Differential antibrucella activity of bovine and murine macrophages

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Brucella abortus is an intracellular pathogen affecting macrophages. Macrophages release some antibrucella components such as lysozymes (LZ), reactive oxygen species (ROS) and reactive nitrite intermediates (RNI) which prevent intracellular survival of Brucella. The present study compared the antibrucella activity of bovine and murine macrophages following stimulation with B. abortus lipopolysaccharides. Our results revealed increased production of these antibrucella substances in murine macrophages as compared to bovine macrophages. The differential production of these antibrucella components explained the differential B. abortus killing ability of these species (bovine and mice) that was measured in terms of intramacrophagic survival of Brucellae in murine and bovine macrophages.

Keywords: Brucella abortus, Cell stimulation, Cytokines, Lysozymes, RNI, ROS

The immune response against Brucella is innate and adaptive in nature that activates antigen-presenting cells including macrophages. The survival or elimination of Brucella depends upon production of some antibrucella substances from macrophages. These antibrucella components from macrophages include lysozymes (LZ), reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) and cytokines that play basic role in elimination of Brucella species from infected hosts. Lysozyme is a glycosidase which hydrolyses N-acetyl hexosaminidase linkages in Brucella cell wall. The ROS and other non-oxygen dependent products of macrophages act as toxicants against Brucella1 while nitric oxide (NO) inhibits mitochondrial respiration of Brucella2. In addition to these, cytokines also have antibrucella activity3.

The present study is based upon the hypothesis that the extent of bactericidal activity of macrophages differs in different macrophages host species such as bovine and murine macrophages. Bovines are the main hosts for Brucella abortus while murine species are laboratory models. Therefore, the comparison of these two species can provide useful insight into the pathogenesis of disease.

Materials and Methods

Isolation of bovine macrophages
Holstein cattle (n=6) from Michigan State University farm, East Lansing, USA were selected for blood collection to isolate bovine macrophages according to the procedure already described4. Murine RAW 264.7 macrophages cell line TIB-71 was procured from American Type culture collection (ATCC). Two strains of Brucella (rough and smooth) were provided by Dr Gerhardt G. Schurig’s laboratory in Virginia Tech., USA, for extraction of lipopolysaccharides.

The rough (RB51) and smooth (S2308) Brucella abortus lipopolysaccharides were extracted according to procedure already described5,6 and subsequently purified7 using minor modifications. The purified LPSs and their combination were used at dose rates (2, 20, 200 µg/mL) for macrophages stimulation8.

Stimulation of Macrophages
Both murine and bovine macrophages were cultured at 37°C for 24-48 h in Dulbecco’s Modified
Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) media, respectively. Both cell types were treated with rough, smooth and combined (1:1) B. abortus LPS and the plates were incubated for another 24 h. The incubation was followed by centrifugation at 4°C at 3000×g for 10 min and the recovered supernatant was frozen prior to the quantification of antibrucella components. E. coli LPS and macrophages alone without any LPS served as positive and negative control, respectively.

**Determination of antibrucella components**

The production of lysozyme, ROS and RNI were determined by lysozyme release assay, nitroblue tetrazolium assay and nitric oxide assay. While the pro-inflammatory (IL-1β, IL-6, IL-12, TNF-α) and anti-inflammatory cytokines (IL-10) in LPS activated macrophages were estimated by sandwich ELISA. The OD was recorded with a microplate reader at 450 nm. "KC4 software (Biotek Instruments Inc. Vinooski, VT, USA) was used to calculate the levels of cytokines present. Recombinant bovine and murine IL1-β, IL-6, IL-10, IL-12 and TNF-α (Bio-Legend) were used as positive control.

**Determination of intracellular survival of Brucella abortus in bovine and murine macrophages**

The colony forming unit assay (cfu) was used to determine intracellular survival of Brucella abortus in macrophages. Twenty four well plates (Becton Dickinson) were seeded with bovine and murine RAW 264.7 macrophages at a concentration of 2.5×10^5 cells/mL and incubated at 37°C for 24 h. After 24 h, the cells were stimulated with rough, smooth and combined Brucella abortus LPS (200 µg/mL) and further incubated at 37°C under 10% CO₂ for 24 h. After incubation, the stimulated macrophages were challenged with B. abortus cells for 1 h at a multiplicity of infection (MOI) of 1:100 with the following treatments of B. abortus cells: (a) RLPS +macrophages+Brucella abortus; (b) SLPS +macrophages+Brucella abortus; (c) RLPS+SLPS +macrophages+Brucella abortus; and (d) Negative control (macrophages+Brucella abortus). Each combination was performed in triplicate.

**Statistical analysis**

The data was statistically analyzed by student’s t test for two-group comparison, P <0.05 was considered to be statistically significant.

**Results**

There was circumstantial evidence from the present study that the outcome of Brucella infection is related to macrophage Brucella interactions. Our results indicated that the macrophages of both susceptible (bovine) and resistant species (mice) were activated by LPS stimulants for antibrucella activity but this activation was lower in susceptible species as compared to resistant species.

Significantly higher (P <0.05) levels of lysozymes, ROS, nitric oxide and pro-inflammatory cytokines (IL-1β, IL-6, IL-12, TNF-α) were induced in murine RAW 264.7 macrophages as compare to bovine macrophages (P <0.05) under the same experimental conditions (Fig. 1 A-C). On contrary, the level of anti-inflammatory cytokine (IL-10) was significantly higher (P <0.05) in bovine macrophages as compare to murine macrophages (Fig. 2). The enhanced release of anti-inflammatory cytokine (IL-10) by bovine macrophages may explain the proportional decrease of pro-inflammatory cytokines in bovines.

The colony forming unit assay (cfu) was used as evidence to support results of lysozyme release assay, nitroblue tetrazolium assay, nitric oxide assay and cytokine ELISA. Initially at 1 h post-infection less viable Brucellae were cultured from murine

![Fig. 1](image_url)—Comparative evaluation of (A) lysozyme; (B) reactive oxygen species (ROS); (C) nitric oxide (NO) induction in bovine and murine macrophages stimulated with various doses of Brucella LPSs.
Fig. 2—Production of pro-inflammatory and anti-inflammatory cytokines in bovine and murine macrophages stimulated with various doses of *Brucella* LPSs. Induction of (A) TNF-α; (B) IL-1β; (C) IL-6; (D) IL-12; and (E) IL-10 from bovine and murine macrophages.

macrophages (1.7×10⁴) as compared to bovine macrophages (5.1×10⁴) pre-treated with rough LPS. This explains the higher activation of murine macrophages as compared to the bovine macrophages. While after 6-24 h post-infection, no *Brucella* cells were retrieved from either type of macrophages suggesting that a majority of the bacteria were phagocytosed and killed by substantial activation of both types of macrophages. Similarly at 1, 6 and 24 h post-infection less viable *Brucella* cells were found in murine macrophages (13×10⁴, 9.5×10⁴, 5.5×10⁴) as compared to bovine macrophages (47×10⁴, 28×10⁴, 19×10⁴) on pre-treatment with smooth LPS. On the other hand pre-treatment of bovine and murine macrophages with a combination of smooth and rough *B. abortus* LPSs (100:100 µg/mL) also resulted in a decreased number of viable *Brucella* cells in murine macrophages (6.6×10⁴, 2.9×10⁴ and 1.8×10⁴) than bovine macrophages (20×10⁴, 9.5×10⁴ and 6.4×10⁴) at 1, 6 and 24 h post-infection, respectively.

**Discussion**

The intracellular survival of *Brucella* depends upon bactericidal activity of immune cells such as macrophages. Increased lysozyme induction from murine macrophages as compared to bovine macrophages in the present study may have several explanations. There seems to be an association in macrophages size and production of lysozymes as murine macrophages are bigger than bovine macrophages. Moreover, bovines are the main reservoirs of brucellosis. It is, therefore, not surprising that they have a high susceptibility and infection rate than the rodents/mice. Previous literature suggests that LPS acts like a toll-like receptor agonist. As in the present study, both the bovine and murine macrophages were stimulated by the same doses and types of LPSs. Hence, the differential production of lysozymes by both types of macrophages could be due to the difference in toll-like receptors of both cells.

When stimulated macrophages increase the utilization of oxygen (respiratory burst) and convert oxygen to reactive oxygen species (ROS). These ROS and related toxic products may contribute significantly to the destruction of extra-cellular as well as intracellular pathogens including *Brucella*. This increased ROS production in murine macrophages could be due to the increased utilization of oxygen by the murine macrophages as compared to bovine macrophages.

In addition, it could also be due to the differential intracellular signaling pathways expressed in these cells. The superoxide anion production depends upon activation of PKC and ERK pathways which contribute to the activation of NADPH-dependent oxidase. This enzyme is highly activated in murine macrophages upon stimulation, and thus, could have contributed to increased production of ROS. These pathways also induce upregulation of TNF-α in murine macrophages. The results of the present study are in agreement with previous report that explained that murine macrophages produced increased ROS levels, particularly superoxide anion and hydrogen peroxide. Our findings also correlate with Zurbrick *et al.* who described that cultured bovine macrophages exhibited decreased release of superoxide anion but did not show detectable lysozyme activity.

The greater nitric oxide induction of murine macrophages than bovine macrophages might be caused by differential intra-macrophagic pH. A previous study reported that the amount of nitric oxide release from murine RAW 264.7 macrophages was increased at acidic pH. Our results concur with previous studies that compared nitric oxide levels in macrophages of different species and found that the level was highest in murine macrophages.
and lowest in bovine macrophages. Similarly in another study, the authors reported more nitric oxide mediated killing of Brucellae in murine macrophages. However, the results of the present study are not in agreement with those of Padgett and Pruett, who found a low induction of nitric oxide in mice. The increased nitric oxide production is dependent upon enzyme called nitric oxide synthase (NOS). The NOS has three isoenzymes of which iNOS (inducible nitric oxide synthase) is present in activated macrophages. It may be predicted that murine macrophages express more iNOS and ultimately higher output of nitric oxide as compared to bovine macrophages. On the other hand, bovine macrophages could have had metabolic inhibitors of iNOS.

The present study showed increased levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-12 & TNF-α) from murine macrophages as compared to bovine macrophages. As the macrophages are also activated by cytokines produced by T cells and there are good numbers of T cells in spleen of mice, it could be the possible reason for increased macrophages activation in mice than bovines. On other hand, the increased TNF-α production from murine macrophages as compared to bovine macrophages is supported by the fact that TNF-α is considered to be necessary for full expression of macrophage’s antibrucella activities and ultimately, macrophages kill intracellular Brucella within 12-24 h following infection. In opposition, IL-10 which was the only anti-inflammatory cytokine measured in this study, showed increased level in bovine macrophages as compared to murine macrophages that could point to the association of antibrucella activities of macrophages with inflammation.

In the present study, the interaction of LPS stimulated bovine and murine macrophages with Brucella abortus rough strain (RB51) revealed prolonged survival of B. abortus in bovine macrophages as compare to murine macrophages. This is in agreement with previous report that the deficiency of clearance factors in bovine macrophages is required for elimination of Brucella. However, they did not mention about the clearance factors that were involved. The present study revealed those clearance factors to be LZ, ROS, RNI and cytokines.

We conclude that the antibrucella activity of macrophages depends upon the production of different intramacrophagic products, such as lysozymes, ROS, nitric oxide and cytokines. This study revealed that murine macrophages are metabolically more active, and hence, resistant to Brucella abortus due to enhanced release of these compounds when compared to bovine macrophages.

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References