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Differentiation between Active and Inactive Human Brucellosis by Measuring Antiprotein Humoral Immune Responses

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A preparation of *Brucella abortus* cytoplasmic proteins was depleted of lipopolysaccharide (LPS) by immunoadsorption with a monoclonal antibody (MAb), BC68, specific for the O antigen of *B. abortus* smooth LPS. Two enzyme-linked immunosorbent assay (ELISA) systems were developed and used in this study. The first system includes an LPS-free cytoplasmic protein preparation; the second one was based on antigen capture on MAb BC68. By using these systems, we have demonstrated that 94% (33 of 35) of the brucellosis patients studied showed immunoglobulin G antiprotein response and also that all of the patients showed a strong anti-LPS reactivity. Thirty-six serologically positive individuals with no active infection at the time of examination (SPI) were also included. No immunoglobulin G antibodies against proteins were detected in 34 of them (92%), whereas 31 SPI (86%) showed various degrees of anti-LPS reactivity. The use of the LPS-free protein extract in ELISAs made it possible to establish differential reactivity patterns between active and inactive brucellosis.

One of the most difficult problems for diagnosing human brucellosis originates in the existence of individuals who are serologically positive, have low titers of both agglutination and complement fixation, and are either asymptomatic or oligosymptomatic. This group, which we have called serologically positive individuals with no active brucellar infection at the time of examination (SPI), usually includes those individuals who have either recovered from a brucellar infection but have not attained negative titers in agglutination and complement fixation tests or those having an epidemiological probability of exposure to brucellar infection but no known history of disease.

Individuals having pathological symptoms caused by cross-reacting bacteria (*Yersinia enterocolitica*, *Francisella tularensis*, or *Vibrio cholerae*) can also exhibit a pattern of serological reactivity similar to that described for SPI.

Current serological procedures for the diagnosis of human brucellosis are based on the detection of antilipopolysaccharide (anti-LPS) antibodies, which, as it is well known, remain high even after recovery from the disease (11). Thus, it is reasonable to assume that the determination of the humoral response against LPS-free *Brucella abortus* proteins could help to circumvent those undesired reactivities.

We have prepared an anti-*B. abortus* monoclonal antibody (MAb), BC68, specific for a repetitive epitope of the O antigen of S-LPS. By using this MAb, an LPS-depleted preparation of *B. abortus* 19-S cytoplasmic proteins (LPS-free CYT) could be obtained by immunoadsorption. We report in this article that this preparation can be used in a standard enzyme-linked immunosorbent assay (ELISA) system to measure antiprotein reactivity in human sera without interference by LPS. Conversely, using MAb BC68 in an antigen capture ELISA, we have been able to measure reactivity against LPS and LPS-associated proteins.

Sera from patients with active brucellosis, either acute or chronic, were the only ones to exhibit significant anti-protein response when these ELISAs were used. Nevertheless, patients showing either active or inactive forms of brucellosis showed anti-LPS reactivity. Patients with brucellosis always have high anti-LPS antibody levels, whereas SPI show a wide range of reactivity.

MATERIALS AND METHODS

Bacterial culture and preparation of cytoplasmic fraction. Suspension cultures of *B. abortus* 19-S were kindly provided by Laboratorios Bagó-San Jorge (Buenos Aires, Argentina). Cells were killed by addition of 0.4% formaldehyde. The cytoplasmic fraction (CYT) was obtained as described by Verstreate et al. (17). Bacterial suspensions were centrifuged at 16,000 × *g* for 10 min and washed three times with 10 mM Tris-HCl, pH 8.0 (Tris buffer). The cells were suspended in Tris buffer (0.1 g [wet weight] of cells per ml) and extruded with an X-Press (type 25; AB BIOS). The assembly was cooled at −26°C, and the bacterial cells were broken by five passages and then digested with DNase and RNase. Unbroken cells were separated by centrifugation. Cell envelopes were harvested by centrifugation at 360,000 × *g* for 2 h. The resulting supernatant (CYT) was stored at −20°C.

MAb. MAb BC68 {immunoglobulin G1 κ light chain [IgG1(κ)]} was derived by somatic cell hybridization as described by Galfré and Milstein, with NSO myeloma cells as fusion partners (4).

The BC68 MAb is specific for a repetitive epitope of the O antigen of LPS, as can be inferred by the following observations. (i) It can agglutinate different smooth *Brucella* strains and *Y. enterocolitica* O9. (ii) Its reactivity against CYT in ELISAs is inhibited by adsorption with the *B. abortus* 19-S but not with the rough strain *B. abortus* 45/20. (iii) ELISA reactivity was not affected by proteinase K

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digestion of CYT. (iv) BC68 precipitated in an Ouchterlony assay with a polysaccharide fraction obtained by mild acid hydrolysis of S-LPS (7).

BC68 was purified by ion-exchange chromatography in DEAE-cellulose.

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (5) with 10% acrylamide gels.

Immunoblotting analysis was performed as described by Tsang et al. (16). Sera from five patients with brucellosis were pooled and used at a 1/200 dilution. Ascitic fluid containing MAb BC68 was diluted 1/100.

Chemical analysis. Protein composition was determined according to the method of Peterson (12), with bovine serum albumin (Sigma) as a standard.

Limulus lysate gelation activities (LLGA) of all fractions were determined as described by Sullivan and Watson (15), with *E. coli* LPS as a standard. *B. abortus* S-LPS contents were estimated by the ratio between *E. coli* and *B. abortus* LPS gelating activities reported by Moreno (6), i.e., 1 ng of *E. coli* LPS is equivalent to 1 ng of *B. abortus* S-LPS.

Immunoadsorption of CYT with Sepharose-BC68. Purified BC68 MAb (5 mg/ml) was coupled to CNBr-activated Sepharose 4B (Pharmacia) according to the supplier's specifications.

With regard to immunoadsorption, 2 ml of BC68-coupled Sepharose 4B gel (Seph-BC68) was incubated with 2 ml of CYT with constant agitation for 24 h at 4°C. After centrifugation, the supernatant was immunoadsorbed once more. The recovered supernatant, named LPS-free CYT, was diluted 1:4 relative to the original CYT and had a protein concentration of 0.86 mg/ml. The Seph-BC68 gel recovered from both immunoadsorption steps was washed three times with phosphate-buffered saline (PBS). To run the SDS-PAGE, the bound antigen was desorbed by boiling in sample buffer.

Capture ELISA. The experimental design of Voller et al. (18) was used for the antigen capture ELISA. Purified BC68 antibody was adsorbed to Immulon II plates (Dynatech). After the plates were blocked with a solution (BS) of PBS containing 3% skim milk and washed with PBS plus 0.05% Tween 20, the wells were incubated with CYT (10 μg per well) in BS containing 0.05% Tween 20 (BST) for 1 h at room temperature. When proteinase K-treated CYT was used, the same conditions as those described for CYT were used. After being washed, the sera under study, diluted 1/100 in BST, were incubated for 1 h at room temperature. The mixture was washed, and 50 μl of a anti-human IgG MAb–horseradish peroxidase conjugate (Janssen Biochimica; diluted 1/3,000 in BST) was added per well. The plates were then incubated for 1 h at room temperature. After the plates were washed, the contents of each well were developed with a solution containing *o*-phenylenediamine (2 μg/μl) and 0.03% H_2O_2 in 0.1 M citrate-phosphate buffer (pH 5), the reaction was stopped with H_2SO_4. The resulting color was read at 495 nm in an ELISA Reader (Dynatech). Control experiments concerning nonspecific adsorption of antigen without addition of the capture monoclonal antibody were performed throughout. Each serum was also tested in the absence of antigen to detect anti-murine IgG activity.

ELISA with LPS-free CYT. Fifty microliters of a 10-μg/ml solution of LPS-free CYT (or 50 μl of proteinase K-treated material) in PBS per well was incubated overnight at 4°C. Washing, blocking, testing of sera and the second antibody, and developing were performed as described above.

Sera. In this study we analyzed sera from 71 outpatients treated at the Brucellosis Department of Hospital F. J. Muñiz, who were classified into three different groups as follows.

(i) Patients with acute or subacute brucellosis. This group included sera from 18 patients who had symptoms compatible with brucellosis (chills, fever, asthenia, arthralgia, sweating, weight loss, lymphadenopathy, and hepatosplenomegaly, etc.) for up to 1 year before diagnosis. The test results for these patients included standard tube and slide agglutination tests with titers equal to or higher than 1/100, a positive rose bengal test, positive agglutination with 2-mercaptoethanol (2-ME), and positive complement fixation. Seven of the patients (39%) had positive blood cultures (14).

(ii) Patients with chronic brucellosis. This group included 17 patients who had symptoms compatible with brucellosis for more than 1 year before diagnosis, positive standard tube and slide agglutination tests with titers equal to or higher than 1/100, a positive rose bengal test, positive agglutination with 2-ME, and positive complement fixation. One of these patients (6%) had a positive blood culture (14).

(iii) SPI. In this group were included 36 individuals who had no clinical picture compatible with brucellosis but who had an epidemiological probability of exposure. Of these patients, 21 had a documented history of brucellosis, and 15 had no documented history of the disease, including 7 slaughterhouse workers, 4 individuals with a history of consumption of nonpasteurized milk products, 2 subjects with a history of long-term contact with cattle in a rural environment, and 2 workers from a cold cut factory.

The sera of all of these patients were positive at titers lower than 1/100 in standard tube and slide agglutination tests. They were also positive in the rose bengal test and negative for agglutination with 2-ME and complement fixation.

Negative control group. This group consisted of sera from 47 healthy volunteers with no history of brucellosis.

Serological tests were performed according to the recommendations of the Centro Panamericano de Zoonosis-Oficina Panamericana Sanitaria (CPZ-OPS) (2a) with antigens provided by the CPZ-OPS laboratory in Buenos Aires, Argentina. 2-ME assays and rose bengal tests were performed as described by Alton et al. (1).

RESULTS

SDS-PAGE and Western blot (immunoblot) analysis of immunoadsorbed CYT. The CYT of *B. abortus* 19-S was immunoadsorbed with Seph-BC68. When the LPS-free CYT was monitored for LLGA, it showed a 1,000-fold reduction in gelating activity with respect to the starting fraction. The LPS concentrations (calculated from the LLGA) were 10.0 mg/ml for CYT and 9×10^{-3} mg/ml for LPS-free CYT. The protein concentrations were 3.6 mg/ml for CYT and 3.44 mg/ml for LPS-free CYT. The concentrations in LPS-free CYT were corrected for dilution due to immunoadsorption.

Figure 1A shows the results of SDS-PAGE analysis of all fractions obtained. It can be seen that the LPS-free CYT leads to a much clearer definition of the bands than the CYT.

LPS-free CYT proteins were analyzed by Western blotting to study their antigenicity in patients with brucellosis. Figure 1B shows the reactivities of sera pooled from such patients against the CYT (lane 1), LPS-free CYT (lane 2), and the retained, LPS-rich fraction (lane 3). Both LPS-containing fractions show broad smears which mask the reactivities of the individual proteins. In contrast, a clear banding pattern is

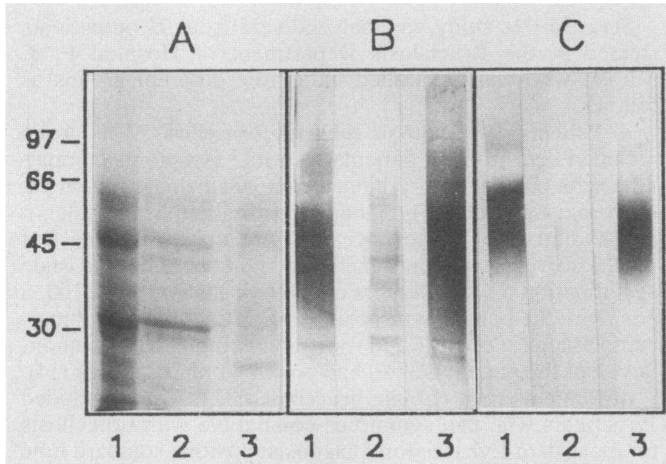

FIG. 1. SDS-PAGE and immunoblot analysis of CYT (lanes 1), LPS-free CYT (lanes 2), and material retained by the Seph-BC68 immunosorbent (lanes 3). (A) SDS-PAGE profiles; (B) immunoblot showing reaction of a pool of sera from human patients with brucellosis; (C) immunoblot showing reaction of anti-LPS MAb BC68.

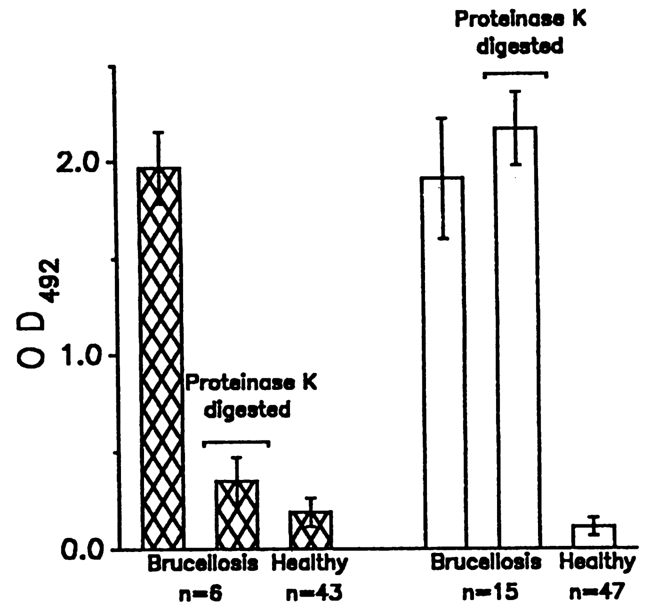

FIG. 2. Reactivity of CYT, by capture ELISA with MAb BC68 (▭) and LPS-free CYT antigen (▩) with and without previous digestion with proteinase K, against sera from patients with brucellosis and from a healthy negative control group. Bars indicate three standard deviations from the mean. OD_{492}, optical density at 492 nm.

observed when LPS-free proteins are allowed to react with the pooled sera (Fig. 1B, lane 2). This result is in agreement with the improvement of band definition in SDS-PAGE that arises from the elimination of LPS.

Figure 1C, lanes 1 and 3, shows the reactivity pattern of MAb BC68 against LPS-containing fractions. The similarity between the patterns in Fig. 1B, lanes 1 and 3, and the similarity between those in Fig. 1C, lanes 1 and 3, suggest that LPS is the main component detected by Western blot analysis of the fractions in which it is present. As expected, it can be seen in Fig. 1C, lane 2, that LPS has been reduced to a level which cannot be detected any longer by this technique.

Determination of anti-LPS and antiprotein serum reactivities by ELISA. An ELISA using LPS-free CYT proteins as the antigen was devised in our laboratory to measure antiprotein reactivity in human sera. The results in Fig. 2 show that a specific antiprotein response can be detected in sera from patients with brucellosis. Therefore, proteinase K digestion of the antigen actually reduces optical densities to the normal values.

When we analyzed reactivity against LPS and LPS-associated proteins by capture ELISA with BC68, all sera from patients having confirmed brucellosis reacted strongly, as shown in Fig. 2. Furthermore, when the cytoplasmic fraction underwent digestion with proteinase K before adsorption to the MAb-coated wells, the reactivities of all sera tested remained unaltered.

Anti-LPS and antiprotein reactivities of sera from SPI and patients with brucellosis. Figure 3, in which a correlation plot is used to compare the reactivities in both systems for each serum tested, shows a clear qualitative difference between the groups of patients. It can be seen that all patients with brucellosis exhibit anti-LPS and antiprotein reactivities of the IgG type. In contrast, sera from SPI react heterogeneously against LPS but do not react against cytoplasmic proteins, so their mean reactivity value is not significantly different from that of the negative controls.

On the other hand, sera of 15 patients with cholera obtained during the Peruvian epidemic (1991) were assayed. None of them showed an antiprotein response in the LPS-free CYT ELISA, whereas three of them had low anti-LPS responses, as determined by capture ELISA (data not shown).

In summary, the capture ELISA was found to be positive for all 35 patients with brucellosis studied (100% sensitivity).

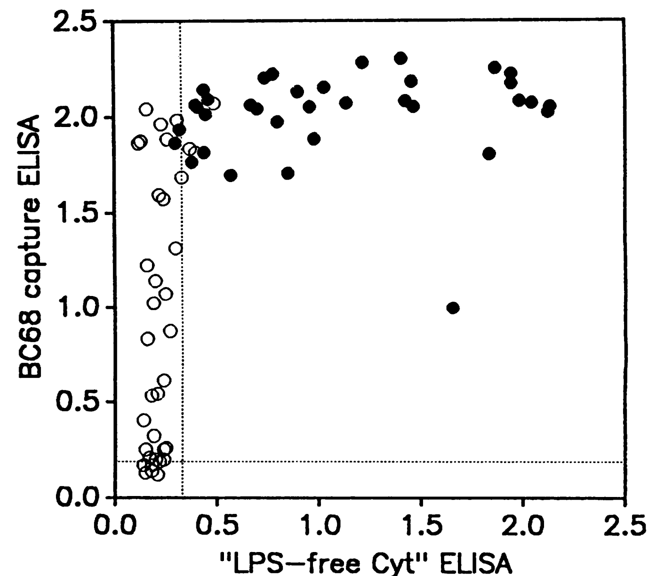

FIG. 3. ELISA reactivity of patients' sera determined by antigen capture with MAb BC68 (*y* axis) and by LPS-free CYT (*x* axis). Broken lines represent the mean + two standard deviations of 47 serum specimens from the negative control group. Symbols: ●, patients with brucellosis; ○, SPI.

The specificity in healthy control individuals was 98% (46 negative results from 47 individuals). The percentage of positive results for the SPI group was 86 (31 of 36 patients).

The antiprotein ELISA was positive for 33 of 35 patients with brucellosis (94% sensitivity), whereas the specificity for healthy controls was 98% (46 negative results from 47 individuals). Only 3 of the 36 serum specimens of the SPI group turned out to be positive for proteins (8% reactivity).

DISCUSSION

Strong associations between proteins and LPS in gram-negative bacteria have been reported; these associations are particularly strong for the genus *Brucella* (8–10). However, very few reports regarding the preparation of LPS-free *Brucella* proteins can be found in the literature (1a, 2, 13). None of the reports mentioned used MAbs as immunoadsorbents. The general approaches used to obtain LPS elimination until now have been extraction of R-LPS by means of chloroform-petroleum ether and phenol (13) and expression in *Escherichia coli* of cloned *B. abortus* proteins (1a, 2).

Two drawbacks of these approaches are that only a limited number of proteins can be obtained and extensive denaturation of the extracted proteins can occur.

Cytoplasmic proteins are the predominant components of the soluble *B. abortus* fractions from both smooth and rough strains. Thus, they are relatively easy to obtain, and it has been reported that both humoral and cellular immune responses against them occur (3). As far as we know, this is the first report of analysis of human humoral immune response against a wide range of *B. abortus* proteins with no interference from LPS.

Most of the patients with brucellosis (33 of 35) developed a significant IgG antiprotein response, and, as would be expected from their agglutination titers, they showed strong anti-LPS responses.

It should be noted that no antiprotein response could be detected for the SPI. However, they showed a variable anti-LPS response, which is in agreement with the above-mentioned variability of titers in agglutination and other LPS-based diagnostic procedures. Some SPI sera react strongly against LPS. High anti-LPS titers in the absence of any detectable anti-protein reactivity could in some cases be explained by contact with other bacteria that cross-react with LPS. Nevertheless, we do not think that this possibility can explain completely the anti-LPS reactivity of SPI. It should also be pointed out that no cross-reactivity has been reported to take place between *B. abortus* cytoplasmic proteins and proteins from bacteria of other genera (3).

Neither an antiprotein nor an anti-LPS response was observed in control sera. Furthermore, patients with cholera showed no antiprotein response, thus indicating that the antiprotein assay is highly specific for diagnosis of brucellosis.

To confirm the usefulness of the antiprotein antibodies in differentiating SPI from patients with brucellosis, a longitudinal follow-up study of the patients from this study is under way.

The results presented here strongly indicate that antiprotein humoral reactivity is useful for differentiation of active from inactive brucellosis.

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