

Immunological Identity of *Brucella* Native Hapten, Polysaccharide B, and *Yersinia enterocolitica* Serotype 9 Native Hapten

L. FERNANDEZ-LAGO, I. MORIYON,† J. TOYOS, AND R. DIAZ*

Department of Microbiology, Faculty of Medicine, University of Navarra, Pamplona, Spain

Received 25 May 1982/Accepted 8 July 1982

Yersinia enterocolitica serotype 9 contained an antigenic component giving a reaction of total identity with *Brucella* native hapten and polysaccharide B. This component was present in a phenol-water extract (fraction 5; M. Redfearn, Ph.D. Thesis, University of Wisconsin, Madison, 1960) along with the smooth lipopolysaccharide. The native hapten could be purified free of lipopolysaccharide and proteins by gel filtration.

It has been shown that phenol-water extracts (fraction 5; M. Redfearn, Ph.D. Thesis, University of Wisconsin, Madison, 1960) of smooth *Brucella* spp. contain two different components (6). The main one has been identified as the smooth lipopolysaccharide (S-LPS) carrying antigens A and M and exhibiting a variety of biological activities (6, 12, 14). It is also responsible for the antigenic cross-reactivity between smooth *Brucella* spp. and *Yersinia enterocolitica* serotype 9 (1, 7). The second component, referred to as native hapten (NH), is a polysaccharide and lacks altogether the properties usually associated with S-LPS (15). It seems to be identical to polysaccharide B (PB) prepared from *Brucella melitensis* 115 rough cells (5).

We have previously reported that phenol-water extracts (fraction 5) of *Y. enterocolitica* serotype 9 could remove the precipitins for both NH and PB of *Brucella* spp. from sera of cattle infected with *Brucella abortus* (4). These results suggested that *Y. enterocolitica* serotype 9 could contain a component with antigenic determinants common to those in NH and PB. In the present study, we show that fraction 5 of *Y. enterocolitica* serotype 9, in addition to the S-LPS, contains a second component immunologically indistinguishable from either NH or PB of *Brucella* spp. This component can be purified free of S-LPS and proteins.

Fraction 5 was prepared from *B. melitensis* 16M and *Y. enterocolitica* serotype 9 (strain MY79) by the phenol-water extraction method developed for *Brucella* spp. by M. S. Redfearn and described by Baker and Wilson (2). PB was extracted with 0.5% trichloroacetic acid (TCA)

from cells of rough *B. melitensis* 115 as described by Diaz et al. (5). NH of *Y. enterocolitica* serotype 9 was prepared by the method described for the extraction of NH from *B. melitensis* 16M (9). Briefly, cells were suspended in distilled water, autoclaved at 121°C for 30 min, cooled, and removed by centrifugation (12,000 × g, 30 min at 5°C). The supernatant of this centrifugation was precipitated with 3 volumes of ethanol at 5°C for 18 h with continuous stirring. The resulting precipitate was removed by centrifugation, and the supernatant was precipitated further with 2 additional volumes of ethanol. The precipitate formed after this second ethanol addition constituted the crude NH. Initial purification of crude NH was achieved by gel filtration on Bio-Gel P300 (100 to 200 mesh). The crude NH (50 mg) was dissolved in borate buffer, pH 8.3, applied to a column (2.8 by 89 cm) and eluted at 20 ml/h with the same buffer. Fractions (1 ml) were collected and monitored by double-gel diffusion against serum from a cow infected with *B. abortus*. Antigen activity was found in fractions 143 to 148 and 228 to 353. Those fractions corresponding to the void volume (143 to 148) were pooled (peak 1), dialyzed, and lyophilized. The yield of lyophilized material was about 0.3 mg. Fractions from 228 to 353 (peak 2) were also pooled, dialyzed, and lyophilized. The yield of antigen was 29 mg.

Protein was determined by the method of Lowry et al. (13), using bovine serum albumin (Sigma Chemical Co.) as a standard. The method of Warren (16) was used to determine 2-keto-deoxyoctonate (KDO) with appropriate mixtures of KDO and deoxyribose as standards (Sigma). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (11), and gels were

† Present address: Department of Veterinary Sciences, College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI 53706.

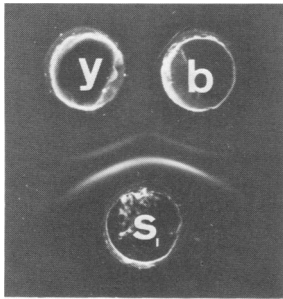


FIG. 1. Immunodiffusion analysis of phenol-water extracts (fraction 5) of *Y. enterocolitica* serotype 9 (well y) and *B. melitensis* 16M (well b).

stained with Coomassie blue. Double immunodiffusion was performed in 1% agarose in borate buffer, pH 8.3, with or without 10% NaCl. Sera from infected cattle were obtained as previously described (5).

When the fraction 5 of *Y. enterocolitica* serotype 9 was examined by double-gel diffusion in gel without 10% NaCl and sera from infected cattle, only one precipitin line was formed. This line, which corresponded to the S-LPS, gave a reaction of partial identity with the S-LPS of smooth *Brucella* spp. These results confirm previous reports of several authors who have demonstrated by gel immunodiffusion that smooth *Brucella* spp. and *Y. enterocolitica* serotype 9 share antigenic determinants in their S-LPS (3, 7, 10). However, when a gel containing 10% NaCl was used for immunodiffusion analysis, a second component giving a reaction of identity with both PB and NH of *Brucella* spp. was observed (Fig. 1). These results indicate that a component equivalent to *Brucella* NH and PB was present in the *Y. enterocolitica* serotype 9 S-LPS preparation obtained by the phenol-water method, and, as it happens with *Brucella* NH and PB, the detection of this second component with most bovine sera is enhanced with 10% NaCl in the immunoprecipitation buffer (5). The reason for this NaCl requirement is not at present well understood but it has been shown (8) that bovine immunoglobulin G₁ reacts better with antigen in hypertonic media.

Crude NH of *Y. enterocolitica* serotype 9 was fractionated on a Bio-Gel P300 column, and two peaks were obtained, the first one being eluted in the void volume. When the individual peaks were analyzed by double immunodiffusion in the gel with 10% NaCl, peak 1 developed a precipitin line that gave a reaction of total identity with *Y. enterocolitica* S-LPS. In contrast, the second peak formed a line of precipitation that gave a reaction of total identity with the NH and PB of *Brucella* spp. and the NH present in fraction 5 of *Y. enterocolitica* and *Brucella* spp. (Fig. 2).

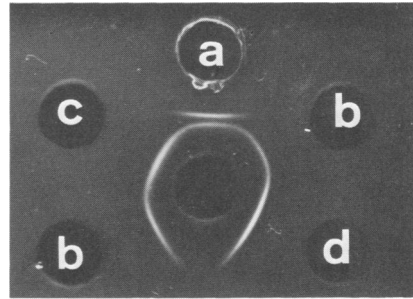


FIG. 2. Immunodiffusion of phenol-water extracts (fraction 5) (well a) of *Y. enterocolitica* serotype 9 reacted with sera from cattle (central well). Well b contained NH of *Y. enterocolitica* serotype 9, well c contained NH of *B. melitensis* 16M, and well d contained PB of *B. melitensis* 115.

The results of chemical analysis of *Y. enterocolitica* NH (peak 2) showed that no S-LPS was present as judged by KDO measurements and immunodiffusion analysis, although 22.9% of the protein was still in it.

Once the results of the chemical analysis were known, the second peak was further treated with TCA as follows. The dried material was suspended in distilled water (2 mg/ml), and TCA was added to a final concentration of 30%. The precipitate formed was discarded, and the supernatant was dialyzed and lyophilized. The final yield varied from 40 to 60% when referred to the weight of the starting material. After precipitation with TCA, the NH was free of contaminating proteins as determined by chemical analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

This work was supported by grant 4411 from the Comisión Asesora de Investigación Científica y Técnica del Ministerio de Universidades e Investigación, Spain.

LITERATURE CITED

1. Ahvonen, P., E. Jansson, and K. Aho. 1969. Marked cross-agglutination between *Brucella* and a subtype of *Yersinia enterocolitica*. *Acta Pathol. Microbiol. Scand.* 75:291-295.
2. Baker, P. J., and J. B. Wilson. 1965. Chemical composition and biological properties of the endotoxin of *Brucella abortus*. *J. Bacteriol.* 90:895-902.
3. Corbel, M. J., and G. A. Cullen. 1970. Differentiation of the serological response to *Yersinia enterocolitica* (serotype IX) and *Brucella abortus* in cattle. *J. Hyg.* 68:519.
4. Díaz, R., and N. Bosseray. 1974. Estudio de las relaciones antigénicas entre *Yersinia enterocolitica* serotipo IX y otras especies bacterianas Gram-negativas. *Microbiol. Esp.* 27:1-14.
5. Díaz, R., P. Garatea, L. M. Jones, and I. Moriyón. 1979. Radial immunodiffusion test with a *Brucella* polysaccharide antigen for differentiating infected from vaccinated cattle. *J. Clin. Microbiol.* 10:37-41.
6. Díaz, R., L. M. Jones, D. Leong, and J. B. Wilson. 1968. Surface antigens of smooth brucellae. *J. Bacteriol.* 96:893-901.
7. Díaz, R., R. Lacalle, M. P. Medrano, and D. Leong. 1970. Immunobiological activities of the endotoxin from *Yer-*

- sinia enterocolitica* strain MY79. Proc. 5th Int. Congr. Infect. Dis. Vienna 2:11-17.
8. **Díaz, R., and D. Levieux.** 1972. Rôle repectif en sérologie de la Brucellose bovine des antigènes et des immunoglobulines G₁ et G₂ dans les tests d'agglutination, de Coombs et au Rose Bengale ainsi que dans le phénomène de zone. C.R. Acad. Sci. 274:1593-1596.
 9. **Díaz, R., J. Toyos, M. D. Salvo, and M. L. Pardo.** 1981. A simple method for the extraction of polysaccharide B from *Brucella* cells for use in the radial immunodiffusion test diagnosis of bovine Brucellosis. Ann. Rech. Vet. 12:35-39.
 10. **Hurvell, D., and A. A. Lindberg.** 1973. Serological cross-reactions between different *Brucella* species and *Yersinia enterocolitica*. An immunological and immunochemical study. Acta Pathol. Microbiol. Scand. Sect. B 81:113-119.
 11. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. Nature (London) 227:680-685.
 12. **Leong, D., R. Díaz, K. Milner, J. Rudbach, and J. B. Wilson.** 1970. Some structural and biological properties of *Brucella* endotoxin. Infect. Immun. 1:174-182.
 13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement by the Folin phenol reagent. J. Biol. Chem. 193:265-175.
 14. **Moreno, E., D. T. Berman, and L. A. Boettcher.** 1981. Biological activities of *Brucella abortus* lipopolysaccharides. Infect. Immun. 31:362-370.
 15. **Moreno, E., S. L. Speth, L. M. Jones, and D. T. Berman.** 1981. Immunochemical characterization of *Brucella* lipopolysaccharides and polysaccharides. Infect. Immun. 31:214-222.
 16. **Warren, L.** 1959. The thiobarbituric acid assay of sialic acids. J. Biol. Chem. 234:1971-1975.