HOST RESPONSE TO INFECTION

Identification and partial characterisation of a new protective antigen of *Brucella abortus*

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Two novel *Brucella abortus* proteins were isolated from *B. abortus* strain RB51 and their immunological properties were determined. These proteins precipitated in the 40–60% saturated concentration range of ammonium sulphate and had a molecular mass of 32.2 kDa and 22.9 kDa, respectively. Both were able to induce a strong in-vitro blast transformation in lymphoid cells obtained from mice previously sensitised with a crude brucella protein extract. The protection studies showed that the 22.9-kDa protein used as a protective immunogen was as effective as the live *B. abortus* RB51 vaccine but the 32.2-kDa protein had a poor protective effect under similar conditions. The amino-terminal sequence of the 22.9-kDa and 32.2-kDa proteins was determined and analysed in a database. The lack of homology with other known *B. abortus* proteins indicated that both proteins were novel antigens.

Introduction

Brucella abortus, a facultative intracellular pathogen, is the aetiological agent of brucellosis, a disease that affects man and several other animal species [1]. *B. abortus* is able to survive and replicate within the cells of the reticulo-endothelial system, thus avoiding the extracellular immune defence mechanisms of the host [2]. Therefore, as for all intracellular bacterial pathogens, a protective immune response against *B. abortus* requires the action of T lymphocytes that mediate cytotoxic effects or induce greater killing capacity in the macrophage population, which is activated to eliminate the pathogen [3]. It is currently accepted that the lipopolysaccharide (LPS) component of *B. abortus* induces a strong humoral response [4], whereas certain protein components induce the T-cell-mediated response responsible for the elimination of the pathogen from the infected host [5]. However, other studies indicate that different proteins purified from *B. abortus* produce different types of immune responses: some proteins induce production of high levels of interferon-gamma (IFN- γ) that clearly correlates with a cellular

immune response, while others induce a pattern of cytokines more compatible with a humoral immune response [6]. Also, it has been observed that *B. abortus* proteins with low or medium molecular mass in the range 34–12 kDa are often the best inducers of cell-mediated immune responses [7, 8]. In this regard, an earlier study demonstrated that Cu/Zn superoxide dismutase extracted from *B. abortus* induces a strong T-cell response with high levels of IFN- γ production [9] and that this protein is able to protect mice against challenge with a pathogenic *B. abortus* strain [10]. Similarly, Splitter and colleagues, in experiments in mice, demonstrated that the purified ribosomal protein L7/L12 induces a pattern of cytokines that correlates with delayed-type hypersensitivity and a protective immune response [3, 5]. For brucellosis, as well as for other infectious diseases, it is important to identify the molecules in the aetiological agent that are able to induce a defensive response. In the future, this knowledge may enable the implementation of new vaccine strategies such as the use of recombinant micro-organisms, peptides or DNA vaccination. At present, the preferred vaccine for bovine brucellosis is based on *B. abortus* RB51, a non-pathogenic mutant of *B. abortus* 2308, described by Schurig in 1991 [11]. Taking these considerations into account, the general objective of this work was to study and to identify proteins from *B. abortus* RB51 able to induce a protective cellular immune response.

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Materials and methods

Animals

Two-month-old female BALB/c mice were obtained from Biosonda SA (Santiago, Chile). They were kept under standard conditions in the animal house facilities.

Bacterial strains

B. abortus strains 2308 from our own culture collection and *B. abortus* RB51 [11], originally obtained from Virginia-Maryland Regional College of Veterinary Medicine (Virginia Polytechnic Institute and State University, Blacksburg, VA, USA), were used throughout. The bacterial cells were grown in aerobic conditions in Trypticase Soy Broth (Difco Laboratories, Michigan, USA) for 72 h at 37°C and later used for inoculation, protein purification or immunological analysis. For inoculation, the bacterial suspensions were adjusted spectrophotometrically to an OD corresponding to 1×10^4 cfu of *B. abortus* strain 2308/ml and 2×10^8 cfu of *B. abortus* RB51/ml.

Extraction, analysis and fractionation of *B. abortus* RB51 protein

To extract the brucella protein for analysis, bulk culture of *B. abortus* RB51 was deposited by centrifugation, washed three times with 0.01 M phosphate-buffered saline (PBS; pH 7.2) and inactivated with methanol 60% for 24 h. After centrifugation, the cells were resuspended and kept for 1 day in a hypertonic salt solution (1 M NaCl with 0.1 M sodium citrate), sonicated, centrifuged at 27 000 g and the supernate containing the protein was recovered. The crude *B. abortus* protein solution was dialysed for 48 h against PBS, and kept at -30°C until use. *B. abortus* protein obtained in this way was tested for contamination with nucleic acids by spectrophotometry at 260 nm [12], for KDO by the thiobarbituric acid method [13] and for LPS content by the Limulus Amoebocyte Lysate E-Toxate Kit (Sigma), used according to the manufacturer's directions. The protein concentration was measured by the Pierce Coomassie protein assay reagent (Pierce, Rockford, USA.) with bovine serum albumin as standard [14]. As a first stage of fractionation, the brucella protein obtained was precipitated with ammonium sulphate 20% saturation, then centrifuged at 12 000 g for 15 min; the precipitate was dissolved and dialysed against PBS for 48 h. The resulting supernate from ammonium sulphate 20%, *B. abortus* protein precipitate, was further sequentially precipitated with 40%, 60% and 80% of saturated ammonium sulphate. In each case the protein obtained was dissolved and centrifuged at 12 000 g to remove insoluble material. In some cases the fractions of *B. abortus* proteins that showed good capacity to induce blast transformation of sensitised cells in vitro were submitted to a second fractionation by ion-exchange chromatography on a 20-ml carboxymethylcellulose column (Sigma). For this

procedure, the column was washed with 50 ml of starting buffer (0.01 M phosphate buffer, 0.1 M NaCl, pH 7.2), then loaded with the brucella protein preparation and run at a flow rate of 0.5 ml/min. The bound protein was eluted from the column with a linear gradient from 0.1 M to 1 M NaCl in 0.01 M phosphate buffer (wash buffer, pH 7.2). Protein was eluted in fractions emerging from 0.2 M to 0.8 M NaCl and the protein content of each fraction was estimated spectrophotometrically at 280 nm. Those fractions with high absorbance and corresponding to a single peak were pooled and analysed by SDS-PAGE. The protein peaks obtained were sterilised by filtration and kept frozen for use in lymphocyte proliferation or mouse protection studies.

N-terminal amino-acid sequencing

Samples (15 µg) of each of the semi-purified proteins from *B. abortus* RB51 were separated by SDS-PAGE and the protein bands were then electrophoretically transferred to polyvinylidene difluoride membrane (Applied Biosystems, Foster City, CA, USA) [15]. The protein bands were visualised by staining the membrane for 1 min with Coomassie Brilliant Blue 0.1% and de-staining with methanol 50%. The stained band was excised, and the N-terminal amino acids sequenced by the method described by Matsudaira [16]. The latter procedure was done at the Biotechnology Resource Laboratory, Medical University of South Carolina, Charleston, SC, USA, and the EMBL nucleic acid sequence database was used for homology searches.

Lymphocyte proliferation assay

This assay was performed as described previously [10]. Briefly, mice were inoculated in the footpad with 25 µg of *B. abortus* protein or PBS as control. In both cases the aqueous solution was emulsified in incomplete Freund's adjuvant (IFA; Sigma), and 7 days after immunisation the animals were killed by cervical dislocation. The popliteal lymph nodes were then removed and teased in cold tissue-culture medium. The cell suspension obtained was allowed to settle for 3 min to remove tissue particles and the free cells remaining in suspension were decanted and washed twice. The lymphocytes were next resuspended in RPMI 1640 medium containing fetal calf serum 10% v/v, plus antibiotics, and cultured in 96-well plates at a concentration of 4×10^5 cells/well. They were cultured alone or in the presence of different amounts of *B. abortus* antigens. After culture for 3 days, the cells were pulsed with thymidine (50 Ci/mmol; Amersham, London) 0.4 µCi/well for 8 h and harvested on glass fibre filters. The thymidine incorporation was measured in each culture and was expressed as counts per minute obtained from triplicate cultures.

Protection experiments

To test the induction of protection with different immunogens, groups of five mice each were vaccinated intraperitoneally (i.p.) with different preparations of *B. abortus* antigen or with 2×10^8 cfu of live *B. abortus* strain RB51 as positive control. Mice inoculated with PBS were used as negative controls. In all cases, the protein preparations and the control PBS were emulsified in IFA. Five weeks after vaccination all mice were challenged by i.p. injection of 10^4 cfu of *B. abortus* strain 2308. Experimentally infected mice were killed 2 weeks later by cervical dislocation, their spleens were disrupted and dilutions were plated to determine the number of *B. abortus* cfu/spleen [10]. The degrees of protection in vaccinated animals and controls were expressed as mean $\log_{10}$ cfu for spleens in each group obtained after challenge [17].

Statistical analysis

Statistical analyses were performed by Student's paired *t* test. $\log_{10}$ units of protection were obtained by subtracting mean $\log_{10}$ cfu for the experimental group from the mean $\log_{10}$ cfu of the corresponding control group [18].

Results

Contaminants of the crude *B. abortus* protein preparation

Before fractionation, the nucleic acid and LPS contaminants in the crude *B. abortus* protein prepara-

tion were determined. The results indicated that it contained 5% nucleic acids, 0.02% KDO and 0.18 U of endotoxin/mg of protein.

Purification of two *B. abortus* proteins

The isolation of two proteins from the crude *B. abortus* RB51 protein preparation was done in two steps. As a first step, brucella protein was precipitated with increasing concentrations of ammonium sulphate. Protein fractions that precipitated with 20% saturated ammonium sulphate and the resulting precipitates at 20–40%, 40–60% or 60–80% of saturation were analysed by SDS-PAGE and compared with the crude brucella protein preparation. The four fractions obtained showed minor differences in the protein pattern when compared with the total brucella proteins (Fig. 1a). The four precipitates obtained induced a lymphoproliferative response in cultures of popliteal lymph node cells obtained from mice immunised with brucella protein (Fig. 1b). Clearly, the fraction that precipitated at 40 and 60% saturated ammonium sulphate was the best inducer of blast transformation; therefore, this fraction was further purified by ion-exchange chromatography. The results of this separation indicated four peaks of material absorbing at 280 nm (Fig. 2a). Pooled fractions from peak A showed one major band of 32.2 kDa when analysed by SDS-PAGE (Fig. 2b, lane 5). Peak B showed two protein bands of 32.2 kDa and 22.9 kDa (Fig. 2b, lane 4), whereas peak C gave only one strong band of 22.9 kDa (Fig. 2b, lane 7). Peak D did not reveal any defined protein band (Fig. 2b, lane 6). The lymphoproliferative response induced by proteins from peaks A, B and C with lymphocytes

Fig. 1. (a) SDS-PAGE analysis of *B. abortus* protein (lane 2) and fractions precipitated with different concentrations of saturated ammonium sulphate: 3, 20%; 4, 20–40%; 5, 40–60%; 6, 60–80%. (b) Blast transformation capacity of the same fractions in lymph node cells obtained from immunised animals ($\square$) or in lymphocytes obtained from non-immunised animals (▨).

Fig. 2. (a) Elution diagram from ion-exchange chromatography of the crude *B. abortus* protein fraction that precipitated between 40 and 60% saturated ammonium sulphate, showing peaks A, B, C and D with high OD_{280} readings. (b) SDS-PAGE analysis of the protein content of peak A (lane 5), peak B (4), peak C (7) and peak D (6); (2) crude *B. abortus* protein; (3) crude *B. abortus* protein fraction precipitated between 40 and 60% saturated ammonium sulphate.

from mice previously sensitised with brucella protein was similar, but less than that observed when the lymphocytes were stimulated in vitro with brucella protein or with the proteins that precipitated between 40 and 60% saturated ammonium sulphate (Fig. 3).

Protection assays

The protection assays were done by immunising BALB/c mice with semi-purified 32.2-kDa or 22.9-kDa protein or crude brucella protein. PBS and viable

Fig. 3. Proliferative response of lymph node cells from mice immunised with total *B. abortus* protein (□) or non-immunised controls (▨), cultured in the presence of total *B. abortus* protein (1), protein fraction precipitated with 40-60% saturated ammonium sulphate (2) or the protein content of peak A (3), B (4) or C (5).

Table 1. Protection of mice against challenge with *B. abortus* 2308 after immunisation with different antigens

Vaccine and dose	Mean (SD) log ₁₀ cfu of <i>B. abortus</i> 2308 in spleen after challenge*	Log ₁₀ units of protection
Saline control	5.43 (0.15)	0
Live RB51 (2×10^8 cfu)	3.58 (0.67)	1.85
Total <i>B. abortus</i> protein (30 µg)	4.95 (0.78)	0.48
32.2-kDa protein (5 µg)	4.60 (0.45)	0.83
22.9-kDa protein (5 µg)	3.51 (0.88) [†]	1.92

*Mice were challenged i.p. with 10^4 cfu of strain 2308 5 weeks after vaccination.

[†] $p < 0.05$ compared with value for control mice.

B. abortus strain RB51 were used as negative and positive controls, respectively. Table 1 shows that, as might be expected, the strain RB51 live vaccine protected efficiently against the pathogenic challenge strain 2308. A similar effect was obtained with the 22.9-kDa protein that was able to induce a significant level of protection (1.92 log₁₀ units) ($p < 0.05$) compared with the negative control inoculated with PBS. In contrast, the protection obtained with crude brucella protein or with the 32.2-kDa protein was poor.

Amino-terminal sequence of 22.9- and 32.2-kDa *B. abortus* proteins

The amino-terminal sequences of the 22.9- and 32.2-kDa *B. abortus* proteins were determined as FRRPFRATPVQKDGP and AGESEVKVIVSGNAI, respectively. When these sequences were compared with the existing data in the EMBL nucleic acid sequence database, no sequences were found to be homologous with previously described *B. abortus* proteins, confirming that both proteins are new antigens not identified previously.

Discussion

The analysis of the crude *B. abortus* protein preparation showed that it contained only traces of endotoxin. This correlates well with the results obtained when the KDO content was measured. This small amount of LPS contamination makes it unlikely that the protection observed in this study was due to antibodies against this component of *B. abortus*. Moreover, if it is considered that the RB51 strain used in this study was essentially free of O-chain, it does not induce antibodies against the immunodominant epitopes located in the N-acylated perosamine polymer of LPS, which is usually responsible for the specific antibodies produced in this disease and detected by standard serological tests [11, 19, 20]. Results not shown in this work indicated that mice immunised in the footpad with the crude extract of *B. abortus* RB51 protein or the purified proteins in IFA did not produce antibody against S-LPS or the crude protein preparation [21].

The blast transformation experiments showed that the two *B. abortus* proteins partially purified in this study

were able to induce a significant cellular response in vitro, which indicates that these antigens were well recognised by the mice. Furthermore, if it is accepted that blast transformation correlates with a CD-4 T-cell response, it could be expected that these two proteins should have the capacity to induce a delayed-type hypersensitivity response, with a possible role in defence. However, the results of the protection assays demonstrated that the 22.9-kDa *B. abortus* protein was far better than the 32.2-kDa protein in this respect. This is consistent with previous observations that not all the protein components of *B. abortus* are good inducers of protection [22]. Antigen recognition, and even an immune response, in this case detected by lymphoproliferation assay, does not necessarily correlate with a protective response. In the present study both purified proteins were good inducers of lymphocyte proliferation in vitro, but only the lower mol. wt protein showed an important protective capacity when used as vaccine. It is important to state that the protective effect of the protein preparation was produced only when the antigen was injected as an emulsion in IFA, as described for the Cu/Zn superoxide dismutase protein (Cu/Zn SOD) and some of its peptides [23]. As demonstrated by the amino-terminal sequence analysis, the two proteins have not been studied before and the results presented in this study showed that at least the 22.9-kDa protein is a novel antigenic entity that deserves further attention.

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