Characterization of an 18-Kilodalton Brucella Cytoplasmic Protein Which Appears To Be a Serological Marker of Active Infection of Both Human and Bovine Brucellosis

FERNANDO A. GOLDBAUM,1 JULIANA LEONI,1 JORGE C. WALLACH,2 AND CARLOS A. FOSSATI1*

Sección Brucelosis, Hospital F. J. Muñiz, Uspallata 2272, 1282 Buenos Aires,2 and Instituto de Estudios de la Inmunidad Humoral (IDEHU-UBA-CONICET), Junín 956, 1113 Buenos Aires,1 Argentina

Received 30 November 1992/Accepted 19 May 1993

Some anticytoplasmic protein monoclonal antibodies (MAbs) from mice immunized by infection with Brucella ovis cells have been obtained. One of these MAbs, BI24, was used to purify by immunoaffinity a protein with a pI of 5.6 and a molecular mass of 18 kDa. This protein was present in all of the rough and smooth Brucella species studied, but it could not be detected in Yersinia enterocolitica 09. Three internal peptides of this protein were partially sequenced; no homology with other bacterial proteins was found. The immunogenicity of the 18-kDa protein was studied with both human and bovine sera by a capture enzyme-linked immunosorbent assay system with MAb BI24.

The serological procedures currently used in the diagnosis of both human and bovine brucellosis are based on the detection of anti-lipopolysaccharide (LPS) antibodies. These antibodies remain high even after patients with brucellosis recover from the disease (17), and moreover, they are unable to differentiate infected cattle from healthy vaccinated cattle (22). On the other hand, tests performed with either LPS or LPS-rich extracts lead to extensive cross-reactions with other gram-negative bacteria (7). Thus, it might be assumed that the determination of the humoral response against LPS-free proteins could help to avoid those undesirable reactivities.

Measuring the humoral immune response against cytoplasmic proteins was previously shown to be important for the diagnosis of human brucellosis (10). A mixture of proteins depleted of LPS by immunoadsorption with an anti-LPS monoclonal antibody (MAb) was prepared. This antigen was used in an enzyme-linked immunosorbent assay (ELISA) to differentiate active infections from inactive infections (10).

Our goal in the present study was to obtain a single protein component from the cytoplasmic fraction which behaves like LPS-free CYT with regard to its ability to detect only active brucellosis.

To this end, we have prepared anti-cytoplasmic protein MAbs according to the protocol of Cloeckaert et al. (6) using spleen cells from Brucella ovis-infected mice.

By using one of these MAbs (B124), we could isolate and partially characterize a protein component specific to the Brucella genus and with a molecular mass of 18 kDa.

MATERIALS AND METHODS

SDS-PAGE and immunoblotting. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (12) with 12.5% acrylamide gels.

Immunoblotting analysis was performed as described by Tsang et al. (18). Ascitic fluid of MAb B124 was diluted 1/100.

Preparation of cytoplasmic fraction. Suspension cultures of Brucella abortus 19S cells were killed by the addition of 0.4% formaldehyde. The cytoplasmic fraction (CYT) was obtained as described by Verstreete et al. (19) with minor modifications as previously described (10). Briefly, bacterial cells were suspended in Tris buffer and broken by extrusion with an X-Press (type 25; AB BIOS) and then digested with DNase and RNase. Cell envelopes were harvested by centrifugation at 360,000 × g for 2 h. The resulting supernatant (CYT) was stored at −20°C.

Preparation of LPS-free CYT antigenic fraction. The LPS-free CYT antigenic fraction was prepared by immunoadsorption of the CYT fraction with the anti-LPS MAb BC68 coupled with CNBr-activated Sepharose 4B (Pharmacia) as previously described (10).

Human sera. In this study, we analyzed the sera from outpatients in the Brucellosis Department of Hospital Muñiz who were classified into three different groups as follows.

(i) Patients with active brucellosis. This group included patients with compatible symptoms (chills, fever, asthenia, arthralgia, sweating, weight loss, lymphadenopathy, and hepatosplenomegaly, etc.). Standard tube and slide agglutination tests with these sera showed titers equal to or higher than 1/100, and sera had positive Rose Bengal tests, positive agglutination with 2-mercaptoethanol, and positive complement fixation, as described earlier (10).

(ii) Patients with inactive brucellosis. This group included individuals with no compatible clinical picture who had epidemiological probabilities of exposure to or documented histories of the disease. The sera of all of those patients were positive at titers of 1/100 or higher by standard tube and slide agglutination tests. They were also positive by the Rose Bengal test and negative by agglutination with 2-mercaptoethanol and complement fixation as previously described (10).

(iii) Negative control group. The negative control group included healthy volunteers with no history of brucellosis.

Bovine sera. In this study, sera from animals experimentally infected with B. abortus bv. 1 (with and without vaccination with B. abortus 19S) and from vaccinated...
healthy animals, kindly provided by O. Rossetti from the Instituto de Biología Molecular de INTA Castelar, were used.

**MAb.** MAb BI24 [immunoglobulin (Ig) G2b (κ)] was derived by somatic cell hybridization as described by Galfré and Milstein with NSO myeloma cells as fusion partners (9). Spleen cells were obtained from BALB/c mouse intraperitoneally infected 30 days previously with 10⁸ B. ovis cells and boosted with 10⁶ inactivated bacterial cells 4 days prior to fusion as described by Cloeckaert et al. (6). The screening assay was performed by indirect ELISA with LPS-free CYT as antigen (1 μg per well) after blocking hybridoma supernatants were assayed without dilution. Rabbit immunoglobulin anti-mouse immunoglobulins, horseradish peroxidase-conjugated (Dako) at a 1:1,000 dilution, was used as a second antibody. Incubation times and development conditions were as described below.

**Immunoadsorption of cytoplasmic fraction with Seph-BI24.** Pulsed MAb BI24 (5 mg/ml) was coupled with CNBr-activated Sepharose 4B (Pharmacia) according to the supplier's specifications. With regard to immunoadsorption, BI24-coupled Sepharose 4B gel (Seph-BI24) was incubated with CYT with constant shaking for 24 h at 4°C. After the gel was washed, the retained fraction was eluted with 0.1 M Tris-HCl (pH 3) buffer.

**Capture ELISA.** The experimental design of Voller et al. (20) was used. Fast protein liquid chromatography-purified BI24 antibody (1 μg per well) was adsorbed onto Immulon II plates (Dynatech). Blocking was done with a blocking solution (BS) of phosphate-buffered saline (PBS) containing 3% skim milk when human sera were assayed or PBS containing 0.2% gelatin when cow sera were used. For washing, PBS plus 0.05% Tween 20 was used. Afterwards, the wells were incubated with LPS-free CYT (10 μg per well) in BS containing 0.05% Tween 20 (BST) for 1 h at room temperature. 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 an anti-human IgG horse-radish peroxidase-conjugated MAb or a rat anti-bovine IgG horse-radish peroxidase-conjugated MAb diluted in BST per well was added for 1 h at room temperature. After being washed, the contents of each well were developed with a solution containing orthophenylenediamine (2 mg/ml)-0.03% H₂O₂ in 0.1 M citrate-phosphate buffer (pH 5), and the reaction was stopped with H₂SO₄. The resulting color at 495 nm was read with an ELISA reader. Controls for nonspecific adsorption of antigen with no addition of the capture MAb were performed throughout. Each serum sample was also tested in parallel in the absence of antigen to detect antimurine IgG activity.

**Two-dimensional gel electrophoresis.** Two-dimensional gel electrophoresis was performed as described by O'Farrell (16). The LPS-free CYT antigen was dialyzed against 1% glycine and diluted in isoelectric focusing sample buffer (30% sucrose, 3% ampholytes [pH 3.5 to 9.5]; 1 M H₂PO₄ and 1 M NaOH were used as anodic and cathodic solutions, respectively. Electrophoresis of 5 μg of the antigen was run at room temperature for 18 h at 300 V. For the second dimension, the capillary gel was extracted and embedded for 5 min with sample buffer for SDS-PAGE and then placed over a running gel of 12.5% polyacrylamide with a stacking gel of 5% acrylamide. After being electrophoresed, the sample was electrotransferred to nitrocellulose and the immunoblot was developed with a 1/1,000 dilution of BI24 ascitic fluid. Horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin serum (Dako) diluted 1/500 was used as the second antibody. As staining agents, 4-Cl-α-naphthol (3 mg/ml) and H₂O₂ (0.03%) in Tris-buffered saline were used.

**Sequence analysis.** The technique described by Matsudaira (14) was used for the amino-terminal sequence. Sequencing by in situ cleavage with trypsin (sequencing-grade trypsin) was performed as described by Aebersold et al. (1). Briefly, after being subjected to SDS-PAGE and being electrotransferred to nitrocellulose paper, the sample was stained with Ponceau S and decolorated with 1% acetic acid. Five bands, including the 18-kDa protein, were cutoff the paper and blocked with 0.5% polyvinylpyrrolidone 40 and incubated overnight at 37°C with 20 μg of sequencing-grade trypsin (Sigma) diluted in 150 μl of 100 mM Tris-HCl (pH 8.2)-acetonitrile (95:5, vol/vol). The peptides resulting from the enzymatic digestion were isolated by high-performance liquid chromatography (HPLC) on a Spheri-S RP18 5-μm column (100 by 1.0 mm) with a 140A solvent delivery system and a model 1000 diodearray detector (Applied Biosystems). Solvent A was trifluoroacetic acid (0.1%), and solvent B was acetonitrile (80%) in solvent A. The gradients were 0% for 6 min, 0 to 20% for 10 min, 20 to 80% for 83 min, and 80 to 100% for 87 min. The flow rate was 50 μl/min.

Sequenceing was performed at LANAI/S-PRO (UBA-COMICET, National Facility for Protein Sequencing) with an Applied Biosystems model 477 A sequintiator.

**RESULTS**

**MAb BI24.** The use of splenocytes of mice infected with rough *Brucella* strains, as in this study with *B. ovis*, for hybridization makes it possible to obtain a very high ratio of anti-protein MAb, as reported by Cloeckaert et al. (6). The availability of an LPS-free protein antigen makes it possible to carry out specific screening of anti-protein antibodies.

Twenty-six hybridomas directed against different cytoplasmic antigens were obtained in this fusion. One of them produced an IgG2b MAb (BI24) which reacted strongly by immunoblotting with a cytoplasmic protein of *B. abortus*. The polypeptidic nature of the epitope recognized by BI24 was evidenced by showing that its reactivity was eliminated by treatment of LPS-free CYT with proteinase K (data not shown).

**Electrophoretic and immunoblotting analyses.** Figure 1A shows the SDS-PAGE profile of the antigen purified from the *B. abortus* cytoplasmic fraction by immunoadsorption with the MAb BI24. As can be seen, this antigen shows a major band of 18 kDa, a minor band of 36 kDa, and other components with greater molecular masses which cannot be dissociated by 2-mercaptoethanol reduction (Fig. 1A).

The same bands are seen by immunoblotting analysis when CYT and LPS-free CYT are used. Nevertheless, the 36-kDa band is masked by the multiplicity of bands developed by MAb BI24. (Fig. 1B, lanes 3 and 4). The antigen recognized by BI24, electrophoretically separated by two-dimensional gel electrophoresis and developed by immunoblotting with the same MAb, shows two spots corresponding to 18 and 36 kDa, both of which have pIs of 5.6 (Fig. 1C).

On the other hand, the 18-kDa component is present in all *Brucella* species studied but could not be detected in *Yersinia enterocolitica* 09 (Fig. 2).

**Microsequencing of the 18-kDa antigen.** The microsequencing technique described by Matsudaira (14) applied to the 18-kDa band of the BI24 antigen showed that the aminoterminal amino acid was blocked. The method of Aebersold et al. (1) was then used as follows: the 18-kDa band was digested in situ with trypsin, and the resulting peptides were
while suis 1330 Brucella whether vaccinated sera. bovine abortus patients with active and controls sera were differentiated from bovine (i) IAFIQADDVLK, (ii) SGYIFDXPG, and (iii) GVEAAXAALQIVSE.

When these sequences were compared with known sequences in a data base (protein data base in GenBank EMBL, Genetics Computer Group, 1991), homology was not observed either with Brucella spp. or with other bacterial proteins.

Reactivities of human and bovine sera. To establish the immunogenicity of this antigen, we have developed an antigen capture ELISA with the BI24 MAb, which is suitable for detecting specific serum antibodies in both human and bovine sera. The results obtained with human sera from patients with active and inactive brucellosis and healthy controls can be seen in Fig. 3A. Notice that sera from patients with active brucellosis produce a strong reaction, while sera from patients with inactive infections cannot be differentiated from sera from healthy individuals. When bovine sera were used, it was observed that infected cattle, whether vaccinated or not with B. abortus 19S, show high reactivities against this antigen, while vaccinated healthy animals do not react (Fig. 3B). On the other hand, all vaccinated healthy animals showed a strong IgG anti-LPS response, as determined by ELISA (results not shown).

**DISCUSSION**

We have shown in a previous paper that Brucella cytoplasmic proteins depleted of LPS were useful for differentiating between active and inactive human brucellosis (10). Our present objective was to analyze whether any individual protein component had the same antigenic properties as the LPS-free Cyt extract.

All of the anti-Brucella MAbS described so far are directed against LPS (5, 11, 21) or membrane proteins (6, 15). Our former attempts to produce MAbS to soluble proteins by hyperimmunization led to the production of anti-LPS MAbS. Nevertheless, we could obtain anti-cytoplasmic protein MAbS by taking advantage of the method described by Cloeckaert et al. (6), which allowed us to obtain 26 specific hybridomas. As far as we know, this is the first article dealing with MAbS specific for cytoplasmic proteins of Brucella spp.

Using one of these MAbS (BI24), we have purified a cytoplasmic protein component of Brucella spp. This antigen shows several bands of different molecular masses. The two major components are those of 18 and 36 kDa. Notice that these components have the same pi and that they are not

---

**FIG. 1.** Electrophoretic analysis of antigen recognized by MAb BI24. (A) Antigen purified by immunoadsorption with MAb BI24 (10 μg per well) (lane 1) and SDS-PAGE of molecular mass markers (lane 2); (B) immunoblotting analysis of Cyt (5 μg per well) (lane 3) and LPS-free Cyt (5 μg per well) (lane 4) developed with MAb BI24; (C) two-dimensional gel electrophoresis of LPS-free Cyt electrotransferred to nitrocellulose and developed by immunoblotting with MAb BI24.

---

**FIG. 2.** Western blot analysis of B. abortus 1119/3 (lane 1), B. abortus 19 (lane 2), B. abortus 544/2 (lane 3), B. abortus 45/20 (lane 4), Brucella melitensis 16M (lane 5), Brucella canis RM6/66 (lane 6), Brucella suis 1330 (lane 7), B. ovis 63-290 (lane 8), and Y. enterocolitica 09 (lane 9) developed with MAb BI24. Before the electrophoresis, 50 μg (wet weight) of bacterial cells was boiled for 1 h in 500 μl of sample buffer and 50 μl was loaded onto the gel. Molecular mass standards (in kilodaltons) are on the left.

---

**FIG. 3.** Analysis of immune response of human (A) and bovine (B) sera by capture ELISA with MAb BI24. OD 492, optical density at 492 nm.
dissociated by treatment with 2-mercaptoethanol under stringent reducing conditions or by 4 M urea in SDS-PAGE (data not shown). As judged by those results, the 36-kDa component does not appear to be a dimeric form of the 18-kDa component, a fact that makes it difficult to explain the multicross components developed by MAb BI24.

The fact that this protein is present in all of the species of Brucella tested but is not detectable in Y. enterocolitica (9) makes it an antigen of special interest for diagnostic purposes.

Among the Brucella proteins already reported (2-4, 6, 8, 13, 19, 23), there are two which have molecular masses similar to that of the one showed here. Zygmunt et al. (23) have recently reported the characterization of the first, a 20-kDa cytoplasmic protein. This protein is immunogenic in infected animals, but its pI (4.9) is different from that of the BI24 antigen. The other is a very basic protein (pI = 8.6) of 20 kDa identified by Bricker et al. (3). It can be assumed, then, that the protein recognized by the MAb BI24 had not been reported at that time.

This antigen, like the LPS-free CYT (10), clearly differentiates between active and inactive human infections when used in an ELISA. The risks of possible interference by LPS in a capture ELISA can be ruled out in our system, since the extract containing the BI24 antigen had been previously depleted of LPS (10).

Because of the great individual variabilities in the humoral immune response among patients, it was surprising to find one individual component whose properties closely resemble those of a heterogeneous protein mixture.

The use of the BI24 antigen for the analysis of human sera demonstrates that this antigen behaves in different immunogenic manners with regard to patients with either active or inactive brucellosis. In fact, while the patients suffering from active infections show high IgG reactivities, those with inactive infections do not react against the BI24 antigen. These results agree with those previously reported by us using LPS-free CYT (10).

In spite of the low number of animals studied, when cow sera were analyzed, only infected animals produced antibodies against the BI24 antigen. The vaccinated healthy animals studied did not show reactivity toward this antigen, while all of them showed anti-LPS antibodies, as determined by ELISA (results not shown). This result is in agreement with the report of Wright and Nielsen (22).

All of the results mentioned above suggest a direct relationship between the bacterial virulence and the immunogenicity of the BI24 antigen.

The results presented here emphasize the importance of studying the humoral immune response against cytoplasmic proteins of Brucella spp., as already reported (10). It is also shown that the use of MABs is an appropriate strategy for the identification, characterization, and application of Brucella protein antigens for diagnostic purposes.

ACKNOWLEDGMENTS

We thank Ana Vigliocco for experimental infection of mice with B. ovis, Osvaldo Rossetti for providing sera from experimentally infected and vaccinated cattle, and Centro Panamericano de Zoonosis-Oficina Panamericana Sanitaria, Buenos Aires, for providing the Brucella and Yersinia strains. We also thank Jorge Wallach, Silvia Miguel, and Pablo Baldí for their help.

This research was financed by grants from CONICET and UBA. Fernando A. Goldbaum is a recipient of a Becas de Perfeccionamiento UBA. Juliana Leoni and Carlos A. Fossati are members of the Carrera del Investigador CONICET. C.A.F. is also a member of the Cátedra de Immunología, Facultad de Ciencias Exactas, UNLP.

REFERENCES


