Original Article

Characteristics of the immune response during acute brucellosis in Sprague-Dawley rats

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Abstract

Background: *Brucella* is a facultative, intracellular pathogen that causes severe disease in animals and humans. Immunity against *Brucella* involves both humoral and cellular responses. To investigate the characteristics of immune response in acute brucellosis in Sprague-Dawley (SD) rats, IgG and its subclass specific immunoglobulins' (IgG1 and IgG2a) response in sera against *B. abortus* biotype 1 infection were studied.

Methodology: Thirty-six rats were inoculated intraperitoneally with 0.1 ml apyrogenic saline containing 1×10^{10} colony forming unit (CFU) of *B. abortus* biotype 1 Korean bovine isolate. Four rats were used as uninfected controls. The sera were collected from infected rats at 3, 7, 14, 21, 28, 35, 42, 49, and 56 days post infection (DPI) and screened for *Brucella* specific antibody response by the rose bengal plate test (RBPT). IgG and its subclass specific immunoglobulins' (IgG1 and IgG2a) response in the sera were measured by a lipopolysaccharide (LPS) based indirect enzyme-linked immunosorbent assay (IELISA).

Results: *Brucella* specific IgG, IgG1 and IgG2a responses in the sera of infected rats were detected from 3 DPI by IELISA**.** IgG and IgG1 concentrations in sera reached the peak level at 35 DPI, and then the concentrations gradually declined to the end of the experiment. IgG2a concentrations in the sera remained almost constant from 7 DPI until the end of this study.

Conclusion: In acute brucellosis, IgG2a response (indicative of a Th1 response) was found to be significantly dominant over IgG1 response (indicative of Th2 response) ($P < 0.001$).

Key Words: immune response, Sprague-Dawley rat, *B. abortus* biotype 1, ELISA, immunoglobulin isotypes.

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Introduction

B. abortus, a gram negative facultative intracellular bacterium, is the etiological agent of an economically important zoonotic disease called brucellosis that affects humans and animals. In humans, undulant fever, chills, sweating, anorexia, fatigue, weight loss, depression, arthralgia, and myalgia are the common clinical symptoms of brucellosis [1]. Brucellosis causes abortion and infertility in domesticated animals [2], resulting in economic losses. Humans are generally infected through direct contact with infected animals or by the consumption of contaminated food, especially unpasteurized milk and milk products [3].

Brucellosis remains endemic in many countries where it undermines animal health and productivity [4]. Bovine brucellosis has emerged as a serious animal and public health issue in Korea [5,6]. Infection in both cattle and people in Korea is commonly due to *B. abortus* biotype 1 [6,7]. Host resistance to *Brucella* spp. is not completely understood but cell-mediated immunity (CMI) seems to play a major role in immune response against virulent *Brucella* infection [8]. Antibody response to *B. abortus* is directed against lipopolysaccharide (LPS) molecules [9].

Immunity against *B. abortus* involves antigen-specific T-cell activation, CD4+ and CD8+ T cells, and humoral responses [8]. The main stimulation of immune response occurring through $CD4^+$ T-helper (Th) lymphocytes is subdivided into Th1 and Th2 responses [10]. The Th1 response stimulates IgG2a production and Th2 response stimulates the production of IgG1 [10]. IgG2a is mostly involved in protection against intracellular pathogens through CMI. IgG1 is mainly responsible for protection against extracellular pathogens through humoral immune response [11]. *Brucella* antigens induce the production of T helper lymphocytes type 1 (Th1) and

adequate Th1 immune response is critical for the clearance of *Brucella* infection [12].

Free-ranging wildlife is the most likely source of transmission of brucellosis to humans and domesticated animals [13] since *Brucella* were isolated from a wide range of wild animals [14]. Rodents, in particular, have received much attention with regard to the epizootiology of brucellosis [15]. Rats are known to harbor *Brucella* in many parts of the world [16] and are found to be infected with *B. abortus* on farms where cattle are infected [14]. Protective immunity against *Brucella* infection has been studied mainly in the mouse model [17]; however, protective immune responses against *B. abortus* have not been studied in rats. In the present study, IgG and its isotypes' (IgG1 and IgG2a) specific immune responses during the acute stage of brucellosis in the SD rat model have been measured by an IELISA using the LPS of *B. abortus* biotype 1.

Materials and Methods

Rats

Adult SD rats (n = 40), weighing 200 to 250 g at eight weeks old, were purchased from a credible specific pathogen free (SPF) laboratory animal company (Koatech, Pyungtaek City, Gyeonggido 451-864, Korea). The rats were housed in a stringently hygienic, climate-controlled environment, and were supplied with commercial feed and water *ad libitum*. All experiments were conducted in compliance with the humane protocols approved by the Chonbuk National University, Jeonju, Republic of Korea.

Bacterial strain

B. abortus biotype 1 Korean bovine isolate was used for the experimental infection. *B. abortus* biotype I lyophilized stock culture was obtained from the laboratory repository. *Brucella* was inoculated into the brucella agar media (Difco, Kansas City, Missouri, USA) and incubated at 37°C for seven days under 5% CO_2. The grown bacteria were harvested in normal saline.

Inoculation into rats

Thirty-six SD rats were inoculated intraperitoneally with 0.1 ml sterile injectable, pyrogen-free solution containing 1×10^{10} CFU/ml of *B. abortus* biotype 1. Four rats were used as uninfected controls.

Clinical examinations

All of the infected rats were examined daily for food and water intake, and rectal temperature was recorded within 72 hours of inoculation by a digital thermometer (Microlife, Switzerland).

Collection of sera

Blood samples were collected from the thirty-six infected and four uninfected control rats throughout the experiment. Additionally, samples were collected from four randomly selected rats out of the 36 infected rats at each time point of infection (specifically, 3, 7, 14, 21, 28, 35, 42, 49 and 56 DPI) through aseptic cardiac puncture under general anesthesia induced by intraperitoneal administration of 10 mg/kg of Tiletamine and Zolazepam (Zoletil 50, Virbac Laboratories-06515, Carros, France). Blood samples were also collected from the four uninfected control rats at 0 DPI. Sera were collected and stored at -20°C until tested. Immediately after bleeding, the rats were euthanized.

Serological test

Sera were screened for detection of anti-*B. abortus* antibodies by the RBPT using *B. abortus* 1119-3 whole cell antigen according to the methods described by Alton *et al*. [18].

Measurement of IgG, IgG1 and IgG2a concentrations

Concentrations of IgG, IgG1 and IgG2a in the sera were measured by the LPS-based IELISA [19]. Briefly, flat-bottomed 96-well polystyrine microtiterplates (Nunc, Denmark) were coated with 100 µl of LPS (5 µg/ml) of *B. abortus* biotype 1 suspended in 0.05 mM sodium bicarbonate buffer (p^H 9.6). Affinity purified rat IgG (Bethyl Laboratories, Inc, USA), rat IgG1 (Bethyl Laboratories, Inc, USA) and rat IgG2a (Bethyl Laboratories, Inc, USA) were used to coat the 96-well plate starting from 500 ng/well to 7.8 ng/well for generation of standard curve, respectively. Each plate was incubated at 37ºC for one hour. Plates were washed three times with wash solution (PBST: PBS, p^H 7.4) with 0.05% (v/v) Tween 20. Each well of the antigen-coated plates were blocked with 200 µl of blocking solution of 1% (w/v) bovine serum albumin (Sigma Aldrich Inc., St. Louis, Missouri, USA) in PBS and incubated at 37ºC for 30 minutes. After three washes with PBST, 100 µl of control and test sera samples diluted 1:100 in sample diluent (50 mM tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20, p^H 8.0) were added to each well in duplicate. The plates were sealed and incubated at

37ºC for one hour. After five washing cycles with PBST, each well was incubated with 100 µl of 1:100,000 dilution of goat anti-rat IgG, IgG1, and IgG2a antibodies conjugated to horseradish peroxidase (Bethyl Laboratories Inc, USA) diluted in conjugate diluent (50 mM tris, 0.14 M Nacl, 1% BSA, 0.05% Tween 20, p^H 8.0), and the plates were incubated at 37ºC for one hour. After five washings as described above, the color reaction was developed by adding 200 µl/well of a solution containing 1.0 mg/ml of O-phenylenediamine dihydrochloride (OPD; Sigma, St. Louis, Missouri, USA) in 0.05 M citrate buffer (p^H 4.0) with 0.04% (v/v) H_2O_2. The plates were incubated in the dark for 30 minutes at room temperature. The colorimetric reaction was stopped by the addition of 50 µl/well of 3 M H_2SO_4. The absorbance measurements were made at 492 nm, using an automatic ELISA reader (Tecan, Austria) and Magellan software program 1.6.

Statistical analysis

IgG1 and IgG2a responses in infected rats at different DPI were analysed for statistical significance by Student's *t* test. A *P* value of < 0.05 was considered significant.

Results

Clinical findings

All rats inoculated with *B. abortus* biotype 1 developed lethargic, anorectic, and febrile conditions within 24 hours. The highest mean rectal temperature of inoculated rats was 38.30 ± 0.152°C within 72 hours.

Serological response

Sera collected from control rats as well as infected rats at 3 DPI were found negative to *Brucella* by RBPT. Sera samples of rats collected at day 7, 14, 21, 28, 35, 42, 49, and 56 DPI were tested positive to *Brucella* by the RBPT.

Production of IgG, IgG1 and IgG2a

Serum IgG, IgG1 and IgG2a responses measured by IELISA at 0 DPI were considered as non-specific to *Brucella*. *Brucella* specific immunoglobulins response (IgG, IgG1 and IgG2a) were detected in sera at 3 DPI by IELISA. The IgG concentration in sera of infected rats reached its peak level at 35 DPI and then gradually decreased until the end of the study. The IgG concentrations in sera at 0, 3, 7, 14, 21, 28, 35, 42, 49 and 56 DPI were 15.03 ± 8.53, 28.86 ± 6.49, 302.95 ± 46.74, 345.82 ± 34.17, 469.16 ± 23.80, 688.93 ± 52.48, 1033.43 ± 96.15, 774.43 ± 92.30, 506.63 ± 43.93 and 411.20 ± 63.76 ng/ml, respectively. The results of IgG concentration measured by IELISA are presented in Table 1.

To further evaluate the immune responses, we determined the magnitude and Th-specificity of antibodies generated against S-LPS of *B. abortus* biotype 1. Serum IgG1 indicative of Th2 response increased until 35 DPI, and then the titers gradually declined up to the end of the experiment. The IgG1 concentrations in sera at day 0, 3, 7, 14, 21, 28, 35, 42, 49, and 56 DPI were 0.67 ± 0.02, 1.67 ± 0.08, 3.62 ± 0.1, 4.73 ± 0.18, 6.50 ± 0.23, 10.79 ± 0.35, 13 ± 0.41, 10.86 ± 0.24, 10.69 ± 0.14 and 9.20 ± 0.21 ng/ml, respectively (Figure 1).

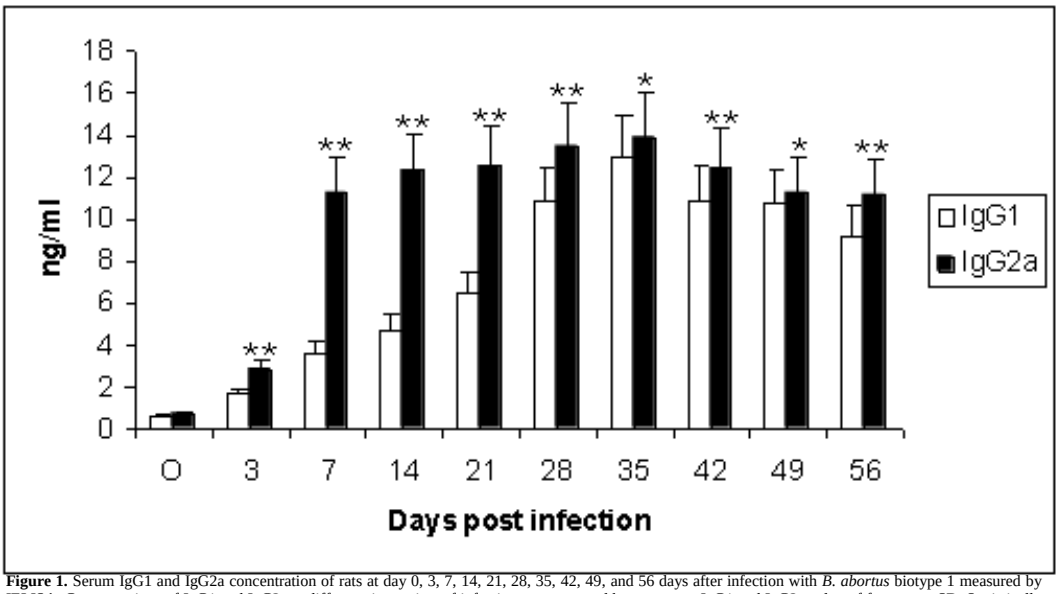

Figure 1. Serum IgG1 and IgG2a concentration of rats at day 0, 3, 7, 14, 21, 28, 35, 42, 49, and 56 days after infection with *B. abortus* biotype 1 measured by IELISA. Concentrations of IgG1 and IgG2a at different time points of infection are expressed here as mean IgG1 and IgG2a value of four rats ± SD. Statistically significant difference between serum IgG1 and IgG2a concentrations in rats at different time points of infection are indicated by asterisks (*, $P < 0.01$ and **, $P < 0.001$)

Table 1. Serum IgG concentrations of rats at 0, 3, 7, 14, 21, 28, 35, 42, 49 and 56 days after infection with *B. abortus* biotype 1 measured by IELISA

Days after infection	Rat no.	Serum IgG concentrations (ng/ml)
0	1	11.27
	2	6.24
	3	16.29
	4	26.35
	Mean ± SD*	15.03±8.53
3	1	21.32
	2	31.38
	3	26.35
	4	36.41
	Mean ± SD	28.86±6.49
7	1	328.10
	2	247.63
	3	283.34
	4	352.74
	Mean ± SD	302.95±46.74
14	1	363.30
	2	307.98
	3	383.92
	4	328.10
	Mean ± SD	345.82±34.17
21	1	484.00
	2	434.21
	3	484.50
	4	473.94
	Mean±SD	469.16±23.80
28	1	685.17
	2	634.87
	3	675.11
	4	760.60
	Mean ± SD	688.93±52.48
35	1	1072.41
	2	1012.06
	3	911.47
	4	1137.78
	Mean ± SD	1033.43±96.15
42	1	740.49
	2	660.02
	3	836.04
	4	861.18
	Mean ± SD	774.43±92.30
49	1	509.15
	2	443.77
	3	539.32
	4	534.29
	Mean ± SD	506.63±43.93
56	1	433.71
	2	393.98
	3	333.13
	4	484.00
	Mean ± SD	411.20±63.76

*Serum IgG concentrations are presented here as mean IgG value of four rats ±SD.

IgG2a indicative of Th1 response was significantly dominant throughout the course of acute infection as compared to Th2 response ($P < 0.001$). The IgG2a antibodies rose significantly from 7 DPI and the response remain almost constant until the end of this study. The IgG2a concentrations in sera at day 0, 3, 7, 14, 21, 28, 35, 42, 49 and 56 DPI were 0.72 ± 0, 2.84 ± 0.08, 11.28 ± 0.2, 12.3 ± 0.22, 12.53 ± 0.18, 13.51 ± 0.39, 13.94 ± 0.21, 12.47 ± 0.27, 11.26 ± 0.2 and 11.14 ± 0.26 ng/ml, respectively (Figure 1).

Discussion

Immunity against *B. abortus* involves both Th1 and Th2 specific immune response. Th1 responses are characterized by cellular immunity and production of IgG2a antibodies, and Th2 responses are characterized by humoral immunity, specifically the production of IgG1 [20]. In this study, experimentally infected rats mounted humoral immune response at 7 DPI in RBPT and 3 DPI in IELISA. Similar results were found by Beh [21], who reported humoral immune response after one week post-infection. The highest recorded humoral immunity measured by IELISA was 35 DPI before the antibody titers gradually decreased until the end of the experiment.

Serological methods have been widely used in evaluating the humoral response [22]. In our study, we evaluated humoral immune response by RBPT and IELISA since these are frequently used confirmatory serological tests for *Brucella* [23,24]. ELISA is very sensitive, highly specific, and detects all the isotypes of IgG in serum [24]. In this study, we evaluated antibody response by an LPS-based IELISA focusing on IgG and its subclasses, such as IgG1 and IgG2a, during the course of acute infection. *B. abortus* infection induces the production of IgG1 and IgG2a, antibody isotypes detectable in both milk and sera of cattle [24]. IgG1 is consistently produced at high levels in *Brucella*-exposed cattle sera [25]. In the current study, the highest IgG1 responses were observed at 35 to 42 DPI after which they declined. Nielsen and Duncan [26] stated that IgG1 antibodies in S19 vaccinated cattle reached peak values at 28 to 42 days, after which they declined and the same general pattern also occurred in experimental infection with virulent strains. There were more Th1 dominant (IgG2a) responses recorded throughout the course of infection in our experiment. The highest IgG2a responses were recorded at 21 to 35 DPI. Similar results were also reported by High *et al*. [27], who also observed the highest IgG2a

response in mice at 21 and 35 days after *B. abortus* infection.

In our study, the presence of high IgG2a and low IgG1 subtype antibodies to the O antigen indicated the induction of Th1 type of immune response during the acute stage of infection. Similar results were also observed by Stevens *et al*. [28], who recorded dominant IgG2a response as compared to IgG1 response after infection with *B. abortus* in the mouse model. IgG2a are preferentially generated in humoral responses against intracellular microorganisms [29]. *Brucella* infection results in Type 1 (Th1) cellular immune response that promotes a clearance of the bacterial organism [8,30]. Th1-type antibody isotypes, such as IgG2a, may also opsonize the pathogen to facilitate phagocytosis [31]. During the course of an infection, *B. abortus* is mainly cell associated; thus infected cells need to kill the bacterium or be killed so that *B. abortus* can be accessed by other mechanisms for clearance, such as those mediated by IgG2a antibodies [8]. Presumably these antibodies have greater facility than other isotypes to recognize microbial antigens on the surface of infected cells. Our study demonstrated that IgG2a response is more prominent in acute *Brucella* infection when compared to IgG1 response.

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Conflict of interest: No conflict of interest is declared.