Short Communication

Comparative Study of Gamma Interferon Production in Mice Immunized with Outer Membrane Proteins and Whole Bacteria of *Brucella abortus*

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**SUMMARY:** *Brucella abortus* is the intracellular bacterium that causes bovine brucellosis and a chronic human disease known as undulant fever. Interferon (IFN)-γ plays critical roles in defending against intracellular bacterial infection. In this experiment, we demonstrated the difference in IFN-γ production between the splenocytes of mice inoculated with outer membrane proteins (OMPs) of *B. abortus* and whole live bacteria. Our results showed that the OMP-inoculated group showed more IFN-γ production than did the bacteria-infected group, suggesting that OMPs are candidates for the induction of immune response.

Brucellosis is a zoonosis caused by bacteria of the genus *Brucella*. These bacteria are non-motile, non-spore-forming small Gram-negative rods. Facultative intracellular organisms that are very difficult to isolate, they have a long latent period that makes early diagnosis impossible, once a host is infected. These microbes are able to grow in phagocytes, where they are inaccessible to bacteriocidal agents, including circulating antibodies, and are very different from the mechanisms of defense against extracellular bacteria (1). Most widely used serological diagnostic methods are based on lipopolysaccharide (LPS), whole cell preparation and cell sonication extracts. LPS of the smooth *Brucella abortus* strains induces especially strong antigenic responses (2); however, O-polysaccharide of LPS from *B. abortus* can cause a cross reaction with *Verronia enterococcolita* O:9, *Salmonella urbana* group, *Vibrio cholerae*, *Francisella tularensis*, *Escherichia coli* O157, and *Stenotrophomonas maltophilia* (3,4). In addition, diagnostic sensitivity could be low due to anti-LPS antibodies, which may persist long after acute brucellosis (5). For these reasons, many researchers have tried to develop a new protective antigen with LPS free proteins (6,7). As protein expression profiles could be changed through endemic environments or growth conditions, the authors of several ongoing studies are making use of local field isolates (8-10).

In light of this, this study was performed to evaluate the interferon (IFN)-γ production from mice inoculated with outer membrane proteins (OMPs) of *B. abortus* or whole live bacteria.

Four field isolates of *B. abortus* (1-7, 1-81, 2-55, and 3-60), *B. abortus* 1119-3, and *B. abortus* RB51 were obtained from the National Veterinary Research & Quarantine Service, Korea, and cultured for 48 h at 37°C in Brucella broth (Difco, Detroit, Mich., USA). OMPs were extracted, and antigenicity was measured by the method described previously (11,12). For immunization and infection, ICR mice (ORIENT BIO Inc., Seongnam, Korea) were used. One group of 21 mice was immunized with 20 μg/100 μl of OMPs by the intraperitoneal route, a second group of 21 mice was inoculated with 2 × 10⁷ CFU/200 μl of bacteria (2 × 10⁷ CFU/200 μl for *B. abortus* RB51) by the same route, and a control group of 21 mice was left untreated. Three mice of each group were sacrificed on the first day and second day and then once weekly for 5 weeks. Blood was collected, spleens were aseptically removed and homogenized, and splenocytes were obtained by mincing the homogenates on a metal mesh. Erythrocytes were lysed with RBC lysis buffer (0.17 M NH₄Cl) and washed twice with Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, Calif., USA), and then 4 × 10⁶ cells/ml of splenocytes were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen) at 37°C under the atmosphere of 5% CO₂. To evaluate the IFN-γ production in splenocytes, we stimulated each group of splenocytes once with OMPs from the same strain or bacteria inoculated into the mice, at the concentration of 1 μg/ml (OMPs) and 1 × 10⁴ CFU/ml (bacteria), respectively. Supernatants were taken at 48 h post inoculation. Concentration of IFN-γ was measured with a Mouse IFN-γ ELISA kit (eBioscience, San Diego, Calif., USA) according to the manufacturer’s protocol. The standard curve was prepared by serial dilutions of recombinant mouse IFN-γ (2,000 pg/ml).

Statistical significance was determined by the Student *t* test using SPSS software (version 17.0). Differences were considered to be significant if a *P* value of <0.05 was obtained.

OMP profiles of the *B. abortus* strains showed different protein expression patterns from each other, and antigenic reactivity with brucellosis-positive serum also showed different patterns among the strains (12). The kinetics of IFN-γ production of each of the groups is shown in Fig. 1. Most strains showed the highest IFN-γ concentration at 1 day post inoculation in OMP-stimulated mice, and the concentration was significantly high compared with that in the bacteria-inoculated mice, except for the 1-81-inoculated mice, which showed a peak concentration at 4 week post inoculation. At 2 days post inoculation, IFN-γ production was very poor in all groups, and only the 1119-3 and 2-55 OMP-stimulated mice showed significant production. Interestingly, in every strain
except 1119-3, bacteria-inoculated mice showed their peak concentration at 2 weeks post inoculation. This result correlates with the IFN-\(\gamma\) concentration in serum shown in Fig. 2, which started to increase at this point, indicating the strongest cellular immune responses against the bacterial infection.

From 1 week to 5 weeks post inoculation, except for 2 weeks and 4 weeks post inoculation in the 1119-3, 1-7, and 3-60 groups, the OMP-stimulated group showed more IFN-\(\gamma\) production than did the bacteria-inoculated group, indicating that OMPs are more effective for producing IFN-\(\gamma\). In both groups, there were no significant differences among the strains. Also there was no significant IFN-\(\gamma\) production in the negative control group.

For immunity and protection against \textit{B. abortus}, a host utilizes its innate and adaptive immune responses (13). In innate immune response, natural killer (NK) cells play a critical role by secreting IFN-\(\gamma\), although NK cells do not play a role in innate immune responses against \textit{B. abortus} infection in mice (14). In adaptive immune response, especially T cell-mediated immune response for intracellular bacteria, IFN-\(\gamma\) plays an important role in activating macrophages and in limiting \textit{Brucella} infection both in vitro and in vivo (15). Because of the vital role of IFN-\(\gamma\) in the infection of intracellular bacteria, it is used in the diagnosis of intracellular bacterial infections such as tuberculosis (16).

There is no previous report on polymorphism of \textit{B. abortus} field isolates, except one on the polymorphism of \textit{B. ovis} field isolates (8). Thus, this experiment was performed to analyze the IFN-\(\gamma\) production by spleen cells of mouse immunized by OMPs or bacteria. Our study results showed that IFN-\(\gamma\) production from splenocytes stimulated with OMP of each strain did not differ significantly among strains. This may have been due to the fact that the field isolates used in our experiment showed differences only in the minor OMPs and the amount of expression. Moreover, the OMP-inoculated mice showed higher production of IFN-\(\gamma\) compared to the bacteria-inoculated mice, indicating that OMPs are more effective than bacteria for inducing IFN-\(\gamma\)-mediated immunity. In related research, subcellular fractions such as inner membrane protein and OMP of \textit{B. ovis} induced cytokine production and a delayed type hypersensitivity (17).

In summary, our experiment provides basic data for the

\[Fig. 1. \text{ Induction of interferon (IFN)-}\gamma\text{ in mouse splenocytes stimulated with outer membrane protein (OMP) or bacteria after 48 h. Dpi, day post inoculation; Wpi, week post inoculation. Significantly different from other antigen stimulated group and control (*}\(P < 0.05\), **\(P < 0.01\)).\]
development of an immunodiagnostic method for detecting brucellosis by measuring IFN-γ. Likewise, OMP could be a useful antigen for the eventual development of a vaccine based on the induction of cellular immune responses.

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