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Preparation by Ultrafiltration and Control by High-Performance Liquid Chromatography of the Native Hapten of *Brucella abortus* for Use in Radial Immunodiffusion Diagnostic Test

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An effective method was developed for the preparation of *Brucella abortus* native hapten by use of an ultrafiltration system. The membranes PM30 and YM5 were used to separate the lipopolysaccharide and the native hapten. The yield of the native hapten was higher than that obtained by a more complex procedure reported previously. This method is economical for the large-scale preparation of *B. abortus* native hapten. The effects of ultrafiltration were evaluated by a quick and sensitive high-performance liquid chromatographic technique.

The diagnosis of human and animal brucellosis is based mainly on the detection of antibodies by standard tests such as the classical serum agglutination tests, the Rose Bengal plate test, the complement fixation test, and the milk ring test (5). Prophylactic measures include serological detection and slaughter of positive animals or vaccination with *Brucella abortus* B19 or 45/20 or *Brucella melitensis* H38 or Rev-1. However, vaccination produces antibodies which cannot be differentiated from those induced by actual infection. This disadvantage could be eliminated by using an antigen which could distinguish between vaccinated and infected animals, and polysaccharide B from the rough strain *B. melitensis* B115 has been shown to precipitate antibodies from infected cattle but has been shown not (or to a lesser degree) to precipitate antibodies from vaccinated cattle in radial immunodiffusion (RID) tests (2). Furthermore, polysaccharide B could be replaced by the native hapten (NH) of *B. melitensis* 16M (4), and NH gave reactions identical to those obtained with polysaccharide B, when examined with sera from infected cattle (9). However, *B. melitensis* 16M is a human pathogen, and the extraction procedure involves several uncontrolled and time-consuming steps, including two ethanol precipitations and centrifugations.

In this report we describe a method in which the virulent *B. melitensis* 16M strain is replaced with the avirulent *B. abortus* B19 vaccine, and the NH was prepared by ultrafiltration and controlled by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Preparation of NH extracts. Fermentor-grown *B. abortus* B19 cells (105 g; supplied by B. Garin-Bastuji, Laboratoire Central de Recherches Vétérinaires, Maisons Alfort, France) were treated with phenol (105 g in 250 ml of saline solution) and then autoclaved (120°C, 30 min). Crude NH was prepared as described by Diaz et al (2). After centrifugation at 12,000 × *g* for 30 min at 5°C, 60% of the supernatant was lyophilized (fraction 1). The weight was 2.29 g. To the other 40% of the supernatant (fraction 2), 2 volumes of ethanol were added, and the mixture was stirred continuously for 18 h at 5°C. The first ethanol precipitate was removed by centrifugation at 5,000 × *g* for 15 min at 4°C, and 2 volumes of ethanol were then added to this supernatant. The precipitate that was formed after this second addition of ethanol was the NH-enriched fraction. The first and second ethanol precipitates were lyophilized. The weight of the first precipitate was 1.15 g, and that of the second was 0.25 g.

Preparation of S-LPS fraction. The smooth lipopolysaccharide (S-LPS) fraction (F5) of *B. abortus* 99 in the smooth phase was prepared by the phenol-water method (8).

Enzymatic treatments. Fraction 1 (200 mg in 400 ml) was treated with enzymes. It was first treated with DNase and RNase (Worthington Diagnostics, Freehold, N.J.) and then with proteinase K (Boehringer GmbH, Mannheim, Federal Republic of Germany). Both treatments were done overnight at room temperature. The concentration of each enzyme was 1 mg/200 mg of extract.

HPLC. Analyses were carried out with a liquid chromatograph (model 8700; Spectra Physics) equipped with a UV/visible variable wavelength detector (SP8440; Spectra Physics) connected to a computing integrator (4200; Spectra Physics). The size-exclusion HPLC column (600 by 7.5 mm; TSK G 2000 SW; Toyo Soda) had a particle size of 10 μm. The eluant was 0.175 mM NaCl (pH 7). The flow rate was 1 ml/min. The eluates were monitored at 200 nm. Fraction 2 was diluted in eluant to a concentration of 4 mg/ml and was injected into the column. During ultrafiltration, the quality of separation was checked by injecting 100 μl of each retentate into the column.

Ultrafiltration. On the basis of the exclusion limits of the membrane given by the manufacturer, a tandem assembly was devised that consisted of two stirred cells (Amicon, Paris, France) placed in series, each with a membrane with a progressively lower cutoff. The volume of each cell was 400 ml. The first precipitate (200 mg; starting solution) was placed in distilled water (pH 7) and treated with DNase, RNase, and proteinase K. The mixture was introduced into the upper cell (the other cell contained distilled water), and a reservoir was attached. Nitrogen pressure (11 lb/in^2) served to circulate distilled water through the fluid system to effect partition. Ultrafiltration was done at 20°C. Four membranes (XM100, PM30, YM10, YM5) were evaluated for their S-LPS and NH retentive properties.

RID. The NH-enriched fraction was evaluated by using a RID test, which was performed as described previously (2).

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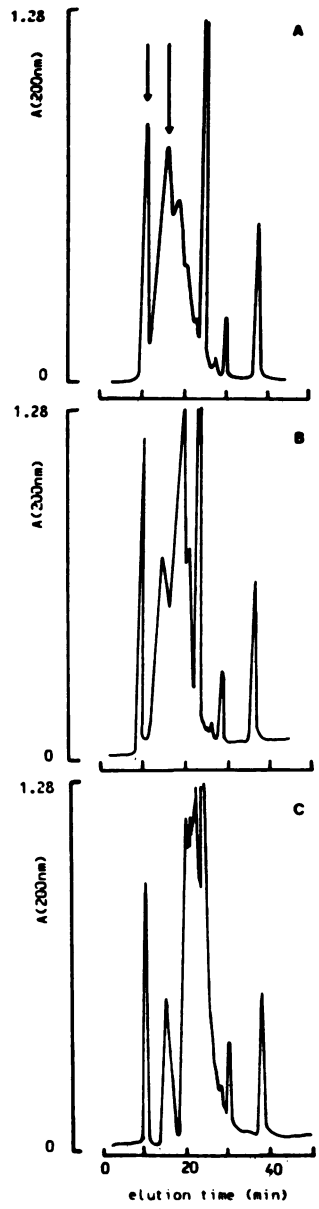

FIG. 1. Effects of hydrolytic treatments on the HPLC elution profile of the supernatant of B19 cells (fraction 1; 200 mg/400 ml). (A) Untreated sample; (B) sample treated with DNase and RNase (1 mg/400 ml); (C) sample treated with DNase, RNase, and proteinase K (1 mg/400 ml). Chromatographic conditions were as follows: column, 60 cm by 7.5 mm (inner diameter); TSK G 2000 SW; eluant, 0.175 mM sodium chloride buffer (pH 7.0); flow rate, 1 ml/min; the eluate was monitored at 200 nm.

The agarose gel contained 10% NaCl, and the laboratory serum standard was obtained from a cow infected with *B. abortus* 544. Yields were estimated by quantitative measurements with dilutions of a NH preparation (0.4 mg/ml) supplied by R. Diaz, which was used as a reference standard.

Bovine sera. The RID test was performed on sera obtained from the following groups of cows: (i) cows which had been infected with *B. abortus* B19 administered by the conjunctival route; (ii) cows which had been experimentally infected with *B. abortus* 544.

RESULTS

Effect of hydrolytic treatment on supernatant of B19 autoclaved cells (fraction 1). The chromatogram of the untreated fraction 1 (Fig. 1A) shows several peaks. The first one, which eluted in the void volume, was identified as a S-LPS-containing fraction, first, because the known S-LPS phenol-extracted fraction (F5) also elutes in the void volume of this column and, second, because of results of immunodiffusion analysis, as described previously by Fernandez-Lago et al. (7). The second peak (14:18) was the NH component because it precipitated only with 10% NaCl in the RID test. The other peaks consisted of a mixture of proteins and oligonucleotides as shown by DNase, RNase, and proteinase K treatments (Fig. 1B and C). DNase and RNase treatments (Fig. 1B) reduced the contaminants in the NH peak, but it was not completely resolved from the other peaks. If DNAse and RNAse treatments were followed by proteinase K treatment (Fig. 1C), the NH peak was easily resolved from the S-LPS and other peaks. So, before ultrafiltration, the enzyme digestion of fraction 1 is recommended.

Comparison of chromatograms of B19 cell supernatant (fraction 1) and the first alcohol precipitate. The chromatogram of the first alcohol precipitate was comparable with that of fraction 1 (Fig. 1A and 2A). This fraction had, in addition to S-LPS, a large amount of NH. Fraction 1 included some peaks in the low-molecular-weight range. The enzymatic treatment of this first precipitate had the same effect as did treatment of fraction 1 (Fig. 1C and 2B), but the NH peak was more easily identifiable. Based on these observations, the removal of NH by ultrafiltration from the first precipitate, which was treated with enzymes (starting solution), was investigated. This fraction contained more NH than fraction 1.

Membrane selection for exclusion of S-LPS. A tandem assembly was devised that consisted of two ultrafiltration units placed in series, each with a different exclusion membrane. This assembly was stable under a nitrogen pressure of 11 lb/in^2. The flow rate was 38 ml/h. The different retentate

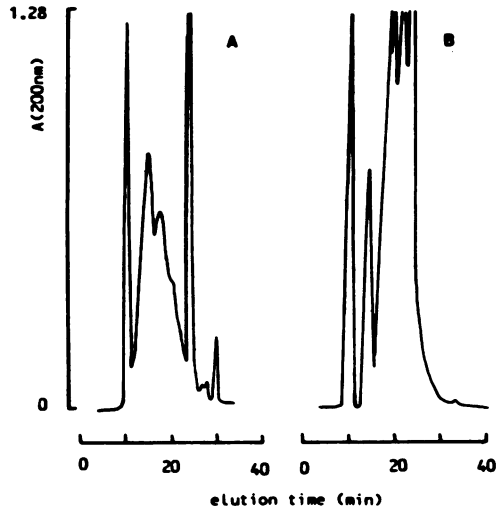

FIG. 2. Comparative chromatogram of the first precipitate prepared by the method described by Diaz et al. (4). (A) Untreated sample; (B) sample treated with DNase, RNase, and proteinase K (1 mg/ml). Chromatographic conditions were as described in the legend to Fig. 1.

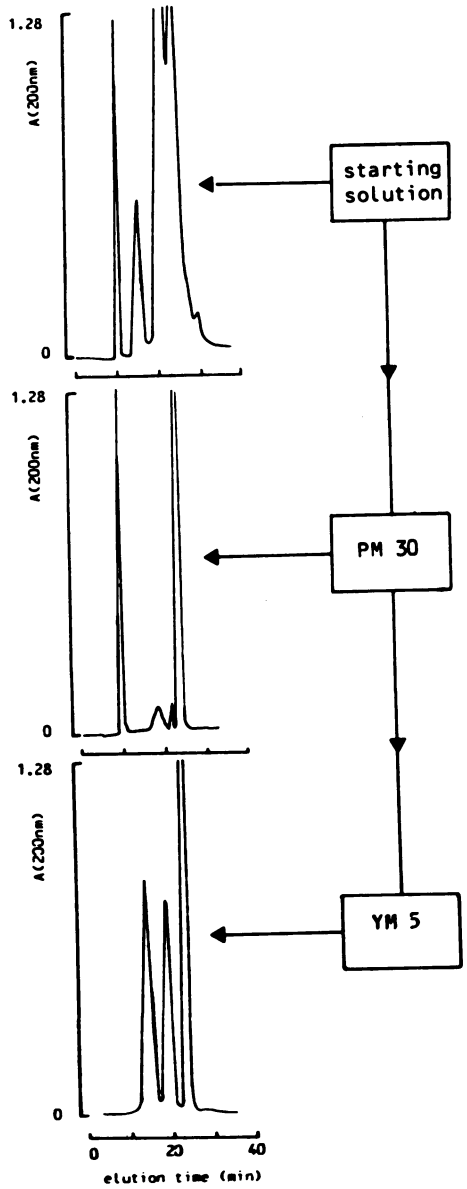

FIG. 3. Membrane separation scheme and the column (TSK G 2000 SW) separation patterns of the first precipitate (starting solution; 200 mg/400 ml). The flow rate was 38 ml/h. The chromatographic conditions were as described in the legend to Fig. 1.

were analyzed by HPLC. The XM100 membrane allowed free passage of NH, which has a molecular weight below the exclusion limit of this membrane, but the retention of S-LPS was not complete. A PM30 membrane, which has a lower

TABLE 1. Effect of membrane type on yield and retention coefficient of NH[a]

Stirred-cell system	NH yield (%) in:		Retention (%) in R2
	R1	R2	
PM30-YM10	0.17	21.5	<90
PM30-YM5	0	79	>98

[a] Abbreviations: R1, Retentate from PM30 (first cell); R2, retentate from YM10 or YM5 (second cell).

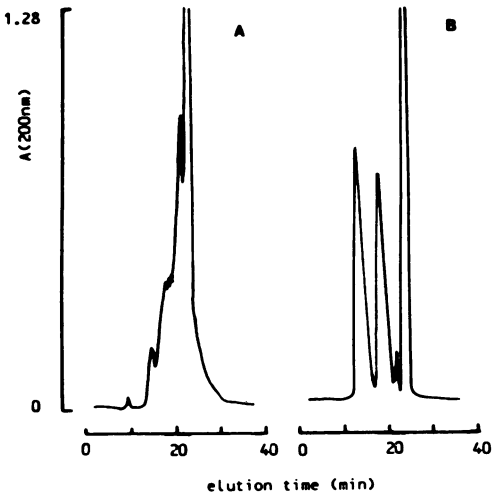

FIG. 4. Comparative chromatogram of the second precipitate prepared by the method described by Diaz et al. (4) (A) and the YM5 fraction (B). Chromatographic conditions were as described in the legend to Fig. 1.

cutoff than that of XM100, was therefore selected for further investigation. With this membrane, S-LPS was retained in the first cell (Fig. 3).

Membrane selection for NH exclusion. The YM10 membrane appeared to be permeable to NH. With this membrane, only 21.5% of the NH of the first precipitate remained in the second cell, and nothing remained in the first cell (<0.2%). This indicates that the rejection coefficient of the NH component was 90% (Table 1). To increase the yield, we chose the lower cutoff YM5 membrane in the second cell. Nearly 80% of the NH remained in the second cell, and the rejection coefficient was elevated to 98% (Table 1). The membrane system (PM30-YM5) was effective at separating S-LPS from NH, as shown by HPLC (Fig. 3). The YM5 retentate prepared by ultrafiltration was compared with the second precipitate prepared by the method described by Diaz et al. (2) (Fig. 4). There was more NH in the YM5 retentate than in the second precipitate, and there was a better separation of NH and other components. The yield of NH obtained with this ultrafiltration system was considerably increased: 80% compared with 19% for the precipitation method.

RID test. Serum samples from infected cows developed a line of precipitation corresponding to that of NH in the RID test with YM5 retentate in gelose (Fig. 5). None of the serum samples from cows vaccinated with strain B19 was positive

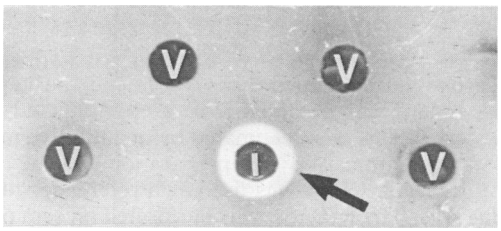

FIG. 5. RID analysis with sera from infected (I) or vaccinated (V) cows. The gel contained 10% NaCl, with YM5 retentate included at a concentration of 400 μg/ml. The ring of precipitation is indicated by an arrow.

by the RID test. The same results were observed with the reference NH.

DISCUSSION

Two haptenic preparations were used in a RID test to distinguish infected from vaccinated cattle. The first, polysaccharide B, was obtained from the trichloroacetic acid extraction of *B. melitensis* 115 rough cells (3). The second (NH) was obtained by two successive ethanol precipitations of the supernatant from smooth cells of *B. melitensis* 16M that were autoclaved and centrifuged (4). Several problems associated with the extraction procedure emerged. The first was the use of ethanol as a precipitating agent. The two precipitation steps did not permit a 100% partition of NH in the second precipitate. The second problem was that the yield of NH was low (1.5%) (4) and that the method was time-consuming (centrifugation, precipitation). In our study, the yield reached 6.06%. By the ultrafiltration technique was used to prepare purified protein derivative from mycobacteria and gave a separation comparable to that obtained by Sephadex gel filtration (1). The same technique was used to prepare a NH-enriched fraction free of S-LPS (Fig. 4), which was at least as good as the *B. melitensis* polysaccharide B used in a previous study (2). The YM5 retentate prepared from *B. abortus* B19 precipitated with sera from infected cows but not with sera from vaccinated cows. The ultrafiltration method described here offers several advantages over ethanol precipitation. First, preparation is easy to perform and no precipitating agent is used. Second, avirulent bacteria (B19 strain vaccine) were used. Third, the yield is better. Fourth, this method is more economical than previous methods. The HPLC analysis allowed rapid and effective control of the ultrafiltration steps. It could be extended to the quantitative measurement of NH. The membrane partition could be an adjunct to other preparative steps, and so fractions for further purification could be enriched by preparative chromatography. Recently, an enzyme-linked immunosorbent assay for the detection of human immunoglobulin against *B. melitensis* 16M was developed (6). NH was used as the antigen, and the purity (LPS-S-free NH) was found to be important for the maximum sensitivity and specificity of this test.

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