Detection of Antibodies to Brucella Cytoplasmic Proteins in the Cerebrospinal Fluid of Patients with Neurobrucellosis

PABLO C. BALDI,1* GEORGE F. ARAJ,2 GRACIELA C. RACARO,1 JORGE C. WALLACH,3 AND CARLOS A. FOSSATI1

Instituto de Estudios de la Inmunidad Humoral (IDEHU), 1113 Buenos Aires,1 and Servicio de Brucelosis, Hospital F. J. Muñiz, 1282 Buenos Aires,2 Argentina, and Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center, Beirut, Lebanon3

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The diagnosis of human neurobrucellosis usually relies on the detection of antibodies to Brucella lipopolysaccharide (LPS) in cerebrospinal fluid (CSF) by agglutination tests or enzyme-linked immunosorbent assay (ELISA). Here we describe the detection of immunoglobulin G (IgG) to cytoplasmic proteins (CP) of Brucella spp. by ELISA and Western blotting in seven CSF samples from five patients with neurobrucellosis. While IgG to CP (titers of 200 to 12,800) and IgG to LPS (800 to 6,400) were found in the CSF of these patients, these antibodies were not detected in CSF samples from two patients who had systemic brucellosis without neurological involvement. The latter, however, had serum IgG and IgM to both LPS and CP. No reactivity to these antigens was found in CSF samples from 14 and 20 patients suffering from nonneurobrucellosis and noninfecitious diseases, respectively. These findings suggest that, in addition to its usefulness in the serological diagnosis of human systemic brucellosis, the ELISA with CP antigen can be used for the specific diagnosis of human neurobrucellosis.

Brucellosis remains a common human zoonotic disease, especially in developing countries. Neurological involvement of the central nervous system (CNS) has been detected in 3 to 5% of the patients with brucellosis, in both the presence and absence of systemic illness (10, 13). Meningitis is the most frequently encountered clinical condition in patients with neurobrucellosis, and it occurs after direct invasion of the CNS by Brucella (7, 10, 13). Acute Brucella meningitis is usually characterized by sudden onset of fever, headache, and nuchal rigidity. Psychiatric and motor sensory disorders are also common. However, since similar symptoms may be present in some patients who have systemic brucellosis without neurological involvement and in patients who have infectious meningitis due to other microorganisms, the diagnosis of neurobrucellosis requires direct or indirect evidence of Brucella in the cerebrospinal fluid (CSF) (7, 10). Because of the low frequency of Brucella isolation from CSF (less than 20% of patients), the diagnosis of neurobrucellosis usually depends on the detection of specific antibodies in that fluid (7, 10, 13). Oligoclonal immunoglobulin G (IgG), indicative of intrathecal antibody production, is frequently detected in CSF but is not exclusively diagnostic of neurobrucellosis (7, 11). The finding of brucellosa-specific antibodies in the CSF is highly indicative of CNS infection; however, since these antibodies are sometimes present at low levels, agglutination tests commonly employed in the diagnosis of neurobrucellosis can give false-negative results (2, 10, 13). In contrast, an enzyme-linked immunosorbent assay (ELISA) for detecting antibodies to heat-killed Brucella antigens showed high sensitivity in the diagnosis of neurobrucellosis (1, 2). These antigens, however, are likely to contain significant amounts of lipopolysaccharide (LPS) and, as indicated by the authors, cross-reactions with other gram-negative bacteria may occur. We have previously shown that the detection of serum antibodies to cytoplasmic proteins (CP [formerly called LPS-free CYT]) of Brucella spp. is useful for the specific diagnosis of human and animal brucellosis (4, 5, 9). Here we present results which indicate that the detection of antibodies to CP in CSF makes possible the differentiation between patients who have neurobrucellosis and those who have systemic brucellosis and neurological manifestations without actual CNS infection.

Two ELISA systems were used. CSF and serum antibodies against Brucella CP antigens were detected by an indirect ELISA as described previously (9). The CP antigen is an LPS-depleted cytoplasmic fraction of Brucella abortus S19, obtained by immunosorption with an anti-LPS monoclonal antibody. Maxisorp polystyrene plates (Nunc, Roskilde, Denmark) were sensitized with 0.5 µg of CP diluted in phosphate-buffered saline (PBS) per well. Plates were blocked with 200 µl of PBS containing 1% skim milk per well. After a wash, human CSF or serum were dispensed in serial dilutions (starting at 1:100) in a solution of PBS, 0.3% skim milk, and 0.05% Tween 20. Specific antibodies were detected with polyclonal anti-human IgG- or anti-human IgM-horseradish peroxidase conjugates (diluted 1:2,000 and 1:1,000, respectively; DAKO, Carpinteria, Calif.) The reaction was developed by adding ortho-phenylenediamine (2 µg µl−1, in 0.1 M citrate-phosphate buffer containing 0.03% H2O2) and was stopped with 4 N H2SO4.

CSF and serum antibodies against LPS were detected by capture ELISA with the anti-LPS monoclonal antibody BC68 as described previously (9). Purified BC68 was absorbed onto the well surfaces of the Maxisorp polystyrene plates, and after blocking, a cytoplasmic fraction of Brucella (9) was added to a
final concentration of 5 μg of LPS per well. The testing of the samples, addition of the conjugates, and development of the reaction were performed as described above.

To establish the cutoff value of the assays, 30 serum samples from healthy subjects and 20 CSF samples from noninfected controls (mostly Alzheimer’s disease patients) were assayed at a 1:100 dilution (anti-CP antibodies) or 1:200 dilution (anti-LPS antibodies) under the conditions described above. The cutoff value of each ELISA system was calculated as the mean specific optical density (OD) plus 3 standard deviations. The titer was calculated as the reciprocal of the last serum or CSF dilution giving an OD higher than the cutoff. For the assays of CSF, the cutoff values were 0.020 for anti-LPS IgM, 0.136 for anti-LPS IgG, 0.028 for anti-CP IgM, and 0.109 for anti-CP IgG.

These assays were used to test seven CSF samples from five patients who had neurobrucellosis, as shown by signs and symptoms indicative of neurological involvement and development of the reaction. The two patients had severe headaches, CSF samples were taken on admission unless otherwise specified. † and ‡, samples taken 60 and 30 days later than the initial sample, respectively; ND, not determined.

### TABLE 1. Clinical and laboratory findings for neurobrucellosis patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Patient sex, age</th>
<th>Clinical condition</th>
<th>Results of CSF analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F, 41</td>
<td>Chronic meningoencephalitis, cranial nerve palsy</td>
<td><em>Culture</em> Brucella sp. <em>Titer determined by:</em> Tube agglutination 80 Indirect Coombs test 160</td>
</tr>
<tr>
<td>2</td>
<td>F, 35</td>
<td>Chronic meningoencephalitis, peripheral nerve involvement</td>
<td>Negative Negative 160 160</td>
</tr>
<tr>
<td>3</td>
<td>M, 29</td>
<td>Chronic myelitis</td>
<td><em>Culture</em> Brucella sp. <em>Titer determined by:</em> Tube agglutination 640 Indirect Coombs test 160</td>
</tr>
<tr>
<td>4</td>
<td>M, 37</td>
<td>Meningitis</td>
<td>Negative Negative Positive Positive† Negative‡ Negative‡ ND ND</td>
</tr>
<tr>
<td>5</td>
<td>M, 35</td>
<td>Meningitis</td>
<td><em>Culture</em> B. suis <em>Titer determined by:</em> Tube agglutination 160 Indirect Coombs test ND</td>
</tr>
</tbody>
</table>

Antibodies to Brucella antigens in CSF and serum. As shown in Fig. 1, CSF samples from noninfected controls assayed at a 1:100 dilution produced very low ODs (below 0.100) in both ELISAs. At the same dilution, in contrast, the CSF from patients with neurobrucellosis produced ODs of 0.223 to 2.068 for anti-CP IgG and 0.563 to 1.882 for anti-LPS IgG. Since the respective cutoff values were 0.109 and 0.136, these samples were all considered positive for IgG to CP and IgG to LPS. Anti-LPS IgG titers from these samples ranged from 800 to 6,400, and anti-CP IgG titers ranged from 200 to 12,800. Conversely, the CSF from the two patients who had brucellosis without neurological involvement were negative for both anti-CP IgG and anti-LPS IgG. Serum samples from these two patients, however, were positive for IgM to LPS (titers of 25,600 and 1,600), IgM to CP (200 and 100), IgG to LPS (800 in both patients), and IgG to CP (200 and 400). CSF samples from patients who had brucellosis with or without neurological involvement were negative for IgM against both CP and LPS except for patient 4, whose titers were 400 and 800, respectively. In addition, CSF samples from the 14 patients with infectious nonbrucellar meningitis were negative for IgG to Brucella CP and LPS (Fig. 1), and the same was true for IgM antibodies to both antigens (not shown).

As shown in Fig. 2, different patterns of reactivity to Brucella CP were observed when CSF samples from patients with neurobrucellosis were assayed by immunoblotting. The reactivity patterns ranged from a few bands developed by the sample from patient 1 to about 20 bands developed by the initial sample of patient 4. All the CSF samples tested reacted with a protein with an apparent molecular mass of 22 kDa. In addition, a protein of 29 kDa was recognized by all samples except that from patient 1, and one band of 66 kDa was revealed by five of the seven samples. Notably, the sample from the B. suis-infected patient reacted with proteins of 13, 26, and 42 kDa that were not recognized by the remaining samples, while it did not react with a group of proteins between 17 and 22 kDa that was recognized by the samples from patients 3 and 4. In
addition, this sample reacted strongly with a protein of 15 kDa that exhibited only a faint reaction with the remaining samples. The sample from patient 1, which reacted only with proteins of 22, 15, and 38 kDa (exhibiting a faint reaction with the last two proteins), was the CSF sample with the lowest anti-CP titer, as determined by ELISA.

Since most patients who have systemic brucellosis without neurological involvement may show signs and symptoms such as headache and neuropsychiatric complaints (10, 11), the differentiation between this condition and neurobrucellosis is of paramount importance. The results reported here suggest that the detection of IgG antibodies against CP of Brucella in CSF allows this differentiation. Both antiprotein and anti-LPS antibodies were detected in the CSF samples from patients with proven neurobrucellosis but not in the CSF samples from patients who had systemic brucellosis without neurological involvement, while serum antibodies to Brucella were present in all these patients. These results agree with those of Araj et al. (1, 2), who detected IgG, IgM, and IgA to antigens from heat-killed Brucella in CSF samples from patients with neurobrucellosis but not in CSF samples from patients who had brucellosis without neurological involvement. It is interesting that all these latter patients had specific IgG and IgA in serum, and 77% of them also had IgM antibodies (2).

The ELISA system used here showed excellent specificity, since antibodies to Brucella CP were not detected in the 14 CSF samples from patients who had CNS infection due to microorganisms other than Brucella. Brucella CP have been previously shown to be specific to that genus and common to all its species (4–6, 8, 9). The CP antigen prepared from B. abortus has been successfully applied to the diagnosis of infections caused by the homologous species (5, 6) and also by B. melitensis (6, 12) and B. canis (4). Moreover, as shown here and in previous studies (6), it is also useful in diagnosing infections caused by B. suis.

Although the incidence of neurological involvement in cases of brucellosis has been reported to range between 3 and 5% (10, 13), the case of B. suis infection presented here was the first case of neurobrucellosis detected in a reference center of Argentina that had more than 300 brucellosis patients referred between 1993 and 1997. While most patients seen in this center are infected by B. abortus and B. suis, almost all the cases of brucellar meningitis reported in the literature were in the Mediterranean area, where B. melitensis predominates. This difference could possibly explain the low incidence of neurobrucellosis among Argentinian patients. Moreover, some authors have proposed a separate meningotropism for B. melitensis (11).

At present we do not know the clinical significance of the different CSF reactivities to Brucella CP shown by the samples from the B. melitensis cases and the B. suis case in Western blotting. This difference could reflect the existence of specific reactivity patterns that could help to distinguish Brucella species or could also be related to different stages of the disease (acute or chronic phase). Both possibilities are currently being assessed in our laboratory.

In conclusion, the ELISA with the Brucella CP antigen is a useful test in the diagnosis of neurobrucellosis. The assay allows distinction between this serious complication, nonbrucellar meningitis, and systemic brucellosis with neurological manifestations but without actual CNS infection. This new application would add to the known usefulness of this test in the diagnosis of systemic human brucellosis.
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REFERENCES