Brucella Ribosomal Protein L7/L12 Is a Major Component in the Antigenicity of Brucellin INRA for Delayed-Type Hypersensitivity in Brucella-Sensitized Guinea Pigs

G. BACHRACH,1 M. BANAI,2 S. BARDENSTEIN,2 G. HOIDA,3 A. GENIZI,4 AND H. BERCOVIER1

Department of Clinical Microbiology, The Hebrew University-Hadassah Medical School, Jerusalem,1 and Department of Bacteriology, The Kimron Veterinary Institute,2 The Israeli Veterinary Services,3 and Department of Statistics, Agricultural Research Organization,4 Beit Dagan 50250, Israel

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A delayed-type hypersensitivity (DTH) reaction in the course of brucellosis in humans and animals can be revealed by the brucellin INRA (Brucellergen) skin test. Brucellergen is composed of more than 20 proteins of different molecular weights. A 12-kDa protein eliciting DTH in Brucella melitensis Rev1-sensitized guinea pigs was found to be a significant component for the allergenic properties of Brucellergen. Sequencing of the gene encoding this protein identified it as the L7/L12 ribosomal protein. The L7/L12 gene of B. melitensis was amplified by PCR and subcloned in the Escherichia coli pQE30 plasmid. The resulting recombinant protein did not produce a DTH reaction in sensitized animals. It was used to raise specific antibodies in a rabbit. Affinity chromatography with these antibodies was used to isolate a single protein from Brucellergen and from B. melitensis cytosol preparations which produced a DTH reaction in guinea pigs sensitized with B. melitensis Rev1. N-terminal amino acid sequencing of the protein confirmed that it was the L7/L12 ribosomal protein. This is the first complete report on the involvement of a defined bacterial ribosomal protein in the DTH response of animals infected with intracellularly multiplying bacteria.

Brucellosis is a zoonotic disease of great public health and economic importance throughout the world (14). Control of the disease depends on the one hand on the ability to identify and remove contaminated animals and on the other hand on prophylactic measures such as vaccination. Identification of brucella infections in animals is based mainly on serological tests. These tests are sensitive and specific, but they are difficult to perform under field conditions (12, 13).

As a facultative intracellular pathogen, a brucella elicits a cellular immune response as well as an antibody response. Delayed-type hypersensitivity (DTH) is one of the expressions of cellular immunity. This reaction can be measured in sensitized animals by the swelling and the erythema resulting from the intradermal injection of the pathogen's allergenic antigens (15, 17, 24).

A skin test to detect DTH and identify brucella-contaminated animals is used widely. However, the use of this test is limited to surveillance of whole herds and does not extend to the detection of the exposure of individual animals in a herd to the pathogen. This is because of the lower sensitivity of the skin test (60 to 80% sensitivity) compared with that of the conventional serum tube agglutination test (80 to 85% sensitivity) and that of the complement fixation test or the Rose Bengal test, which both have 90 to 95% sensitivities (12). Nevertheless, the specificity of the skin test is superior to that of the seroagglutination test and equal to that of the complement fixation test (14, 27). Brucella protein preparations (such as brucellin INRA, also called Brucellergen) have proved to be able to reveal a DTH reaction in sensitized animals (14). The protein composition of these preparations has been poorly characterized (18). Brucellergen is composed of 20 to 30 proteins. Only one fraction, composed of at least 3 not functionally defined proteins each with a molecular weight of $3 \times 10^4$, has been shown to be immunogenic in the DTH reaction (7). For the reasons stated above, the standardization of the existing skin test has not been yet achieved. Recombinant brucella-allergenic proteins could be good candidates for use in a skin test that could be standardized. In addition, the use of recombinant proteins would reduce the risks due to exposure to brucella during production of such reagents.

Filters bearing a 12-kDa protein isolated from Brucella abortus were able to reveal a DTH reaction in sensitized guinea pigs (5). This protein was characterized as an immunodominant protein by Brooks-Worrell and Splitter using a T-lymphocyte Western blot (immunoblot) technique (9). The N-terminal amino acid sequence of this protein was reported by Brooks-Worrell and Splitter (10). An oligonucleotide corresponding to the N-terminal amino acid sequence of the B. abortus 12-kDa protein was used as a probe to screen a Brucella melitensis genomic library. The gene encoding the 12-kDa B. melitensis protein was identified, sequenced, and found to be similar to the Escherichia coli ribosomal protein L7/L12 (2) and identical to the B. abortus L7/L12 protein (22).

In the work presented here, we describe the isolation and the identification of the brucella L7/L12 protein from brucellin INRA (Brucellergen). We also characterize the antigenic properties of this protein in the DTH reaction of brucella-sensitized guinea pigs.

**MATERIALS AND METHODS**

**Bacterial strains and DNA preparation.** B. melitensis Rev1, B115, and 16M were grown on tryptic soy agar supplemented with glucose serum (1). The strains were from freeze-dried bacterial stocks maintained at the Israeli Brucellosis Reference Center, Kimron Veterinary Institute. The DNA of strain 16M was isolated as previously described (6).
Expression and purification in E. coli of the brucella 12-kDa protein. The primers p512 (GGGGGATCCCTCGGTATCTCG CAAAGATTGTGAA) and p312 (CCAGTACCCTCA CCTACCTAGTTCACCT) were selected from the B. melitensis L7/L12 sequence (GenBank L27819) and synthesized with the Applied Biosystems 380 B DNA synthesizer. These primers included a BamHI site at the 5' end (p512) of the sense strand, and a BspHI site at the 3' end (p312). B. melitensis 16M DNA encoding the L7/L12 protein was amplified by PCR (VentR DNA polymerase; New England Biolabs, Beverly, Mass.). After amplification, the PCR product was digested with KpnI and BamHI simultaneously and the resulting DNA fragment was purified by agarose gel separation. Purification was followed by gene clean extraction of the DNA (Geneclean II kit; Bio 101, La Jolla, Calif.). The purified L7/L12 gene was ligated to the E. coli pQE30 plasmid expression vector (Qiagen; Diagen, Hilden, Germany) predigested by KpnI and BamHI. After ligation (2 h at 20°C with 2 U of T4 ligase, IBI, USA), the resulting plasmid containing the L7/L12 sequence, named pQE3012, was used to transform E. coli SG13009. The plasmid pQE3012 encodes for six histidines which are tagged to the C-terminus of the expressed protein. These histidines enable easy purification, under denaturation conditions, of the recombinant protein by affinity chromatography with immobilized nickel chelate (nickel-nitrilotriacetic acid [Ni-NTA] column; patent by Hoffmann-La Roche, Basel, Switzerland). The product of the cloned gene was expressed in high quantities by IPTG (isopropyl-β-D-thiogalactopyranoside) induction and purified on an Ni-NTA column as specified by the manufacturer’s instructions (Diagen). The purified protein was dialyzed against saline and submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (26) with anti-L7/L12 hyperimmune rabbit serum (described below) at a dilution of 1/2,000.

Preparation of rabbit anti-recombinant brucella L7/L12 antiserum. Two hundred micrograms of the recombinant L7/L12 protein (1 ml in a 0.9% NaCl solution) was emulsified in 1 ml of Freund complete adjuvant. The mixture was administered to a New Zealand White rabbit by subcutaneous injection (neck) and intramuscular injection (hind legs). Booster injections of the recombinant L7/L12 protein (200 μg) mixed with Freund incomplete adjuvant were given on day 14 and day 28. Blood was collected on days 38 and 43. The serum was separated by centrifugation (3,000 x g for 15 min) and stored at 4°C with 0.02% sodium azide.

Preparation of the anti-L7/L12 affinity column. One milliliter of Affi-Gel 10 (Bio-Rad Laboratories, Hercules, Calif.) was filtered, washed with distilled water, and equilibrated with 10 volumes of phosphate-buffered saline (PBS). The activated beads were added to a mixture containing 1 ml of serum (rabbit anti-recombinant brucella L7/L12) and 3 ml of 0.167 M MOPS (3-[N-morpholino]propanesulfonic acid) (pH 7). The mixture was agitated for 4 h at 4°C. The remaining free binding sites on the beads were blocked by the addition of 0.5 ml of 1 M ethanolamine (pH 7), and then the mixture was agitated at 4°C for 1 h. The agarose beads were transferred to a disposable plastic column apparatus and washed with 10 volumes of PBS before use.

Purification of the L7/L12 antigen from brucellin INRA and from B. melitensis. Tryptic soy broth (500 ml) was inoculated with B. melitensis B115 and incubated for 48 h at 37°C. Cells were harvested by centrifugation at 5,000 x g for 15 min, washed with PBS, and suspended in 15 ml of lysis buffer (0.44 M sucrose, 0.03 M MgCl₂, 25 μg of DNase per ml). Sand grains (12.5 g) were added, and the cells were crushed with a Braun homogenizer in five cycles of 50 sec each, with 10-sec interrupptions between cycles. The sand grains were removed by centrifugation (5,000 x g for 5 min). Whole cells and large aggregates were removed by centrifugation (14,000 x g for 20 min), and the supernatant was collected. The supernatant was loaded on the anti-L7/L12 affinity column. The column was then washed with 10 volumes of PBS followed by 3 volumes of NaCl solution (1 M) at a rate of 1 ml/min. The L7/L12 antigen was released from the column by the addition of 0.2 M glycine buffer (pH 2.3). Fractions of 1.35 ml were collected in tubes containing 150 μl of 2 M Tris (pH 9) neutralizing buffer. Samples (10 μl) from each fraction were loaded on SDS–15% polyacrylamide gels (19) to determine the purity of each fraction and were subjected to Western blot analysis as described above. The L7/L12 protein was also isolated from Brucellegon (batch 96 G 091; Rhone-Merieux, Lyon, France) by affinity column purification and subjected to Western blot analysis in accordance with the protocols described above. In addition, immunoaffinity-purified L7/L12 was further purified by SDS-PAGE. The band corresponding to the 12-kDa protein (the L7/L12 protein as identified by Western immunoblot analysis) was excised from the gel. The resulting highly purified L7/L12 protein was renatured by dialysis and then dialyzed against saline. The protein concentration was determined according to the technique of Bradford (8).

Amino acid sequencing. The purified L7/L12 protein isolated from the brucelin INRA with the affinity purification column was subjected to N-terminal amino acid sequencing by the automatic Edman degradation procedure with an Applied Biosystems 475A gas-phase protein sequencer coupled with a synchronized high-performance liquid chromatography system.

Skin reaction test. Forty male Hartley guinea pigs (250 g each at the beginning of the experiment) were sensitized by subcutaneous injection of 10⁶ B. melitensis Revl organisms per animal (1). Three weeks later, recombinant L7/L12, affinity-purified L7/L12, Brucellegon (Rhone-Merieux), and L7/L12-depleted Brucellegon were injected intradermally at various doses (in 100 μl). The L7/L12 protein (150 ng) purified on the immunoaffinity column and then subjected to SDS-PAGE and electrophoresis was injected into three sensitized guinea pigs and one control guinea pig. Diameters (in millimeters) of the areas of erythema were read 24 h later. The immediate nonspecific response was checked after 4 h.

The statistical analysis was done by the SAS (1990) generalized linear models procedure.

RESULTS

Antigenicity of the B. melitensis L7/L12 protein expressed in E. coli. The DNA of B. melitensis 16M encoding the L7/L12 protein was amplified by PCR with the primers p512 and p312. The resulting product had a size of 375 bp (data not shown), compatible with the expected size of the gene. It was subcloned in plasmid pQE30 to give plasmid pQE3012, which subsequently was transformed into E. coli SG13009. The six histidines tagged at the N terminus of the recombinant protein enabled purification of the product with the Ni-NTA column. As can be seen in Fig. 1 (lane b), the recombinant protein was expressed in E. coli at a high level (8 mg of recombinant protein per liter of E. coli culture) and was purified to complete homogeneity (6 mg of pure recombinant protein per liter of bacterial culture) by passage of the E. coli lysate through the Ni-NTA column (Fig. 1, lanes d to h).

This purified L7/L12 protein was used to immunize a rabbit. The rabbit antiserum prepared against the recombinant L7/L12 protein was collected twice on days 38 and 43 of the
immunization treatment. The sera reacted identically against the recombinant protein. A titer of 1:2,000 with the recombinant protein as the sole antigen was found optimal for Western blot analysis (data not shown). The serum was tested against Brucellergen and the brucella lysate, and as shown in Fig. 2 (lanes b and d, respectively), it was highly specific and sensitive for the detection of the L7/L12 protein.

Identification and purification of L7/L12 from Brucellergen and brucella lysate. Brucellergen L7/L12 protein was purified from Brucellergen and from the B. melitensis B115 lysates with the rabbit antibodies bound to the Affi-Gel column. Figure 3 depicts the various stages of purification of the protein from Brucellergen. Purification from whole-cell lysates produced the same results (data not shown). A single protein band was obtained after elution of the column and was subjected to N-terminal amino acid sequencing for definitive identification. The resulting sequence confirmed that this protein was the L7/L12 protein; the sequence was identical for the first 9 amino acids (ADLAKIVED) to the amino acid sequences deduced from the L7/L12 B. melitensis and B. abortus DNA sequences. It was also identical to the N-terminal amino acid sequence of the 12-kDa immunodominant protein (10).

Activity of the ribosomal L7/L12 protein in sensitized guinea pigs. The antigenicity of the various preparations of the L7/L12 ribosomal protein in the DTH reaction was compared with that of Brucellergen. The sensitization of guinea pigs inoculated with live B. melitensis Rev1 was checked 3 weeks after inoculation with Brucellergen. A typical reaction in these guinea pigs, which were injected with a 1-to-15 dilution of Brucellergen (7 μg of protein in 100 μl), was an area of erythema with a mean diameter of 19 mm. As shown in Fig. 4, further dilutions of Brucellergen led to reductions in the sizes of the areas of erythema. The regression of the sizes of the areas of erythema on log (dose) calculated from the results up to the dose of 2 μg was not significant (P = 0.1367). One outlying result (an area of erythema with a diameter of 24.5 mm after a protein dose of 0.41 μg) was excluded from the calculation.

Doses of 16 and 6 μg of the recombinant L7/L12 protein produced in E. coli and purified with the Ni-NTA column (as described above) were intradermally injected into five guinea pigs sensitized to B. melitensis and into two naïve control guinea pigs. No detectable DTH reaction to the recombinant protein was observed in sensitized guinea pigs, whereas the same guinea pigs reacted to Brucellergen injected into the contralateral side, as described above.

B. melitensis L7/L12 proteins purified from either Brucellergen or whole-cell brucella lysate as described above were intradermally injected into sensitized guinea pigs. As can be seen in Fig. 4, the L7/L12 protein purified from Brucellergen elicited a strong DTH response at amounts as small as 62.5 ng. The DTH response of the guinea pigs to the L7/L12 protein was dose dependent up to the 1-μg dose (P = 0.0001), with a slope much steeper than that obtained for Brucellergen. The result obtained with 2 μg of the L7/L12 protein was not included. The 500-ng dose of L7/L12 gave a reaction similar to that for 2,500 ng of Brucellergen, demonstrating that the L7/L12 protein was a potent DTH-revealing antigen. Similar results (data not shown) were obtained with the L7/L12 protein isolated from whole-cell brucella lysate. The injection of the L7/L12 protein (150 ng) purified by immunoadsorption and

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**FIG. 1.** Expression and purification of recombinant brucella L7/L12 protein. E. coli SG13009 cells harboring plasmid pQE3012 were grown to an optical density at 600 nm of 0.8. IPTG (1 mM) was added to induce the recombinant protein. Recombinant L7/L12 was purified from the E. coli lysate by affinity purification on an Ni-NTA column (see Materials and Methods). Samples were analyzed on SDS-14% polyacrylamide gels and stained with Coomassie blue. Lanes: a, molecular mass markers; b, total lysate of induced cells (10 μl); c, total lysate of noninduced cells (10 μl); d to h, fractions 1 to 4 of recombinant L7/L12 from induced lysate purified on an Ni-NTA column.

**FIG. 2.** Analysis of rabbit anti-L7/L12 serum specificity. A rabbit was immunized with the purified recombinant brucella L7/L12 protein (see the legend to Fig. 1). Brucellergen (lanes a and b) and B. melitensis lysate (lanes c and d) were subjected to SDS-PAGE and then to Western blotting (lanes b and d) as described in Materials and Methods. The hyperimmune anti-L7/L12 serum (1:2,000) was used to detect the presence of the L7/L12 protein in Brucellergen (lane b) and in the B. melitensis lysate (lane d). The total protein (Coomassie blue stain) profiles of Brucellergen (7 μg) and the B. melitensis lysate (5 μg) are depicted in lanes a and c, respectively.

**FIG. 3.** Chromatography purification of L7/L12 from Brucellergen. Brucellergen was loaded on an anti-L7/L12 immunoaffinity column. The bound L7/L12 was released from the column as described in Materials and Methods. Fractions were collected, analyzed on SDS-14% polyacrylamide gels, and stained with Coomassie blue. Lanes: a, molecular mass markers; b, Brucellergen (7 μl); c, unbound Brucellergen (7 μl) after passage through the immunoaffinity column; d to h, glycine elution fractions 1 to 5. The purified brucella L7/L12 ribosomal protein is depicted in lane f as a single 12-kDa band.
then subjected to SDS-PAGE and electroelution resulted also in a significant DTH reaction (erythema areas with diameters of 10 mm ± 1 mm versus no reaction in the control guinea pig). In order to evaluate the relative activity of the L7/L12 protein in Brucellergen, whole Brucellergen and Brucellergen from which the L7/L12 has been depleted by the affinity column method were intradermally injected into sensitized guinea pigs. Depletion of the L7/L12 antigen from Brucellergen resulted in a reduction of the allergenic activity of the preparation. A significant regression on log (dose) was demonstrated (P = 0.038). There was no significant difference between the slopes (P = 0.817) of the lines of the two preparations (regular or depleted Brucellergen). Therefore, a model with a common slope (P = 0.011) was adopted, and the lines in Fig. 4 were drawn accordingly. A constant difference of 2.24 mm between the DTH responses elicited by the two preparations was found.

Five control naive guinea pigs which were inoculated with either the L7/L12 preparations (400 ng in 0.1 ml), the whole Brucellergen, or the L7/L12-depleted Brucellergen did not show any reactions after 4, 24, and 48 h.

**DISCUSSION**

Ribosomal vaccines against different diseases have been proposed, but their rationale has been controversial (16, 20, 25, 31–34). It has been shown that brucella (11) and mycobacteria (3, 4, 23) ribosomes can elicit a DTH response when injected into sensitized animals. However, what exactly in the ribosomes induced the DTH was not definitely identified. The results reported in our present work clearly identify brucella ribosomal protein L7/L12 as an antigen capable of eliciting DTH in brucella-sensitized guinea pigs. We showed that this protein is present in Brucellergen (Fig. 2). The depletion of this protein from Brucellergen reduced the antigenicity of the preparation in the DTH test by a considerable (15%) but not significant amount (P = 0.16). The purified L7/L12 protein elicited a DTH response in sensitized guinea pigs, and its activity was dose dependent, with a slope of the regression line much steeper than that observed with Brucellergen. This probably indicates that the purified L7/L12 protein has a higher specific activity in the DTH reaction than the Brucellergen composed of a mixture of proteins. In a recent report, a partially purified L7/L12 protein from *Mycobacterium bovis* BCG was shown to be antigenic by the DTH reaction of guinea pigs sensitized with either live *M. bovis* BCG or killed *M. tuberculosis* (29). It seems therefore that during infection by intracellularly multiplying bacteria such as brucelae and mycobacteria, some of the bacterial structural proteins, such as the L7/L12 protein, are involved in the cellular immune response developed in the host. The abundance of the L7/L12 protein, which can reach as much as 45% of the ribosomal mass in *E. coli* (21), may be the reason for its recognition in infected guinea pigs. However, the highly conserved sequences of this protein among bacteria and even eukaryotes (21) raise questions about the nature of the antigenic motif that is recognized by the immune response of the infected host.

Ribosomal proteins L7/L12 are encoded by the same gene and differ only by acetylation of the NH3 terminus of the L12 protein, which then becomes the L7 protein (21, 30). Which one of these two proteins, if not both, is responsible for the DTH reaction remains to be determined.

The reason(s) why the recombinant L7/L12 protein was not antigenic in the DTH reaction is not yet understood. The lack of brucella-specific posttranslational modification in *E. coli* could be one explanation. It seems more reasonable to suggest...
that the six histidines added to the protein N terminus in the pQE3012 construct might have interfered with protein antigenicity. We are now building new recombinant proteins lacking these histidines and trying specific polypeptides for DTH-inducing activity.

It has been suggested that the immunological properties of ribosomal proteins depend on contaminating molecules such as rRNA or cellular RNA (11). The purified \textit{B. melitensis} L7/L12 protein was treated with RNase (500 ng of L7/L12 in 0.1 ml with 5 mg of RNase for 1 h at 37°C) (data not shown), and it did not lose its DTH activity after treatment. In contrast, the DTH reaction was nearly nullified when the L7/L12 protein was treated with proteinase K (500 ng of L7/L12 in 0.1 ml with 5 µg of proteinase K for 1 h at 50°C, followed by inactivation at 80°C for 10 min). The DTH reaction obtained with the electroeluted L7/L12 protein isolated after immunoadfinity column purification and SDS-PAGE of \textit{Brucella} rules out the possibility of contamination by an additional protein. These preliminary data showed that only the protein L7/L12 and not other molecules attached to it was responsible for the DTH activity.

So far, a major drawback in the use of \textit{Brucella} has been the difficulty in standardization of the antigen (1). The identification of a single protein with DTH antigenicity will contribute to the improvement of a skin test which can be standardized for the diagnosis of brucellosis. This would result in a skin test with better diagnostic and epidemiological values than the existing one. However, the correlation in DTH reactions between guinea pigs and large animals must be assessed (28) before the L7/L12 protein can be used as a diagnostic tool. Finally, a new strategy to develop a vaccine which would enable a skin test that could differentiate between vaccinated and infected animals is now conceivable. This could be achieved by the alteration of the L7/L12 protein in \textit{Brucella} strains used for vaccines, such as \textit{B. melitensis} RevI and \textit{B. abortus} S19, in such a way that the DTH reaction of the animals towards this protein would be abolished.

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**REFERENCES**


