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Invasive *Escherichia coli* vaccines expressing *Brucella melitensis* outer membrane proteins 31 or 16 or periplasmic protein BP26 confer protection in mice challenged with *B. melitensis*

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Abstract

Because of the serious economic and medical consequences of brucellosis, efforts are to prevent infection of domestic animals through vaccines. Many disadvantages are associated with the current *B. melitensis* Rev.1 vaccine prompting development of alternative vaccines and delivery. *E. coli* (DH5α) was engineered to express a plasmid containing the *inv* gene from *Yersinia pseudotuberculosis* and the *hly* gene from *Listeria monocytogenes*. These recombinant invasive *E. coli* expressing *B. melitensis* outer membrane proteins (Omp31 or 16) or the periplasmic protein BP26 were evaluated for protection of mice against virulent *B. melitensis*. Importantly, these invasive *E. coli* vaccines induced significant protection against *B. melitensis* challenged mice. Invasive *E. coli* may be an ideal vaccine platform with natural adjuvant properties for application against *B. melitensis* since the *E. coli* delivery system is non-pathogenic and can deliver antigens to antigen-presenting cells promoting cellular immune responses.

Keywords

Brucella melitensis; mice; Invasive *E. coli* vaccine; *Brucella* Omps 31, 16; periplasmic protein BP26

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1. Introduction

Brucellosis caused by *Brucella melitensis* has a high incidence in developing countries, and the World Health Organization considers brucellosis as one of the seven neglected zoonoses, a group of diseases that contribute to the perpetuation of poverty [1]. Current vaccination relies on the live attenuated strain Rev.1 developed decades ago [2]. Live *Brucella* vaccines are considered advantageous over killed vaccines because they stimulate the immune system while replicating in the host [3]. Clearance of the vaccine strain is necessary to ensure safety of the vaccine [4]. However, *B. melitensis* Rev.1 vaccine induces abortions when applied during pregnancy, is virulent for humans, and elicits antibodies to smooth lipopolysaccharide (LPS) of *Brucella* interfering in serodiagnosis. Moreover, Rev.1 is resistant to streptomycin, an antibiotic used to treat the disease. Effective brucellosis vaccines not interfering in the diagnosis of infected animals would represent a major breakthrough [5,6].

Alternative methods of vaccine delivery and design to safely immunize animals need to be developed to maximally reduce the prevalence of brucellosis. The *Brucella* cell wall consists of a peptidoglycan layer strongly associated with the outer membrane [7] consisting of at least 75 proteins with several outer membrane proteins (Omps) reported [8]. One gene encoding a periplasmic protein BP26 has been useful in differentiating *Brucella* spp. infected from vaccinated animals [9, 28, 30, 32]. The genes *omp25*, *omp31*, and *omp2b*, encoding the major 25-, 31-, and 36-kDa *Brucella* Omps, respectively, have been cloned and sequenced [9–14]. The 16-kDa Omp, named Omp16, shows significant similarity to the peptidoglycan-associated lipoproteins of many Gram-negative bacteria [15]. Major Omps from *Brucella* have been classified in group 2 (Omp2a and Omp2b [36 to 38 kDa]) and group 3 (Omp25 and Omp31 [25 to 27 and 31 to 34 kDa, respectively]) [16].

The aim of the present study was to evaluate recombinant invasive *E. coli* possessing the *inv* gene from *Yersinia pseudotuberculosis* and the *hly* gene from *Listeria monocytogenes* [17] and expressing *B. melitensis* outer membrane proteins 31 or 16 or the periplasmic protein BP26 as a vaccine platform against brucellosis. Invasin, encoded by *inv* is located on the bacterial surface, binds β 1-integrin of a wide variety of target cells triggering entry of *Yersinia* into host cells [18] leading to intracellular delivery of plasmids and recombinant proteins [17,19,20]. Also, recombinant *E. coli* expressing the *hly* gene (listeriolysin O) from *L. monocytogenes* delivers antigens to antigen-presenting cells [21,22]. The mechanism involves a listeriolysin O-dependent release of the bacterial content into the cytosol followed by proteasome processing and presentation of the antigenic motifs by MHC class I [22,23]. In the present study these invasive *E. coli* vaccines induce a cellular immune response and protect BALB/c mice against infectious *B. melitensis* 16M challenge.

2. Materials and methods

2.1. Bacterial strains and growth conditions

B. melitensis 16M (virulent strain) isolated from an aborted goat fetus [24] was confirmed by the Veterinary laboratory, Weybridge, Surrey, U.K.

2.2. Plasmid constructs

Prokaryotic expression vector pGB2 Ω inv-hly [19] (10.05 kb; spectinomycin resistance) was gifted by C. Grillot-Courvalin and expresses *invasin* from *Yersinia pseudotuberculosis* and *listeriolysin O* (LLO) from *Listeria monocytogenes*. *Brucella* BMEI0844 (*omp31*), BMEI0340 (*omp16*) and BMEI0536 (*bp26*) genes were isolated from the *Brucella* ORFeome (OPEN Biosystems) [25]. Prokaryotic expression vectors pDEST17/Omp31,

pDEST17/Omp16, and pDEST17/BP26 were generated by shuttling the insert from pDONR201/Omps using the Gateway LR recombination system (Invitrogen).

2.3. Construction of inv *E. coli* vaccines

Construction of inv *E. coli* vaccines used co-transformation of BL21-AI cells (Invitrogen) with pGB2Qinv-hly and pDEST17/Omps. Overnight grown *E. coli* BL21-AI cells harboring pDEST17/Omp and pGB2Qinv-hly plasmids were inoculated into LB. The culture was grown and induced with 20% L-arabinose when the OD₆₀₀ reached 0.6. After induction, cells were grown for 5 h, centrifuged, and washed twice with phosphate buffered saline (PBS). SDS-PAGE to confirm expression of *B. melitensis* Omps. Resuspended bacteria were enumerated by serial dilution plating and counting, and 2×10^7 *E. coli* BL2-AI were injected into mice.

2.4. Expression and purification of outer membrane proteins

Bacteria were centrifuged, washed, resuspended in lysis buffer (10 mM Tris-HCl [pH 8], 5 mM EDTA and 100 mg/ml lysozyme), sonicated, and lysate centrifuged. The pellet fraction containing His6-tagged Omps in inclusion bodies was collected and solubilised in 50 ml 6 M guanidine-HCl (pH 6.5) containing 0.05% Triton X-100. Extract was centrifuged, the supernatant passed through a Ni-affinity column (Pharmacia Biotech), and protein eluted in elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl [pH 7.0]). Purified protein aliquots were stored at -70°C until use. Purified recombinant Omps were analysed by SDS-PAGE and western blotting.

2.5. Mouse vaccination and challenge

Mouse experiments were performed according to institutional guidelines and approved by an ethics committee. BALB/c female mice 4–6 weeks old were divided into five groups in duplicate consisting of five mice per group. Group 1 was immunized with *E. coli* inv-Omp31, group 2 with *E. coli* inv-Omp16, group 3 with *E. coli* inv-BP26, group 4 with *E. coli* inv empty control, and group 5 with saline only. One day prior to vaccination mice were injected with 0.2 ml PBS. *E. coli* (2×10^7) containing the respective *omp* (0.2 ml) was injected intraperitoneally (i.p.) in mice of groups 1, 2 and 3, while group 4 received empty inv *E. coli* and group 5 saline only. Animals received a booster with the same dose two weeks later and sacrificed after 4 weeks to study the immune response following vaccination but before challenge. To evaluate protection, mice were given inv-*E. coli*-Omp vaccines, boosted two weeks later, and challenged four weeks afterwards with 2×10^4 CFU of *B. melitensis* 16M i.p. On day 21 after challenge immunized and nonimmunized mice were bled to evaluate antibody titers and euthanized by CO₂ inhalation. Experiments were repeated twice and representative results presented.

2.6. Quantity of bacteria in spleens

Following 21 days after virulent bacterial challenge spleens were removed and individually homogenized. One-half ml of neat homogenates and 10 ml of serial 10-fold dilutions of homogenates were cultured on trypticase soy agar, and *Brucella* colonies enumerated 3–5 days later as log₁₀ per spleen. Vaccine efficacy is presented in units of protection, representing *B. melitensis* in the spleen (log₁₀ *B. melitensis* recovered from saline vaccinated mice minus log₁₀ *B. melitensis* recovered from vaccinates).

2.7. Isotype-specific Ig ELISAs

Wells of plates (Nunc, USA) were coated overnight at 4°C with purified Omps (final concentration 1 µg/ml in PBS, pH 7.4). After washes, plates were saturated for 2 h at RT with 150 µl blocking buffer (2.5% casein). Wells were washed (PBS containing 0.1%

Tween 20). Serial twofold-diluted serum (50 μ l) from immunized mice prior to *Brucella* challenge, starting at 1/100 dilution in buffer (PBS with 1.25% casein, 50 mM EDTA, and 0.05% Tween 20), was added and incubated for 1 h at RT. Serum from nonimmunized mice served as a negative control. After five washes, plates were incubated with 1,000-fold dilution of goat biotinylated anti-mouse IgG1 (Amersham) for 1 h at RT. Finally TMB (3,3', 5,5'-tetramethyl benzidine) in citrate-phosphate buffer (0.05 M Na_2HPO_4 , 0.025 M citric acid, pH 5.0) and 2 mM H_2O_2 were added. Reaction was stopped after 20 min by 2 M H_2SO_4 and measured on an ELISA Reader (LabSystems, Finland). Titer was defined as the highest serum dilution giving an optical reading of two times the negative control.

2.8. Lymphocyte proliferation assay

Splenocytes from immunized mice prior to *Brucella* challenge were adjusted to 2×10^6 cells/ml, and dispensed (0.5 ml) into flat bottom 96 well plates in triplicate. Wells were pulsed with the respective Omp (10 μ g/ml) and incubated for 48 h. Four hours before the end of incubation, 50 μ l of supernatant was removed and 50 μ l DMEM medium containing 5% fetal calf serum and MTT (3-[4,5dimethylthiazole-2yl]-2,5 diphenyl tetrazolium bromide, 5 mg/ml) was added. After 48 h, 150 μ l of dimethyl sulfoxide (DMSO) was added followed by incubation overnight. The test was read at 570 nm wavelength with reference reduction at 650 nm. Lymphocyte proliferation was determined by optical density of wells containing test samples compared to wells containing no Omp. A signal to noise ratio used the formula: average OD at maximally stimulated wells / average OD in non-stimulated wells.

2.9. Cell Cytotoxicity Assays

Cytotoxicity assays used autologous primed macrophages as target cells and splenocytes from vaccinated and control mice as effector cells. Splenocytes (3×10^5 cells/well) were plated in 96 well flat bottom plates. After 2 h, approximately 10% of macrophages adhered and were used to calculate the effector:target (E:T) cell ratio. Recombinant Omp antigens (10 μ g/ml) were used to prime macrophages overnight. E:T ratio (25:1) was adjusted using autologous splenocytes as cytotoxic T cell effectors in DMEM medium and plates were incubated for 24 h. MTT dye was added to wells (25 μ l), and plates incubated 4 h after which 150 μ l of DMSO was added. Optical density at 492 nm was recorded after overnight incubation with reference reduction at 650 nm. Percent cytotoxicity was calculated using the formula:

$$\% \text{cytotoxicity} = 100 - \frac{(\text{O.D}_{\text{E+T}} - \text{O.D}_{\text{E}})}{\text{O.D}_{\text{T}}} \times 100$$

where

$\text{OD}_{\text{E+T}}$ = Optical density of wells with effector and targets, OD_{E} = Optical density of wells with effectors alone, and OD_{T} = Optical density of wells with targets alone.

2.10. Cytokine determination

IFN- γ in splenocyte culture supernatants was measured after 96 h with antigen as described for the lymphocyte proliferation assay. IFN- γ was assayed according to manufacturer's instructions using an enzyme-linked immunosorbent assay (ELISA) (Pharmingen, USA).

2.11. Statistical analysis

The *p* value was calculated using Student's *t* test and a *p* value of 0.05 was considered significant.

3. Results

3.1. Invasive *E. coli* vaccines

E. coli BL21AI was transformed with a plasmid encoding invasin from *Y. pseudotuberculosis* and LLO from *L. monocytogenes* (*E. coli* *inv+hly*) and three invasive *E. coli* vaccines were prepared (Table S1). Omps expression was analysed by SDS-PAGE and western blot analysis of whole-cell lysates (Figure 1).

3.2. Humoral response to Omps induced by invasive *E. coli* vaccines

Sera from invasive *E. coli* vaccinated and control mice 4 weeks after the final boost was assayed for Omp specific antibodies by indirect ELISA using the relevant purified Omp as antigen. Mice injected with invasive *E. coli* had considerable IgG1 titers to Omps (Figure 2), while mice immunized with empty invasive *E. coli* vaccine had undetectable anti-Omp antibodies. Humoral response measured post-vaccination indicates invasive *E. coli* generate IgG1 suggestive of a Th2 response.

3.3. Splenocyte proliferative response

To further investigate the cellular immune response, the proliferative splenocyte response to individual Omps was analyzed. Invasive *E. coli* vaccination resulted in antigen specific cell proliferation in response to individual Omps (Figure 3). In contrast, immunization with empty invasive *E. coli* had no detectable effect on cell proliferation (Figure 3).

3.4. Cytotoxic response from invasive *E. coli* vaccinated mice

To study the cytotoxic response by splenocytes from invasive *E. coli* vaccinated and control mice, individual autologous Omp antigen primed macrophages were used as target cells. Splenocytes from invasive *E. coli* vaccinated mice elicited a marked cytotoxic response against target cells compared to control mice (Figure 4).

3.5. Cytokine assay

Cytokine production from vaccinated mice 4 weeks following the final boost was examined after stimulating splenocytes *in vitro* with individual Omps. Splenic lymphocytes from invasive *E. coli* vaccinated mice produced up to 1851 pg of IFN- γ per ml upon restimulation with Omps. By contrast, minimal IFN- γ was detected from mice vaccinated with *E. coli* vector only or from saline immunized animals (Figure 5). Taken together, these results indicate a considerable Th1 response was induced after immunization with invasive *E. coli* Omp containing vaccines.

3.6. Protection against *B. melitensis* 16M challenge

Finally, to determine if immunization with the invasive *E. coli* vaccine platform expressing Omps would induce protection, mice immunized with invasive *E. coli* vaccine or empty vaccine vector were infected 4 weeks later with virulent *B. melitensis* 16M. Twenty-one days after challenge, mice were killed and splenic CFU quantified. Mice immunized with *E. coli* expressing individual *B. melitensis* Omps possessed significant protection (*p* 0.05) 21 days after challenge (Figure 6). In contrast, no significant difference in bacterial numbers isolated from spleens of saline and empty *E. coli* vector vaccinated animals was observed. At 21 days after challenge spleens were weighed. Vaccinated mice had higher spleen weights compared to controls as Omp31, Omp16 and BP26 ranged from 115 to 152, 120 to

174 and 107 to 159, respectively, compared to empty *E. coli* vaccinated mice (103 to 108 mg) (Table S2). Immunization of mice with invasive *E. coli* expressing individual *Brucella* Omps suggests significant anti-*Brucella* activity following Omp vaccination.

4. Discussion

Our aim was to evaluate a novel vaccine approach engineering *E. coli* to express *B. melitensis* Omp31, BP26 or Omp16 and mimic invasion of *Brucella* without its pathogenicity. Recombinant *E. coli* expressing listeriolysin O (LLO) from *L. monocytogenes* can deliver antigens to antigen-presenting cells [21,22]. Mice immunized with invasive *E. coli* vaccines expressing an individual Omp conferred protection against virulent *B. melitensis*. Splenocytes from vaccinated animals induced cytotoxic cell activity leading to *in vitro* lysis of macrophages expressing the relevant Omp. *In vitro* stimulation of splenocytes from Omps immunized mice also induced strong IFN- γ secretion as well as antigen specific IgG1 response suggesting induction of Th1 and Th2 responses.

Brucella spp. Omps are potential immunogenic antigens. Omp31 cloned from *B. melitensis* 16M [14] forms oligomers suggesting that it is a porin [16]. Omp31 is expressed in all *Brucella* species, except *B. abortus*, which has a 25-kb chromosomal deletion comprising *omp31* [26]. *B. melitensis* Omp31 has been cloned and expressed by *E. coli* [14] and can protect mice against a *B. ovis* challenge [27]. Previously, *Brucella* BP26 was identified as an immunodominant antigen in infected cattle, sheep, goats, and humans [9,10,28–32]. To date, Omp16 is the first *Brucella* protein without the requirement for external adjuvants to induce protection similar to live *B. abortus* strain S19 vaccine [33]. Moreover, the protein portion of Omp16 elicits a protective response when administered orally [33].

Protection against *Brucella*, requires potent cell-mediated immunity linked to a Th1-type immune response [34–36]. IFN- γ , Th1 cytokine, plays a prominent function in up-regulating macrophage anti-*Brucella* activity and, with tumor necrosis factor alpha (TNF- α), is considered crucial for protection against *Brucella* spp. [37–41]. Also, IFN- γ enhances cytotoxic T-lymphocyte activity as well as IgG2a and IgG3 isotype switching. The significance of CD4⁺ and/or CD8⁺ T cells in *Brucella* immunity has been controversial [42] both T cell populations are likely important in *Brucella* immunity [43].

In the present study *E. coli* was modified as a live vector to invade host cells, release antigen (Omp31, 16 or BP26) that was efficiently produced by the prokaryotic expression system, and then stimulate an immune response. *E. coli* infection would not persist, unlike live *Brucella*, being cleared as we have previously reported [20]. Further, smooth *Brucella* infection of macrophages is inefficient with only 40–60% of cells infected *in vitro* after 1 h [44]. In contrast, the *E. coli* vector expressing invasins from *Yersinia* can effectively invade 80–100% of professional phagocytic cells and a broad spectrum of cells expressing β 1-integrin [20].

In addition to *invasin* of *Y. pseudotuberculosis* our recombinant *E. coli* vaccine vector co-expressed the *hly* gene of *L. monocytogenes*. As reported [45], bacteria containing LLO would be lysed by phagosome-lysosome fusion. Through pore-forming action of LLO, cytoplasmic contents of the bacterial vaccine vector (including over-expressed antigen) escape into the cytosol and are processed by proteasomes promoting MHC I antigen presentation [20]. Using chicken ovalbumin (OVA) as a model antigen, *E. coli* expressing cytoplasmic LLO and OVA proteins can deliver the OVA H-2K^b restricted epitope SIINFEKL for MHC class I presentation by murine dendritic cells. The mechanism involves the LLO-dependent release of bacterial contents into the cytosol followed by proteasomal processing and presentation of antigenic motifs onto MHC class I [21,23].

Vaccination using bacterial vectors expressing subunit antigens has several distinct benefits over other methods. First, they are inexpensive to manufacture on a large scale. Second, multiple recombinant proteins can be conveniently expressed and laborious protein purification is not required. Third, *E. coli* are well studied and are easier to manipulate than viral vectors. Fourth, bacteria contain factors such as LPS that stimulate APC via Toll-like receptors, activating APC appropriately during antigen uptake. Fifth, the *E. coli* vector platform permits exclusion of *Brucella* proteins that inhibit an immune response such as Omp25 that suppresses TNF α [46]. Last, directed targeting to APCs *in vivo* occurs normally and requires no further manipulation. Recombinant bacterial vaccine vectors have been tested in mouse models suggesting the potential value of these vectors and raising the possibility of human and domestic animal application.

In summary, our data demonstrate an invasive *E. coli* vector platform can deliver antigens of *B. melitensis* to the immune system. Invasive *E. coli* may be an ideal vaccine since they are non-pathogenic, can deliver antigens to antigen-presenting cells, and contain natural adjuvant properties to promote cellular immune responses. Immunization of mice by direct injection of invasive *E. coli* carrying *Brucella* genes provided a potent anti-Omp response, resulting in high levels of protection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Non-pathogenic invasive *E. coli* used as a vaccine platform to mimic *B. melitensis*
- Invasive *E. coli* expressing *B. melitensis* Omps induce IFN- γ and cytotoxic T cells
- Vaccine platform expressing Omps induce antibodies and T cell proliferation
- Invasive *E. coli* expressing Omps protect against *B. melitensis* challenge

Figure 1. SDS-PAGE and western blot analysis of *E. coli* BL21-AI cells expressing *B. melitensis* Omp31, BP26, or 16. Panel A. SDS-PAGE analysis of *E. coli* BL21-AI cells harbouring pDESTOMPs of *B. melitensis*. (M) Marker lane, (1) uninduced, (2), (3) and (4) are Omp31, BP26, and Omp16, respectively. Panel B. Western blot analysis of induced samples using anti-HIS probe. Lanes (1), (2) and (3) are *B. melitensis* Omp31, BP26, and Omp16, respectively.

Figure 2.

Omp or BP26 specific IgG1 production. Mice were immunized i.p. with 2×10^7 inv *E. coli* containing the respective *omp* or *bp26*, boosted with same dose of inv-*E. coli* vaccine two weeks later, and sacrificed after 4 weeks. Serum from saline (dark column) or inv-*E. coli* vaccinated (gray column) mice was evaluated by ELISA using specific Omp or BP26 coated wells. Data are the mean $\pm$ SE of 5 mice per group. *Significantly different at $p < 0.01$ in IgG1 titer compared to mice immunized with saline.

Figure 3.

Lymphocyte proliferation to individual Omps or BP26. Mice were immunized i.p. with 2×10^7 *inv-E. coli* containing the respective *omp* or *bp26*, boosted with same dose of *inv-E. coli* vaccine two weeks later, and sacrificed after 4 weeks. Splenocytes from saline (dark column) or *inv-E. coli* vaccinated (gray column) mice were cultured with individual Omps or BP26 for 48 h and proliferation assessed by MTT assay. Data are the mean $\pm$ SE of 5 mice per group. *Significantly different at $p < 0.05$ in lymphoproliferation compared to mice immunized with saline.

Figure 4.

Cytotoxic T lymphocyte response to autologous adherent splenocytes pulsed with individual Omps or BP26. Mice were immunized i.p. with 2×10^7 inv-*E. coli* containing the respective *omp* or *bp26*, boosted with same dose of inv-*E. coli* vaccine two weeks later, and sacrificed after 4 weeks. Splenocytes from saline (dark column) or inv-*E. coli* vaccinated (gray column) mice were cultured at 25:1 effector:target ratio with adherent splenocytes pulsed with individual *Brucella* protein. Cytotoxicity was determined at 4 h by MTT assay. Data are the mean $\pm$ SE of 5 mice per group. *Significantly different at $p < 0.05$ in CTL response compared to mice immunized with saline.

Figure 5.

IFN- γ production from mice immunized with *inv-E. coli* containing the respective Omp or BP26. Mice were immunized i.p. with 2×10^7 *inv-E. coli* containing the respective *omp* or *bp26* and boosted with same dose of *inv-E. coli* vaccine two weeks later and sacrificed after 4 weeks. Splenocytes from saline (dark column) or *inv-E. coli* vaccinated (gray column) mice were cultured with individual protein for 48 h and IFN- γ determined by ELISA. Data are the mean $\pm$ SE of 5 mice per group. *Significantly different at $p < 0.05$ in IFN- γ production compared to mice immunized with saline.

Figure 6.

Protection against *B. melitensis* 16M challenge conferred by inv-*E. coli*-Omp or BP26 vaccines. Mice were immunized i.p. with 2×10^7 inv *E. coli* containing the respective *omp* or *bp26*, boosted with same dose of inv-*E. coli* vaccine two weeks later, challenged 4 weeks later with *B. melitensis* 16M (2×10^4 CFU), and sacrificed at day 21 after challenge. Bacteria were determined by colony counts from the spleens of saline (dark column) or inv-*E. coli* vaccinated (gray column) mice. Data are the mean $\pm$ SE of 5 mice per group.

*Significantly different at $p = 0.05$ in protection compared to mice immunized with saline.