INFECTION AND IMMUNITY, Mar. 1988, p. 716–718
0019-9567/88/030716-03$02.00/0

Vol. 56, No. 3

Serological Response to the Outer Membrane Lipoprotein in Animal Brucellosis

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Received 26 May 1987/Accepted 28 November 1987

The presence of antibodies to *Brucella* outer membrane lipoprotein was investigated in cattle and rams. Low but significant amounts of antibody were detected in sera from *B. abortus*-infected cattle and from *B. ovis*-infected rams which had developed epididymitis. Strain-19-vaccinated cattle also showed a weak albeit transient antibody response.

The outer membrane (OM) of *Brucella* spp. is composed of lipopolysaccharide (LPS), phospholipid, and several major proteins (8, 15, 17, 21), including a lipoprotein (LPP) which bears an overall biochemical resemblance to the peptidoglycan-linked LPP of *Escherichia coli* (9, 10). However, in contrast to the *E. coli* LPP, which is not exposed on the surface of smooth cells, evidence has been presented showing the presence of antigenic determinants of *Brucella* LPP on the surface of both *Brucella abortus* and *B. melitensis* (10). The antibody response to LPS has been studied extensively, and it has been shown that the polysaccharide moiety of this molecule bears the main antigenic determinants involved in the standard serological tests for smooth brucellae (3, 5). In contrast, the immunogenic role of *Brucella* OM proteins in natural infection is largely unknown. In the present study, we report results concerning the antibody response to *Brucella* LPP in vaccinated and naturally infected animals.

The 8-kilodalton fragment of the LPP was prepared from the rough strain *B. abortus* 45/20 by exhaustive sodium dodecyl sulfate extraction of cell envelopes, trypsin digestion, and gel filtration under conditions described previously (9). The absence of contaminants such as proteins or LPS was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, isolectric focusing, lack of reactivity with anti-rough LPS in immunoenzymatic tests, lack of ability to elicit anti-LPS antibodies, and lack of hydroxylated fatty acids (9, 10). We have shown that the LPP fragment obtained from *B. abortus* 45/20 cross-reacts with the LPPs of *B. ovis* and *E. coli* (10). Smooth LPS (fraction 5) was prepared as described elsewhere (14) and partially purified by digestion with proteinase K (E. Merck, Darmstadt, Federal Republic of Germany) and by ultracentrifugation. Rough LPS was extracted as described by Galanos et al. (6) and coupled to bovine serum albumin to make it soluble (7). Antibody specific for the LPP was measured in an enzyme-linked immunosorbent assay. Polystyrene plates were coated by overnight incubation of the corresponding antigen in carbonate buffer (LPP at 5 μg/ml; rough and smooth LPSs at 5 and 2.5 μg/ml, respectively). We have shown that under those conditions the 8-kilodalton LPP fragment binds efficiently to polystyrene plates (10). The assay was done as described elsewhere (13) with peroxidase-conjugated goat anti-cow immunoglobulin G (IgG; heavy and light chain specific) or peroxidase-conjugated rabbit anti-sheep IgG (heavy and light chain specific; Nordic Immunological Laboratories, Tilburg, The Netherlands), and the reaction was assessed colorimetrically in a Titertek Multiscan spectrophotometer (Flow Laboratories, Inc., McLean, Va.). For each antigen and animal species, it was determined which dilution of the sera showed the greatest difference between the average optical density readings of positive and negative controls (see below). The mean and standard deviation of the optical density readings of this dilution were calculated for each group of sera (see below) and used for statistical analysis (11). The Tukey test was used to analyze the differences between the results obtained with the same antigen (19). Bacteriological methods and standard serological tests for *B. abortus* and *B. ovis* are described elsewhere (1, 12).

The serological response to the LPP was studied first in cattle infected with *B. abortus* or vaccinated with the standard dose of the attenuated strain 19. The sera analyzed belonged to three different groups: (i) 15 negative controls (healthy *Brucella* sp.-free heifers), (ii) 15 cows with milk cultures positive for *B. abortus*, and (iii) 15 calves vaccinated with *B. abortus* 19 and bled at 15 days, 4 months, and 1 year after vaccination; the last 15 animals were maintained in a *Brucella* sp.-free herd throughout the experiment. The results of this first set of experiments are presented in Table 1. The levels of anti-LPP antibody in the infected animals were significantly higher than in the healthy animals. With respect to the vaccinated animals, there were significant anti-LPP antibody levels only 4 months after vaccination. The assays performed with smooth LPS showed that there was always a more intense response to this surface antigen (Table 1).

We reported previously that rams infected by *B. ovis* develop a serological response to group 2 OM major proteins as intense as the one directed to the rough LPS (16). To determine whether this was also true for the LPP, we measured the levels of antibody specific to the LPP and the rough LPS in three groups of rams: (i) 15 healthy rams as negative controls, (ii) 10 rams bacteriologically and serologically positive for *B. ovis*, and (iii) 30 animals positive in the standard serological tests for *B. ovis*, including 15 that presented epididymitis and 15 that had not developed palpable lesions at the time of bleeding. No bacteriological analysis could be performed with the last group of 30 animals.

The results obtained with the ram sera are summarized in Table 2. There were significant differences between the healthy and the *B. ovis*-infected rams (infection demonstrated bacteriologically). In addition, since epididymitis develops late after infection, a more detailed study on the development of the response could be made with the 30

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TABLE 1. Analysis by enzyme-linked immunosorbent assay of the levels of antibody specific for the LPP or the smooth LPS in cattle

Antigen	Antibody level (mean OD ± SD) in[a]:				
	Healthy cattle	Infected cattle	Cattle vaccinated and bled after:		
			15 days	4 mo	1 yr
LPP	0.191 ± 0.064	0.305 ± 0.122*	0.194 ± 0.080	0.280 ± 0.134*	0.202 ± 0.146
Smooth LPS	0.121 ± 0.067	1.011 ± 0.047*	0.563 ± 0.244*	0.535 ± 0.168*	0.299 ± 0.193

[a] Figures are from the dilution showing the greatest difference between healthy and infected animals (1:400). *, Values significantly different ($P < 0.05$) from those for the healthy controls. OD, Optical density.

TABLE 2. Analysis by enzyme-linked immunosorbent assay of the levels of antibody specific for the LPP or the rough LPS in rams

Antigen	Antibody level (mean OD ± SD) in[a]:			
	Healthy rams	Infected rams	Seropositive rams	
			With epididymitis	Without epididymitis
LPP	0.178 ± 0.082	0.340 ± 0.237*	0.284 ± 0.099*	0.136 ± 0.151
Rough LPS	0.412 ± 0.150	0.889 ± 0.135*	0.754 ± 0.754*	0.664 ± 0.182*

[a] Figures are from the dilution showing the greatest difference between healthy and infected animals (1:40 and 1:100 for the LPP and the LPS, respectively). *, Values significantly different ($P < 0.05$) from those for the healthy controls. OD, Optical density.

seropositive rams. The results showed that although in the rams without symptoms the antibody response was similar to that of the negative controls, it was significantly higher in the rams with epididymitis (Table 2). However, even in the subgroup with epididymitis, the serological response to the LPP in rams was not important compared with that elicited by the rough LPS (Table 2).

We have shown previously that there is a serological cross-reaction between the LPPs of *Brucella* spp. and *E. coli* when they are examined with sera from hyperimmunized rabbits (10). However, it is very unlikely that the anti-*Brucella* LPP antibody detected in both rams and cattle could be the result of antigenic stimuli by *E. coli*, because we would have to admit that undetected *E. coli* infections occurred more often in the *Brucella* sp.-infected animals than in the *Brucella* sp.-free ones. Moreover, there was an overall correlation between the magnitude of the LPP and LPS readings in both rams and cattle, an observation that demonstrates that the anti-LPP antibody relates to *Brucella* infection and not to inapparent infections due to other gram-negative bacteria. It has to be stressed that LPS contamination of the LPP could not be detected by either biochemical or immunoenzymatic methods (9, 10).

In addition to the LPS, soluble proteins also stimulate an important serological response in cattle infected by *Brucella* spp. (5, 18, 20). Likewise, in patients afflicted with brucellosis, there is an antibody response to both LPS and soluble proteins (4, 5). In rams infected by the rough species *B. ovis*, the serological response is directed not only to the rough LPS and soluble proteins, but also to OM proteins of group 2 (16). In contrast to soluble proteins and LPS, the observations reported here demonstrate that the LPP plays a minor role as an antigen during infection, either in cattle or in rams. Moreover, the data obtained with the vaccinated cattle and the rams with and without epididymitis suggest that a prolonged antigenic stimulus is necessary for the development of the antibody response. This would also explain the negative results obtained with a limited number of sera from patients with acute brucellosis (M. J. Gómez-Miguel, I. Moriyón, and R. Díaz, unpublished results), since effective antibiotic therapy precludes such prolonged stimulus.

To the best of our knowledge, this is the first report on the serological response to the OM LPP of a bacterial pathogen. Comparative studies with infections by other gram-negative bacteria would clarify whether the low response to the LPP pertains to the characteristics of *Brucella* infection or, on the other hand, if it relates to the intrinsic properties of this low-molecular-weight protein. Since the purified 8-kilodalton LPP fragment is poorly immunogenic in laboratory animals (2, 9; unpublished results), we currently favor the second hypothesis.

This investigation was supported in part by the Comisión Asesora de Investigación Científica y Tecnológica of Spain (grant 2910/83) and by the U.S.A.-Spanish Joint Committee on Scientific and Technical Cooperation (grant CCA-8510/085). Fellowship support for M. J. Gómez-Miguel from the Fondo de Investigaciones Sanitarias de la Seguridad Social is also acknowledged.

LITERATURE CITED

1. **Alton, G. C., L. M. Jones, and D. E. Pietz.** 1975. Laboratory techniques in brucellosis. W.H.O. Monogr. Ser. **55**:73–79.
2. **Bosch, V., and V. Braun.** 1975. Distribution of murein-lipoprotein between the cytoplasmic and outer membrane of *Escherichia coli*. FEBS Lett. **34**:307–310.
3. **Díaz, R., and D. Levieux.** 1972. Rôle respectif en sérologie de la brucellose bovine des antigènes et des immunoglobulines G_1 et G_2 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.
4. **Díaz, R., E. Maraví-Poma, and A. Rivero.** 1976. Comparison of counter-immunoelectrophoresis with other serological tests in the diagnosis of human brucellosis. Bull. W.H.O. **53**:417–424.
5. **Dubray, G.** 1985. Antigens of diagnostic significance in *Brucella*, p. 123–138. *In* J. M. Verger and M. Plommet (ed.), *Brucella melitensis*, a CEE seminar. Martinus Nijhoff Publishing, The Hague.
6. **Galanos, C., O. Lüderitz, and O. Westphal.** 1969. A new method for the extraction of R lipopolysaccharide. Eur. J. Biochem. **9**:245–249.
7. **Galanos, C., E. T. Rietschel, O. Lüderitz, and O. Westphal.** 1972. Biological activities of lipid A complexed with bovine serum albumin. Eur. J. Biochem. **31**:230–233.
8. **Gamazo, C., and I. Moriyón.** 1987. Release of outer membrane fragments by exponentially growing *Brucella melitensis* cells. Infect. Immun. **55**:609–615.

9. **Gómez-Miguel, M. J., and I. Moriyón.** 1986. Demonstration of peptidoglycan-linked lipoprotein and characterization of its trypsin fragment in the outer membrane of *Brucella* spp. Infect. Immun. **53**:678–684.
10. **Gómez-Miguel, M. J., I. Moriyón, and J. López.** 1987. *Brucella* outer membrane lipoprotein shares antigenic determinants with *Escherichia coli* Braun lipoprotein and is exposed on the cell surface. Infect. Immun. **55**:258–262.
11. **Heck, F. C., J. D. Williams, and J. Pruett.** 1980. Interpretation of spectrophotometric absorbance values to define results of enzyme-linked immunosorbent assays. J. Clin. Microbiol. **11**:398–401.
12. **Jones, L. M., G. Dubray, and J. Marly.** 1975. Comparison of methods of diagnosis of *Brucella ovis* infection of rams. Ann. Rech. Vet. **6**:11–12.
13. **Lamb, V. L., L. M. Jones, G. Schurig, and D. T. Berman.** 1979. Enzyme-linked immunosorbent assay for bovine immunoglobulin subclass-specific response to *Brucella abortus* lipopolysaccharides. Infect. Immun. **26**:240–247.
14. **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.
15. **Moriyón, I., and D. T. Berman.** 1982. Effects of nonionic, ionic, and dipolar ionic detergents and EDTA on the *Brucella* cell envelope. J. Bacteriol. **152**:822–828.
16. **Riezu-Boj, J. I., I. Moriyón, J. M. Blasco, C. Marín, and R. Díaz.** 1986. Comparison of lipopolysaccharide and outer membrane protein-lipopolysaccharide extracts in an enzyme-linked immunosorbent assay for the diagnosis of *Brucella ovis* infection. J. Clin. Microbiol. **23**:938–942.
17. **Santos, J. M., D. R. Verstreate, V. Y. Perera, and A. J. Winter.** 1984. Outer membrane proteins from rough strains of four *Brucella* species. Infect. Immun. **46**:188–194.
18. **Schurig, G. G., L. M. Jones, S. L. Speth, and D. T. Berman.** 1978. Antibody response to antigens distinct from smooth lipopolysaccharide complex in *Brucella* infection. Infect. Immun. **21**:994–1002.
19. **Sokal, R. R., and F. J. Rohlf.** 1969. Biometry: the principles and practice of statistics in biological research, p. 417–421. W. H. Freeman and Co., San Francisco.
20. **Stemshorn, B., and K. Nielsen.** 1977. The bovine immune response to *Brucella abortus*. I. A water-soluble antigen precipitated by sera of some naturally infected cattle. Can. J. Comp. Med. **41**:152–159.
21. **Verstreate, D. R., M. T. Creasy, N. T. Caveney, C. L. Baldwin, M. W. Blab, and A. J. Winter.** 1982. Outer membrane proteins of *Brucella abortus*: isolation and characterization. Infect. Immun. **35**:979–989.