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Demonstration of Antibodies against *Brucella melitensis* 16M Lipopolysaccharide and Native Hapten in Human Sera by Enzyme-Linked Immunosorbent Assay

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An enzyme-linked immunosorbent assay (ELISA) for the detection and quantification of human immunoglobulin G (IgG), IgA, and IgM antibodies against *Brucella melitensis* 16M by using lipopolysaccharide (LPS) and native hapten (NH) as antigens is described. The results obtained with the LPS ELISA were compared with the results of the NH ELISA. A good statistically significant correlation was established between the antibody titers of the IgG class against both antigens. A total of 104 (99%) of the 105 serum samples of patients with brucellosis exhibited specific anti-NH antibodies by the ELISA technique. In 52 (50%) of these positive samples, antibodies against NH were detected by radial immunodiffusion (RID). In 100% of these RID-positive sera, the antibody titers of the IgG class with ELISA-determined anti-NH specificity were equal to or greater than 160. These results point to a higher sensitivity of the ELISA technique as compared with RID. Inhibition experiments revealed that the assay was specific for LPS and NH from *B. melitensis* 16M.

Infections caused by *Brucella* organisms in humans are generally associated with a high humoral response. Isolation of the bacteria is possible only in a minority of infected patients in the acute phase of the disease (30). Accordingly, diagnosis often depends on serology. The tests most commonly used include direct agglutination in the presence and absence of 2-mercaptoethanol, a complement fixation test, and an anti-human globulin (Coombs) test, by which it is possible to distinguish specific antibodies of the immunoglobulin M (IgM) class, associated with acute brucellosis, and of the IgG class, associated with chronic infections (10, 17). The results obtained with these tests are not always easy to interpret, because none of them is specific for a single immunoglobulin class. Furthermore, because whole bacterial cells are used as the antigen, in which there is a large amount of antigenic determinants, great limitations are seen in their sensitivity and specificity.

In recent years, more sensitive techniques have been introduced, such as radioimmunoassay (25) and enzyme-linked immunosorbent assays (ELISAs) (3, 6, 14, 15, 19–22, 26, 28) to perform the serological diagnosis of human brucellosis. The sensitivity and specificity of the latter technique for the detection of anti-*Brucella* antibodies is directly related to the degree of purity of the different antigenic preparations used (5, 19), such that one previous and necessary step for standardization is to obtain *Brucella* antigens which are sufficiently characterized, such as the lipopolysaccharide (S-LPS) and the native hapten (NH). Because the S-LPS is an immunodominant antigen, the anti-LPS antibodies are the first to appear after infection by *Brucella abortus* (7, 10). However, NH is a polysaccharide which is present together with S-LPS on the external surface of *Brucella* organisms in the smooth phase (8, 24). Specific anti-NH antibodies have been demonstrated by radial immunodiffusion (RID) in the serum of cattle infected by *B. abortus*. Its appearance seems to be related to active infection (7, 11, 16).

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In this study, the ELISA was used to examine the occurrence of antibodies of the IgG, IgA, and IgM class against *Brucella melitensis* 16M S-LPS and NH in the sera of patients with *Brucella* infection. A further objective was to compare these results with those recorded by studying the sera by the RID technique with NH of *B. melitensis* 16M as antigen.

MATERIALS AND METHODS

Bacterial cultures. The strains used in this study and their characteristics and culture conditions were described previously (8, 10).

Extraction and purification of S-LPS. Extraction of the S-LPS from smooth strains of *B. melitensis* 16M was done by the hot phenol-water technique modified for *Brucella* organisms by M. Redfearn (Ph.D. thesis, University of Wisconsin, Madison, 1960) and described by Leong et al. (18). Purification of crude S-LPS isolated in the phenolic phase (f5) was performed by a technique described previously (23). Briefly, 12.5-mg batches of f5 were dissolved in 2.5 ml of dimethyl sulfoxide and stirred for 20 min at room temperature. Then, to eliminate noncovalent associations between the S-LPS and other bacterial components, 2.5 ml of 4 M NaI was added as a chaotropic agent. The solution was maintained at room temperature for 20 min, after which the S-LPS was precipitated with 3 volumes of cold methanol reagent and stirred for 1 h at 4°C. The precipitate thus obtained was dissolved in 2.5 ml of dimethyl sulfoxide–2.5 ml of 4 M NaI and precipitated again. This process was repeated a further eight times. Finally, the last precipitate was suspended in 5 ml of *dd*-H_2O, dialyzed for 3 days at 4°C against *dd*-H_2O, and then lyophilized. A sample (30 mg) of the material thus obtained was dissolved in 2 ml of 10 mM Tris hydrochloride buffer (pH 8.1)–2 M NaI–5% Tween 40 and chromatographed on Sephacryl S-300. The purified S-LPS obtained in the void volume was dialyzed and then lyophilized.

NH extraction and purification. To obtain NH, the technique described previously by Diaz et al. (12) was followed.

Purification of the second precipitate, the NH-enriched fraction, was performed by filtration on Sephacryl S-300. Crude NH (40 mg) was dissolved in 2.5 ml of 10 mM Tris hydrochloride (pH 8.1) and chromatographed on Sephacryl S-300. The fractions thus obtained were studied for S-LPS and NH by double gel diffusion in agarose borate containing 10% NaCl (7). Once the fractions showing the presence of NH had been collected, they were fractionated on a PM-30 (Diaflo) membrane. The diffusion volume obtained, containing NH, was dialyzed for 3 days at 4°C against *dd*-H_2O and then lyophilized.

Preparation of poly B. The polysaccharide B (poly B) of *B. melitensis* 115 was prepared by trichloroacetic acid extraction as described before (7). This antigen was used without further purification.

Serum specimens. The human sera were mostly obtained from our diagnostic laboratory. All samples had been tested by Rose Bengal (RB) and immunodiffusion tests for antibodies against *Brucella* organisms. The 105 specimens selected were positive in both tests. As negative controls, serum samples were collected from 50 healthy persons. As specificity controls, serum samples from 30 patients affected with diseases other than brucellosis (yersiniosis [serotype O3], salmonellosis, and tuberculosis) were used. All the specimens were stored at −20°C until assessed.

Serological tests. The RB test was performed as described by Alton et al. (1). For double gel diffusion, the gels were prepared with 1% agarose in borate buffer (pH 8.3) containing 10% NaCl (7). RID was performed by a previously described technique (7, 16), by using in each case five different concentrations of purified NH (2, 4, 8, 16, and 32 μg/ml). In this test, the sera which developed a precipitation ring in less than 8 h were considered positive.

ELISA procedure. The technique used for the ELISA was the indirect method initially described by Engvall and Perlmann (13) and van Veemen and Schurs (29) using the microtechnique proposed by Ruitemberg et al. (27), with some modifications introduced for adaption to the antigen-antibody system under study.

The test was performed in disposable polystyrene microtiter plates with flat-bottomed wells (LIMBRO; Flow Laboratories, Inc., McLean, Va.). SLS-LPS from *B. melitensis* 16M was dissolved in phosphate-buffered saline (pH 7.2) with 0.02% sodium azide; for NH, a barbital-acetate buffer (pH 4.6) with 0.02% sodium azide was used. The optimum antigen concentration (1 μg [dry weight] of S-LPS and 20 μg [dry weight] of NH per ml) was determined by prior titrations. Higher concentrations of both antigens resulted in a slight prozone effect and did not increase the specificity or sensitivity of the assay, and the use of lower concentrations resulted in decreased sensitivity. Antigen solution (100 μl) was placed in each well. Absorption was allowed to take place over 12 h at 37°C. The sensitized wells were previously washed three times with physiological saline containing 0.05% Tween 20 (PS-T) before the sera were added.

Once inactivated for 30 min at 56°C, the clinically positive controls were diluted in phosphate-buffered saline (pH 7.2) containing 0.5% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.). The use of a lower concentration of bovine serum albumin or its exclusion led to a pronounced unspecificity for the assay. The initial dilution of each serum was 1:10, from which double serial dilutions were made until 1:20,480 in each of the successive wells. Volumes (100 μl) of these dilutions were incubated for 1 h at 37°C. The cuvettes were then washed three times with PS-T and 100 μl of goat anti-human IgG + IgA + IgM-peroxidase conjugate (Fc specificity; Cooper Biomedical, Inc., West Chester, Pa.). To determine the most suitable working dilution, different dilutions were made of each peroxidase conjugate (1:50, 1:100, 1:200, 1:400, 1:800, 1:1,200, 1:1,400, and 1:1,600), which were studied in the test against each antigen by using a constant dilution of a positive and negative reference serum. When the S-LPS of *B. melitensis* 16M was used as antigen, the optimum dilution of the different peroxidase conjugates (anti-IgG, -IgA, and -IgM) proved to be 1:1,200, and in the case of NH it was 1:600. After incubation for 1 h at 37°C, the cuvettes were again washed three times with PS-T. Following this, 100 μl of substrate (a solution of 5-aminosalicylic acid [5-AS] and H_2O_2) was placed in each well. The substrate was prepared by dissolving 80 mg of 5-AS in 100 ml of warm distilled water and adjusting the pH to 6.0 with NaOH (1 N). Immediately before the substrate was used, 0.05% H_2O_2 was added to the 5-AS solution at a ratio of 1 ml to 9 ml of the 5-AS solution.

The plates were then maintained for 1 h at room temperature in the dark, after which the reaction was halted by adding 0.025 ml of NaOH (1 N) to each well. The A_{450} of the contents of each well was read on a Titertek Multiskan (Flow Laboratories, Finland). The photometer was adjusted to zero by using wells containing control serum samples. The antibody titer was determined in serial dilutions of the test sera. The reciprocal of the dilution corresponding to 50% of the maximum absorbance reading was considered to be the titer of the serum.

Inhibition studies. Inhibition experiments were performed with different sera from patients with brucellosis to determine the capacity of the purified NH of *B. melitensis* 16M and of the poly B of *B. melitensis* 115 to absorb specific antibodies directed against the S-LPS of *B. melitensis* 16M. Briefly, 1 ml of undiluted serum was added to 1 ml of phosphate-buffered saline with 0.5% bovine serum albumin and 0.05% Tween 20 containing purified NH (4 mg/ml) or poly B (8 mg/ml). The mixtures were incubated for 1 h at 37°C and later for 18 h at 4°C. The immunoprecipitate was then collected by centrifugation at 8,000 × *g* for 60 min at room temperature. The supernatant fluid thus obtained in each case was studied by the ELISA technique using goat anti-human IgG+IgA+IgM-peroxidase conjugate (Fc specificity; Cooper Biomedical).

RESULTS

Specificity of the assay. When 50 serum samples from healthy donors were studied, the ELISA (anti-LPS and anti-NH) was always negative. Similar results were obtained with 30 serum samples from patients with yersiniosis (serotype O3), salmonellosis, and tuberculosis. Complete inhibition of antibody binding to *B. melitensis* 16M NH-coated cuvettes was seen after absorption of sera with *B. melitensis* 16M NH, whereas no effect was observed in the antibodies directed against the S-LPS of *B. melitensis* 16M (Table 1). Similar results were obtained after absorption of the sera with the poly B of *B. melitensis* 115 (Table 1). In all cases, the degree of reaction inhibition was related to the amount of antigen used in absorption.

ELISA antibody titers to *B. melitensis* 16M S-LPS antigen. In 100% of the serum samples studied, antibodies of the IgG class were demonstrated at a titer equal to or greater than 80 (Table 2). All these were positive in the RB test and precipitated the S-LPS antigen used in the immunodiffusion test. In 52 serum samples (49%) which showed IgG titers in the LPS ELISA ranging between 640 and 20,480, the pres-

TABLE 1. Specificity of the assay for antibodies against *B. melitensis* 16M S-LPS and NH

Human serum no.	Serum absorbed with (mg):	ELISA[a] titer with given antigen on the plate[b]:	
		S-LPS[c]	NH
11	None	10,240	10,240
	NH (2)	10,240	0
	Poly B (4)	10,240	0
21	None	320	20
	NH (2)	320	0
	Poly B (4)	320	0
85	None	5,120	80
	NH (2)	5,120	0
	Poly B (4)	5,120	0
92	None	5,120	2,560
	NH (2)	5,120	0
	Poly B (4)	5,120	0

[a] Goat anti-human IgG+IgA+IgM-peroxidase conjugated (Fc specificity).
[b] Titers are expressed as reciprocals.
[c] *B. melitensis* 16M S-LPS free from NH.

ence was observed of antibodies against the NH of *B. melitensis* 16M by the RID test. However, 38 serum samples (36%) which were negative in RID were positive in the LPS ELISA, with titers ranging between 640 and 20,560 (Fig. 1). Antibodies of the IgA and IgM class were determined, respectively, in 103 (98%) and 82 (78%) of the serum samples examined (Table 3). No kind of correlation could be established between the levels in each sample of IgA and IgM anti-LPS antibodies and the presence of anti-NH antibodies as determined by RID.

ELISA antibody titers to *B. melitensis* 16M NH antigen. Antibodies of the IgG class against NH extracted from *B. melitensis* 16M were detected in 99% of the serum samples from the patients with brucellosis (Table 4). Ninety-eight percent of the serum samples presenting IgG titers equal to or greater than 160 were positive by RID (Fig. 1). In only 92 (87%) and 25 (23%) of the 105 serum samples tested could

TABLE 2. Results of LPS ELISA IgG antibody and other tests on sera from patients with clinical symptoms of brucellosis

ELISA[a] titer	No. of serum samples	No. of serum samples giving positive reactions in:		
		RB	Immunodiffusion[b] (A&M)	RID[c] (NH)
80	1	1	1	0
160	3	3	3	0
320	11	11	11	0
640	23	23	23	2
1,280	23	23	23	9
2,560	23	23	23	20
5,120	11	11	11	11
10,240	4	4	4	4
20,480	6	6	6	6
% Positive	100	100	100	49

[a] For polystyrene binding in the ELISA, 1 μg of S-LPS in carbonate buffer was incubated at 37°C overnight in microtiter plates (100 μl per well).
[b] Three concentrations of S-LPS (10, 20, and 40 mg/ml) were used.
[c] Each serum sample was tested with five concentrations of NH (2, 4, 8, 16, and 32 μg/ml) in the RID test. Positive reactions were recorded in less than 8 h.

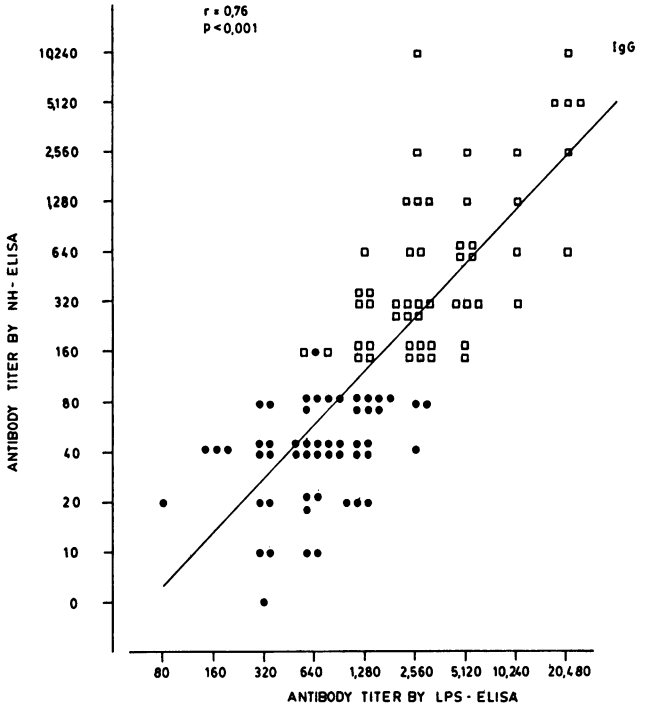

FIG. 1. Correlation between anti-*B. melitensis* 16M IgG titers determined by S-LPS and NH ELISA. Symbols: ●, sera negative by RID; □, sera positive by RID.

antibodies of the IgA and IgM classes with anti-NH specificity be detected (Table 3).

Correlation of IgG antibody titers obtained with the LPS ELISA and the NH ELISA. When the levels of antibody of the IgG class obtained with the LPS ELISA and the NH ELISA were compared, a good statistically significant correlation was seen. In this case, the correlation coefficient was 0.76 ($P < 0.001$) (Fig. 1). In contrast, a poorer correlation was observed for the immunoglobulins of the IgA class anti-LPS and anti-NH, and this correlation was null in the case of the IgM-class antibodies.

DISCUSSION

Although antibody affinity affects the results of the ELISA (4), the method can be regarded as quantitative. Being sensitive, specific, and easy to perform, the ELISA offers many advantages compared with other serological methods. Nevertheless, in this assay the specificity of the antigenic preparation used is of great importance with respect to taking advantage of its greater sensitivity and specificity (2, 5).

We developed an ELISA for antibodies against *B. melitensis* 16M S-LPS and NH and used it to quantify

TABLE 3. IgM and IgA antibody responses to *B. melitensis* S-LPS and NH with sera from 105 patients with clinical symptoms of brucellosis

ELISA antigen	No. of serum samples positive by ELISA in:		No. of serum samples tested
	IgM test[a]	IgA test[a]	
S-LPS	82	103	105
NH	25	92	105

[a] Titers ≥10 (expressed as reciprocals).

TABLE 4. Results of NH-ELISA IgG antibody and other tests on sera from patients with clinical symptoms of brucellosis

ELISA[a] titer	No. of serum samples	No. of serum samples giving positive reactions in:		
		RB	Immunodiffusion[b] (A&M)	RID[c] (NH)
0	1	1	1	0
10	4	4	4	0
20	9	9	9	0
40	22	22	22	0
80	16	16	16	0
160	15	15	15	14
320	15	15	15	15
640	9	9	9	9
1,280	5	5	5	5
2,560	4	4	4	4
5,120	3	3	3	3
10,240	2	2	2	2
% Positive	99	100	100	49

[a] For polystyrene binding in the ELISA, 20 μg of NH in barbital-acetate buffer (pH 4.6) was incubated at 37°C overnight in microtiter plates (100 μl per well). Titers are expressed as reciprocals.

[b] Three concentrations of S-LPS (10, 20, and 40 mg/ml) were used.

[c] Each serum sample was tested with five concentrations of NH (2, 4, 8, 16, and 32 μg/ml) in the RID test. Positive reactions were recorded in less than 8 h.

antibodies of the IgG, IgA, and IgM class in sera from patients. The results presented here suggest that by using both antigens in sufficiently purified form it is possible to do an adequate serological diagnosis of human brucellosis. When this technique was used to study 50 serum samples from healthy donors, all negative according to the classic serological methods used in the diagnosis of brucellosis, its specificity, assessed as the percentage of correctly identified uninfected individuals, was 100%. Similar results were obtained when the technique was used to study 30 serum samples from patients with diseases other than brucellosis.

Furthermore, the results obtained in the inhibition experiments performed with NH and the poly B of *B. melitensis* 16M (Table 1) showed that when the ELISA technique is used with the S-LPS and NH of *B. melitensis* 16M, specific antibodies directed against one or the other antigen were determined. The capacity of poly B to absorb all the antibodies directed against NH showed that with this technique it is not possible to demonstrate the existence of antigenic determinants that differ between the polysaccharides. Such results are in agreement with those obtained with double gel diffusion (results not shown), because there are no differences in precipitation when both antigens are studied with different sera from patients with brucellosis.

Because S-LPS is an immunodominant antigen, the antibodies against S-LPS are the first to appear once infection by *B. melitensis* 16M has taken place (7, 10). In this study, we detected in 100% of samples the presence of antibodies of the IgG class against the S-LPS of *B. melitensis* 16M at titers greater or equal to 80. The fact that all samples were positive by the RB test confirmed the results of other workers (9) regarding the major role of antigen A&M in this test. Nevertheless, we are unaware whether the presence of specific antibodies against NH contributes in some way to the positivity of the reaction. Further studies should be done to shed more light on this aspect.

In 99% of the serum samples assayed, it was possible by ELISA to detect IgG-class antibodies against the NH of *B. melitensis* 16M. However, it is difficult to evaluate the biological significance of this, although results obtained with bovine sera (7, 16) clearly point to the existence of a relationship between the appearance of these antibodies and active infection. Similar results were recently reported for rabbits infected with virulent and attenuated strains of *B. abortus*. The existence of a statistically significant correlation between IgG-class antibody titers against NH and S-LPS (Fig. 1) suggested that the levels of anti-NH antibodies are initially related to the intensity of infection. Nevertheless, additional research is necessary to determine the time of appearance of these antibodies, their duration, and their exact relationship to any given state during the course of the disease.

By the RID technique we demonstrated antibodies against the NH of *B. melitensis* in 49% of the serum samples studied. In contrast, the ELISA technique revealed the presence in 99% of the same samples of IgG-class antibodies against this antigen. These results evidently show the greater sensitivity of the ELISA compared with the RID test. However, it should be borne in mind that 98% of the serum samples positive in RID had anti-NH antibody titers of the IgG class greater than or equal to 160. Furthermore, a concentration of 10% NaCl in the gels was necessary to precipitate the NH in nearly all the human serum samples studied. Under these conditions, it has been shown (9) that IgG1 of bovine origin precipitates *Brucella* antigens faster than in isotonic gels. Our results show that this is due to a relatively higher proportion of IgG with anti-NH specificity; hence, the presence of NaCl in the gels facilitates the precipitation of soluble antigen-antibody complexes.

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