Removal of LPS from a *Brucella* cytoplasmic fraction by affinity chromatography with an anti-LPS monoclonal antibody as immunosorbent

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Summary. Affinity chromatography on polymyxin B-Sepharose 4B is one of the most commonly used methods for the removal of contaminating lipopolysaccharides (LPS). However, the LPS of *Brucella* spp. do not bind to polymyxin B. An affinity chromatography method with an anti-O antigen of *Brucella* LPS monoclonal antibody as immunosorbent was developed. The method produced a 1000-fold reduction in the LPS content of the cytoplasmic fraction of *B. abortus*. The eluted proteins retained their antigenicity. The method, which uses mild physiological conditions, is simple, effective and reproducible.

Introduction

Lipopolysaccharide (LPS) is the immunodominant antigen of the genus *Brucella*.¹ Most serological procedures currently used in the diagnosis of brucellosis are based on the detection of anti-LPS antibodies. However, the use of these anti-LPS antibodies in diagnosis presents difficulties: in man, antibody titres remain high even after recovery from the disease;² in bovine brucellosis, they cannot be used to differentiate between infected and vaccinated animals;³ and more importantly, they are highly cross-reactive with other gram-negative bacteria.⁴ Consequently, the assay of *Brucella*-protein antibodies provides an alternative approach in the diagnosis of brucellosis.

Since *Brucella* LPS binds very strongly to both cytoplasmic and membrane proteins,⁵ it is difficult to obtain protein preparations free of LPS contamination. Unlike other gram-negative bacteria, *Brucella* LPS does not bind to polymyxin B⁶ and therefore affinity chromatography with polymyxin B cannot be used.

Two strategies have been used in attempts to solve this problem; the extraction of the LPS moiety by chemical procedures^{7–9}, the cloning and expression of brucella proteins in *Escherichia coli*.^{10,11}

We report a method to eliminate LPS from a cytoplasmic extract of *B. abortus* by affinity chromatography with an anti-LPS monoclonal antibody (MAb) as immunosorbent.

Materials and methods

Bacterial culture and preparation of cytoplasmic fraction

A culture suspension of *B. abortus* strain 19-S was kindly provided by Laboratories Bagó San Jorge (Argentina). Cells were killed by the addition of formaldehyde 0.4% v/v. The cytoplasmic fraction (CYT) was obtained as described by Verstrete *et al.*¹² Bacterial suspensions were centrifuged at 16000 *g* for 10 min and washed three times with 10 mM Tris-HCl, pH 8.0. Cells were suspended in Tris buffer (0.1 g wet weight/ml) and extruded with an X-Press (Type 25, AB BIOS). The apparatus was cooled to –26°C and the bacterial cells were broken by five successive passages. They were then digested with DNAase 1% w/v and RNAase 1% w/v (Sigma). Unbroken cells were removed by centrifugation at 8000 *g*. Cell envelopes were harvested by centrifugation at 360000 *g* for 2 h. The supernate (CYT) was stored at –20°C.

Monoclonal antibody

MAb BC 68 (IgG, Kappa) was derived by somatic cell hybridisation as described by Galfré and Milstein¹³ with NSO myeloma cells as fusion partners. Mice used for fusion were hyperimmunised with *B. abortus* 19S cells which had been inactivated by autoclaving at 121°C for 15 min. MAb BC68 was purified by ion exchange chromatography on Q-sepharose in an FPLC P-500 system (Pharmacia).

Polysaccharide fraction of Brucella S-LPS

The polysaccharide fraction was obtained by mild

acid hydrolysis of brucella S-LPS, by the technique described by Moreno *et al.*⁶

ELISA

The method of Voller *et al.*¹⁴ was used for the ELISA. All the antigens were adsorbed to Immulon II plates (Dynatech)—1 µg of protein in 50 µl of 0.15 M phosphate-buffered saline (PBS), pH 7.2, into each well. After blocking with PBS containing skim milk 3% v/v (BS) and washing with PBS plus Tween 20 0.05% v/v (BST), serum from hyperimmunised mice or ascitic fluid of MAb BC68 diluted 1 in 100 in BST was added and the plates were incubated for 1 h at room temperature. After washing with BST, 50 µl of a peroxidase-conjugated rabbit anti-mouse IgG (Dakopatts) diluted 1 in 1000 in BST was added to each well and the plates were incubated for 1 h at room temperature. After a further wash, the plates were developed by adding a solution containing *o*-phenylenediamine 2 mg/ml and H₂O₂ 0.03% v/v in 0.1 M citrate-phosphate buffer, pH 5.0. The reaction was stopped by adding 50 µl of 2 M H₂SO₄ to each well and absorbance was measured at 495 nm in an ELISA reader (Metrolab).

SDS-PAGE and immunoblotting

SDS-PAGE was performed with acrylamide 10% slab gels.¹⁵

Immunoblotting analysis was performed as described by Tsang *et al.*¹⁶ Serum from five patients with brucellosis was pooled and used at a dilution of 1 in 200 in BS. Ascitic fluid containing MAb BC68 was diluted 1 in 100 in BS.

Chemical analysis

Proteins were measured by the method of Peterson¹⁷ with bovine serum albumin (BSA; Sigma) as standard.

The limulus lysate gelation activity (LLGA) of all fractions was determined as described by Sullivan and Watson.¹⁸ *E. coli* O111:B4 LPS (Sigma) was used as standard. *B. abortus* S-LPS was estimated from the ratio of *E. coli* and *B. abortus* LPS gelation activity, i.e., 1 ng of *E. coli* LPS is equivalent to 1 ng of *B. abortus* S-LPS.⁶

Immunoabsorption of cytoplasmic fraction with BC68-Sepharose 4B

Purified MAb BC68 (5 mg/ml) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia), according to the manufacturer's specifications.

For immunoabsorption, 2 ml of BC68-coupled Sepharose 4B gel (BC68-Seph 4B) was incubated with 2 ml of CYT with gentle shaking for 24 h at 4°C. After centrifugation or 300 *g*, the supernate (called "first

adsorption") was immunoabsorbed as above, once more. The supernate, named "LPS-free CYT", was diluted 1 in 4 in PBS with respect to the original cytoplasmic fraction. The protein concentration was 0.86 mg/ml.

Agglutination tests

Direct agglutination tests were performed with equal volumes (50 µl) of a suspension of phenol inactivated *B. abortus* 19S 3% v/v in saline 0.85% containing phenol 0.5% v/v and antibody.

Results

MAb BC68 is specific for the repetitive epitope of the O antigen of brucella LPS. It agglutinates smooth cells of different *Brucella* species and also *Yersinia enterocolitica* O9 in which the O antigen is identical to brucella O antigen. MAb BC68 reactivity against CYT was inhibited by adsorption with the smooth *B. abortus* 19-S strain, but not with the rough *B. abortus* 45/20 strain. The reactivity of MAb BC68 against CYT was not abolished by previous digestion of this antigen

Fig. 1. Ouchterlony analysis of MAb BC68. CYT, *B. abortus* cytoplasmic fraction; RS, serum from a rabbit hyperimmunised with *B. abortus*; P, polysaccharide fraction of *B. abortus* S-LPS obtained by mild acid hydrolysis.

Table. Removal of LPS from CYT with BC68-Sepharose 4B

Fraction analysed	Protein concentration (mg/ml)	LPS concentration* (mg/ml)
CYT	3.6	8.9–12.5
LPS-free† CYT	3.4	7.2–10.0 × 10 ⁻³

* Calculated from the LLGA test.

† Concentrations in LPS-free CYT were corrected for dilution due to immunoabsorption.

Fig. 2. ELISA analysis of CYT and the eluted material of the two steps of affinity chromatography with BC68-Sepharose B4: ■, ascitic fluid of MAb BC68; ▨, serum of a mouse hyperimmunised with *B. abortus*; □, normal mouse sera.

Fig. 3. Immunoblot analysis of CYT and "LPS-free CYT" with: 1, pooled serum from patients with brucellosis; 2, ascitic fluid containing MAb BC68.

with proteinase K (Sigma) 50 µg/ml (results not shown). In the Ouchterlony assay, MAb BC68 precipitated with the polysaccharide fraction obtained by mild acid hydrolysis of S-LPS (fig. 1). However, MAb BC68 did not precipitate CYT fraction although it has a high content of LPS. This may be due to the low diffusion of the LPS molecule. Rabbit antiserum, on the other hand, produced a diffuse precipitation band against both CYT and the polysaccharide fraction.

The high avidity shown by MAb BC68, manifest by the high titres developed in both ELISA and agglutination systems (> 200 000 and 2000, respectively) make this MAb a suitable immunosorbent. Therefore, we used it coupled to Sepharose 4B in affinity chromatography to deplete the CYT fraction of LPS. The eluted material of the second step of immuno-

adsorption ("LPS-free CYT") showed a 1000-fold reduction in LPS content (table).

CYT and the eluted material obtained after the two steps of affinity chromatography were analysed by ELISA with MAb BC68 and serum from a mouse hyperimmunised with *B. abortus* (fig. 2). The reactivity with MAb BC68 decreased by 75% after the first step of adsorption and even further after the second step ("LPS-free CYT"). The material eluted from affinity chromatography retained its antigenicity and reactivity against the hyperimmune serum.

Immunoblotting with CYT and "LPS-free CYT" studies (fig. 3) gave similar results. Pooled serum from patients with brucellosis produced a broad smear with some well-defined protein bands against CYT. In contrast, a clear well-defined pattern of protein bands

was obtained with "LPS-free CYT". This showed that the protein bands were masked by the LPS present in CYT. Most of the protein bands developed by the hyperimmune serum remain after immunoadsorption with MAb BC68 (fig. 2).

As a control, both antigens were developed with MAb BC68. The results show that the LPS content of CYT had been reduced by immunoadsorption to a level which was no longer detectable.

Discussion

Brucella LPS differs from the LPS of other gram-negative bacteria. These differences involve both lipid A and the "backbone".^{6,19} Consequently, brucella LPS does not bind to polymyxin B⁶, an antibiotic capable of irreversibly binding LPS from most gram-negative bacteria. Polymyxin B-sepharose is often used to reduce the LPS contamination of macromolecular solutions.²⁰⁻²²

Removal of brucella LPS by chloroform-petroleum ether-phenol extraction⁹ or trichloroacetic acid (TCA) precipitation followed by sonication^{7,8} has been reported. However, these methods produce extensive protein denaturation, especially TCA precipitation.

Although there are several anti-brucella LPS MAbs, there are no reports of these being used to remove brucella LPS.

Immunoadsorption with BC68-Sepharose 4B, in common with affinity chromatography with poly-

myxin B, employs mild physiological conditions, which permit the recovery of proteins without denaturation.²¹ By using MAb BC68 as immunoadsorbent, a 10³-fold reduction of LPS content of CYT was achieved. Studies using polymyxin B to remove LPS from other gram-negative genera have reported reductions of 10³-10⁵-fold.²⁰⁻²² However, in these reports the LPS was merely a contaminant (up to µg/ml quantities), whereas in this work brucella LPS was present in concentrations in the order of mg/ml in the cytoplasmic fraction.

It is not clear whether the remaining LLGA present in "LPS-free CYT" is due to a trace amount of non-extracted S-LPS, or to the presence of either R-LPS or lipid A, either of which can produce gelation. However, this was not detected by the immunochemical methods and, consequently, did not interfere with the detection of anti-protein antibodies. We have shown previously that the "LPS-free CYT" antigenic fraction prepared by this method can be used to measure the humoral immune response to brucella proteins without interference by anti-LPS antibodies.²³

In conclusion, removal of LPS by affinity chromatography with an anti-LPS MAb can be used to prepare brucella LPS-free protein extracts. The method is simple, effective and reproducible.

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