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Cutting Edge: *Brucella abortus* Exploits a Cellular Prion Protein on Intestinal M Cells as an Invasive Receptor

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*Brucella abortus* is a Gram-negative bacterium causing brucellosis. Although *B. abortus* is known to infect via the oral route, the entry site in the gastrointestinal tract has been unclear. We found that *B. abortus* was selectively internalized by microfold cells (M cells), a subset of epithelial cells specialized for mucosal Ag uptake. During this process, colocalization of cellular prion protein (PrPC) and *B. abortus* was evident on the apical surface as well as in subapical vacuolar structures in M cells. Internalization of *B. abortus* by M cells of PrPC-deficient (*Prnp^−/−*) mice was greatly reduced compared with that in wild-type mice. Furthermore, an oral infection study revealed that translocation of *B. abortus* into the Peyer's patch was significantly lower in *Prnp^−/−* than in wild-type mice. These observations suggest that orally infected *B. abortus* invades the host through M cells by using PrPC on the apical surface of M cells as an uptake receptor. *The Journal of Immunology*, 2012, 189: 1540–1544.

The mucosal surface of the gastrointestinal tract is continuously exposed to vast numbers of commensal microorganisms and sporadically to pathogens. In this context, GALT such as Peyer’s patches (PPs) serve as sentinels for the recognition and initiation of the immune responses against those microbes (1). One of the unique features of GALT is the lack of afferent lymphatic ducts, which necessitates the sampling of luminal Ags across the mucosal epithelium. The luminal side of the GALT lymphoid follicles is covered by the dome-shaped follicle-associated epithelium (FAE), within which are microfold cells (M cells). M cells are a unique subset of epithelial cells that actively transport luminal macromolecules through transepithelial membrane traffic, a process referred to as transcytosis (2, 3). Luminal contents transported via M cells are in turn captured by dendritic cells (DCs) residing beneath M cells to initiate mucosal immune responses, which ultimately leads to the production of Ag-specific IgA by B cells (1). Ag delivery through M cells is thus important for host defense. In contrast, the M cell-dependent Ag uptake process can be exploited by diverse pathogenic microbes, including *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*) and *Yersinia enterocolitica* as a portal for host invasion (4). Proteins and/or oligosaccharides on the M-cell apical surface, including GPI-anchored proteins (5), are postulated to serve as receptors for these pathogens (6). In this regard, we have recently shown that the GPI-anchored protein gp2 (GP2) is specifically expressed on the apical plasma membrane of M cells and serves as an endocytic receptor for *S. Typhimurium* and *Escherichia coli* (7). We have also discovered that another GPI-anchored protein, cellular prion protein (PrPC), is predominantly expressed on the M-cell apical surface among the intestinal epithelial cells (8), suggesting its role as a similar endocytic receptor.

*Brucella abortus* is a Gram-negative bacterium that causes brucellosis, a major zoonotic infection. Brucellosis manifests as undulant fever, arthritis, endocarditis, and meningitis in humans, as well as abortion and infertility in domestic and wild animals. *B. abortus* is a facultative intracellular pathogen that replicates within both phagocytic and nonphagocytic host cells (9). The organism is taken up by macrophages through a process involving initial movement on the cell surface and generalized membrane ruffling, leading to swimming internalization (10). The internalized *B. abortus* are enclosed by phagosomes with accumulated lipid rafts to form replicative vacuoles that do not fuse with lysosomes (11). The Type IV secretion system (T4SS) encoded by the *VirB* genes appears to

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Abbreviations used in this article: DC, dendritic cell; FAE, follicle-associated epithelium; GP2, gp2; h, human; Hsp60, heat shock protein 60; m, mouse; M cell, microfold cell; PP, Peyer’s patch; Prnp^−/−, PrPC-deficient; PrPC, cellular prion protein; T4SS, Type IV secretion system; WT, wild-type.

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be essential for replicative vacuole formation, because phagosomes containing a virB mutant strain of *B. abortus* fuse with lysosomes to form conventional phagolysosomes (12). The inhibition of phagolysosome formation by T4SS has thus been implicated as a mechanism for the intracellular survival of *B. abortus*. Of interest, *B. abortus* expresses heat shock protein 60 (Hsp60) on its cell surface, probably via T4SS-mediated secretion. The surface-expressed Hsp60 binds to the PrPC on macrophages (13). This interaction facilitates macropinosome formation and subsequent intracellular replication of *B. abortus* within macrophages. Although the above-mentioned in vitro studies have revealed the intracellular survival mechanisms of *B. abortus*, the in vivo infectious route of this bacterium is still unclear. *B. abortus* is classified as a food-borne pathogen; however, it remains to be elucidated how this bacterium can translocate across the mucosal epithelial barrier.

In this article, we report that *B. abortus* is efficiently internalized only into M cells among intestinal epithelial cells, suggesting a role for M cells as an entry portal for this bacterium after oral infection. We also observed colocalization of PrP<sup>C</sup> and *B. abortus* on the apical surface of M cells. Importantly, the translocation of *B. abortus* into PPs after oral administration was significantly reduced in PrP<sup>C</sup>-deficient mice. These observations indicate that PrP<sup>C</sup> on M cells serves as a major uptake receptor for *B. abortus* during oral infection.

**Materials and Methods**

**Animals**

BALB/c and C57BL/6 mice were purchased from CLEA Japan. Prnp<sup>−/−</sup> mice (14) were backcrossed onto a C57BL/6 background. Other PrP<sup>C</sup>-deficient (Prnp<sup>−/−</sup>) mice (RRBC00437) were provided by RIKEN BioResource Center through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology of Japan (15). These mice were maintained under specific pathogen-free conditions. Animal experiments were approved by the Animal Research Committees of all institutions.

**Recombinant mouse prion protein preparation**

To obtain constructs for fusion proteins of mouse (m) PrP<sup>C</sup> (mPrP<sup>C</sup>) with the Fc segment of human (h) IgG, hlgG<sub>m</sub> (mPrP<sup>C</sup>-Fc), cDNA prepared from FAE were used as a template for PCR amplification. Primers used were as follows: 5′-CGGGATCCACCATGGCGAACCTTGGCTACT-3′ (forward) and 5′-CGCCTGAGGATCTTCTCGTAATAG-3′ (reverse). CDNA fragments were inserted into the BamHI/Xhol cloning sites of a pcDNA3 expression vector (Invitrogen) containing a fragment encoding the Fc segment of hlgG<sub>m</sub>, to obtain mPrP<sup>C</sup>-Fc. Recombinant protein was prepared as described (7).

**In vitro Brucella spp. binding assay**

A total of 0.25 μg mPrP<sup>C</sup>-Fc or control hlgG–Fc proteins were immobilized in 96-well flat-bottom plates (Nunc) by incubation overnight at 4°C. After washing, the wells were incubated with 1% BSA in PBS for 2 h for blocking, and then incubated for 2 h with 1 × 10<sup>6</sup> CFUs *B. abortus* 544 at 25°C. For Ab blocking, *B. abortus* were incubated with 0.5 μg/ml anti-Hsp60 Ab (Enzo Life Science) or isotype-matched control Ab (Jackson Immunolaboratory) for 15 min before binding assay. After washing five times with sterile PBS, genomic DNA was extracted from bound bacteria with a DNeasy Blood & Tissue Kit (Qiagen). Quantitative PCR was performed to quantify copy numbers of *hcp31* (16), using the SYBR Premix Ex Taq and the Thermal Cycler Dice Real Time System (TAKARA).

**Ligated intestinal loop assay**

Mice were anesthetized with Avertin (0.4 mg/g) and placed on a warming pad during the procedure. Next, 50 μg/ml mPrP mAb (SAF-32; Cayman Chemical) or isotype-matched control IgG (BD Biosciences) was injected into the ligated intestinal loop containing PPs. After incubation for 30–60 min, PPs were excised and fixed with Cytofix/Cytoperm (BD Biosciences) for 1 h at 4°C. Intracellular localization of primary Abs was probed with 10 μg/ml Alexa Fluor 488-conjugated anti-mouse IgG Ab (Molecular Probes). The specimens were further treated with 20 μg/ml Rhodamine *Ulex europeus* agglutinin-1 (UEA-1) (Vector Laboratories).

To assess the blocking effect of anti-Hsp60 Ab on *B. abortus* uptake by M cells, 1 million GFP- B. abortus (17), *B. abortus* 544, and *B. abortus* 544 pretreated with anti-Hsp60 Ab were injected into a ligated intestinal loop of C57BL/6 or Prnp<sup>−/−</sup> mice (14). After incubation, whole-mount specimens of PPs were immunostained with anti-*B. abortus*-specific rabbit antiserum (1:100 dilution) (17), together with anti-PrP mAb (44B1; Ref. 18) or GP2 mAb, followed by Alexa Fluor 594-conjugated anti-rat IgG. The specimens were analyzed with a DeltaVision Restoration deconvolution microscope (Applied Precision).

**Evaluation of oral infection**

C57BL/6 or Prnp<sup>−/−</sup> mice (four mice per group), 8–10 wk old were anesthetized with isoflurane or 50 mg/kg sodium pentobarbital. Then, mice were inoculated intragastrically by gavage with 0.2 ml 0.1 M sodium bicarbonate containing 1 million *B. abortus* 544. After 4 h, PPs were dissected and incubated at 25°C in sterile PBS containing 20 μg/ml gentamicin for 30 min. The tissues were weighed and homogenized in sterile PBS. The homogenates were plated on Thayer–Martin Selective Agar (BD) to determine CFUs.

**Statistics**

Statistical analysis was performed with the Mann–Whitney *U* test. Differences were considered significant at *p* < 0.01.

**Results and Discussion**

PrPC on M cells serves as an endocytic receptor

Given that PrP<sup>C</sup> is a GPI-anchored protein highly expressed on the M-cell apical surface (8) and that diverse infectious agents often use GPI-anchored proteins to gain entry into host cells (5), we hypothesized that it serves as an endocytic receptor for Ag sampling by M cells. This possibility was first examined by means of an in vivo Ab-uptake assay. We injected an anti-PrP mAb into a ligated intestinal loop containing PPs, to explore whether the mAb bound to PrPC on the M-cell surface is internalized into M cells. The subcellular localization of the mAb was analyzed by deconvolution microscopy to obtain high-resolution images. Serial X–Y images demonstrated that the PrP mAb was efficiently internalized into vesicular structures in the cytoplasm of M cells (Fig. 1A, 1B). Internalization of the PrP mAb was observed only in M cells, and not in the surrounding FAE cells. It is unlikely that the Ab uptake was mediated by a nonspecific pinocytic pathway, because no internalization of an isotype-matched
control IgG took place at all (Fig. 1A, 1C). These observations suggest that PrP<sup>C</sup> on M cells can serve as an endocytic receptor for the luminal constituents to which it can bind.

**B. abortus are selectively taken up by M cells through interaction with PrP<sup>C</sup>**

Because PrP<sup>C</sup> plays an important role in the uptake of *B. abortus* by macrophages (13), we examined whether this is also the case in M cells. Our in vitro binding assay using a rmPrP<sup>C</sup>–hIgG–Fc fusion protein (mPrP<sup>C</sup>–Fc) confirmed the interaction between PrP<sup>C</sup> and *B. abortus* (Fig. 2A). To further examine the interaction between PrP<sup>C</sup> and Hsp60 on *B. abortus*, the bacteria were pretreated with anti-Hsp60 Ab before the binding assay. The binding efficiency of *B. abortus* to mPrP<sup>C</sup>–Fc was profoundly impaired in anti-Hsp60–treated *B. abortus* compared with that in bacteria not treated or pretreated with isotype-matched control Ab (Fig. 2B).

We then asked whether PrP<sup>C</sup> expressed on M cells binds the bacterium. The ligated intestinal loop assay verified that *B. abortus* bound exclusively to M cells among epithelial cells in FAE and villous regions (Fig. 2C and data not shown). In addition, the X–Y images indicated that *B. abortus* were internalized into the cytoplasm of M cells, where colocalization of the *B. abortus* and PrP<sup>C</sup> was evident (Fig. 2D). These results support the idea that *B. abortus* can be taken up by M cells through its interaction with PrP<sup>C</sup>. To further confirm this possibility, we tested whether ablation of PrP<sup>C</sup> affects the efficiency of *B. abortus* uptake by M cells. In the ligated intestinal loop assay, we found that the number of surface-bound *B. abortus* in PrP<sup>C</sup>-deficient (*Prnp<sup>−/−</sup>*) mice was less than half that in wild-type (WT) mice (Fig. 3A, 3B). Moreover, the internalization of *B. abortus* into M cells was markedly reduced in *Prnp<sup>−/−</sup>* compared with WT mice (Fig. 3B). We also examined the effect of anti-Hsp60 Ab on the interaction between PrP<sup>C</sup> on M cell and *B. abortus*. Binding and internalization of *B. abortus* to M cells were decreased in anti-Hsp60–treated *B. abortus* (Fig. 3C). Taken together, these observations suggest an important role for PrP<sup>C</sup>, via interaction with Hsp60 on the bacterial surface, in the entry of *B. abortus* into M cells.

**B. abortus enters the host via M cells**

To gain further evidence for PrP<sup>C</sup>-dependent uptake of *B. abortus*, we performed oral infection with *B. abortus* in *Prnp<sup>−/−</sup>* and WT mice. After oral administration, a substantial number of viable *B. abortus* organisms were detectable in PP's of WT mice; by contrast, the bacteria were nearly undetectable in *Prnp<sup>−/−</sup>* mice (Fig. 4). Taken together with the above observations made with the ligated loop assay, this result underscores the biological significance of PrP<sup>C</sup> in the uptake of *B. abortus*, as well as supports the idea that *B. abortus* enters the host through M cells, using PrP<sup>C</sup>.

Prion protein is the causative agent of the transmissible spongiform encephalopathies. According to the “prion hypothesis,” the infectious isof orm of prion protein, termed...
PrP<sup>Sc</sup>, replicates by interacting with cellular PrP<sup>C</sup> and mediating its conformational change into the disease-causing PrP<sup>Sc</sup> (19). Compared with its well-defined pathological significance, the physiological function of PrP<sup>C</sup> remains unclear. PrP<sup>C</sup> is highly expressed not only by cells in the CNS but also by follicular DCs, mature myeloid cells, and activated T cells. This distribution suggests involvement of PrP<sup>C</sup> in immune surveillance (20).

Our present study defines a novel role for PrP<sup>C</sup> as an M-cell receptor for the uptake of pathogenic bacteria. PrP<sup>C</sup> on macrophages has been reported to recognize surface-exposed Hsp60 of <i>B. abortus</i> and to facilitate internalization of the bacteria (13); however, Fontes et al. (21) reported a contradictory result. Using Prnp<sup>-/-</sup> mice, they showed that <i>B. suis</i> infection is independent of PrP<sup>C</sup> expression. By contrast, WT macrophages had a greater tendency to be infected with <i>B. abortus</i> than did Prnp<sup>-/-</sup> macrophages, although no significant difference between WT and Prnp<sup>-/-</sup> macrophages was found in intracellular multiplication of <i>B. abortus</i>. To shed more light on these observations, we performed oral infection with <i>Brucella</i> spp. in WT and Prnp<sup>-/-</sup> mice. After oral administration, a substantial number of <i>B. suis</i> organisms were detectable in both WT and Prnp<sup>-/-</sup> PPs (data not shown), whereas the translocation of <i>B. abortus</i> into PPVs was significantly reduced in Prnp<sup>-/-</sup> mice (Fig. 4). These data are consistent with the observation by Fontes et al. (21). In addition, these authors discussed the spatial proximity of <i>Brucella</i> spp. and PrP<sup>C</sup> during the early stage of infection. In accordance, our ligated loop assay showed that the internalized <i>B. abortus</i> were surrounded by PrP<sup>C</sup> in the cytoplasmic vacuolar compartment of M cells (Fig. 2D). Taken together, these results suggest that <i>B. abortus</i> is efficiently taken up by M cells in a PrP<sup>C</sup>-dependent manner. However, <i>B. suis</i> might invade the host independently of PrP<sup>C</sup>

Interaction of PrP<sup>C</sup> and Hsp60 family proteins has been demonstrated by several approaches, including a yeast two-hybrid screening as well as a pull-down assay (22). Our in vitro binding assay confirmed the interaction between PrP<sup>C</sup> and Hsp60 on <i>B. abortus</i> (Fig. 2B). We also showed that PrP<sup>C</sup> on M cells interacts with Hsp60 on <i>B. abortus</i> (Fig. 3C). Nevertheless, we cannot formally exclude an alternative and mutually not exclusive possibility that PrP<sup>C</sup> on M cells acts as a scaffold to coordinate several proteins in a complex, with the complex mediating <i>B. abortus</i> internalization (23). The Hsp60 proteins have been recognized as immunodominant Ags of many microbes (24). Hsp60 normally resides in the bacterial cytoplasm, but the protein can be secreted via T4SS and expressed on the outer membrane of the bacteria. In fact, the presence of Hsp60 on the bacterial surface is not restricted to <i>B. abortus</i>. A similar phenomenon has also been shown for other bacteria (25–28). The exposure of Hsp60 on the surface appears to increase bacterial adherence to host cells. PrP<sup>C</sup> expressed on the apical plasma membrane of M cells in the GALT thus may contribute to immunosurveillance on the mucosal surface by promoting transcytosis of bacteria that express Hsp60 at their surface. This idea is concordant with the observation that <i>H. pylori</i> translocation from the intestinal mucosa into PPVs, possibly via the M cells, is essential for the induction of humoral and cellular immunity against this pathogen (29).

Accumulating evidence supports the idea that many infectious agents and their toxins use GPI-anchored proteins to gain entry into host cells (5). For example, CD48 on macrophages and mast cells contributes to phagocytosis of <i>E. coli</i> via FimH recognition (30). On the basis of our observations that PrP<sup>C</sup> and GP2 proteins on the apical plasma membrane of M cells can serve as receptors (this study and Ref. 7), M cells also seem to use GPI-anchored proteins for intestinal immunosurveillance. In conclusion, our findings indicate that the PrP<sup>C</sup>-dependent route of bacterial uptake by M cells can be exploited for <i>B. abortus</i> invasion into the host. Once <i>B. abortus</i> penetrate M cells, the bacteria are capable of surviving inside DCs, which accumulate beneath the M cells, by forming replicative vacuoles with subsequent systemic spread to other organs. This model offers a new insight into the pathogenesis of <i>B. abortus</i> infection, a disease that leads to significant economic losses for cattle and other domestic animals and, in turn, transmission to humans. The disruption of the Hsp60–PrP<sup>C</sup> interaction on the mucosal surface may provide a useful therapeutic target for protection against <i>B. abortus</i> infection.

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Disclosures
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