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Limited Diagnostic Usefulness of Antibodies to Cytoplasmic Proteins of Brucella in Early-treated Human Brucellosis

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Antibodies to cytoplasmic proteins (CP) of Brucella have been shown to be useful for the diagnosis of human brucellosis; however, some early-diagnosed patients lack such an antibody response while having high titers of antibodies to lipopolysaccharide (LPS). To address which factors determine this serological discrepancy in the early stages of brucellosis we examined the antibody response to CP and LPS of 21 patients involved in an outbreak of B. melitensis infection who had a short duration of clinical illness at diagnosis (3–40 d). At diagnosis, antibodies to LPS (IgM and/or IgG) were found in all patients, while anti-CP antibodies were detected in 16 subjects (76%). At 6 weeks post-diagnosis IgG to CP (with or without IgM) had been detected in 13 patients and IgM alone had been found in 4; however, 4 other patients (19%) had no response to CP. No significant differences were found between these 3 groups in terms of age, gender, antimicrobial agents or factors that could hamper the immune response. Notably, however, the 4 non-responders and 3 of the 4 patients having only IgM to CP had started antibiotic therapy within 14 d post-symptoms, while treatment was started later in 9 of 13 patients who developed anti-CP IgG. In addition, maximum titers of IgG to CP tended to be lower in early-treated patients. These results suggest that very early antibiotic therapy hampers the antibody response to Brucella CP but has little impact on the anti-LPS response. Given the higher specificity of the former and the higher sensitivity of the latter, both reactivities should be measured in order to diagnose human brucellosis.

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INTRODUCTION

Human brucellosis still occurs frequently in many developing countries. Serology has proved to be an important tool for the diagnosis, prognosis and management of this infection. Most of the work found in the literature has focused on antibodies directed to Brucella lipopolysaccharide (LPS), in spite of several studies showing the diagnostic drawbacks associated with the measurement of this response. While the sensitivity of tests measuring anti-LPS antibodies (agglutination and complement fixation) is usually $>90\%$, several studies have documented the serological cross-reactivity between the LPS of smooth brucellae and that of other Gram-negative bacteria, including Vibrio cholerae, Salmonella group N, Pseudomonas maltophilia and Yersinia enterocolitica O : 9 (1, 2). The epitopes responsible for these cross-reactivities, which have been fully characterized, are located in the O-polysaccharide chain of the LPS (3). Another diagnostic drawback of the anti-LPS response is the marked persistence of antibody titers, as measured by tube agglutination, after the clinical resolution of human brucellosis (4).

We have previously shown the diagnostic usefulness of the detection of serum antibodies directed to cytoplasmic proteins of Brucella spp. An indirect enzyme-linked immunosorbent assay (ELISA) using a preparation of cytoplasmic proteins (CP; formerly LPS-free CYT) of B. abortus, depleted of LPS by specific immunosorption, proved to be useful to differentiate active from inactive human brucellosis. While anti-CP antibodies were detected in 94% of patients having active brucellosis, this response was absent in 92% of patients with inactive brucellosis (5). In contrast, this differentiation was not possible when anti-LPS antibodies were measured. No serum reactivity against CP has been found in patients infected with V. cholerae (5) or in cattle infected with Y. enterocolitica O : 9 (6). Similarly, while anti-CP antibodies were readily detected in the cerebrospinal fluid (CSF) of patients having neurobrucellosis, no reactivity to CP was detected in the CSF of patients with meningitis caused by Mycobacterium tuberculosis, Staphylococcus aureus, Neisseria meningitidis, etc. (7).

When we analyzed retrospectively the serology of patients having different post-antibiotic outcomes of brucellosis (recovery, persistent illness, relapse) the kinetics of the serum antibody response to proteins were similar to those against LPS and both responses correlated with the clinical outcome of patients (8). Since then, the ELISA with CP antigen has been routinely used in our laboratory, in conjunction with classical serology, to diagnose human brucellosis. Along with the wider application of the assay we noted a few cases in which no antibodies to CP were detected in spite of the high titers obtained by classical agglutination tests (indicative of high anti-LPS titers). The analysis of the clinical records revealed that virtually all these cases corresponded to patients with recent onset of symptoms. This led us to hypothesize the existence of a

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"window" during the early stages of human brucellosis in which no antibodies to CP are detected while anti-LPS antibodies are already present. Surprisingly, however, some of these patients never developed anti-CP antibodies during a follow-up of up to 6 months, although no apparent cause could be identified. Thus, a priority for our group was to identify those factors that could potentially determine the absence of anti-CP antibodies in some patients and, in consequence, could impose some limitations on the usefulness of the ELISA based on the CP antigen. The occurrence of a human outbreak of B. melitensis infection (9) gave us the opportunity to address this question. Given the epidemic nature of the infection and the virulence of the strain involved, most patients were diagnosed a few days after the onset of symptoms. Here we describe the IgM, IgG and IgA responses of these patients to CP and LPS of Brucella at initial diagnosis and during the following 30 weeks. The influence of demographic, epidemiologic and therapeutic factors on these responses is analyzed and possible implications for diagnosis are discussed.

MATERIALS AND METHODS

Patients

Patients included in the present study were involved in an outbreak of B. melitensis infection that occurred on a ranch in Argentina, where an epidemic of caprine abortions due to this bacterium had begun 15 d before the presentation of the first human case. Clinical, epidemiologic and therapeutic aspects of this outbreak are described elsewhere (9). Briefly, active brucellosis was diagnosed in 33 of the 60 subjects who lived and/or worked on the ranch where the outbreak occurred. According to the epidemiologic studies, these patients had acquired the infection by direct contact with goats and/or goat manure, but none of them had ingested goat's milk or goat's cheese. Human brucellosis was diagnosed on the basis of clinical, serologic, bacteriologic and epidemiologic data. Classical serological tests included standard tube agglutination (STA), 2-mercaptoethanol agglutination test (2ME) and Rose Bengal test (2). In addition, ELISA tests were performed to detect IgM, IgG and IgA antibodies to LPS and CP of Brucella (see below).

As the purpose of the study was to assess the antibody response to Brucella antigens in the early phase of acute brucellosis, a serological follow-up of 30 weeks duration was performed in 21 patients (20 men) whose illness had begun up to 40 d before initial diagnosis. The majority of these patients were engaged in rural activities (mainly goat breeding) which, on this ranch, were performed exclusively by men (5). This explains the large proportion of male patients in our cohort. At this time, blood cultures were performed in 20 patients and B. melitensis biovar 1 was isolated in 12 cases.

Antibiotic therapy

Therapy with antimicrobials was initiated as soon as the diagnosis of brucellosis was established. In 12 patients the initial treatment was oral doxycycline, 100 mg every 12 h for 42 d, plus parenteral streptomycin, 1.0 g every 24 h for 21 d. The other 9 patients were initially treated with the same regimen of doxycycline combined with oral rifampin, 300 mg every 12 h for 42 d.

Serological follow-up

Serum samples were obtained from all patients at the time of diagnosis and at 6, 18 and 30 weeks after the first sample. The time from the onset of symptoms to the first serum sample being taken was 17 ± 12 d (range 3–40 d).

Agglutination tests

At diagnosis and during the follow-up serum samples were assayed by STA, 2ME and Rose Bengal test as described by Alton et al. (2). For the first 2 tests, the USDA technique (serial twofold dilution of sera starting at 1 : 25) was used.

ELISA for detecting antibodies to Brucella LPS

Serum reactivity against LPS and LPS-associated proteins was titrated by capture ELISA, using the LPS-specific monoclonal antibody BC68, as described previously (8). Briefly, Maxisorp polystyrene plates (Nunc, Roskilde, Denmark) were sensitized with purified BC68 and unbound sites were blocked with phosphate-buffered saline (PBS) containing 3% skimmed milk. After washing, plates were incubated with a B. abortus-soluble fraction (equivalent to 5 μg/well of LPS), followed by the addition of serial twofold dilutions of the patient sera (initially 1 : 200). Specific antibodies were detected with a horseradish peroxidase (HRP)-conjugated monoclonal antibody to human IgG (anti-γ chain; Sigma, St. Louis, MO), a HRP-conjugated polyclonal antibody to human IgM (anti-μ chain; Sigma) or a HRP-conjugated polyclonal antibody to human IgA (anti-α chain; Sigma). The reaction was developed with ortho-phenylenediamine/H_2O_2 in 0.1 M citrate–phosphate buffer, and was stopped with 4 N H_2SO_4. To establish the cut-off value of the assay, 50 sera from healthy controls were assayed at a dilution of 1 : 200 under the same conditions used for patient sera. The cut-off value was calculated as the mean optical density (OD) plus 3 standard deviations. Serum titer was established as the reciprocal of the last serum dilution yielding an OD higher than the cut-off.

ELISA for detecting antibodies to CP of Brucella

Serum IgG, IgM and IgA reactivities against Brucella proteins were assayed by indirect ELISA using the CP antigen of B. abortus (5). This antigen was prepared by immunoadsorption of the cytoplasmic fraction of Brucella with the anti-LPS monoclonal antibody BC68 coupled to Sepharose-4B gel (Pharmacia, Piscataway, NJ) as previously described (10).

The plates were sensitized with 0.5 μg/well of CP diluted in PBS. Sera were assayed at twofold serial dilutions beginning at 1 : 100. Washing, blocking, addition of the conjugates, development of the reaction and determinations of the cut-off value and serum titers were performed as described above.

Statistical analysis

Data were analyzed using the Kruskal–Wallis Nonparametric ANOVA Test and Dunn's Multiple Comparisons Test with InStat 2 software (GraphPad Software Inc., San Diego, CA).

RESULTS

Detection of antibodies to Brucella antigens at initial diagnosis

At diagnosis, both IgM and IgG to LPS were detected in 18 patients, while the other 3 patients had only anti-LPS IgM. The STA test, which detects both anti-LPS isotypes, was positive in all the cases studied. Although 2ME is deemed to detect mainly anti-LPS IgG, this test was positive in only

10 of the 18 patients positive for anti-LPS IgG by ELISA. Anti-LPS IgA was detected in 19 patients, including 1 patient negative for IgG to LPS. Regarding anti-CP antibodies, both IgM and IgG were found in 3 patients, while 7 patients had only IgM and 6 had only IgG. No response to CP was detected at diagnosis in 5 patients.

As shown in Table I, the proportion of patients with a positive result for the initial serum sample was higher for IgM and IgG to LPS than for the corresponding anti-CP antibodies. All isotypes of anti-CP antibodies were detected less frequently than their anti-LPS counterparts. At diagnosis, ≥ 1 isotype of anti-LPS antibodies had been detected in all patients, while only 76% of the individuals were positive for ≥ 1 isotype of anti-CP antibodies. While most patients (90%) tested positive for anti-LPS IgA, only 2 patients had IgA to CP.

Serological follow-up

All 21 patients were serologically followed for at least 30 weeks. For the purposes of the present study, however, only the results of patients who, at diagnosis, were negative for both antibody isotypes or showed only specific IgM are discussed.

As mentioned above, 3 patients were negative for anti-LPS IgG at diagnosis. Two of them were positive for this reactivity at 6 weeks post-diagnosis but the remaining patient was persistently negative during the whole follow-up. In addition, the 2 patients who were negative for IgA to LPS at diagnosis did not develop this reactivity during the remainder of the study.

As stated earlier, only 1 isotype of antibodies to CP was detected at diagnosis in 13 patients (IgM in 7, IgG in 6), while no response was found in 5 patients. Three of the 7 patients having only IgM were positive for anti-CP IgG at 6 weeks post-diagnosis, but the remaining 4 did not develop this reactivity during the whole follow-up. In addition, 1 of the 5 patients showing no response to CP at diagnosis was found positive for IgG at 6 weeks. In consequence, 4 patients initially negative for IgG to CP became positive for this reactivity between diagnosis and 6 weeks post-diagnosis. At the end of the follow-up both IgM and IgG to CP had been detected in 6 patients, IgM without switching to IgG had been found in 4, IgG without IgM had been detected in 7 and no response to CP was found in 4 patients.

Factors possibly interfering with the antibody response to Brucella proteins

In order to analyze which factors were possibly associated with the absence of antibody response to CP or the lack of switching from IgM to IgG in those patients in which specific IgM was detected, some characteristics were compared between these 2 groups and those patients who had developed IgG to CP at some point of the follow-up (with or without detection of anti-CP IgM). As shown in Table II, no significant differences were found between these 3 groups in terms of mean age, gender or mean duration of illness at onset of antibiotic therapy. In addition, antibiotic regimens were evenly distributed among the different groups of anti-CP response. While not statistically significant, the time elapsed from first symptoms to the onset of therapy tended to be lower in patients having no response to CP than in those who developed anti-CP IgG (7.7 d vs. 20.2 d). In addition, in the group having only anti-CP IgM, the time elapsed from presentation to therapy averaged 8.3 d in 3 patients and was 48 d for the remaining patient.

Some interesting trends were noted when patients were stratified according to the duration of illness at the onset of antibiotic therapy. Notably, 3 of the 4 patients who did not show a switching of anti-protein antibodies and the 4 patients having no response to CP in the first 6 weeks had begun antibiotic treatment within 14 d post-symptoms. In contrast, 9 of the 13 patients (69%) showing IgG to CP had started antibiotic therapy at least 15 d post-symptoms. Maximum titers of IgG to CP during follow-up tended to be lower in patients treated within 14 d post-symptoms than in patients who began treatment later (median 50 vs. 300, $p = \mathrm{NS}$), but these trends were less marked regarding

Table I. *Serological findings from 21 patients with acute active brucellosis*

Serological test[a]	Cumulative number of positive results (% of patients)			Median titer at diagnosis (range)
	At diagnosis	At 6 weeks	At 30 weeks	
STA	21 (100)	21 (100)	21 (100)	800 (25–6400)
2ME–STA	10 (48)	13 (62)	14 (67)	12.5 (0–400)
IgM to LPS	21 (100)	21 (100)	21 (100)	3200 (200–25,600)
IgG to LPS	18 (82)	20 (95)	20 (95)	400 (200–3200)
IgA to LPS	19 (90)	19 (90)	19 (90)	1600 (100–6400)
Antibodies to LPS (any class)	21 (100)	21 (100)	21 (100)	–
IgM to CP	10 (48)	10 (48)	10 (48)	200 (100–400)
IgG to CP	9 (43)	13 (62)	13 (62)	200 (100–800)
IgA to CP	2 (9)	2 (9)	2 (9)	150 (100–200)
Antibodies to CP (any class)	16 (76)	17 (81)	17 (81)	–

[a] IgM, IgG and IgA were determined by ELISA.

Table II. *Characteristics of patients showing different patterns of antibody response to Brucella CP*

Isotype detected	No. of patients	Gender (M/F)	Age (y; mean, range)	Days from clinical onset to antibiotics (mean, range)	Antibiotic regimen (DS/DR)[b]
IgG (with or without IgM)[a]	13	13/0	23.1, 10–58*	20.2, 3–45*	7/6
IgM only	4	3/1	28.7, 14–40*	18.2, 3–48*	2/2
No response[c]	4	4/0	41.2, 27–56*	7.7, 3–11*	3/1

[a] This group includes 7 cases in which both isotypes were detected and 6 cases in which only IgG was found (first detected at diagnosis in 5 cases and at 6 weeks in 1).
[b] Antibiotic therapy: doxycycline–streptomycin (DS) or doxycycline–rifampin (DR).
[c] Includes 1 patient who developed anti-CP IgG only after suffering a relapse (18 weeks).
* No statistically significant difference between groups ($p > 0.05$).

anti-LPS IgG response (median 1200 vs. 2400, $p = \mathrm{NS}$). Although the difference in mean age between the groups depicted in Table II approached statistical significance ($p = 0.08$), the stratification by age showed that, by chance, the mean time elapsed from first symptoms to the onset of antibiotic therapy was higher in patients < 30 y old than in older patients (23.7 and 9 d, respectively). Therefore, although a role for the patient's age cannot be excluded, the time elapsed to antibiotic therapy seems to be the main factor affecting the anti-CP response.

Other factors that could potentially influence the immune response did not seem to be associated with the diversity of anti-LPS or anti-CP responses. No patient had a previous history of brucellosis, and no intercurrent diseases or clinical conditions that could impact on the immune response were detected. In addition, no patient was taking immunosuppressive agents or any drug known to modify the leukocyte count. The frequency and degree of smoking and alcohol consumption were similar among the groups. The only woman included in the study did not become pregnant during the whole follow-up.

DISCUSSION

The diagnosis of human brucellosis has traditionally been based on the detection of antibodies to Brucella LPS (2, 4). In view of the diagnostic drawbacks of this strategy, our group has been investigating the potential usefulness of antibodies directed to CP of Brucella. We have developed an ELISA test using LPS-free CP of B. abortus, which has been successfully used to differentiate active human brucellosis from inactive forms of the disease and from infections caused by other bacteria (5). In the present study, we took advantage of an outbreak of human brucellosis to assess the performance of this test in patients diagnosed and treated at very early stages of the disease. For comparison, the antibody response to LPS was also followed.

As shown in Table I, antibodies to CP were detected less frequently than antibodies to LPS, both at initial diagnosis and during the whole follow-up. At first examination anti-LPS antibodies of ≥ 1 isotype were detected in all patients, while anti-CP antibodies of any class were found in only 76% of the subjects. As some patients became positive for anti-CP antibodies during the following 6 weeks, the percentage of anti-CP detection reached 81%. However, 4 patients had only IgM to CP without subsequent switching to IgG and no anti-CP response was detected in 4 other patients.

The analysis of clinical records revealed that most of the patients showing no switching of isotypes or no response to CP had initiated antibiotic therapy within 14 d from the onset of symptoms. In addition, in these early-treated patients, maximum titers of antibodies to both LPS and CP were slightly lower than those of patients treated later. These observations suggest that, at least in some cases, the early onset of antibiotic treatment could hinder the antibody response to CP of Brucella and, to a lesser extent, that to LPS. The bacterial clearance at the initial stages of the disease would reduce the chances of interaction between Brucella antigens and the immune system. Given the lower immunogenicity of Brucella CP compared to LPS, this clearance is more likely to interfere with the immune response to proteins. Using a murine model of B. melitensis infection, we have recently shown that early antibiotic therapy abolishes the antibody response to Brucella proteins and reduces the antibody response to LPS (11).

In a previous study in patients who acquired brucellosis by ingestion of unpasteurized goat's cheese we found anti-CP antibodies in all 9 individuals examined (12). While these patients were infected with the same pathogen than those included here (i.e. B. melitensis biovar 1), their duration of illness at the time of diagnosis was considerably longer (8–21 weeks). Those findings suggest that the interplay between the immune system and the pathogen must be longer than in the present study in order for an appropriate anti-CP response to be developed.

Besides therapy, other factors may also contribute to the diversity of the antibody response to proteins, as some patients showed switching of anti-CP isotypes despite receiving an early antibiotic treatment. Patients included in this study constituted a homogeneous group, as all of them were infected by the same bacteria, and probably by the same route (9), and all were diagnosed and studied at early

stages of the disease, i.e. shortly after the onset of symptoms. No significant differences in age, gender, pregnancy, previous history of brucellosis or intercurrent diseases were found among patients having different anti-protein patterns. It is possible that other factors not assessed in this study (e.g. size of the inoculum) could be related to the diversity of anti-protein responses observed here.

Anti-CP IgG has been shown to be useful not only from a diagnostic but also from a prognostic point of view. After therapy, declining anti-CP titers correlate with an evolution to clinical recovery, while unchanging titers indicate persistent illness (8). Obviously, the prognostic usefulness of anti-CP IgG is lost in early-treated patients who do not develop these antibodies. When present, however, the anti-protein IgG response was a marker of clinical outcome in most patients included here (not shown), in agreement with our previous findings.

Several similarities were found between our study and previous studies regarding the antibody response to LPS. Most patients diagnosed shortly before the onset of symptoms had high initial titers of IgM to LPS but low titers of IgG to this antigen, in accordance with results reported by Ariza et al. (13) and by Gazapo et al. (14). Notably, in 7 of our patients anti-LPS IgG was detected by ELISA but not by 2ME, a discrepancy probably related to the higher sensitivity of the ELISA. These findings suggest potential limitations of the 2ME test in the diagnosis and serological follow-up of human brucellosis. While this test has been advocated as a marker of active infection by some authors (15), others have questioned its diagnostic value as there is evidence that the 2ME concentration used in the assay destroys not only IgM but also some IgG (4).

In summary, the present study shows that antibodies to CP can be absent in some patients infected with B. melitensis if antibiotic treatment is initiated very early in the course of the disease. As a consequence of the epidemic presentation and the high virulence of the pathogen, most patients were diagnosed and treated shortly after the onset of symptoms, precluding the development of a significant immune response against Brucella proteins. While these results apply only to patients with a very short course of the infection, they suggest a potential limitation for the diagnosis based on anti-CP antibodies. In clinical practice, human brucellosis is seldom diagnosed within 14 d of initial symptoms, specially in non-epidemic cases. In fact, we have observed a lack of anti-CP response in only $\approx 2\%$ of the patients treated in our hospital. When an outbreak is studied, however, there is an increased likelihood of finding patients in the early stages of the disease who, in consequence, have not yet developed an anti-CP response. Given the low frequency of this limitation and the high specificity of anti-CP antibodies, the detection of this reactivity should be routinely included in the battery of tests used to diagnose human brucellosis. In view of the lack of switching to anti-CP IgG in some patients, and the failure to detect IgM to CP in others, it seems advisable to measure both isotypes of anti-CP antibodies. The detection of specific IgA, in contrast, does not increase the overall sensitivity of the anti-CP response. In spite of their known cross-reactivities, anti-LPS antibodies as measured by ELISA would be useful for diagnosis given their higher sensitivity relative to anti-CP antibodies. The combined but independent determination of anti-LPS and anti-CP antibodies appears to be the best option to compensate for the relative weaknesses of each test.

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