IMMUNE RESPONSE TO INFECTION

Evaluation of immunogenicity and protective activity in BALB/c mice of the 25-kDa major outer-membrane protein of *Brucella melitensis* (Omp25) expressed in *Escherichia coli*

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The antibody response specific to the 25-kDa major outer-membrane protein (Omp25) of *Brucella melitensis* expressed in *Escherichia coli* was assessed in BALB/c mice. Groups of mice were immunised and boosted either with sonicated *E. coli* carrying plasmid pAC2533 – *E. coli* (pAC2533) – expressing the gene coding for Omp25 (*omp25* gene) of *B. melitensis*, or with *E. coli* carrying plasmid pUC19 – *E. coli* (pUC19). One control group received saline. The evolution of antibody responses was investigated by indirect ELISA with whole rough (R) *B. melitensis* H38 cells as antigen. Serum antibody titres of mice immunised with *E. coli* (pAC2533) were appreciably higher than those of mice immunised with *E. coli* (pUC19). The specificity to Omp25 of murine antibodies induced by *E. coli* (pAC2533) was demonstrated by SDS-PAGE and immunoblotting of five *B. melitensis* strains. Binding of antibody in *E. coli* (pAC2533) immune sera to the surface of *B. melitensis* strains differing in their smooth lipopolysaccharide (S-LPS) expression was also studied by whole-cell ELISA and by flow cytometry. Antibody reactivity to R and smooth-rough (S-R) was much stronger than that to smooth (S) *B. melitensis* strains, indicating a much better accessibility of Omp25 to antibody on strains lacking or expressing less O-polysaccharide on their surface. The antibodies to Omp25 were predominantly of IgG2a isotype. The capacity of *E. coli* (pAC2533) to induce protective immune responses against four challenge strains of *B. melitensis* was further evaluated in mice. Significant reductions in splenic infections, in comparison with mice immunised with *E. coli* (pUC19) and unimmunised (saline injection) mice, were observed in R *B. melitensis* B115, S-R *B. melitensis* EP and S *B. melitensis* H38 infected mice. Protection against S *B. melitensis* 16M was not significant. The data from the present study, together with previous results, suggest that humoral immunity against probably conformational, well-exposed epitopes of the Omp25 could contribute to protective mechanisms against *B. melitensis* infection in mice.

Introduction

The search for new vaccines against *Brucella* spp., overcoming the drawbacks of those currently used, is a goal for several laboratories worldwide [for reviews see refs 1 and 2]. They have directed efforts to the identification, characterisation and isolation of bacterial fractions capable of retaining protective activity. Several antigens that induce protective responses have been localised in the outer membrane of brucella cells

[3–7]. Bacterial surface antigens are prime candidates for vaccines as they represent the initial point of contact between the pathogen and their host [8].

Two brucella fractions able to induce protection in mice under different experimental conditions contain major components of the outer membrane: the sodium-dodecyl-sulphate-insoluble (SDS-I) fraction [3–5, 7, 9, 10] and the hot-saline (HS) extract [6, 11]. The SDS-I fraction contains smooth (S) and rough (R) lipopolysaccharides (LPS), depending on the strain used to obtain it, peptidoglycan (PG) and the major outer-membrane proteins (OMPs) of 25–27 kDa, 31–34 kDa and 36–38 kDa tightly bound to PG [4, 9, 10, 12]. The major OMPs are also called group 3 (25–30 kDa) and group 2 (36–38 kDa) proteins, respectively [10, 13].

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Furthermore, HS extract, obtained from *B. ovis* was shown to contain mainly R-LPS and group 3 OMPs [11]. The protective activity of purified brucella 25-kDa OMP [3] or the analogous group 3 OMP [7] has been studied in mouse models as a potential vaccine candidate. However, the evaluation of the importance of these proteins in protection has so far been hampered by the presence of brucella LPS in all these fractions, which is strongly associated with OMPs [5, 14, 15]. Moreover, the proteins used in the trials had been extracted from brucella cell envelopes by harsh, denaturing treatments. These problems were addressed by passive protection experiments in mice with OMP-specific monoclonal antibodies (MAbs). MAbs directed to at least three epitopes of the O-chain of brucella S-LPS were protective in mice [16–20]. However, in similar experiments MAbs specific for OMPs and R-LPS either failed to protect or conferred very limited protection against smooth *Brucella* strains [19, 21–23], most likely because of the shielding effect of the O-chains of S-LPS [21, 22, 24, 25]. As the use of MAbs which probably cover only a few specificities of the 25-kDa OMP could have led to underestimation of the protective activity of this molecule, active protection tests were reconsidered.

The 25-kDa OMP (Omp25) is a major OMP in *B. melitensis* [9, 25] among the proteins so far identified as being surface-exposed [24, 25]. The gene coding for Omp25 of *B. abortus* (*omp25*) was recently cloned and sequenced [26]. Polymerase chain reaction and restriction fragment length polymorphism analysis showed that the *omp25* gene is highly conserved among *Brucella* spp. and strains [27]. This was further confirmed by nucleotide sequence analysis of the *omp25* gene of the different *Brucella* spp. [28]. The *omp25* gene of each *Brucella* spp. was cloned and expressed in *E. coli* [28]. By use of anti-Omp25 MAbs it has been shown that Omp25 was expressed in the *E. coli* outer membrane [28]. Moreover, insertion of Omp25 in the *E. coli* outer membrane allowed the correct folding and preservation of potentially important conformational epitopes [28]. Thus, the construction enabled more precisely investigation of the role played by this major OMP in protective immunity.

This report describes the first results obtained in BALB/c mice on the immunogenicity of Omp25 of *B. melitensis* expressed at the surface of *E. coli* and an evaluation of its protection inducing activity against *B. melitensis* infection.

Materials and methods

Bacteria and plasmids

The *Brucella* strains, from the INRA Nouzilly *Brucella* culture collection, were S *B. melitensis* 53 H38, H38 (S), currently used as the challenge strain in experi-

mental infections, and its R variant, H38 (R); S *B. melitensis* 16M, the type strain of the genus *Brucella* and reference for biovar 1 of *B. melitensis* [29], *B. melitensis* EP, behaving as a S-R strain because of a small amount of surface S-LPS [30]; and *B. melitensis* B115, a R strain isolated from goat's milk in Malta (G.G. Alton, personal communication) which contains intracellularly expressed O-polysaccharide chain [31]. Plasmid pAC2533 containing the *omp25* gene of *B. melitensis* 16M was constructed as described previously [28]. Expression of the *omp25* gene of *B. melitensis* was under the control of the Plac promoter. Cultures of *E. coli* (pAC2533) or *E. coli* (pUC19) (TG1 strains) were prepared as follows. Overnight cultures grown at 37°C were adjusted to an absorbance value (A_{600}) of 0.1 in 50 ml of Luria Bertani medium. After incubation for 2 h at 37°C with stirring, IPTG was added to a final concentration of 1.0 mM. Cultures were allowed to continue growth for 4 h at 37°C with stirring and were then harvested by centrifugation ($4000 \times g$, 30 min at 4°C). Pellets were suspended in phosphate-buffered saline (PBS) and prepared for inoculation. Expression of *omp25* was checked with MAbs by ELISA and immunoblotting [28].

Monoclonal antibodies

The generation and characteristics of MAbs have been described previously [25]. MAbs used were: A59/05F05/C09 (IgG2a), specific for Omp25; MAb A76/08C03/G03 (IgG2a), specific for the 16.5 kDa OMP; and MAb A68/03F03/D05 (IgG2b) specific for R-LPS of *Brucella*. MAbs were used as ascitic fluids.

Mice and immunisation

Female, 5–6-week-old BALB/c mice (IFFA Credo, Orléans France) were randomised into groups of six and acclimatised for 1 week before starting immunisations. They received water and food *ad libitum*. Suspensions of *E. coli* (pAC2533) and *E. coli* (pUC19) cells were sonicated (amplitude 5, power setting: high; MSE 150 W Ultrasonic Disintegrator, Sussex) before injection. Animals were first inoculated intravenously (i.v.) either with *E. coli* (pAC2533) or *E. coli* (pUC19); 0.2 ml of suspensions with an A_{600} value of 5.0 before sonication were used (A_{600} of 10.0 corresponds to $c. 10^9$ *E. coli* cells/ml). Fifty days later the mice were given booster injections intraperitoneally (i.p.) of the respective antigens (0.2 ml suspensions with A_{600} values of 0.5 before sonication). A third group of mice, inoculated with saline, served as control. Sera were obtained by retro-orbital bleeding under ether anaesthesia at 15, 30, 45, 65 and 80 days after the first immunisation.

ELISA

The brucella suspensions used to coat plates were cells grown for 24 h on tryptose soy agar (TSA), harvested

and heat-inactivated (65°C, 60 min). Sterility was checked by subculture on TSA. For antibody determination, 96-well flat-bottomed plates (Greiner, Stuttgart, Germany) were coated overnight with 100 µl of a suspension of *E. coli* (pAC2533) or *E. coli* (pUC19) (1 in 100 dilution of a suspension at an A_{600} value of 0.5), or *B. melitensis* strains H38 (R), H38 (S), EP (S-R), 16M (S), or B115 (R) respectively (suspensions at an A_{600} value of 1.0) diluted in 0.1 M sodium bicarbonate buffer, pH 9.6. The plates were washed five times with Tween 20 0.05% in 0.15 M NaCl between incubations. Three-fold serum dilutions (1 in 30 to 1 in 177147, in Tween 0.05% PBS) were distributed at 100 µl per well and incubated for 2 h at 37°C. Bound antibodies were detected by successive incubation with biotinylated sheep anti-mouse IgG immunoglobulins (Amersham, Aylesbury; 1 in 1000 dilution, 1 h at room temperature – 20–22°C) and peroxidase-conjugated streptavidin (Amersham; 1 in 1000 dilution, 1 h at room temperature). Enzyme activity was revealed by adding 100 µl of a solution containing 4 mM H_2O_2 and 1 mM 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and developing for 60 min at room temperature with continuous shaking. Colour was read at 414 nm. Titres were calculated by interpolating in the linear response part of dilution curves at $A = 0.2$. Conditions were the same for isotype determination of the antibody, except that biotinylated sheep anti-mouse IgG1, IgG2a, IgG2b, IgG3 and IgM (Amersham; 1 in 1000 dilution) were used.

For binding determinations with anti-R-LPS MAb A68/03F03/D05 and anti-Omp25 MAb A59/5F05/C09, MAb dilutions (1 in 30 to 1 in 177147) were distributed on plates coated under the same conditions as for serum antibody determination. After incubation for 2 h at 37°C, bound MAb was detected with a peroxidase-conjugated goat anti-mouse IgG (H+L) affinity purified serum (BioRad, Richmond) incubated for 1 h at room temperature. Enzyme activity and plate reading were carried out as above.

Flow cytometry

For flow cytometric analysis, 200 µl of a suspension (A_{600} value of 0.165) of brucella cells (prepared as described for ELISA) were incubated (1 h, 37°C) with pooled immune sera diluted 1 in 300 in 0.01M PBS, pH 7.2, in Eppendorf polystyrene conical vials. After two washes with 0.5 ml of PBS, bound serum antibodies were detected by incubating cells in 200 µl of a 1 in 150 dilution of FITC-conjugated goat anti-mouse IgG (H+L) serum (Sigma), washed twice and resuspended to a volume of 0.5 ml. Suspensions were then gently sonicated for 5 s to disaggregate possible clumping due to repeated centrifugation and finally filtered through a fine nylon mesh. Negative controls were bacteria incubated only with the conjugate and these cells were used to set the acquisition parameters; 20 000 brucella cells were analysed in each sample. Background and

non-specific fluorescence signals were excluded from the analysis. Sample population parameters were obtained by LYSIS II software (Becton Dickinson, Mountain View, CA, USA).

SDS-PAGE and immunoblotting

Immunoblotting was done after separating the antigens in SDS-PAGE. Acrylamide 14% gels were used, following the method of Lugtenberg as previously described [30]. Samples were whole-cell lysates of *E. coli* (pAC2533), *E. coli* (pUC19), or *Brucella* strains, suspended at an A_{600} value of 5.0 (for *Brucella* suspensions) or 2.0 (for *E. coli* suspensions) in sample buffer, boiled for 10 min, sonicated for 10 s and centrifuged. The supernates (30 µl per lane) were then loaded. After electrophoresis, antigens in gels were electroblotted (0.8 mA/cm²) for 1 h. Nitrocellulose sheets were saturated (skimmed milk 3%, Tween 0.05% PBS) for 1 h and incubated overnight with a pool of six immune sera from mice diluted 1 in 100. All antibody dilutions were in skimmed milk 1%, Tween 0.05% PBS if not otherwise indicated. Detection was by incubating blots successively in rabbit anti-mouse serum (Nordic, Tilburg, The Netherlands) diluted 1 in 500 and protein A-peroxidase (Sigma) diluted 1 in 2000. The blots were developed in a solution containing 4.4 mM H_2O_2 and α -chloronaphthol (Sigma) 0.5 mg/ml. In some assays, pooled sera were first absorbed with a suspension of *E. coli* or *Brucella* cells. Adsorption was performed on sera diluted 1 in 100 in PBS, pH 7.2, with a 10:1 serum:packed cell volume ratio, by incubating once with agitation at room temperature for 2 h. MAbs A76/08C03/G03 and A59/05F01/C09 were incubated together at a 1 in 500 dilution overnight and detected in the same conditions as indicated for immune sera.

IFN- γ determination

IFN- γ was determined in supernates of in-vitro restimulated splenocytes. Splenocytes were cultured in 24-well plates (Falcon, USA) at 2×10^6 cells/ml in a total volume of 1.5 ml. Stimulating antigens were suspended in complete medium by sonication and distributed at 100 µl well (equivalent to 10^7 bacteria/ml). Cell culture supernates were harvested at 72 h. IFN- γ was determined by capture ELISA [32] in supernates pooled from duplicate wells (24-well culture plates). Immunocapture was achieved with purified anti-mouse IFN- γ MAb (Pharmingen, USA) and detection was by incubating sequentially with rabbit (polyclonal) anti-murine IFN- γ antibody (Immunogenex, USA) and alkaline phosphatase-conjugated goat anti-rabbit IgG (H+L) antibody (ICN, USA). Standard curves were constructed with murine recombinant IFN- γ (Genzyme, USA). Cytokine concentrations in the samples were obtained by interpolating in the standard curve. Data from three determinations were analysed.

Mouse protection tests

To evaluate the potential protective role of Omp25 expressed in *E. coli* against *B. melitensis*, mice (six per group) vaccinated with *E. coli* (pAC2533) and *E. coli* (pUC19) and control groups were challenged 45 days after the booster injection with four strains of *B. melitensis* differing in their mode of S-LPS expression (S *B. melitensis* H38, S *B. melitensis* 16M, S-R *B. melitensis* EP and R *B. melitensis* B115). The inocula were prepared from 24 h slants of each strain, and suspensions were adjusted taking an A_{600} value of 0.165 as equivalent to 10^9 *Brucella* cells. Exact counts were determined retrospectively by plating on TSA. Mice were thus inoculated (i.v.) with 0.88×10^4 *B. melitensis* H38 (S), 0.57×10^4 *B. melitensis* 16M (S), 4.5×10^7 *B. melitensis* EP (S-R) or 3.24×10^7 *B. melitensis* B115 (R). At 15 days after infection, mice were killed by cervical dislocation and their spleens were removed aseptically, weighed and kept frozen (-20°C) until further processing. For enumeration of cfu/spleen, the spleens were homogenised in stomacher bags, serially diluted and plated on TSA. Results were represented as the mean log cfu and SD per group.

Statistical analysis

Differences in spleen infection levels between the different groups were estimated through ANOVA by Fisher's protected least significant difference test (SuperANOVA software; Abacus Concepts, CA, USA).

Results

Antibody responses

Antibody reactivity and specificity. Antibodies to *B. melitensis* H38 (R) in sera of all mice immunised with *E. coli* (pAC2533) were detected as early as 15 days after infection, and peaked at 30–45 days (IgG titre = 7842) (Fig. 1). The booster further increased the antibody response (IgG titre = 61 520), which peaked at around 60 days after infection. Sera from mice immunised with *E. coli* (pUC19) showed a much lower antibody reactivity to *B. melitensis* H38 (R) cells (mean IgG titres of 285 SD 57 and 845 SD 123 at 30 and 60 days after infection, respectively). Reactivity of immune sera to whole *B. melitensis* H38 (R) cells indicated the presence of antibodies to brucella surface-exposed antigens. The specificity of antibodies was suggested by their higher binding activity in ELISA when sera against *E. coli* (pAC2533) were tested on *E. coli* (pAC2533) relative to *E. coli* (pUC19) cells (not shown).

Immunoblotting after SDS-PAGE was used to identify the molecules of *B. melitensis* reactive with anti-*E. coli* (pAC2533) immune sera. Antibodies of *E. coli* (pAC2533) immune sera reacted with a band at 25 kDa, corresponding to Omp25, on the five strains of *B. melitensis* tested (Fig. 2). This band was also

Fig. 1. Antibody response, measured by ELISA, to *B. melitensis* H38 (R) cells of mice immunised with *E. coli* (pAC2533) or *E. coli* (pUC19). Mice were first immunised i.v. at day 0 and boosted i.p. on day 52 with *E. coli* (pAC2533) (●) or *E. coli* (pUC19) (○) which express or do not express Omp25 of *B. melitensis*, respectively. Blood samples were taken at days 15, 30, 45, 60 and 80. Individual sera were tested in indirect ELISA for antibodies to *B. melitensis* H38 (R) whole cells. Plotted values represented the mean and SD ($n = 6$) of serum titres obtained by interpolating from logarithmic regression curves at an A_{600} value of 0.2.

observed in the *E. coli* (pAC2533) lysate, but was absent from the *E. coli* (pUC19) lysate. In addition, among several faint bands, a second intense band was revealed in the *B. melitensis* lysates, which migrated between the 14-kDa and 20.1-kDa markers (Fig. 2). The origin of this additional band was then studied. For this, antibody reactivity in immunoblots of anti-*E. coli* (pUC19) sera was analysed before and after adsorption with whole *B. melitensis* B115 cells (Fig. 3). Before adsorption, pooled sera detected many bands on *E. coli* lysates, among which was the additional band mentioned above (Fig. 2). This pool did not reveal Omp25 which was detected on the blot by anti-Omp25 MAb A59/05F01/C09. Adsorption of *E. coli* (pUC19) immune sera with *B. melitensis* B115 resulted in removal of antibodies tagging the additional band, which migrated to a position quite close to that of the 16.5-kDa brucella OMP, as detected by MAb A76/08C03/G03. Furthermore, adsorption of *E. coli* (pAC2533) immune sera with *E. coli* (pUC19) cells also removed the cross-reacting antibodies and left 90% of the antibody binding on to *B. melitensis* H38 (R) whole-cells, as determined by indirect ELISA (data not shown).

These results indicated, first, that antibodies in sera of mice immunised with *E. coli* (pAC2533) recognised Omp25 of *B. melitensis*, and that a high proportion of

Fig. 2. Antibody reactivity of anti-*E. coli* (pAC2533) immune sera in immunoblotting. Whole-cell lysates of *B. melitensis* B115 (R) (lane 1); *B. melitensis* H38 (R) (2); *B. melitensis* EP (S-R) (3); *B. melitensis* H38 (S) (4); *B. melitensis* 16M (S) (5); *E. coli* (pAC2533) (6) and *E. coli* (pUC19) (7) were electrophoresed, blotted and incubated with a 1 in 100 dilution of pooled immune sera ($n=6$). Molecular mass markers, positioned as indicated on the left, were: 14.2 kDa, α -lactalbumin; 20.1 kDa, soybean trypsin inhibitor; 29 kDa, carbonic anhydrase; 36 kDa, glyceraldehyde 3 phosphate dehydrogenase; 45 kDa, ovalbumin; 66 kDa, bovine serum albumin.

the induced antibodies reacting with the *B. melitensis* cell surface were directed to this OMP and, second, that a proportion of the antibodies induced reacted with the denatured protein in immunoblotting.

Influence of S-LPS on the binding of serum anti-Omp25 antibody to *B. melitensis*. The influence of the S-LPS of *B. melitensis* on the accessibility of Omp25 to specific antibodies was investigated. An indirect ELISA was devised which used suspensions of five *B. melitensis* strains for coating, all belonging to biovar 1 of *B. melitensis*, including S, S-R and R forms. The dilution curves indicated important differences in antibody binding (Fig. 4). The curves on R and S-R *B. melitensis* strains (B115 and H38 (R) and EP) plateaued (absorbances ≥ 3.0) at $c. 1$ in 1000 dilution whereas those of S *B. melitensis* strains 16M and H38 (S) presented much lower absorbance values at that dilution, indicating a much better binding of anti-Omp25 antibody on to *B. melitensis* R and S-R than on

Fig. 3. Adsorption experiments with anti-*E. coli* immune sera. A pool ($n=6$) of anti-*E. coli* (pUC19) sera was reacted with electroblotted whole-cell lysates of *B. melitensis* H38 (R) (lanes a); *E. coli* (pAC2533) (b); *E. coli* (pUC19) (c). Nitrocellulose sheets were incubated with non-adsorbed anti-*E. coli* (pUC19) sera (1 in 100 dilution) (centre), or the same pool sera adsorbed with *B. melitensis* B115 (R) whole-cells (serum:cells ratio = 10:1 v:v) (right). As a control (left) blots were incubated with a mixture of two MAbs specific for the brucella 16.5-kDa OMP (MAb A76/08C03/G03) and brucella Omp25 (A59/05F01/C09).

Fig. 4. Antibody reactivity to Omp 25 of *E. coli* (pAC2533) immune sera on to *B. melitensis* cells differing in their LPS expression. Immune sera were serially diluted and tested by whole-cell indirect ELISA on to *B. melitensis* 16M (S) ($\square$), *B. melitensis* H38 (S) ($\circ$), *B. melitensis* H38 (R) ($\bullet$), *B. melitensis* EP (S-R) ($\blacktriangle$), and *B. melitensis* B115 (R) ($\blacksquare$). Points represent the mean absorbance values and SD per groups of mice ($n=6$).

B. melitensis S strains. However, the binding on to *B. melitensis* H38 (S) was slightly higher than that on to *B. melitensis* 16M.

Indirect ELISA with MAb A68/03F03/D05 specific for brucella R-LPS was used to control further the accessibility to epitopes located relatively deep in the surface of strains of *B. melitensis*. This MAb showed much better binding on to R and S-R strains than on to S *B. melitensis* strains H38 (S) and 16M (not shown). In addition, MAb A59/05F01/C09, specific for Omp25, showed a similar result (data not shown), comparable with that of binding of the polyclonal anti-Omp25 antibody.

Flow cytometry was used to monitor the binding of

anti-*E. coli* (pAC2533) sera antibodies to whole brucella cells in suspension. Brucella cells were incubated with an appropriate dilution of pooled immune sera ($n = 6$). The bacterial cell populations stained differently (Fig. 5); strains B115 (R), H38 (R) and EP (S-R) showed strong fluorescence signals (mean values: 714 SEM 10, 521 SEM 7 and 423 SEM 6, respectively). In contrast, S strains H38 (S) and 16M (S) gave low values (71 SEM 3 and 83 SEM 4, respectively). Control suspensions gave consistently low values (< 10 fluorescence units). These results confirmed much better binding of anti-Omp25 antibodies on to individual cells of R and S-R strains than on to S strains.

Taken together, these data strongly suggested that the

Fig. 5. Antibody reactivity, studied by flow cytometry, of *E. coli* (pAC2533) immune sera on to *B. melitensis* cells differing in their LPS expression. Suspensions of *B. melitensis* cells were successively incubated with anti-*E. coli* (pAC2533) pooled immune sera ($n = 6$) diluted 1 in 300 and a FITC-goat anti-mouse IgG. Control cells were incubated with the FITC conjugate only. **a**, *B. melitensis* B115 (R); **b**, *B. melitensis* EP (S-R); **c**, *B. melitensis* H38 (R); **d**, *B. melitensis* H38 (S); **e**, *B. melitensis* 16M (S).

accessibility of Omp25 to antibodies in *E. coli* (pAC2533) immune sera was greatly influenced by the S-LPS covering each of the *B. melitensis* strains tested.

Antibody isotypes. The antibody isotypes generated by immunisation with *E. coli* (pAC2533) and *E. coli* (pUC19) and reacting with *B. melitensis* H38 (R) cells were characterised. The results are shown in Table 1. Individual sera from mice immunised with *E. coli* (pAC2533) (taken before the time of challenge) showed a clear predominance of IgG2a isotype in every case. The ratio of IgG2a:IgG1 ranged between 1.41 and 9.53. Mice were not uniform in their IgG1 and IgG2b antibody responses. Furthermore, IgG3 antibody levels were always the lowest. On the other hand, immunisation with *E. coli* (pUC19) was shown to provoke a mainly low titre IgM response similar to that observed with *E. coli* (pAC2533).

IFN- γ production

IFN- γ secretion from spleen cells was studied *in vitro* after re-stimulation. Splenocytes from both immunised and non-immunised mice prepared at the time of challenge were cultured with different antigens (Table 2). There were no significant differences ($p > 0.05$) between unstimulated cells from mice given either *E. coli* or saline. Cells from mice given *E. coli* (pAC2533) or *E. coli* (pUC19) increased IFN- γ secretion when stimulated with either *E. coli* (pAC2533) ($p < 0.001$ and $p > 0.05$, respectively) or *E. coli* (pUC19) ($p < 0.001$ and $p < 0.05$, respectively). On the contrary,

cells from unimmunised (saline inoculated) mice were unable to increase their IFN- γ production significantly upon re-stimulation ($p < 0.05$).

Protective activity

The potential protective activity induced by *E. coli* (pAC2533) was evaluated by challenging mice with virulent and less virulent *B. melitensis* strains. The experiment was performed with the four *B. melitensis* strains which differed in their S-LPS expression.

Spleen weights of mice vaccinated with *E. coli* (pAC2533) and challenged with *B. melitensis* EP (S-R) and B115 (R) strains showed significant reduction – $p < 0.05$ for EP (S-R) and $p < 0.01$ for B115 (R) challenge – at 15 days after infection compared with groups immunised with *E. coli* (pUC19) or PBS controls (Table 3). No significant differences ($p > 0.05$) were found in mice infected with the S strains H38 (S) and 16M (S). Finally, no significant differences ($p > 0.05$) were detected between the two control groups – *E. coli* (pUC19) or PBS.

The splenic infection levels were also determined 15 days after infection (Table 2). Immunisation with *E. coli* (pAC2533) significantly reduced the number of brucella cfu compared with mice receiving PBS ($p < 0.01$) or *E. coli* (pUC19) ($p < 0.05$) when challenged with R strain B115, S-R strain EP and S strain H38 (S). No statistically significant differences ($p > 0.05$) were observed among the immunisation groups challenged with the S strain 16M. Finally,

Table 1. Isotypes in immune sera of mice immunised with *E. coli* (pAC2533) expressing Omp25 of *B. melitensis*

Mouse no.	Antibody titre*					
	IgM	IgG1	IgG2a	IgG2b	IgG3	IgG2a/G1 ratio
1	905	203	6825	193	778	3.36
2	950	2097	10 121	1782	1192	4.82
3	847	6967	9805	2801	1275	1.41
4	882	1463	5133	2007	882	3.51
5	856	2335	16 887	2298	832	7.23
6	1071	3139	29 922	5764	2099	9.53

* Antibody isotypes were determined by indirect ELISA on *B. melitensis* H38(R) whole cells.

Secondary antibodies were biotinylated anti-mouse isotypes.

Titres were calculated as the intercept from calculated slopes at A = 0.2.

Table 2. In-vitro IFN- γ secretion from spleen cells of mice immunised with *E. coli* expressing brucella Omp25

Stimulating antigen	Mean (SEM) IFN- γ *	Secretion after immunisation with	
	None (saline)	<i>E. coli</i> (pAC2533)	<i>E. coli</i> (pUC19)
<i>E. coli</i> (pAC2533)	9.2 (1.4)	25.1 (2.8) [†]	12.9 (5.0)
<i>E. coli</i> (pUC19)	6.9 (2.6)	22.7 (3.2) [†]	14.6 (4.2) [‡]
None (saline)	7.1 (2.9)	2.9 (1.7)	6.3 (1.6)

Unfractionated spleen cells (2×10^6 /ml) were cultured for 72 h in the presence or absence of sonicated *E. coli* cells (equivalent to $A_{600} = 0.1$). Supernates were then assayed for IFN- γ by a capture ELISA.

* (ng/ml).

[†] $p < 0.001$.

[‡] $p < 0.05$.

Table 3. Evaluation of protection induced by *E. coli* (pAC2533) in BALB/c mice

Vaccine		1. Mean (SD) cfu/spleen* and 2. spleen weight (g) with <i>B. melitensis</i> challenge strain			
		B115	EP	H38 (S)	16M
<i>E. coli</i> (pAC2533)	1.	6.41 (0.18) [†]	5.10 (0.55) [†]	5.61 (0.33) [†]	4.63 (0.72)
	2.	0.269 (0.033)	0.360 (0.062)	0.230 (0.028)	0.285 (0.050)
<i>E. coli</i> (pUC19)	1.	6.78 (0.28) [‡]	5.70 (0.27) [‡]	6.31 (0.21) [‡]	4.98 (0.58) [‡]
	2.	0.371 (0.037)	0.448 (0.038)	0.277 (0.086)	0.287 (0.034)
PBS	1.	7.06 (0.20)	6.15 (0.25)	6.18 (0.56)	4.78 (0.13)
	2.	0.402 (0.058)	0.456 (0.063)	0.235 (0.120)	0.289 (0.031)

Mice vaccinated with *E. coli* (pAC2533) and *E. coli* (pUC19) expressing or not expressing, respectively the Omp25 of *B. melitensis* were infected intravenously 45 days after the booster injection. Controls received PBS. Exact doses injected were: 0.88×10^4 *B. melitensis* H38 (S); 0.57×10^4 *B. melitensis* 16M (S); 4.5×10^7 *B. melitensis* EP (S-R) or 3.24×10^7 *B. melitensis* B115 (R). Mice were killed 15 days after infection and the level of spleen infection was determined.

*Groups of six mice.

[†]Different (Fisher's PLSD test) ($p < 0.05$) from *E. coli* (pUC19) vaccinated group and from controls inoculated with PBS.

[‡]Not statistically different from PBS-injected mice.

immunisation with *E. coli* (pUC19) did not diminish the number of cfu ($p > 0.05$) compared with controls receiving PBS.

Discussion

The immunogenicity and active protection against *B. melitensis* induced in mice by *E. coli* expressing the major Omp25 of *B. melitensis* was evaluated. This construct provided firstly the Omp25 free of other brucella antigens and, secondly, an outer-membrane framework beneficial for appropriate folding and exposure of the protein [28].

Whole-cell sonicates of *E. coli* (pAC2533) cells expressing the *omp25* gene of *B. melitensis* 16M were used to obtain a high antibody response to Omp25 in mice without the addition of extrinsic adjuvants. Antibodies reacted at high titre on to *B. melitensis* H38 (R). Immunoblotting revealed Omp25 and another band found to be a cross-reactive surface antigen, probably brucella 16.5-kDa OMP which shows sequence homology to *E. coli* peptidoglycan-associated lipoprotein (PAL) [33]. Furthermore, ELISA and immunoblotting showed that there were probably antibodies to both non-denatured, surface-exposed protein and to denatured protein, most likely representing conformational and linear epitopes, respectively [28, 34, 35]. The amount of antigen injected could have contributed to the strong response to Omp25. When *Salmonella* was used as expression vector for a plasmid-encoded antigen, it was observed that the initial amount of antigen priming the immune system had more impact than the persistence of the vector strain in the mouse tissues [36].

On the basis of earlier reports [37], both the i.v. and i.p. routes were used for immunisation. This has been shown to favour the production of antibodies to conformational, exposed protein epitopes [37], which was of interest in the present model. As other routes were not compared, it is not possible to deduce

whether this accounted for the type of response obtained. However, insertion of OMPs into bilayer membranes could have important consequences for the quality of antibody responses obtained. For instance, when inserted into liposomes, the Por protein of the gonococcus induced a greater proportion of antibodies able to bind to whole neisseria cells than when injected as proteosomes or as an alum-adsorbed preparation [38]. Moreover, a porin from *Salmonella* has been shown to induce higher protection in mice when associated with LPS; this effect was even stronger than when monophosphoryl lipid A, the moiety of LPS with adjuvant activity, was used [39]. Class 1 OMP from *Neisseria meningitidis* produced in *Bacillus subtilis* and purified under denaturing conditions was shown to refold adequately when complexed with salmonella LPS [40]; furthermore, antisera prepared with LPS-associated OMP induced bactericidal and protective antibodies in contrast to unfolded protein [40]. This supported the idea that LPS had a role in the conformation, and hence the presentation of OMPs to the immune system. In addition, it has been shown that purified brucella OMPs were capable of integrating into lipid bilayers of heterologous LPS preparations [41] and multilayers of brucella R-LPS [35]; the latter was also shown to restore antigenicity. The results of the present study are consistent, in that higher responses to Omp25 on whole brucella cells were obtained when immunising with membrane-associated Omp25 instead of a recombinant, purified fusion Omp25 (unpublished results). This could be due to a bias in antibody specificity of immune sera to epitopes located deeper in Omp25, as above, or to a loss of tertiary structure (antigenicity) of the cytoplasm-derived constructs compared to the properly folded, membrane-associated protein, or both.

Mice immunised with *E. coli* (pAC2533) showed a limited but significant reduction in splenic infection levels when challenged with R B115, S-R EP and S H38 strains, but not with strain 16M. The amount of S-LPS expressed by these strains greatly influenced

the binding of polyclonal antibodies specific for Omp25. The results agreed with previous data that showed greater accessibility of OMP epitopes – as well as of core LPS – to MAbs in R strains of several *Brucella* spp. [21, 22, 24, 25]. The difference in protective activity observed against the two S strains could probably be linked with the relatively higher binding of anti-Omp25 antibodies on *B. melitensis* H38 (S) than on *B. melitensis* 16M. However, yet unexplored in-vivo modifications of the expression of outer-membrane antigens could also account for different antibody activity. Alternatively, this might have reflected intrinsic virulence differences between strains. Although small, protection obtained with *E. coli* (pAC2533) was greater than that reported with killed whole-cell rough brucella vaccines against *B. melitensis* and *B. suis* [42].

It is as yet premature to speculate about the mechanisms responsible for the protection observed. Macrophages could have been activated by IFN- γ , becoming more bactericidal [43, 44]. However, this sole mechanism could not explain the reduction in splenic infection, as high IFN- γ secretion was found with cells from mice immunised with both *E. coli* (pAC2533) and *E. coli* (pUC19). Adjuvants may affect both specificity and isotype of antibody responses [45]. So, *E. coli* LPS could have modulated the antibody switching by favouring IgG2a over the other isotypes. LPS is recognised as a powerful inducer of tumour necrosis factor and IFN- γ , and the latter has been shown to drive B cells to IgG2a synthesis [46]. Therefore, IgG2a antibodies could have bound brucella cells through Omp25 mediating opsonic phagocytosis or complement killing [47]; an earlier study has already shown that IgG2a MAbs against OMPs, including anti-Omp25 MAbs, protect mice against *B. ovis* [48]. Experiments including passive transfer of immune sera and cells to mice as well as in-vitro testing for bacteriolysis and opsonophagocytic killing should be done to clarify these points.

Few protection trials have been reported concerning recombinant *Brucella* proteins. Fusion BaOMP were tested in lymphocyte proliferation tests on *B. abortus*-infected cattle [49]. However, neither antibody nor protection tests were considered. A recombinant salmonella expressing a soluble *B. abortus* protein (BCSP-31) was tested in mice [50, 51] and swine [51], but no protective activity was registered. Protection was demonstrated against *B. abortus* by immunising mice with a peptide derived from the sequence of the *B. abortus* Cu-Zn superoxide-dismutase; however, the whole recombinant protein was not efficacious [52]. More recently a purified recombinant ribosomal protein was shown to be protective in mice, inducing both immune cells and antibodies [53]. This would indicate that immune (protective) mechanisms against brucella might involve both internal and outer-membrane antigens.

This is the first report on immunogenicity and protective activity of an *E. coli*-expressed recombinant OMP of brucella. Although the protection reported here with Omp25 was limited, the approach is promising for the evaluation of other brucella OMPs, especially where conservation of folding is critical. Work is in progress to examine further the immune responses to Omp25 and other recombinant brucella proteins and to evaluate them as protective antigens against smooth and rough *Brucella* strains.

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