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The effects of red ginseng saponin fraction-A (RGSF-A) on phagocytosis and intracellular signaling in Brucella abortus infected RAW 264.7 cells

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One sentence summary: This study highlights the phagocytic and intracellular modulating effect of RGSF-A and its potential as an alternative remedy to control Brucella abortus infection.

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ABSTRACT

This study indicated that RGSF-A caused a marked reduction in the adherence, internalization and intracellular growth of Brucella abortus in RGSF-A-treated cells. Furthermore, a decline in the intensity of F-actin fluorescence was observed in RGSF-A-treated cells compared with untreated B. abortus-infected cells. In addition, an evaluation of phagocytic signaling proteins by Western blot analysis revealed an apparent reduction of ERK and p38α phosphorylation levels in B. abortus-infected RGSF-A-treated cells compared with the control. Upon intracellular trafficking of the pathogen, a higher number of B. abortus-containing phagosomes colocalized with LAMP-1 in RGSF-A-treated cells compared with control cells. These results strongly suggest that inhibition of B. abortus uptake could be mediated by suppression in the activation of MAPKs signaling proteins phospho-ERK 1/2, and p38 levels. On the other hand, inhibition of intracellular replication results from the enhancement of phagolysosome fusion in host macrophages. This study highlights the phagocytic and intracellular modulating effect of RGSF-A and its potential as an alternative remedy to control B. abortus infection.

Keywords: Brucella abortus; red ginseng saponin fraction-A; phagocytosis; macrophage

INTRODUCTION

Brucella spp. are included in the Centers for Disease Control and Prevention (CDC, USA) select agent list because of its eminent infective rate (Gomez et al. 2013). The most important routes of infection are respiratory and gastrointestinal exposure to direct animal tissue or unpasteurized products (Gomez et al. 2013). Internalization of Brucella abortus into macrophages is facilitated by the pathogen swimming on the cell surface, which causes a generalized membrane ruffling that lasts for a few
minutes. After being internalized, the bacteria are then enclosed in macropinosomes (Kim et al. 2005). This pathogen multiplies within membrane-bound replicative-competent compartments called phagosomes in phagocytic cells, but it can also replicate in non-phagocytic cells (Siadat, Salmani and Aghasadeghi 2012). Due to the highly adapted survival strategies developed by Brucella, new biological therapeutic interventions would be extremely advantageous. Furthermore, there is an increasing need to establish a biological therapy other than conventional antibiotics for the treatment of infectious diseases due to the number of side effects and financial burdens of conventional therapy (Attele, Wu and Yuan 1999).

Bacterial infections have been efficiently controlled through vaccination, surveillance and confinement programs. However, no human vaccine exists for brucellosis (Avila-Calderon et al. 2013). The widespread use of antibiotics with elevated doses and prolonged treatment to eradicate brucellosis is controversial (Pappas et al. 2005). This infection requires extended periods of synergistic or additive combination antibiotic treatments that often lead to irreversible sequelae (Siadat, Salmani and Aghasadeghi 2012).

Ginseng is a valuable herb in Asia that spans a very long history of use as a ‘panacea’ that can cure all diseases and thereby promote longevity. A wide range of pharmacological effects of ginseng has been established in several studies. The effects of saponin on the mechanism of cellular immune responses and the complex interactions between specific receptors on the cell membrane and the respective moieties of saponin have not been extensively studied.

The aim of the current study was to investigate the effect of RGSF-A on phagocytosis and intracellular signaling in RAW 264.7 infected with B. abortus. The results of this study suggest possible immune modulation by RGSF-A as manifested in the inhibition of bacterial uptake and intracellular replication of B. abortus in host macrophages.

**MATERIALS AND METHODS**

**RGSF-A preparation**

RGSF-A was isolated from Korean red ginseng supplied by the Institute of Technology, Korea Ginseng Corporation (Daejeon, Korea) as previously described (Yayeh et al. 2012). In brief, RGSF-A was extracted with ethanol, and the extract was air-dried at 60°C for 2 days. An aqueous extraction at 95–100°C was performed on the extract powder, and the resulting water extract was filtered through a 100 μm filter. The final filtrate was harvested and concentrated via evaporation and finally freeze-dried. When used in experiments, it was dissolved in DMSO, filtered through a 0.45 μm filter. The final filtrate was harvested and concentrated via evaporation and finally freeze-dried. When used in experiments, it was dissolved in DMSO, filtered through a 0.45 μm membrane and diluted using phosphate-buffered saline (PBS; pH 7.4).

**Bacteria and cell culture**

Standard wild-type strains were derived from B. abortus 544 (ATCC 23448), a smooth, virulent B. abortus biovar 1 strain. Brucella abortus strain was cultivated in Brucella broth or in Brucella agar. Bacteria were grown at 37°C with vigorous shaking until they reached stationary phase. RAW 264.7 cells, a murine macrophage cell line, were grown at 37°C in 5% CO2 atmosphere in RPMI 1640 containing 10% heat-inactivated fetal bovine serum (FBS), with or without 100 U ml−1 penicillin and 100 μg ml−1 streptomycin (Gibco, USA).

**Bactericidal analysis**

Bacteria were diluted with PBS to a concentration of 2 × 10⁴ colony forming units (CFU)/ml, added to PBS containing different concentrations of RGSF-A (1, 10 and 100 μg ml⁻¹) and incubated at 37°C for 0, 4, 8 and 24h. Each diluent was plated onto Brucella agar after incubation and cultured for 2 days at 37°C. The bacterial survival rates were expressed as the percentage of the survival rate of the treated sample relative to an untreated control, which was set at 100%.

**Cytotoxicity assay**

Different concentrations of RGSF-A (0, 1, 10 and 100 μg ml⁻¹) were selected to evaluate its cytotoxic effect on RAW 264.7 macrophages. Briefly, cells were incubated with different concentrations of RGSF-A for 48h in a 96-well culture plate. The cell cytotoxic effect of RGSF-A was evaluated by a 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay.

**Determination of efficiency of bacterial uptake and intracellular growth**

RAW 264.7 cells, overnight culture in 96-well plate at 2 × 10⁴ cells well⁻¹, were pretreated with the highest non-cytotoxic dose of RGSF-A (10 μg ml⁻¹) or PBS (negative control) for 2 h before infection. Bacteria were then deposited onto cells at a multiplicity of infection of 100, centrifuged at 150 × g for 10 min at room temperature and incubated at 37°C in 5% CO₂ for 0 or 30 min. The infected cells were washed once with PBS and then incubated at 37°C in RPMI 1640 containing 10% (v/v) FBS and gentamicin (30 μg ml⁻¹) for 30 min to kill any remaining extracellular bacteria. The cells were washed twice with PBS and lysed with distilled water. For intracellular growth efficiency, bacterial infection was done as described above for bacterial uptake. Infected cells were incubated for 2, 24 and 48h with RPMI 1640 containing 10% (v/v) FBS and gentamicin (30 μg ml⁻¹) containing RGSF-A (10 μg ml⁻¹) or PBS. The same protocol for washing, lysis and plating was conducted to analyze the efficiency of bacterial internalization (Lee et al. 2012).

**F-actin staining**

RAW 264.7 cells were stained for F-actin and examined by immunofluorescence microscopy as previously described, with slight modifications (Watarai et al. 2002). Briefly, overnight culture of bacteria was harvested and suspended into PBS, and adjusted to 1 × 10⁵ bacteria ml⁻¹. Bacteria were transferred to a microcentrifuge tube, pelleted and suspended in 1 ml fluorescein isothiocyanate (FITC, 0.5 mg ml⁻¹) (Sigma-Aldrich, USA) in 50 mM sodium carbonate-100 mM sodium chloride at pH 9.0. Bacterial suspension was incubated for 20 min at room temperature and washed twice with PBS (Weingart et al. 1999). RAW 264.7 cells were infected with FITC-conjugated B. abortus. To observe for F-actin reorganization, bacterial infection was monitored for 10 min by previously described methods (Watarai et al. 2002). The infected cells were fixed with 4% paraformaldehyde, incubated at 37°C for 1h, permeabilized with 0.1% Triton X-100 for 10 min at 22°C and incubated with blocking buffer (2% goat serum in PBS) for 30 min. The cells were then incubated with 0.1 μM rhodamine-phalloidin (Cytoskeleton, USA) for 30 min at 22°C.
LAMP-1 staining

To determine the colocalization of BCPs with LAMP-1, infected RAW 264.7 cells were incubated at 37 °C for 1 h, washed once with medium, incubated with RPMI 1640 containing 10% FBS with gentamicin 30 μg ml⁻¹ and treated with RGSF-A 10 μg ml⁻¹ and then incubated for 2 and 24 h. Fixation, permeabilization and blocking procedure were performed as with F-actin staining. The samples were stained with anti-LAMP-1 rat monoclonal antibody (Santa Cruz Biotech, USA) that was diluted 1:100 in blocking buffer. After three washing cycles of 5 min each with PBS, the samples were stained with Texas red-goat anti-rat IgG (1:1000). The samples were stained with anti-B. abortus rabbit serum and FITC-conjugated anti-rabbit IgG to identify the bacteria and placed in mounting media. Fluorescence images were captured using a laser scanning confocal microscope (Olympus FV1000, Japan) and processed using FV10-ASW Viewer 3.1 software. For LAMP-1 staining, 100 bacteria within macrophages were randomly selected, and the extent of LAMP-1 colocalization with the bacteria was determined.

Bacterial adherence assay

To evaluate bacterial adherence, RAW 264.7 cells were pretreated with RGSF-A (10 μg ml⁻¹) for 4 h before infection. During the last 40 min of incubation, 2.5 μl of cytochalasin D (500 μg ml⁻¹) with a final concentration of 1.25 μg ml⁻¹ was added to inhibit bacterial internalization. Bacterial infection, fixation, staining and microscopic evaluation were done as described above for bacterial detection. One hundred macrophages were randomly selected, and the bacteria that adhered to these macrophages were counted.

FACS assay

The relative content of F-actin in B. abortus-infected and uninfected cells in the presence or absence of RGSF-A (10 μg ml⁻¹) was assessed. In brief, RAW 264.7 cells were cultured in 6-well plates and pretreated with RGSF-A as described for the bacterial adherence assay. The cells were infected for 30 min, fixed with 4% paraformaldehyde at 22 °C for 30 min, permeabilized and stained with 20 μg ml⁻¹ lysoctphansidylcholyl containing 1 μM tetramethyl rhodamine isothiocyanate–phalloidin (Sigma-Aldrich, USA) for 1 h at 22 °C. The cells were centrifuged at 300 × g for 5 min at 4 °C and washed with PBS. F-actin content was quantified by FACS analysis using a FACSCalibur flow cytometer (BD Biosciences, USA) and is represented on log-scale histograms depicting 10 000 cells. The average F-actin content of a population was expressed as the mean fluorescence intensity. All assays were conducted in triplicate.

Immunoblot analysis

Immunoblot analyses were performed as described previously by MacPhee (2010). RAW 264.7 cells were cultured in 6-well plates, incubated with RGSF-A (10 μg ml⁻¹), washed and then infected with B. abortus for the indicated times. The cells were then washed twice with ice-cold PBS. Ice-cold Radioimmuno-precipitation assay buffer with 1% protease inhibitor cocktail was used to lyse the cells for 30 min at 4 °C. Samples were separated by SDS-PAGE. The proteins were electrically transferred onto Immobilon-P membranes (Millipore, USA) using 1X transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) with a constant current of 2 mA cm⁻² for 1.5 h in a semi-dry electroblot assembly (Bio-Rad, USA). The membrane was blocked with 5% (w/v) bovine serum albumin in 1X Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris-HCl, 150 mM NaCl, Tween 0.1%, pH 7.6) for 30 min at room temperature. Then, it was incubated with phospho-specific antibodies (Cell Signaling, USA) against ERK1/2 (Thr183/Tyr185), JNK (Thr183/Tyr185) and p38 (Thr180/Thr182) overnight at 4 °C. Fan antibodies and β-actin antibody were also used to demonstrate equal amounts of protein loaded between all samples. Primary antibody binding to the membrane was detected by incubating the membrane in horseradish peroxidase-conjugated protein G (Thermo Scientific, USA; 1:1000 dilution) in 5% blocking buffer for 1 h and washing with 1X TBS-T. Finally, the signal was detected with a luminal-coumaric acid-H₂O₂ detection solution (Atto Corporation, Japan) and a Molecular Imager Chemidoc XR+ system machine (Bio-Rad Laboratories, USA). The immunoblot signals were quantified using the NIH ImageJ software.

Statistical analysis

The results of each experiment are expressed as the mean ± standard deviation (SD). A Student’s t-test or one-way ANOVA was performed to compare differences between the groups. Differences of P < 0.05 were considered statistically significantly.

RESULTS

Bactericidal and cytotoxicity assay

Treatment of bacterial cells with different concentrations of RGSF-A (1, 10 and 100 μg ml⁻¹) did not cause a significant change in bacterial survival rate, which indicates that RGSF-A has no bactericidal effect on B. abortus. In addition, treatment and incubation of RAW 264.7 cells with various concentrations of RGSF-A (0, 1, 10 and 100 μg ml⁻¹) indicated that 10 μg ml⁻¹ was an optimal dose that could be given without a decrease in the OD value (cell survival) compared with untreated cells. Doses higher than 10 μg ml⁻¹ caused increasing levels of cytotoxicity. A dose of 10 μg ml⁻¹ was used in all subsequent experiments.

Influence of RGSF-A on bacterial internalization and intracellular growth

The results indicate that B. abortus invasion into RGSF-A-treated cells was significantly inhibited after both 0 and 30 min of incubation (P < 0.05) (Fig. 1a). Similarly, intracellular replication was reduced, particularly between 2 and 24 h of incubation, but was rapidly increased after 24 h (Fig. 1b).

Interference of RGSF-A on B. abortus phagocytosis

The results demonstrated that the number of bacteria that adhered to cells pretreated with RGSF-A (24.4 ± 2.12) was significantly decreased compared with untreated control cells (48 ± 4.24; 48.96% ± 1.50 reduction) (P < 0.05) (Fig. 2). When the macrophages were examined by fluorescent microscopy, a reduction in the formation of filopodial structures and microspikes were apparent in RGSF-A–infected and untreated cells compared with untreated control cells. The results indicate that RGSF-A significantly inhibited F-actin polymerization. Quantification of F-actin through FACS analysis confirmed the inhibitory effect of RGSF-A on the F-actin polymerization that is required for phagocytosis of B. abortus. In accordance with the initial result from F-actin microscopy,
Figure 1. Effect of RGSF-A on the invasion and intracellular growth of *B. abortus*. RAW 264.7 macrophages were incubated with RGSF-A (10 μg ml⁻¹), infected for 0 and 30 min and further incubated for 2, 24 and 48 h. Bacterial internalization (a) and bacterial intracellular growth (b) were evaluated. Data represent the mean ± SD of duplicate experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (∗P < 0.05; ∗∗P < 0.01).

Figure 2. Effect of RGSF-A on the adherence of *B. abortus* to macrophages. RAW 264.7 macrophages were pre-incubated with RGSF-A (10 μg ml⁻¹) for 4 h and simultaneously incubated with cytochalasin D (500 μg ml⁻¹) for 40 min at 37°C, and then were infected for 30 min. Infected cells were fixed, washed and stained with anti-*B. abortus* polyclonal rabbit serum (1:500) and FITC-conjugated goat anti-rabbit immunoglobulin G (IgG) (1:500) for 1 h at 37°C. Fluorescence images were collected using a microscope equipped with a camera. One hundred macrophages were randomly selected and the bacteria that adhered to the cells were counted. Data represent the mean ± SD of triplicate experiments. Statistically significant differences relative to the untreated control are indicated by asterisks (∗P < 0.05).

DISCUSSION

*Brucella* spp. must endure intramacrophagic conditions during a prolonged infection, but it appears well adapted to the environmental conditions in the intracellular niche of this host (Gomez et al. 2013). However, there is currently no safe vaccine that can be used to control brucellosis and treatment of this disease with antibiotics is controversial (Lee et al. 2012). Korean red ginseng has been traditionally cultivated and used as an herbal medicine in East Asia. Different mechanisms of action have been described in both physiologic and pathologic states (Lu, Yao and Chen 2009). In particular, several effects of ginseng can be attributed to its saponin component, including anti-allergic, immunomodulating activity, antiviral, molluscidal, antifungal activities and improvement of phagocytic activity of macrophages (Kim, Germolec and Luster 1990; Lacaille-Dubois and Wagner 1996; Scaglione et al. 1996).

RGSF-A contains a high percentage of panaxadiol saponins and was further analyzed by HPLC to identify seven major panaxadiol (Rb1, Rb2, Rb3, Rc, Rd, F2 and Rg3) and three panaxatriol ginsenoside components (Re, Rf and Rg1) (Endale et al. 2014).

In the current study, bacterial adhesion and internalization was significantly reduced in the RGSF-A-treated cells compared with controls, suggesting that RGSF-A inhibit *Brucella*—host cell interaction. A previous study has revealed that anti-adhesive activity against different microorganisms such as *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, *Propionibacterium acnes* and *Staphylococcus aureus* was demonstrated by pectin-type polysaccharides from *Panax ginseng* (Lee et al. 2009). In addition, bacterial load and lung pathology in chronic pneumonia caused by *Pseudomonas aeruginosa* in rats were significantly

Influence of RGSF-A on the phagocytic signals and intracellular pathways

Phosphorylation levels of ERK1/2 and p38α in the RGSF-A-treated cells were significantly reduced 30 min after infection compared with those in the infected control cells. However, no difference was observed in the phosphorylation levels of JNK between the treated and control cells. After RGSF-A treatment, ERK1/2 and p38α were reduced by 48.80% and 22.77%, respectively (Fig. 5). These findings indicated the inhibitory effect of RGSF-A on *B. abortus* infection, which acted by reducing the activation of mitogen-activated protein kinases (MAPKs). In addition, colocalization of LAMP-1 with BCPs in RGSF-A-treated cells was increased 1.77-fold after 2 h of incubation in comparison with untreated control cells (∗P < 0.05) (Fig. 6).
Figure 3. F-actin polymerization and bacterial colocalization. Shown in white arrows are the formation of filopodial structures and microspikes which are significantly reduced with infected FITC-conjugated \( B.\) \textit{abortus} RGSF-A-treated cells compared with the control. Lesser number of internalized (yellow arrows) and adherent bacteria (blue arrows) are also apparent with RGSF-A-treated cells. The results are representative of three separate experiments (Scale bars \( = 5 \mu m\)).

Figure 4. Effect of RGSF-A on the phagocytosis of \( B.\) \textit{abortus} by F-actin polymerization modulation and analyzed using FACS. RAW 264.7 cells were infected with \( B.\) \textit{abortus}, fixed and stained with 0.1 \( \mu M \) rhodamine-phalloidin for 30 min at 22\( ^\circ \)C. Statistically significant differences relative to untreated control cells are indicated by asterisks (*) \( P < 0.05 \); \( ** P < 0.01 \).

Figure 5. Effect of RGSF-A on the activation of intracellular signaling pathways required for the phagocytosis of \( B.\) \textit{abortus}. RAW 264.7 cells were incubated with RGSF-A (10 \( \mu \)g ml\(^{-1}\)), infected for 30 min and lysed for 30 min at 4\( ^\circ \)C. Samples were separated by SDS-PAGE, electrically transferred onto Immobilon-P membranes, blocked and incubated with phospho-specific antibodies against ERK1/2 (Thr183/Tyr185) and p38\( \alpha \) (Thr180/Thr182) overnight at 4\( ^\circ \)C. Immunoblot analysis of total RAW 264.7 cell lysates pretreated with RGSF-A was performed with phospho-specific ERK1/2 and p38\( \alpha \) antibodies at the indicated times.

Reduced upon subcutaneous treatment with ginseng (Song et al. 1997). Thus, our results suggest that RGSF-A as classed under ginsenoside, a major constituent of Korean red ginseng could have its effect on the inhibition of \( Brucella \) infection.

A study by Lee et al. (1997) conducted a receptor-binding assay that demonstrated that Rg1 from \( P.\) \textit{ginseng} was a functional ligand of glucocorticoid receptor (GR). Therefore, it may cause receptor signaling and induce the regulation of the transcription of target genes containing specific DNA sequences termed glucocorticoid response elements (Dong et al. 1990). A critical step in the initiation of early immune responses is the activation of the MAPK signaling cascade and transcriptional reprogramming that is prompted in plants and animals by microbial-associated molecular patterns such as bacterial lipopolysaccharide, peptidoglycan and flagellins (Dong, Davis and Flavell 2002).

In this study, RGSF-A resulted in the downregulation of MAPKs and inhibited bacterial penetration by restricting F-actin polymerization. The inhibitory effect of RGSF-A on the invasion of \( B.\) \textit{abortus} into host macrophage can be attributed to the downregulation of MAPKs (ERK1/2 and p38\( \alpha \)). Additionally, it was demonstrated that p38 MAPK is selectively glucocorticoid sensitive due to the activation of MPK-1, which inhibits p38 MAPK by GR (Bhattacharyya et al. 2007). In this study, we have demonstrated that RGSF-A affects the intracellular trafficking ability of \( B.\) \textit{abortus} by promoting its colocalization with the late endosome marker LAMP-1. Lysosome-associated membrane proteins are transmembrane proteins that are characterized to have huge glycosylated luminal domain and short cytosolic tail which are
delivered to the phagosomes during the phagosome maturation process that eventually culminates in the fusion of phagosomes with lysosomes. This fusion creates an organelle that acquires degradative and antimicrobial components such as reactive oxygen species and hydrolytic enzymes that are essential elements for the killing of internalized microorganisms [Huynh et al. 2007].

In this study, inhibition of intracellular replication is significantly evident up to 24 h of incubation; however beyond 24 h, there was a rapid increase in the intracellular growth. In the phagosome–lysosome fusion analysis, the fusion rate at 2 h post-infection was increased but was decreased at 24 h post-infection by RGSF-A treatment. These results suggest that RGSF-A affects the early phagosome–lysosome fusion in Brucella infection within macrophage. The observed inhibition of intracellular replication clearly implies that saponins may affect the innate immune system and particularly suggests that immune modulation occurs in the presence of RGSF-A. However, this requires further evaluation given that little is known about the function of the other components of RGSF-A.

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**Conflict of interest.** None declared.

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