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Enzyme-Linked Immunosorbent Assay with *Brucella* Native Hapten Polysaccharide and Smooth Lipopolysaccharide

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Brucella melitensis **native haptens (NH) are polysaccharides identical to the O-side chain of the smooth lipopolysaccharide (S-LPS) (E. Moreno, H. Mayer, and I. Moriyón, Infect. Immun. 55:2850–2853, 1987) which precipitate with sera from infected cattle but not from strain 19-vaccinated cattle. In the present work, NH was extracted by the hot-water method (R. Díaz, J. Toyos, M. D. Salvo, and M. L. Pardo, Ann. Rech. Vet. 12:35–39, 1981) and purified free of S-LPS and protein. Purified NH lacked the ability to coat polystyrene and sheep erythrocytes. In contrast, NH acylated with stearoyl chloride bound to both polystyrene and erythrocytes. By hemagglutination and enzyme-linked immunosorbent assay (ELISA), S-LPS and acylated NH gave similar results with blood sera from brucellosis-free, strain 19-vaccinated, and infected cattle. Moreover, a significant correlation between the results of NH ELISA and S-LPS ELISA was demonstrated with milk sera. However, in a competitive ELISA with milk sera, S-LPS in the liquid phase abrogated the binding of antibodies to acylated NH adsorbed to polystyrene, while NH in the liquid phase did not influence the binding of antibodies to polystyrene-adsorbed S-LPS. It is hypothesized that the different precipitations of NH and S-LPS with sera from infected or strain 19-vaccinated cattle are due to differences in the affinity of the antibodies produced upon vaccination or infection and in the physical state of aggregation of NH and S-LPS in aqueous solutions.**

The smooth lipopolysaccharide (S-LPS) of *Brucella abortus* elicits a vigorous antibody response in infected and strain 19-vaccinated cattle (6, 9, 13, 30). This S-LPS is the major molecule of the cell surface (7, 13), and since standard serological tests use suspensions of smooth bacteria, it is the antibody response to the S-LPS that is usually measured (9, 20). Consequently, it is often difficult to know whether a positive result in such tests is due to infection or to vaccination with strain 19. To solve this problem, alternative tests and antigens other than the S-LPS have been investigated (13, 34), and it has been found that the polysaccharide haptens of *B. melitensis* are precipitated by sera from infected cattle but not by sera from vaccinated cattle bled as early as 2 months after vaccination (3, 6, 10, 12, 17, 21, 30). In contrast, precipitins to the S-LPS persist longer after vaccination.

The native polysaccharide hapten (NH) of *B. melitensis* has been shown to be identical to the O-side chain of the homologous S-LPS both by chemical and immunological means (4, 5, 11, 27–29). This identity implies that the antigenic determinants of NH should be contained in the S-LPS, and, therefore, the different reactivities of NH and S-LPS in precipitation tests with sera from vaccinated animals is puzzling. To reexamine this problem, we have developed an enzyme-linked immunosorbent assay (ELISA) with acylated NH adsorbed to the solid phase and have performed a comparative study with the homologous S-LPS.

MATERIALS AND METHODS

Bacterial strains and growth. Strains 115 (rough, avirulent) and 16M (smooth, virulent) of *B. melitensis* used in this work have been described previously (8). To assure the smoothness of *B. melitensis* 16M, the strain was inoculated into guinea pigs, recovered from the spleens 7 days later, and stored in skim milk at −75°C. The conditions of cell propagation and inactivation (strain 16M) with phenol have been described elsewhere (12).

Extraction and purification of NH. Saline-washed *B. melitensis* 16M cells were extracted with hot water at 120°C (12), and the extract was precipitated first with 3 volumes of ethanol at 4°C for 18 h with continuous stirring. The precipitate was collected (5,000 × *g* for 15 min at 4°C), dialyzed, and freeze-dried. This first precipitate contains S-LPS, NH, nucleic acids, and denatured proteins and, by double gel diffusion, shows the precipitin lines of S-LPS and NH (12). The supernatant fluid obtained after the first precipitation was mixed with 2 more volumes of ethanol (−20°C overnight), and the second precipitate was collected (5,000 × *g*, 15 min, 4°C), dialyzed, and freeze-dried. This second precipitate is enriched in NH and, although suitable for precipitation tests (12), contains trace amounts of S-LPS which would interfere in the ELISA. To purify the NH, 100 mg of the second precipitate was dissolved in 50 ml of 4.0% sodium dodecyl sulfate and incubated in a water bath at 80°C for 15 min. The sodium dodecyl sulfate-protein and sodium dodecyl sulfate-S-LPS complexes which formed were removed by the addition of potassium acetate (5.0% final concentration), followed by incubation at 4°C for 12 h and centrifugation (5,000 × *g*, 10 min, 4°C). The supernatant was then mixed with 5 volumes of ethanol, and the precipitate was suspended in 150 ml of distilled water, treated batchwise twice with 4 g of Dowex 1X4-200 (Sigma Chemical Co., St. Louis, Mo.), dialyzed, and freeze-dried. The freeze-dried product was dissolved in 10 mM borate-NaOH (pH 8.3), filtered, and chromatographed at 10 ml/h through a Bio-Gel P-300 column (70.0 by 2.6 cm) (Bio-Rad Laboratories, Richmond, Calif.) equilibrated in borate buffer. Fractions (2.5 ml) were collected and tested by double gel diffusion in 10% NaCl agarose-borate (6). The void volume fractions showed the precipitin line of the S-LPS and a faint line similar to that of the NH. The fractions of a second peak (Kav, 0.35)

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containing only NH were pooled, precipitated with 5 volumes of ethanol, dialyzed, and freeze-dried.

Acylation of purified NH. The method of Hämmerling and Westphal was used (18). Fifty milligrams of purified NH was dissolved in 4.0 ml of dimethylformamide at 50°C. To this solution, 0.6 ml of pyridine and 10 mg of stearoyl chloride (Fluka AG, Buchs, Switzerland) in 0.1 ml of dimethylformamide were added; the atmosphere was replaced by N_2, and the reaction vessel was sealed and kept at room temperature in the darkness for 3 days. After this time, 0.5 ml of water was added, and the mixture was precipitated with 25 ml of ethanol at −20°C for several hours. Finally, the precipitate was dialyzed and freeze-dried.

LPS preparations. *B. melitensis* 115 rough LPS (R-LPS) was extracted from acetone-dried cells by the petroleum ether-chloroform-phenol method (15); these extracts contained less than 1% protein. For ELISAs, R-LPS was coupled to bovine serum albumin (16).

S-LPS (fraction 5) was obtained from the phenol phase after water-phenol extraction of *B. melitensis* 16M cells (24) and was partially purified by digestion with proteinase K (E. Merck AG, Darmstadt, Federal Republic of Germany) and ultracentrifugation.

Analytical methods. Standard colorimetric methods were used to determine total protein (25) and fatty acid (19), with bovine serum albumin or stearic acid as the respective standards. The thiobarbituric acid method (36) was used for 2-keto-3-deoxyoctulosonic acid with the pure sugar and deoxyribose as standards. Polyacrylamide gel electrophoresis in sodium dodecyl sulfate and Coomassie blue and alkaline-silver stainings were performed as described elsewhere (22, 26). High-performance liquid chromatography on size-exclusion columns (TSK G 2000 SW) was kindly performed by G. Dubray (Institut National de la Recherche Agronomique, Station de Patologie de la Reproduction, Nouzilly, France) as described previously (37).

ELISA. Stock solutions (1 mg/ml) of S-LPS, NH, and acylated NH were made in distilled water and stored at −20°C. Solutions of bovine serum albumin–R-LPS were prepared directly in coating buffer. Coating solutions were made in 60 mM carbonate (pH 9.6) (1 to 2.5 μg/ml, 2.5 μg/ml, and 10.0 μg/ml for NH, S-LPS, and R-LPS, respectively), dispensed in the wells, and incubated overnight at 37°C. The assay was performed as described by Voller et al. (35) with peroxidase-conjugated rabbit anti-bovine immunoglobulin G or goat anti-rabbit immunoglobulin G (both of heavy- and light-chain specificity; Nordic Immunological Laboratories, Tilburg, Holland) and H_2O_2–0.09% 5-amino-2-hydroxybenzoic acid (pH 6.0) as substrate. The reaction was assessed colorimetrically at 450 nm (Titertek Multiscan; Flow Laboratories, Inc., McLean, Va.). Sera from healthy unvaccinated controls and infected controls (groups 1 and 4; see below) were used to find which dilution showed the greatest average difference in optical density (OD) between both sets of controls (1:400 to 1:800 under the conditions used). The OD of such a dilution was also measured in other groups.

Inotech ELISA plates (Bioreba, Basel, Switzerland) were used in most of the work. Other polystyrene plates also tested for adsorption of NH were the Limbro EIA microtitration plate (Flow Laboratories, Ltd., Irvine, Ayrshire, Scotland), the Cooke Microtiter (Dynatech Laboratories, Inc., Alexandria, Va.), the Microtest 96 (Nunc, Roskilde, Denmark), and the Serocluster EIA plate (Costar, Cambridge, Mass.).

Competition between NH and S-LPS in ELISA. After the plates were coated with either S-LPS or acylated NH, 50 μl of a solution of the heterologous molecule (200 μg/ml) and 50 μl of the corresponding serum dilution were added to the wells. Thus, incubation with the antibody was performed in the presence of both the adsorbed antigen (S-LPS or acylated NH) and an excess of unbound heterologous antigen (purified NH or S-LPS, respectively). The remaining steps were performed as in the standard indirect ELISA.

Other serological tests. Precipitation in 10% NaCl agarose gels and hemagglutination tests were performed as described elsewhere (6, 12, 19).

Sera. The following groups of bovine blood sera were used: group 1, sera of 24 unvaccinated animals from a brucella-free herd; group 2, sera from 16 calves vaccinated conjunctivally with the standard dose of strain 19 and bled 1, 2, and 3 months after vaccination; group 3, sera from 44 calves vaccinated subcutaneously with the standard dose of strain 19 and bled a year later; and group 4, sera of 108 cattle that tested positive in rose bengal and complement fixation tests (1) and from whose milk *B. abortus* had been isolated. In addition, milk sera from 88 animals of group 4 were also used.

Antibodies to the R-LPS were produced in rabbits by intravenous infection with 10^{10} viable *B. melitensis* 115 cells. Rabbits were bled 20 days later.

RESULTS

NH characterization. No 2-keto-3-deoxyoctulosonic acid, protein, or fatty acid could be detected by colorimetric methods in purified NH preparations. Moreover, in contrast with controls performed with S-LPS, Coomassie blue or silver staining of polyacrylamide gels loaded with purified NH did not detect any material in the gels. High-performance liquid chromatography of representative preparations showed (220 nm) a single sharp peak which contained over 99.0% of the material and had a retention time of 23.46 min (retention time of S-LPS was 9.61 min). Finally, purified NH gave a single precipitin line with sera from infected cattle (group 4) but did not precipitate with any sera from vaccinated animals bled 2 months after vaccination (group 3).

Acylation of purified NH with stearoyl chloride resulted in a material (acylated NH) containing between 0.7 and 2.1% fatty acid. Like purified NH, acylated NH was also precipitated with sera from the infected animals but not with sera from the vaccinated animals. By ELISA (see below), acylated NH did not react with sera from *B. melitensis* 115-infected rabbits, although these sera reacted strongly with bovine serum albumin-coupled R-LPS.

Adsorption of purified NH to polystyrene and erythrocytes. The adsorption of NH and acylated NH was compared by using the sera of healthy unvaccinated animals and infected animals. Purified NH did not absorb to polystyrene since, after incubation of the plates with concentrations of NH as high as 20 μg/ml in coating buffer, similar background OD readings were obtained with negative and positive sera. In contrast, 2.5 μg of acylated NH per ml in coating buffer (preparations with 1.5 to 2.0% stearate) gave results similar to those obtained with the same concentration of S-LPS, with both brucella-exposed and healthy unvaccinated animals (Table 1). In the hemagglutination test, erythrocytes treated with acylated NH at 10 μg/ml were agglutinated by sera from infected and vaccinated cattle, while erythrocytes treated with NH were not (maximal NH concentration tested was 100 μg/ml).

Reactivity of sera from infected or strain 19-vaccinated cattle with acylated NH and S-LPS. As expected, the sera of

TABLE 1. Analysis by ELISA of the levels of antibody specific for S-LPS and NH in cattle

Antigen	Antibody level (mean OD ± SD)[a] in cattle					
	Healthy	Infected	Vaccinated and bled after:			
			1 mo	2 mo	3 mo	1 yr
S-LPS	0.121 ± 0.067	1.011 ± 0.047	0.623 ± 0.217	0.557 ± 0.266	0.412 ± 0.176	0.299 ± 0.193
NH	0.210 ± 0.068	0.947 ± 0.068	0.596 ± 0.218	0.488 ± 0.243	0.411 ± 0.194	0.263 ± 0.169

[a] OD readings from the dilution showing the greatest difference between healthy and infected animals (1:400). SD, Standard deviation.

conjunctivally vaccinated animals which were bled between 15 days and 4 months after vaccination had levels of antibodies to the S-LPS well above the background (healthy unvaccinated controls) (Table 1). A year after subcutaneous vaccination, the OD readings obtained with S-LPS were very low. In all groups of sera, the ELISA with acylated NH gave results close to those obtained with the S-LPS (Table 1). In spite of this, the sera of vaccinated animals precipitated the S-LPS but not the NH (Fig. 1), whereas the sera from most infected animals precipitated both the NH and the S-LPS (Fig. 1). A few sera of infected animals precipitated only the NH (Fig. 1). However, these few sera had antibodies which reacted with the S-LPS in ELISA (average OD reading, 0.823).

Correlation between the antibody responses to S-LPS and to NH in blood and milk sera. The OD readings obtained with acylated NH and S-LPS and blood sera from infected animals showed no significant correlation ($P < 0.05$) (Fig. 2A). Since milk tests (such as the ring test) also detect antibodies to S-LPS, the ELISA with acylated NH and S-LPS was also performed with milk sera from infected animals. The analysis of these new data (Fig. 2B) showed a significant correlation between the OD readings of NH and S-LPS ($P < 0.01$).

ELISA with R-LPS. The sera from *B. melitensis* 115-infected rabbits did not react with acylated NH in ELISA, a result showing that this R-LPS would detect exclusively antibodies to core-lipid A antigenic determinants when used with cattle sera. The results obtained with blood sera of infected cattle showed antibody levels corresponding to an average OD of 0.592 (standard deviation, 0.138) for the 1:400 dilution. Lower levels of antibodies to core-lipid A determinants were found in milk sera (OD, 0.176 ± 0.031 for the 1:400 dilution).

Competition between NH and S-LPS for antibodies reacting with common antigenic determinants. Since milk sera had lower levels of antibody to LPS determinants not present in the NH, they were used in these experiments. As expected, incubation with an excess of either free S-LPS or free NH completely removed the antibodies reacting with the homologous molecule adsorbed to polystyrene. On the other hand, while an excess of free S-LPS removed the antibodies reacting with the NH adsorbed to polystyrene, an excess of free NH did not reduce the binding of antibodies to polystyrene-adsorbed S-LPS.

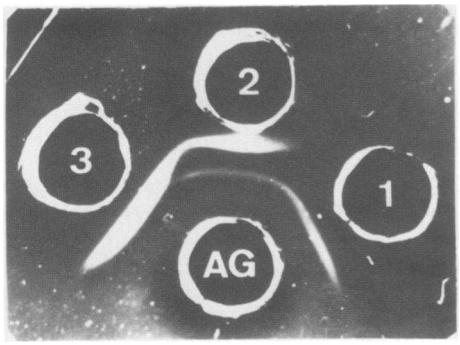

FIG. 1. Double gel diffusion analysis of representative sera from strain 19-vaccinated (well 1) and *B. abortus*-infected (well 2) cattle. Well 3 contained the serum of an infected animal which precipitated only the NH. The antigen (AG) used was the first ethanol precipitate of *B. melitensis* 16M heat extract (S-LPS plus NH) at 5 mg/ml.

DISCUSSION

The data presented here show that while S-LPS or acylated NH bound efficiently to polystyrene or erythrocytes, purified NH did not, a result in contrast to those of other works in which adsorption of NH to polystyrene has been reported (15, 29). In the gel filtration step, we observed that a fraction of NH (as defined by gel precipitation) eluted in the void volume associated with the S-LPS, far apart from the purified NH (Kav, 0.35). This same observation has been made before (11, 28), and it has been reported that such LPS-associated NH has small amounts of lipid and that it adsorbs to plastic and erythrocytes (28). Thus, it seems likely that differences in the methods of extraction and purification could account for those discrepancies. Contamination of NH preparations with S-LPS is also a possible explanation, since S-LPS binds readily to polystyrene and our results show that it cannot be distinguished from NH by ELISA.

Acylated NH retained the ability to react with specific antibody. It has been shown that purified *B. melitensis* 16M NH is made up only of *N*-formyl perosamine (4,6-dideoxy-4-formamido-α-D-mannopyranose) with C-1,2 and C-1,3 linkages (27, 28). Accordingly, this NH has unsubstituted hydroxyl groups that can be acylated and, in addition, only a few antigenic determinants, all of them repeated many times along the polymer. These features and the relatively low (up to 2.1%) degree of acylation achieved under the conditions described explain why the antibody reactivity of NH was not destroyed.

The observation that cattle vaccinated with strain 19 seldom show precipitating antibodies to the NH 3 months after vaccination has been used to develop a radial immunodiffusion test for the differential diagnosis of cattle brucellosis (3, 6, 10–12, 17, 21, 30). However, the results of both hemagglutination and ELISA with acylated NH demonstrate that vaccinated animals produce antibodies to the NH. Furthermore, the average ELISA OD readings of S-LPS and NH were similar for the different groups of cattle tested, and there was significant correlation between the responses to NH and S-LPS in milk sera. Statistically significant correlation was not observed in blood sera, suggesting that in a given individual the relationship between NH and S-LPS in blood sera is less predictable. However, it is obvious that any explanation of the results of the correlation analyses has to take into account the fact that S-LPS contains antigenic

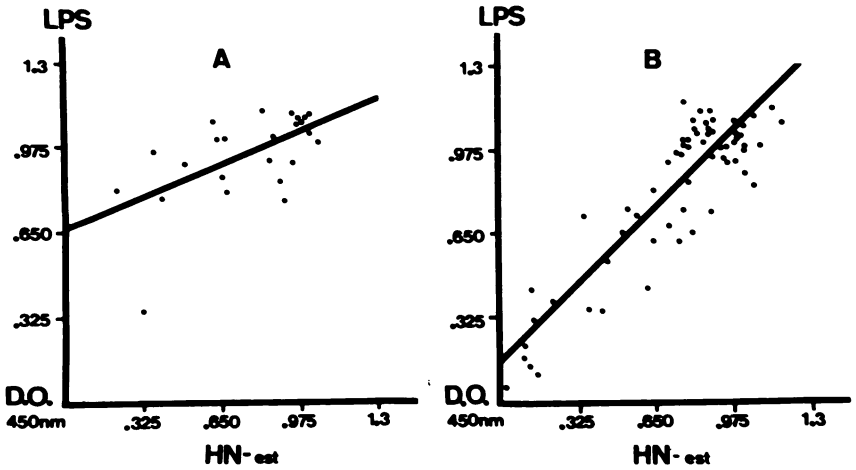

FIG. 2. Correlation between the levels of antibody (OD of the 1:400 dilution) to the S-LPS and to the NH in (A) 24 blood sera ($r = 0.357$, not significant; $P < 0.05$) and (B) 71 milk sera ($r = 0.812$, significant; $P < 0.01$). D.O., Optical density; HN, native hapten.

determinants absent from NH. Thus, although no definitive conclusion can be reached in this regard, the presence in blood sera, compared with that in milk sera, of a higher and more variable level (ODs, 0.592 ± 0.138 versus 0.176 ± 0.031) of antibody to core-lipid A determinants could explain the lack of correlation. It has been shown before that antibodies to R-LPS are detected in ELISA when S-LPS is used as the antigen, even though the cross-reactivity cannot be observed by gel precipitation methods (23).

Our results present for the first time an agreement between the data of the chemical analysis of NH and S-LPS and those of the serological tests. However, it remains to be explained why antibody reacting with the NH in ELISA is not detected by precipitation. It has been shown that both hemagglutination and ELISA detect low-affinity immunoglobulin G more efficiently than precipitation tests (31) and that the presence of precipitating antibodies to NH correlates with the intensity of the antigenic stimulus elicited by *Brucella* species (11). Both observations are consistent with the hypothesis that in most animals vaccination would elicit low-affinity antibodies that, although unable to precipitate the NH, would be detected by ELISA. On the other hand, the more vigorous stimulus brought about by infection would produce high-affinity antibody reacting in both ELISA and precipitation tests. Low amounts of high-affinity antibodies could also explain why a few sera of infected animals precipitated only the NH, since this component migrates closer to the antibody wells.

Another question that remains to be answered is why, despite sharing the relevant antigenic determinants (4, 5, 11, 27–29), S-LPS is precipitated by sera from vaccinated animals while NH is not. As suggested by Moreno et al. (29), antibody to lipid A-core determinants (absent from the NH) could contribute to the precipitation of the S-LPS. In addition, the different states of aggregation of S-LPSs and NHs (micelles versus dispersed molecules) (32, 33) could affect the precipitation by antibodies specific for both, since it has been shown that the precipitation curves for the S-LPS and NH of enterobacteria are different (2). This hypothesis would explain the results of the competitive ELISA which showed that, in the presence of both NH and S-LPS, antibody specific for both molecules would bind preferentially to S-LPS. The fact that this same observation has also been made with blood sera from patients afflicted with brucellosis (14) lends further support to this hypothesis.

Finally, the results of the present work demonstrate that, for diagnostic purposes, the ELISA with NH offers no advantage over the same assay with S-LPS.

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