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Purification of Nonlipopolysaccharide Antigen from *Brucella abortus* during Preparation of Antigen Used for Indirect Hemolysis Test†

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The indirect hemolysis test (IHLT) for the diagnosis of brucellosis uses a lipopolysaccharide (LPS) antigen obtained by dimethyl sulfoxide extraction of *Brucella abortus*. We showed that a non-LPS antigen can be obtained as a by-product of the IHLT antigen preparation. The antigen was purified to homogeneity by a combination of gel-filtration chromatography and ion-exchange chromatography. The substance contained 8% protein and about 65% carbohydrate. The molecular weight of the primary unit was 19,750, when analyzed by polyacrylamide gel electrophoresis under denaturing conditions. The non-LPS antigen, which is serologically identical to *B. abortus* smooth LPS O antigen, did not bind to cell membranes. However, it could be used to detect specific antibodies by complement fixation, precipitation in agarose gels, and inhibition of the IHLT.

The complement fixation (CF) test is considered to be the most useful and definitive procedure for serodiagnosis of bovine brucellosis (1, 14). In recent years, an indirect hemolysis test (IHLT) has been developed which appears to be as reliable as the CF test, and it does not have some of the shortcomings of the CF test (16). For example, the IHLT can be carried out at great complement excess. Therefore, accurate titration of complement is not required. Also, the prozone phenomenon (frequently encountered with the CF test) is less of a problem (6, 16).

The IHLT is a passive hemolysis assay which utilizes a *Brucella abortus* antigen noncovalently bound to bovine erythrocyte targets. In a previous study (6), we established optimal conditions for conducting the IHLT. Lipopolysaccharide (LPS) produced by dimethyl sulfoxide (DMSO) extraction of *Brucella abortus* 1119-3 (U.S. Department of Agriculture strain 19 used for preparation of diagnostic antigens) (6) was employed as the cell-coating antigen. Purification of LPS includes an extraction with water. Ordinarily, the water-soluble material is discarded. However, we noticed that the extract was quite opalescent, which suggested the presence of water-miscible LPS. We felt that the yields of antigen could be increased if LPS could be recovered from the water phase. We demonstrate in this study that the aqueous phase contains LPS. In addition, this fraction contains a non-LPS antigen (NLA) which reacts with antibodies to *B. abortus* and inhibits the IHLT. The NLA was isolated and purified. This discrete compound shares an antigenic determinant with smooth *B. abortus* LPS, yet it does not bind to cell membranes and is devoid of 2-keto-3-deoxyoctonate (KDO), which is a characteristic moiety of LPS (12). Because the material can be produced and purified rather easily, it should be suitable for use in serological procedures for diagnosis of brucellosis.

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MATERIALS AND METHODS

Bacteria. *B. abortus* 1119-3 was kindly supplied by George Brown of the U.S. Department of Agriculture, National Animal Disease Center, Ames, Iowa.

Buffered isotonic solutions. Phosphate-buffered isotonic sodium chloride (PBS) and isotonic Veronal-buffered sodium chloride that contained 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.1% gelatin (GVB) were prepared as described previously (6).

Antiserum. Serum samples from five *B. abortus* culture-positive Holstein cows, that were CF test positive, were pooled, and the immunoglobulin fraction was obtained by ammonium sulfate fractionation (7). Immunoprecipitation analysis revealed that the fraction contained mainly immunoglobulin G1 (IgG1) with trace amounts of IgG2.

Complement. Fresh frozen guinea pig serum (Pel Freeze, Rogers, Ark.) was used as the complement source. The serum was adsorbed three times at 0°C with J-antigen-negative bovine erythrocytes (17). The complement was titrated by the procedure described by Mayer (10). Twenty times the amount of complement necessary for lysis of 50% of antibody-sensitized erythrocytes (20 CH_{50}) was used, unless otherwise indicated.

Antigens. The IHLT antigen used for this study was prepared from *B. abortus* 1119-3. Briefly, the cells were washed sequentially with water, ethanol (95%), acetone, and ethyl ether. The washed cells were dried in vacuo at room temperature (23 to 24°C) and then extracted for 3 h at 60°C with DMSO. The material was centrifuged at 12,000 × *g* for 5 min at 23°C, the supernatant was saved, and the pellet was reextracted with DMSO for 10 min at 60°C. The DMSO extracts were pooled, and crude LPS was precipitated with a solution of 1% sodium acetate prepared in methanol (1% MSA). The precipitate was suspended in water and stirred for 60 min at room temperature. Insoluble material was recovered by centrifugation at 12,000 × *g* for 10 min at room temperature. The aqueous phase that resulted from this step was saved, and it served as the starting material for purification of the NLA. The insoluble LPS-containing phase was extracted two additional times with DMSO for 10 min each

at 60°C. LPS was precipitated from the combined DMSO extracts with 1% MSA. The reprecipitated LPS was dialyzed against water and lyophilized. The LPS was dissolved in 0.25 N NaOH, heated at 56°C for 60 min, neutralized with 1.0 M acetic acid, and precipitated with three volumes of cold 95% ethanol. The precipitate was dissolved in water, dialyzed against water, and lyophilized (16).

Authentic *B. abortus* smooth LPS was also prepared by the hot phenol extraction procedure described by Westphal et al. (19). The LPS fraction that appeared in the phenol phase was further purified by nuclease treatment and extensive deproteinization (12).

A sample of purified smooth *B. abortus* LPS O antigen was kindly provided by John Cherwonogrodsky (Animal Disease Research Institute, Nepean, Ontario, Canada). This was used in comparison assays with NLA during polyacrylamide gel electrophoresis (PAGE) and serological analyses.

Serologic procedures. The IHLT was conducted as described previously (6). Bovine erythrocytes in PBS (4.5×10^8/ml) were coated by incubation in an equal volume of alkali-treated IHLT antigen (800 μg/ml in PBS) for 60 min at 37°C. The coated cells were washed three times in GVB and diluted to 4.5×10^7/ml in the same buffer. The immunoglobulin fraction of bovine anti-*B. abortus* immunoglobulin was diluted in GVB, and 25 μl was placed into microtiter plate wells (Cooke Engineering Co., Alexandria, Va.). A total of 25 μl of antigen-coated erythrocytes was added to each well, the plates were incubated at 37°C for 15 min, and 25 μl of adsorbed guinea pig complement (20 CH_{50}/ml) was added. The plates were held at 37°C for an additional 60 min, and the reciprocal of the highest dilution of anti-*B. abortus* immunoglobulin capable of causing 100% hemolysis was taken as the endpoint.

An indirect hemolysis inhibition test (IHL-I) was developed for assaying *B. abortus* fractions for antigens identical to those found on the IHLT antigen. For this procedure, 25 μl of the test fraction was incubated for 15 min at 37°C with 25 μl of the greatest dilution of the immunoglobulin fraction of anti-*B. abortus* capable of causing 100% lysis of LPS-antigen coated bovine erythrocytes in the presence of excess complement (20 CH_{50}). Twenty-five microliters of antigen-coated bovine erythrocytes was added to each reaction mixture, and the incubation was continued for an additional 10 min at 37°C. Then, 20 CH_{50} units of guinea pig complement (in 25 μl) was added to each mixture, and the tests were incubated for 60 min at 37°C. Fractions that inhibited the ability of complement to lyse the cells were presumed to contain an antigen similar to that found on LPS-coated bovine erythrocytes.

A modified CF test (11) was also used to assay *B. abortus* fractions for antigenicity. Dilutions of the immunoglobulin fraction of bovine anti-*B.-abortus* (0.4 ml) were incubated with 0.4 ml of antigen (0.1 mg/ml) and 0.5 ml of guinea pig complement diluted to contain five times the amount of complement required to lyse 50% of 2×10^7 erythrocytes coated with specific antibodies (10, 11). After the primary incubation, 0.2 ml of erythrocytes coated with specific antibodies (10^8/ml) was added to each tube, and the mixtures were incubated for an additional 60 min at 37°C. The optical densities of the supernatant fluids were measured at a wavelength of 541 nm in a spectrophotometer (DU-50; Beckman Instruments, Inc., Fullerton, Calif.). The average optical density value for complete lysis was 0.727 (established from triplicate samples of three separate experiments; standard deviation, 0.013). If antibodies bound to *B. abortus* antigenic determinants during the first incubation, complement was consumed. Therefore, because more than 3 CH_{50} units would have to be consumed before there was less than 100% of the erythrocytes coated with specific antibodies lysed during the second incubation, any test giving less than 100% hemolysis was considered positive.

A 0.75% agarose gel in 10% sodium chloride buffered to pH 8.6 with 0.1 M sodium barbital was employed for detecting precipitating antibodies by immunodiffusion (3).

Chemical assays. The purified *B. abortus* antigen was analyzed for KDO by the thiobarbituric assay procedure (8). Total carbohydrate was estimated by the phenol-sulfuric acid method (4), and protein was measured by the procedure described by Lowry et al. (9).

PAGE. *B. abortus* extracts were subjected to electrophoresis (model 500/200 Protean system and power supply; Bio-Rad Laboratories Richmond, Calif.) on slabs of 14% polyacrylamide; a sodium dodecyl sulfate (SDS)-gel system incorporating 4 M urea was employed (18). Samples were diluted in 0.1 M Tris hydrochloride (pH 6.8) that contained 2% SDS, 20% sucrose, 1% 2-mercaptoethanol, and 0.001% bromphenol blue. The samples were boiled for 5 min and then cooled to room temperature before application to the gels. The samples (40 μl) were subjected to 20 mA per slab gel until the bromphenol blue marker had migrated 10 cm. The completed PAGE slabs were developed with the modified silver stain described by Tsai and Frasch (18) for detecting LPS in polyacrylamide gels.

The molecular weights of antigens were estimated by comparison of electrophoretic mobilities with known protein markers (Sigma Chemical Co., St. Louis, Mo.). The authentic markers for PAGE and the molecular weights were bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and α-lactalbumin (14,200).

Gel-filtration chromatography and ion-exchange chromatography. The water-soluble material obtained from the first DMSO extraction of *B. abortus* 1119-3 was prepared in 0.1 M ammonium carbonate (pH 8.1 to 8.2) at 2 mg/ml. A total of 10 ml was applied to a G-200 Sephadex column (1.5 by 90.0 cm) which was equilibrated with 0.1 M ammonium carbonate. The column was eluted with 0.1 M ammonium carbonate, and the IHL-I assay was used to test column fractions for *B. abortus* antigen(s).

Fractions that contained *B. abortus* antigen were pooled, dialyzed against water, and freeze-dried. A total of 10 ml of the material at 1 mg/ml in 0.01 M potassium phosphate (pH 7.8) was applied to a column (2.5 by 30.0 cm) of DEAE cellulose (0.6 mEq/g; BioRad) which had been equilibrated with 0.01 M potassium phosphate (pH 7.8). A spectrophotometer (DU-50; Beckman) was used to determine the optical densities of the fractions at a wavelength of 220 nm (see Fig. 4). A total of 300 ml of 0.01 M potassium phosphate was passed through the column before a linear sodium chloride gradient, which consisted of 400 ml of 0.01 M potassium phosphate (pH 7.8) and 400 ml of 0.5 M sodium chloride in 0.01 M potassium phosphate (pH 7.8), was applied. Fractions of 6 ml were collected and assayed for *B. abortus* antigen. Fractions that possessed antigen were pooled, dialyzed against water, and freeze-dried.

RESULTS

Extraction of a putative NLA from *B. abortus* 1119-3. The DMSO procedure described above was employed to extract *B. abortus* 1119-3 cells. The IHLT antigen (LPS) yielded two

bands of precipitation when it was allowed to react with the immunoglobulin fraction of bovine anti-*B. abortus* in an immunodiffusion assay (Fig. 1). The water-soluble fraction (Fig. 1, well D) also possessed two antigens which precipitated with the immunoglobulin fraction of anti-*B. abortus*. One of these showed serological identity with one of the antigens in the crude LPS preparation (IHLT antigen). The IHL-I test also revealed that there was at least one antigen in the water-soluble phase. Dilutions of up to 1:128 were capable of completely inhibiting hemolysis. The antigen that caused the line closest to well A in Fig. 1 was apparently LPS which was minimally soluble and did not diffuse well under the conditions used for analysis. Deposition of insoluble LPS in the agarose was observed when the gel diffusion plates were stained with Coomassie blue (Fig. 1). The water-soluble antigen preparation (Fig. 1, well D) was chosen for further purification of the second antigen (NLA).

Purification of NLA. The water-soluble extract (described above) resolved into two distinct peaks when applied to G-200 Sephadex in 0.1 M ammonium carbonate (Fig. 2). The first peak eluted immediately after the void volume, and it contained KDO; the individual fractions were capable of inhibiting the IHLT. Two precipitating antigens were present in this peak (Fig. 1, well C). One of the antigens appeared to be LPS because it was minimally soluble and diffused poorly in the agarose gel; precipitation was seen along the leading edge of the zone before the gel was stained with Coomassie blue. The second peak was KDO negative, it inhibited the IHLT, and it contained one precipitating antigen which was identical to the putative NLA component in the IHLT antigen preparation (Fig. 1, well B).

The material in peak two was designated NLA. This antigen was analyzed for purity by PAGE. It can be seen from the results shown in Fig. 3 (lane 5) that the NLA was impure. At least three components were present.

Additional purification of the NLA (peak two from the G-200 Sephadex column) was achieved by chromatography on DEAE-cellulose. Individual fractions were tested for IHLT inhibition, and it was found that all antigenic activity was associated with a peak of unbound material which came through the column during the wash with equilibrating buffer (Fig. 4). The material in the first DEAE peak was isolated, dialyzed against water, and freeze-dried. This material gave a single band of precipitation when it was allowed to react against the immunoglobulin fraction of anti-*B. abortus* in an immunodiffusion test. The antigen appeared to be pure since only a single band was seen when the silver stain was applied after PAGE in the presence of SDS and 4 M urea (Fig. 3, lane 6).

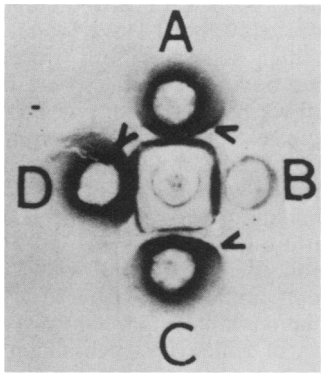

FIG. 1. Coomassie blue-stained agar gel diffusion assays showing the immunoglobulin fraction of bovine anti-*B. abortus* that reacted with IHLT antigen (well A), peak two obtained after G-200 Sephadex gel filtration of the DMSO extraction aqueous fraction (well B), peak one obtained after G-200 Sephadex gel filtration of the DMSO extraction aqueous fraction (well C), and the aqueous fraction obtained by DMSO extraction of *B. abortus* 1119-3 (well D). The arrowheads point to bands of immunoprecipitation on the edges of the LPS zones.

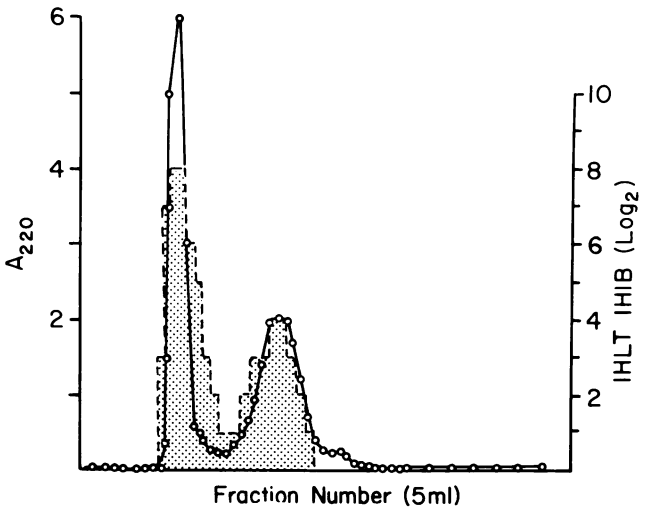

FIG. 2. Gel filtration chromatography on a G-200 Sephadex column of the aqueous phase obtained by DMSO extraction of *B. abortus* 1119-3. Symbols: open circles, optical densities of the fractions at a wavelength of 220 nm; shaded area, antigen assays using inhibition of the IHLT.

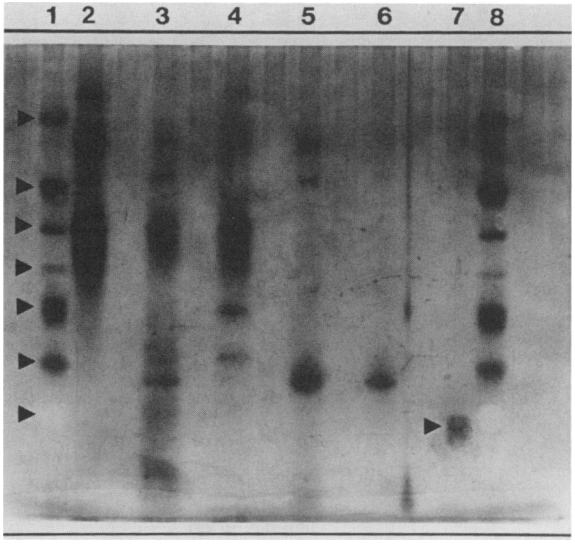

FIG. 3. PAGE of *B. abortus* 1119-3 extracts and fractions. Separation was carried out in a 14% gel that contained 2% SDS and 4 M urea. Samples were heated at 100°C for 5 min in 1% 2-mercaptoethanol and 2% SDS before application to the gel. Lanes 1 (arrowheads) and 8, authentic molecular weight standards (from top to bottom: bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase, trypsinogen, trypsin inhibitor, and α-lactalbumin); lane 2, authentic smooth LPS from strain 1119-3; lane 3, Sephadex G-200 column input; lane 4, Sephadex G-200 column peak one; lane 5, Sephadex G-200 column peak two; lane 6, DEAE peak one; lane 7, authenic O polysaccharide (arrowhead).

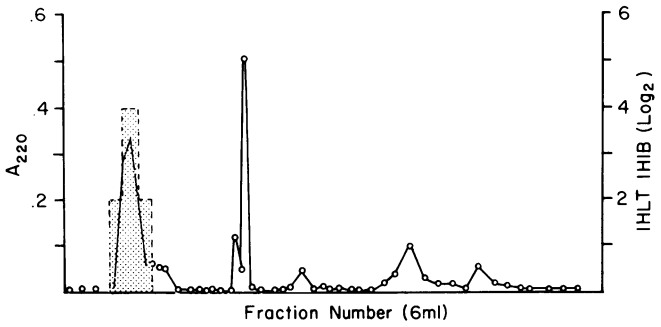

FIG. 4. DEAE chromatography of G-200 Sephadex peak two antigen. A total of 10 mg of starting material in 0.01 M potassium phosphate (pH 7.8) was applied to a column (2.5 by 30.0 cm) equilibrated with the same buffer. After the column was washed with 300 ml of 0.01 M potassium phosphate (pH 7.8), a linear gradient that consisted of 400 ml of 0.01 M potassium phosphate (pH 7.8) and 400 ml of 0.5 M sodium chloride in 0.01 M potassium phosphate (pH 7.8) was applied. The optical densities of column fractions were determined at a wavelength of 220 nm, and the presence of antigen was monitored by the ability of individual fractions to inhibit the IHLT.

Serological characteristics of NLA. NLA inhibited the IHLT, which indicates that the antigen possessed at least one determinant that was similar to an antigen in the crude LPS preparation that was coated onto bovine erythrocytes. Therefore, experiments were conducted to determine if

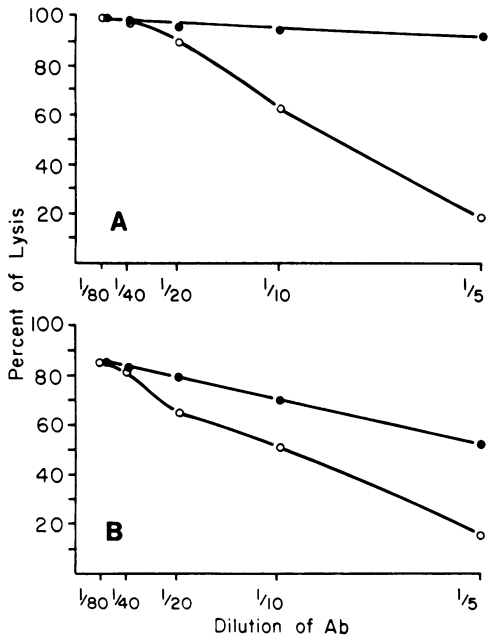

FIG. 5. Adsorption of NLA-specific complement-fixing antibodies (Ab) from the immunoglobulin fraction of bovine anti-*B. abortus*. (A) The unadsorbed antiserum (○) was capable of fixing guinea pig complement and thus inhibiting hemolysis of sheep erythrocytes coated with antibodies. Adsorption of the antiserum with *B. abortus* 19 removed the complement-fixing antibodies, and sheep erythrocytes coated with antibodies were completely lysed at every antiserum concentration (●). (B) The unadsorbed antiserum preparation (○) was capable of fixing guinea pig complement. Adsorption of the antiserum with bovine erythrocytes coated with *B. abortus* LPS caused a reduction in the concentration of complement-fixing antibodies (●).

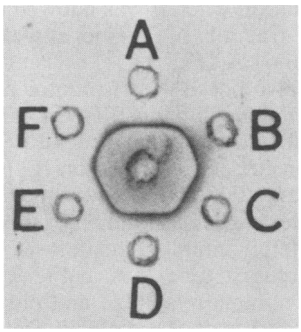

FIG. 6. Immunoprecipitation in a gel that consisted of 0.75% agarose–0.1 M barbital buffer (pH 8.6)–1.0% Triton X-100–10% sodium chloride–0.01 M EDTA. Center well, immunoglobulin fraction of bovine anti-*B. abortus*; wells A and D, authentic, purified *B. abortus* 1119-3 LPS; wells B, C, and F, purified NLA; well E, Sephadex G-200 column peak two.

NLA shared a determinant with the smooth *B. abortus* LPS complex or if NLA was an amphipathic molecule present in the crude DMSO-produced LPS preparation which could bind independently to bovine erythrocytes.

We found that the NLA could still inhibit the ability of the immunoglobulin fraction of anti-*B. abortus* and complement to lyse bovine erythrocytes that were coated with purified preparations of *B. abortus* 1119-3 smooth LPS which had been prepared by the procedure described by Westphal et al. (19). A dilution of 1:128 caused complete inhibition of lysis of bovine erythrocytes coated with purified *B. abortus* LPS. Furthermore, purified NLA would not bind to bovine erythrocytes. Cells treated with NLA at 2,000 μg/ml and washed could not be lysed by the immunoglobulin fraction of anti-*B. abortus* and guinea pig complement.

Antibodies specific for purified NLA could be detected by the CF test. Adsorption of the antiserum three times with 10^9 *B. abortus* 19 cells per adsorption resulted in complete removal of the antibodies detectable by the CF test (Fig. 5A). We also found that five adsorptions of the antiserum with bovine erythrocytes coated with authentic *B. abortus* LPS (0.05 ml of packed, coated cells per ml of serum per adsorption) reduced the level of antibodies (Fig. 5B).

Immunodiffusion assays were carried out in agarose at pH 8.6 in the presence of 10% NaCl. We found, as reported by others (3), that high salt conditions were necessary for precipitation when bovine antibodies were used. When the assay plates were washed thoroughly in PBS and dried and stained with Coomassie blue, it was apparent that LPS was minimally soluble because a zone of precipitated material was seen surrounding wells which had contained LPS (Fig. 1). Immunoprecipitation, when it occurred, was present on the leading edge of the LPS zone, between the sample and the antiserum well. Before staining with Coomassie blue, only the immunoprecipitate was visible.

A modified immunodiffusion procedure was subsequently applied to assays in which LPS-containing antigens were used. Agarose gels were prepared which included 1.0% Triton X-100, 10% NaCl, and 0.01 M EDTA. LPS was soluble in the gel under these conditions. A line of serological identity with purified NLA was obtained when LPS was allowed to react with the immunoglobulin fraction of bovine anti-*B. abortus* (Fig. 6). This finding indicates that purified NLA possesses an antigenic determinant that is serologically identical to a determinant present on purified *B. abortus* smooth LPS.

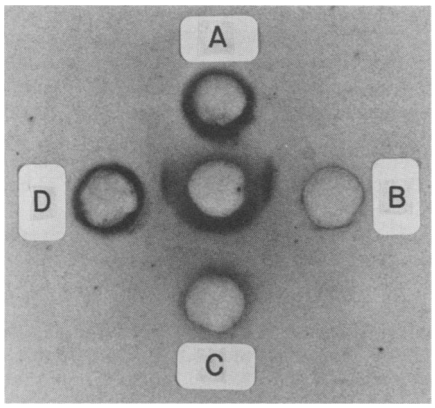

FIG. 7. Immunoprecipitation comparing NLA (wells B and D) with authentic smooth LPS O polysaccharide antigen (well C). The center well contained the immunoglobulin fraction of bovine anti-*B. abortus*. Well A was blank.

Partial characterization of NLA. The accumulated data indicate that NLA is antigenically related to a component of smooth LPS. It is possible that NLA is identical to the O antigen or one of the serologically related polysaccharides (polysaccharide B or native hapten) which have been isolated from *B. abortus* (3, 5).

Authentic *B. abortus* O polysaccharide, which consists of repeating units of 4,6-dideoxy-4-formamido-α-D-mannopyranoside (perosamine), was compared with the NLA by PAGE. It can be seen (Fig. 3, lane 7) that the O-polysaccharide moved further in the gel than did the NLA (Fig. 3, lane 6). The approximate molecular weight of the O polysaccharide was 12,000 and that of the NLA was about 19,750. Bands corresponding to the free O polysaccharide and the NLA were not evident in a smooth LPS sample which was also subjected to PAGE electrophoresis at the same time (Fig. 3, lane 2).

Purified NLA was assayed for KDO (8), protein (9), and carbohydrate (4) content. There was no detectable KDO, about 8% of the dry weight was protein, and approximately 65% was carbohydrate. The protein was apparently strongly associated with the carbohydrate because there was only one stainable band after polyacrylamide gel electrophoresis under conditions which should have broken disulfide bridges and noncovalent bonds.

The serologic relationship between purified *B. abortus* smooth-LPS O-polysaccharide antigen and NLA was established by allowing the purified antigen to react with the immunoglobulin fraction of bovine anti-*B. abortus* in an agarose gel along with authentic O-polysaccharide side chain antigen; a line of serological identity resulted (Fig. 7).

DISCUSSION

A serologically active substance which was extracted and purified from smooth *B. abortus* 1119-3 has been described. The substance reacted with antibodies to field strain *B. abortus* and inhibited the IHLT which uses LPS antigen-coated bovine erythrocytes. The purified substance gave a single, well-defined band on PAGE when developed with the silver stain. The apparent molecular weight of the unit, under denaturing conditions, was 19,750. The purified NLA was also capable of precipitating with the immunoglobulin fraction of bovine anti-*B. abortus*.

There are two explanations for the fact that NLA inhibited the IHLT. The antigen used for the IHLT consists of a partially purified LPS preparation. Presumably, it is the LPS antigen that binds to the target cells. Therefore, the NLA may inhibit the IHLT because it is structurally identical (or similar) to some component of smooth LPS. On the other hand, the crude LPS preparation used for the IHLT could contain another amphipathic antigen capable of binding to erythrocyte membranes. Thus, the pure NLA would bind-up those antibodies in the immunoglobulin fraction of bovine anti-*B. abortus* specific for the second antigen, thereby inhibiting the IHLT.

The latter possibility does not seem to be the case. NLA still inhibited the IHLT when purified LPS was used to coat bovine erythrocytes, and the NLA did not appear to bind to bovine erythrocytes because cells treated with the antigen were not lysed by the immunoglobulin fraction of bovine anti-*B. abortus* and guinea pig complement. Therefore, it seems that the IHLT detects only antibodies specific for LPS complex determinants, and no other amphipathic *B. abortus* antigens are present on erythrocytes coated with a DMSO extract of *B. abortus* 1119-3.

Adsorption of bovine anti-*B. abortus* with live strain 19 bacteria removed antibodies specific for the NLA. This shows that the antigenic determinant is expressed on the surfaces of smooth *B. abortus* bacteria. Some of the complement-fixing antibodies in the immunoglobulin fraction of bovine anti-*B. abortus* could also be adsorbed by bovine erythrocytes coated with purified *B. abortus* smooth LPS. This ability of purified LPS to adsorb antibodies specific for the NLA further pointed out the similarity between NLA and some determinant present on smooth LPS. In view of other data showing serological identity of the NLA and the O-antigen side chain of smooth LPS, the failure of adsorption to completely remove all antibodies specific for the NLA is probably due to the incomplete removal by limiting amounts of LPS bound to bovine erythrocytes. However, there is a possibility that a second epitope exists on NLA which is not found (or exposed) on smooth LPS. Adsorption of the antiserum with LPS would remove antibodies to a common epitope, but antibodies to a nonshared determinant would remain. These latter antibodies would then be detected by CF.

Antigenic similarities between smooth LPS and NLA were also recognized by an immunodiffusion procedure. An agarose gel system containing a nonionic detergent (Triton X-100) and 0.01 M EDTA was used to keep the LPS soluble so that free diffusion could take place. This system did not inhibit immunoprecipitation, and a band of identity between the smooth LPS and NLA was observed again demonstrating antigenic identity between the latter substance and some component of LPS.

Results of our experiments also showed that a purified preparation of authentic *B. abortus* polysaccharide O antigen gave a line of serological identity with the NLA when it was reacted against the immunoglobulin fraction of bovine anti-*B. abortus*. Because the O antigen is a homopolymer of perosamine (2), we conclude that NLA has at least one antigenic determinant which consists of an oligomer of perosamine. However, the primary unit of NLA has a higher molecular weight than the O antigen, it is more homogenous, and it possesses about 8.0% tightly bound protein. NLA may represent a precursor form of the O-antigen side chain of smooth LPS, a role also suggested for a *B. abortus* polysaccharide known as polysaccharide B (13) which is synthesized de novo and added to LPS during assembly of the complex. Polysaccharide B has been shown to be serologically identical to another *B. abortus* polysaccharide called native

hapten (5, 13). It is possible that NLA is similar to (or identical to) native hapten because Perera et al. (15) provided evidence that, like NLA, some native hapten is covalently bound to protein, and serological identity of native hapten and a component of smooth LPS has been demonstrated (13). However, data showing characteristics such as purity and the molecular weight of native hapten (as well as polysaccharide B) are not available. Future studies to compare NLA with native hapten and polysaccharide B are planned.

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