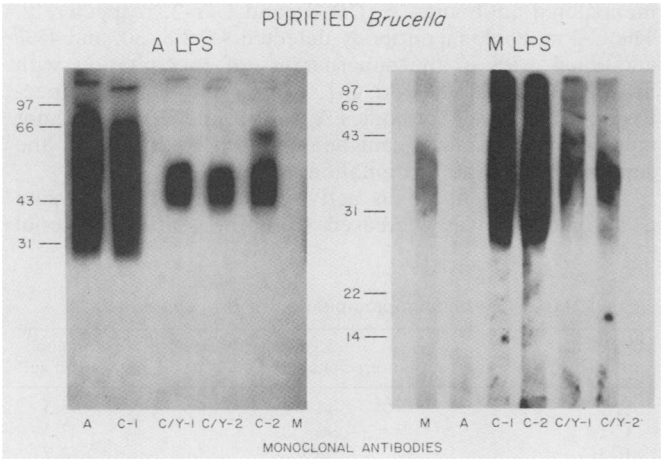

FIG. 1. Purified *Brucella* A and M LPSs were run on 12 and 10% SDS-PAGE gels, respectively, and Western blotted. Strips of the Western blotted LPSs were incubated with the monoclonal antibodies A, M, C-1, C-2, C/Y-1, and C/Y-2. Bound monoclonal antibodies were sandwiched with rabbit anti-mouse IgG and detected with ^{125}I-labeled protein A when exposed to Kodak X-AR5 film. The numbers refer to molecular weights (10^3) of protein standards included but not shown.

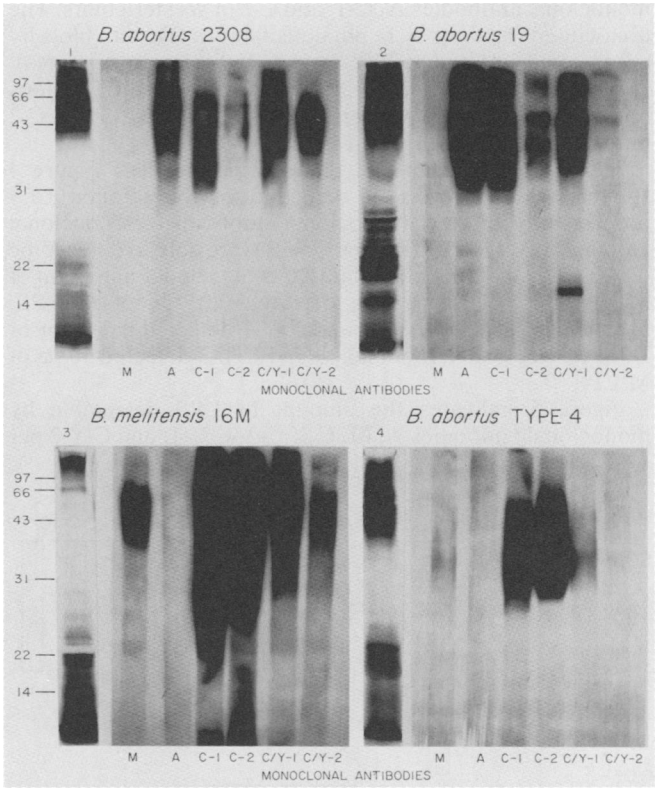

FIG. 2. Chloroform-killed *Brucella* whole cells were proteinase K extracted, and the extracts were run on 10% SDS-PAGE gels and Western blotted. The blotted extracts were reacted with the monoclonal antibodies and detected as described in the text and in the legend to Fig. 1. Lanes 1, 2, 3, and 4 are silver-stained SDS-PAGE extracts (32). The numbers represent molecular weight markers (in thousands), which were run but are not shown.

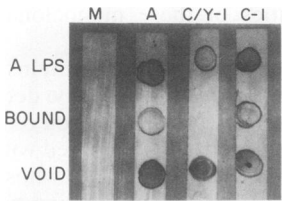

FIG. 3. Purified *Brucella* LPS was immunoaffinity purified with monoclonal antibody C-1. The A LPS applied to the affinity column, antibody-bound A LPS, and void material were diluted 1:500 and assayed in dot blots with monoclonal antibodies A, M, C-1, and C/Y-1, as described in the text (also see Fig. 4 legend).

Extract from *B. melitensis* 16M showed a distribution of binding of monoclonal antibodies C-1 and C-2 from below 14,000 to above 97,000, relative to marker proteins (Fig. 2). The monoclonal antibody M bound *B. melitensis* 16M extract migrating from 40,000 to 60,000. Binding to the *B. melitensis* extract by monoclonal antibodies C/Y-1 and C/Y-2 occurred above 25,000.

Extract from *B. abortus* biotype 4 weakly bound monoclonal antibodies M, C/Y-1, and C/Y-2. Monoclonal antibodies C-1 and C-2 were bound by epitopes of *B. abortus* biotype 4 migrating from 30,000 to 50,000. No reactivity with monoclonal antibody A was observed.

Affinity chromatography. Fractions which eluted with urea from the C-1 monoclonal antibody column and contained detectable carbohydrate in PAGE gels were reactive with monoclonal antibodies A, C-1, and C-2 in Western blots. The urea-eluted fractions were not detectable in Western blots by monoclonal antibodies C/Y-1 or C/Y-2 (data not shown). Likewise, when diluted 1:500, the urea-eluted fractions reacted in dot blots with monoclonal antibodies C-1 and C-2 but did not react with C/Y-1 or C/Y-2 (Fig. 3).

Immunoprecipitation and quantitative dot blot. Figure 4 summarizes the levels of detection of the purified LPS preparations by the monoclonal antibodies. Monoclonal antibodies A, C-1, C/Y-1, and C/Y-2 were able to detect 5 pg of A LPS. Monoclonal antibody C-2 detected a minimum of 50 pg of A LPS and also detected 0.05 pg of M LPS. Monoclonal antibodies C-1 and C/Y-1 detected 0.5 pg of M LPS. Monoclonal antibodies M and C/Y-2 detected 50 pg of M LPS.

Figure 5 indicates the amount of LPS detectable by monoclonal antibodies A, M, C-1, C-2, C/Y-1, and C/Y-2 per

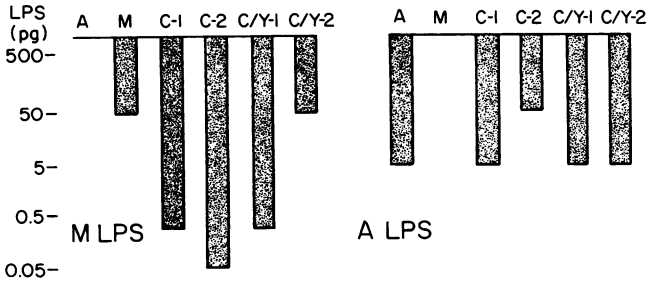

FIG. 4. Quantitation of *Brucella* LPS preparations by the monoclonal antibodies was performed in dot blots. Purified LPS preparations were diluted in 0.9% saline, dotted in 10-μl volumes, and incubated with monoclonal antibody. Bound monoclonal antibodies were sandwiched with rabbit anti-mouse IgG and detected by enhanced protein A-gold staining. The levels of minimal detection with each monoclonal antibody are presented.

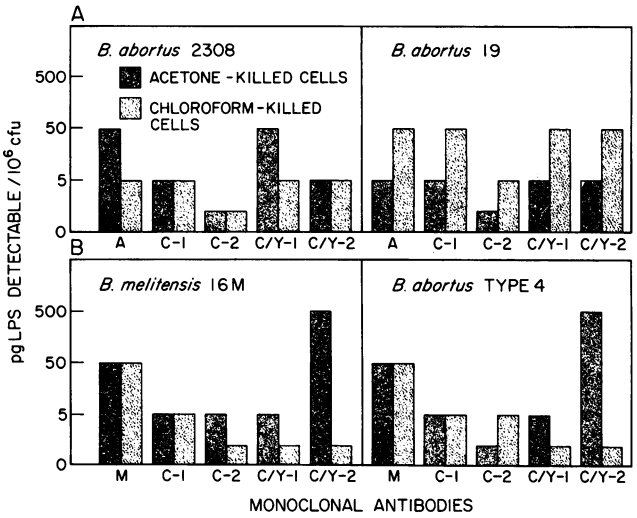

FIG. 5. Suspensions of acetone- and chloroform-killed *Brucella* serotype A (A) and serotype M (B) cells adjusted to A_{420} corresponding to 5×10^9 CFU/ml (1). Dilutions of the whole cells were dotted on nitrocellulose and detected as described in the text and in the legend to Fig. 4. The endpoints of cells detectable with the monoclonal antibodies are presented as the amount of LPS detectable per 10^6 CFU.

10^6 bacterial cells. Differences in the detection of acetone- versus chloroform-killed cells by this bank of monoclonal antibodies are evident.

The results of immunoprecipitation of purified A LPS from *B. abortus* are summarized in Tables 2 and 3. Monoclonal antibodies C-1 and C-2 precipitated 96 and 90% of the total amount of LPS, respectively. Monoclonal antibodies A, C/Y-1, and C/Y-2 precipitated 44, 45, and 41% of the total LPS, respectively. Less than 1% of the total amount of LPS was detectable in the supernatant with the corresponding antibodies used for the precipitation. The C-1 monoclonal antibody detected 32, 45, and 42% additional LPSs in the supernatants of monoclonal antibody precipitations with monoclonal antibodies A, C/Y-1, and C/Y-2, respectively. The C-2 monoclonal antibody detected 44, 2.8, 30, and 45% additional LPS in the supernatants of precipitation with monoclonal antibodies A, C-1, C/Y-1, and C/Y-2, respectively. Monoclonal antibodies A, C/Y-1, and C/Y-2 were not able to detect a significant amount of LPS in any of the supernatants of the precipitations.

The specific endotoxin activity in the *Limulus* lysate gelation assay was increased from the starting material

TABLE 2. Immunoprecipitation of *Brucella* A LPS[a]

Monoclonal antibody	% LPS precipitated[b]	Sp endotoxin act[c]
A	44	5.5
C-1	96	2.5
C-2	90	2.7
C/Y-1	45	5.3
C/Y-2	41	5.9

[a] Purified *B. abortus* 1119.3 A LPS.

[b] Percent precipitation of LPS in micrograms from a total of 100 μg with the monoclonal antibody listed. The precipitates were washed, proteinase K treated, and quantitated by dot blot assay.

[c] EU/0.1 g of precipitated LPS determined by *Limulus* lysate gelation assay. The starting material contained 2.4 EU/0.1 g.

TABLE 3. *Brucella* A LPS[a] in immunoprecipitate supernatants

Monoclonal antibody[b]	Amt of LSP (μg)[c] in supernatant from precipitation with monoclonal antibody:				
	A	C-1	C-2	C/Y-1	C/Y-2
A	<1	<1	<1	<1	<1
C-1	32	<1	<1	45	42
C-2	44	2.8	<1	30	45
C/Y-1	<1	<1	<1	<1	<1
C/Y-2	<1	<1	<1	<1	<1

[a] Purified *Brucella* 1119.3 LPS precipitated as described in Table 2 and the text.

[b] Used in the quantitative dot blot.

[c] Micrograms of LPS in the supernatant detectable after precipitation of 100 μg of LPS as described in Table 2.

containing 2.4 endotoxin units (EU) per 0.1 g to 5.5, 5.3, and 5.9 EU/0.1 g by precipitation with monoclonal antibodies A, C/Y-1, and C/Y-2, respectively. The specific activities of the precipitates from monoclonal antibodies C-1 and C-2 were 2.5 and 2.7 EU/0.1 g, respectively.

No quantitative difference was observed in the immunoprecipitation of the purified *Brucella* M LPS preparation. More than 90% of the total M LPS was precipitated by all of the monoclonal antibodies. Likewise, the M LPS precipitates contained 100% of the endotoxin activity prior to precipitation (1.25 EU/0.1 g).

DISCUSSION

This study of smooth *Brucella* LPS used well-characterized monoclonal antibodies to address questions such as the following. (i) What mixture of LPS and PS molecules do brucellae express? (ii) How can smooth LPS and its complexes with PS be defined? (iii) What are the criteria to establish purity? (iv) What comparisons of the structural relationships of *Brucella* LPS and PS can be made?

We have previously shown with monoclonal antibodies A and M that *Brucella* A and M LPSs are distinct, separate antigenic structures with unique and common epitopes (13). Most serological cross-reactions observed between the LPS types and whole organisms have been attributed to common determinants present on A and M LPS molecules. Monoclonal antibodies C-1 and C-2 detect epitopes common to both A and M LPS types. C/Y-1 and C/Y-2 also detect epitopes common to both *Brucella* A and M LPS as well as *Y. enterocolitica* O:9. Furthermore, the epitopes bound by these monoclonal antibodies are not associated with the oligosaccharides present on rough *Brucella* spp.

Studies of the A LPS side chain have established that at least part of the serological cross-reactivity of smooth *B. abortus* 1119.3 and *Y. enterocolitica* O:9 is associated with the 1,2-linked *N*-acylated 4-amino-4,6-dideoxy-α-D-mannopyranosyl unit in the O-chain polysaccharide of their LPSs (9, 10). The NHs share this O-chain polymer but lack the lipid A found in LPS (35). In contrast, purified polyB consists of a family of nonimmunogenic, cyclic 1,2-linked polymers of glucopyranosyl residues (7). LPS complexes of NH and polyB form immunogens that strongly cross-react and also induce serological responses which, in some cases, may be used to indicate whether animals have been either infected or vaccinated (11, 22).

Biological as well as immunochemical heterogeneity of membrane-bound smooth LPS complex has previously been demonstrated (10, 29, 33, 34). *Brucella* LPS profiles in SDS-PAGE have shown heterogeneous patterns from strain to strain, as well as between LPS types (14). The heterogeneity of purified A LPS observed by nuclear magnetic resonance spectroscopy is attributed to the presence of both E and Z conformations of the *N*-formyl group (10). Quantitative precipitation with *B. abortus*-infected cow serum demonstrates a highly heterogeneous population of membrane-bound LPS. Binding of antibody by LPS varies from strain to strain of *Brucella* and from fraction to fraction of purified preparations of LPS extracts (33).

Wu and Mackenzie observed the purified LPS fraction f5A of strain 1119.3 to be the most homogeneous LPS preparation (34). Yet they found that group f5A LPSs of strains 2308 and 19 yield O chains ranging in size, as determined by ultrafiltration, from molecular weight cutoff 8.0×10^3 to 10.0×10^3, 3.5×10^3 to 5.0×10^3, and $<1.0 \times 10^3$. These f5A fractions constitute more than 85% of the total immunoreactive materials.

Fractionated *Brucella* LPS extracts, however, display a very high degree of immunochemical cross-reactivity. Better definition of this mixed but antigenically related population of *Brucella* polysaccharides and their structures is needed to help explain the cross-reactivities of these molecules.

Monoclonal antibodies C/Y-1 and C/Y-2 detect a population of molecules in the purified A LPS preparation migrating between 40,000 and 50,000, relative to marker proteins (Fig. 2). Monoclonal antibody C-2 does not recognize epitopes below 40,000 which monoclonal antibodies A and C-1 bind. Four unique epitopes in *Brucella* LPS extracts are identified by these monoclonal antibodies. The O-chain, type-specific monoclonal antibodies recognize either A or M LPS only. The type C monoclonal antibodies bind epitopes common to both A and M LPS that are not shared with *Y. enterocolitica* O:9 LPS. The type C/Y monoclonal antibodies are specific for epitopes which do cross-react with *Y. enterocolitica* O:9.

The Western blot patterns of the type A, C, and C/Y epitopes indicate that two distinct populations of molecules are in the purified A LPS preparation. The differences in immunoprecipitation profiles, *Limulus* lysate gelation assay activity (Tables 2 and 3), and binding patterns with *B. abortus* extract (Fig. 2) indicate that the type C/Y monoclonal antibodies recognize molecules possessing the biologically active lipid A portion of the LPS molecule. The restricted binding pattern of the type C/Y monoclonal antibodies in Western blots of the purified LPS preparation may be attributed to a finite O chain length on the complete A LPS molecule selected for by the purification scheme. The epitopes reactive with monoclonal antibody A are distributed throughout the blotted purified LPS preparation. This indicates that the specific A LPS epitope may occur on molecules other than complete LPS. The specific endotoxin activities of monoclonal antibody A immunoprecipitates, however, indicate that the majority of the A epitopes in this preparation are associated with lipid A. The type C monoclonal antibodies bind epitopes in the purified A LPS preparation not precipitated by the A or C/Y monoclonal antibodies and remove all A and C/Y reactivity. The type C epitopes appear to be common to all PS molecules in the preparation.

The specific reactivities of the monoclonal antibodies described here with epitopes of *Brucella* native hapten and polysaccharide poly B was not determined. These carbohydrates, as mentioned previously, merit investigation. The availability of well-characterized, purified preparations of these antigens, however, is a limiting factor in their study.

The monoclonal antibodies described here provide tools

with which to monitor the expression of LPS epitopes by *Brucella* spp. and determine the content of preparations intended for use in the control of brucellosis. The comparative study of chloroform- versus acetone-killed *Brucella* spp. yielded some interesting results demonstrating that the antigenic profile of the bacteria can be altered by such treatments that may cause preferential extraction of the outer membrane oligosaccharides.

We have shown that immunoprecipitation and immunoaffinity chromatography can be exploited to separate epitopes recognized by the common monoclonal antibodies. Separating the molecular populations in *Brucella* LPS with monoclonal antibodies provides an approach which may be useful in purifying improved antigens for use as *Brucella* diagnostics or vaccines.

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