Original Article

Comparative Analysis of Immune Responses to Outer Membrane Antigens OMP10, OMP19, and OMP28 of *Brucella abortus*

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SUMMARY: *Brucella* infection is accompanied by cytokine production, which serves as an important factor to evaluate the innate and adaptive immune responses. Several researchers have been investigating the mechanisms involved in *Brucella* infection in the host. Here, we conducted an analytical study to define pathogenic pathways and immune mechanisms involved in *Brucella* infection by investigating the antigenic efficacy of recombinant outer membrane protein 10 (rOMP10), outer membrane protein 19 (rOMP19), and outer membrane protein 28 (rOMP28) in vitro and in vivo upon stimulation/immunization. Cytokine production was analyzed by nitric oxide (NO) assay and enzyme-linked immunosorbent assay (ELISA) after stimulation of RAW 264.7 cells and naive splenocytes with the recombinant proteins. Our results show that levels of NO, tumor necrosis factor (TNF)- α , and interleukin (IL)-6 increased in RAW 264.7 cells in a time-dependent manner following recombinant protein stimulation. In contrast, levels of interferon (IFN)- γ and IL-2 increased in naive splenocytes after stimulation with proteins. ELISA and ELISpot assays were performed after immunization of mice with recombinant proteins. rOMP28 greatly increased IFN- γ , IL-2, and TNF- α levels than IL-4 and IL-6 levels in vitro. Of the recombinant proteins, rOMP19 elicited a mixed Th1/Th2 immune response by increasing the number of IgG-secreting cells in vivo.

INTRODUCTION

Brucella is a gram-negative bacterium and causative agent of a serious zoonotic disease, brucellosis. The genus *Brucella* has been classified according to its primary preferred host and antigenic variations (1–4). *Brucella* infections induce a variety of clinical symptoms in both animals and humans (5).

Brucella spp. are able to easily establish chronic infections in the host cell by avoiding lysosomal degradation and maintaining proliferation within macrophages (6,7). Lipopolysaccharide (LPS) is the most important virulence factor that activates the innate immune system; *Brucella* strains are deficient in LPS and, hence, have lesser virulence and stronger ability to survive. However, LPS-based serological tests may give false-positive results, owing to the similarity between *Brucella* and gram-negative bacteria, such as *Yersinia enterocolitica* O:9 (8), *Escherichia coli* (9), and *Salmonella urbana* (10). Therefore, several studies have aimed to investigate other factors, such as outer membrane proteins (OMPs) and their potential applications as diagnostic markers and vaccines against brucellosis (11–13). Recent studies have focused on various OMPs, such as OMP28, OMP2b, OMP10, OMP16, and OMP19 (14–16). Of these, OMP28 displays significant potential as a diagnostic candidate that may trigger protective effects in vivo and show high efficiency in sero-

logical tests against brucellosis (17–20). Attempts have been made to clone these OMPs and other proteins to investigate if their immunogenicities may allow LPS-free diagnosis following infection with *Brucella* spp.

Immune responses in the host are caused following pathogenic infection, which triggers the production of proinflammatory cytokines, such as interleukin (IL)-1 β , IL-4, IL-6, IL-12, interferon (IFN)- γ , and tumor necrosis factor (TNF)- α . The production of these cytokines results in the disturbance of immune responses in the host, as these molecules participate in cellular and/or humoral immune responses (21,22). Therefore, the analysis of the cytokine production may explain the correlation between the invasion and localized inflammation by *B. abortus* infection. Many researchers have reported Th1 (cellular) and Th2 (humoral) immune responses following infection (22,23). Although several immunogenic antigens have been investigated, only a few immune mechanisms have been identified in brucellosis.

In this study, 3 recombinant OMPs, rOMP10, rOMP19, and rOMP28, were selected to study the pathogenic pathways and evaluate their immunogenicities in hosts through the investigation of immune responses elicited in vitro and in vivo tests.

MATERIALS AND METHODS

Construction of OMP10, OMP19, and OMP28 expression clones: Total genomic DNA was prepared from *B. abortus* 544 (ATCC 23448) culture using a G-spinTM Genomic DNA extraction kit for bacteria (Intron, Gyeonggi-do, Korea). Genes encoding OMP10, OMP19, and OMP28 of *B. abortus* were amplified by polymerase chain reaction (PCR) with following primer pairs: OMP10 sense 5'-AGCAGAATTCATGAAACGCTTCCGCA-3',

Received January 17, 2017. Accepted December 22, 2017.

J-STAGE Advance Publication April 27, 2018.

DOI: 10.7883/yoken.JJID.2017.019

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OMP10 anti-sense 5'-ATTACTGCAGTCAGCCGGCGTT GC-3', OMP19 sense 5'-AGCAGGATCCATGGGAATTT CAAAAGCAAG-3', OMP19 anti-sense 5'-ATACTGCAG TCAGCGCGACAGCG-3', OMP28 sense 5'-GATCGGAT CCAACACTCGTGCTAGCAATTTT-3', and OMP28 anti-sense 5'-GATCAAGCTTTTACTTGATTTCAAAAACGA C-3'. The amplified DNA samples were digested with appropriate restriction enzymes and ligated into pCold Trigger factor (TF) vector (Takara, Shiga, Japan), wherein the recombinant proteins were expressed as fusion proteins to TF agent.

Expression and purification of rOMP10, rOMP19, and rOMP28 proteins: The expression and purification of recombinant proteins were performed as previously described (18) with a few modifications. Briefly, *E. coli* containing the fusion plasmid were cultured by inoculating 20 mL bacteria into 1 L ampicillin-containing Luria Bertani broth and incubated at 37°C for 7 h. Isopropyl β -D-1-thiogalactopyranoside (IPTG, Amresco, Radnor, PA, USA) was added at a final concentration of 0.3 mM and the culture incubated at 37°C for additional 2 h to induce protein synthesis. Bacterial cells were subsequently harvested by centrifugation at $4,400 \times g$ for 20 min. The supernatant was discarded, and the pellet was resuspended in 40 mL column buffer (20 mM Tris HCl, 8 M urea, 500 mM sodium chloride [NaCl], 20 mM imidazole, and 1 mM β -mercaptoethanol; pH 8.0). The samples were sonicated at 10,000 Hz in an ice-water bath and centrifuged at $4,400 \times g$ for 20 min to collect the supernatant. The supernatant was loaded onto a His SpinTrap (GE Healthcare, Buckinghamshire, UK) column according to the manufacturer's instructions.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis: The purified recombinant proteins were diluted with a sample buffer and boiled for 10 min at 100°C. After electrophoresis, samples were visualized by staining with Coomassie Brilliant Blue R-250 (Intron). Proteins resolved by SDS-PAGE were transferred onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA) for 25 min using an iBlot® transfer device (Invitrogen). Next, membranes were blocked by incubation in 5% skim milk (BD, Franklin Lakes, NJ, USA) for 1 h at room temperature (RT), washed 3 times with TBS, and incubated with anti-histidine antibody (1:2,000 dilution, AprilBio Co., Ltd, Seoul, Korea) for 3 h at RT. The membranes were washed 3 times (10 min each) and incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse IgG antibody (1:2,000 dilution, Bethyl Laboratories, Montgomery, TX, USA,) for 1 h at RT. After the final wash, proteins were visualized with an AP conjugate substrate kit (Bio-Rad, Hercules, CA, USA).

Production of NO and cytokines in RAW 264.7 cells: After incubating RAW 264.7 cells for 8 h in 12-well plates at a seeding density of 1×10^6 cells/mL, cells were stimulated with 10 μ g/mL rOMP10, rOMP19, or rOMP28. Contamination by LPS in recombinant proteins was inhibited by treatment with polymyxin B (10 μ g/mL) for 30 min before stimulation of macrophages. The culture supernatants were collected at 4, 8, and 24 h after stimulation. The levels of TNF- α , IL-6, IL-1 β , IL-12p70, and IFN- γ were measured using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction (eBioscience Inc., San Diego, CA, USA). The

level of NO was determined by measuring the nitrite accumulation based on Griess reagent system.

Production of cytokines in naive splenocytes of mice: For IFN- γ , IL-2, IL-4, and IL-5 assays, splenocytes (1×10^6 cells/mL) were isolated from healthy female BALB/c mice (5-week old, Orient-Bio, Gyeonggi-do, Korea) and stimulated with 10 μ g/mL rOMP10, rOMP19, or rOMP28. After 24 h, the level of IFN- γ , IL-2, IL-4, and IL-5 in culture supernatants was measured by ELISA, according to the manufacturer's instructions (eBioscience Inc.). LPS (Sigma, St. Louis, Mo, USA) and media were used as a positive and negative control, respectively. All cells were pretreated with polymyxin B to avoid the activity of LPS contamination. The care and handling of animals was performed with the approval of the Seoul National University Institutional Animal Care and Use Committee (IACUC), and the approval number was SNU-150302-2-1.

Production of immunoglobulins and antigen (OMPs)-specific antibodies in mice immunized with recombinant proteins: We immunized 6-week-old BALB/c mice with an intraperitoneal injection of 30 μ g purified recombinant proteins, rOMP10, rOMP19, or rOMP28 mixed with complete Freund's Adjuvant (CFA, Sigma) on day 0 and incomplete Freund's Adjuvant (IFA, Sigma) on day 14. Five mice were immunized with recombinant proteins for each group. Sera for antibody response detection were obtained on 3, 7, 14, and 28 days after the first immunization. Briefly, 96-well microplates were coated by incubating purified recombinant proteins (5 ng/well) in a coating buffer (14.2 mM sodium carbonate [Na₂CO₃], 34.9 mM sodium bicarbonate [NaHCO₃], and 3.1 mM sodium azide [NaN₃], pH 9.6) overnight at 4°C for ELISA. The plates were blocked with 1% bovine serum albumin (BSA; Sigma) in a solution of phosphate-buffered saline (PBS) containing 0.1% Triton X-100 (PBST) for 2 h at 37°C. After washing with PBST, 1/200 diluted serum sample was added to the wells and incubated for 1 h at 37°C. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgG1, IgG2a (1:2,000, Bio-Rad), and IgM (1:3,000, Jackson Immuno Research, West Grove, PA, USA) diluted in PBS containing 1% BSA were used to detect IgG, IgG1, IgG2a, and IgM, respectively. Color developed when the substrate, 3,3',5,5'-tetramethyl-benzidine (TMB) (Sigma), was added. The absorbance was measured at 450 nm using an automatic microplate reader (Molecular Device Co., Silicon Valley, CA, USA). The levels of antibodies specific to rOMP10, rOMP19, and rOMP28 were measured after incubation of sera from mice immunized with the recombinant proteins at 37°C for 1 h. For the detection of antibodies specific to 3 recombinant proteins, 96-well microplates were coated with purified recombinant proteins (25 ng/well) in the coating buffer overnight at 4°C. The plates were blocked with 1% BSA in PBST for 2 h at 37°C. After washing with PBST, 1/160 diluted serum sample was added to the wells and incubated for 1 h at 37°C. HRP-conjugated goat anti-mouse IgG (H + L) (1:2,000, Bio-Rad) diluted in PBS containing 1% BSA was used to detect IgG. Color developed when the substrate, TMB, was added, and the absorbance was measured at 450 nm using an automatic microplate reader.

Measurement of cells secreting IgG, IFN- γ , and IL-4 in mouse splenocytes: Recombinant rOMP10-, rOMP19-,

and rOMP28-specific IgG memory B cells and T cells secreting IFN- γ and IL-4 from spleens of immunized mice were measured using an enzyme-linked immunospot (ELISpot Basic) assay kit according to the manufacturer's instructions (Mabtech AB, Stockholm, Sweden). The number of cells secreting IgG, IFN- γ , and IL-4 were counted using Eli. Scan + (A.EL.VIS, Hannover, Germany).

Statistical analysis: Statistical significance was calculated with Student's t-test using the Statistical Package for Social Science (SPSS) software version 4.0 (MS, Chicago, IL, USA). Differences were considered to be significant if a value of $P < 0.05$ was obtained. All experiments were repeated at least 3 times.

RESULTS

Cloning and purification of recombinant OMP10, OMP19, and OMP28 of *B. abortus*: Cloning of genes encoding OMP10, OMP19, and OMP28 was performed as described in a previous study (16) with some modifications. SDS-PAGE profiles revealed the size of purified rOMP10, rOMP19, and rOMP28 to be approximately 65.86, 71, and 80 kDa, respectively (Fig. 1A). Western blot analysis was performed to evaluate the recombinant proteins after protein purification, and the expected sizes of rOMP10, rOMP19, and rOMP28 were determined (Fig. 1B).

Production of NO and inflammatory cytokines in RAW 264.7 cells: We failed to observe any increase in NO production in groups stimulated with rOMP10 and rOMP28 at 24 h. On the other hand, cells stimulated with rOMP19 showed an increase in NO production at 24 h as compared to the control group ($**P < 0.01$) (Fig. 2A). The level of TNF- α detected in groups stimulated with all 3 recombinant proteins was higher than that observed in the control group and these changes occurred in a time-dependent manner (Fig. 2B). The production of IL-6 increased after 8 h in response to stimulation with rOMP10 and rOMP19. In contrast, stimulation with rOMP28 induced the production of IL-6 at 24 h after stimulation (Fig. 2C). We failed to detect IL-1 β , IL-12, and IFN- γ in cells stimulated with the recombinant proteins. Thus, the 3 recombinant proteins showed immune-stimulating activities in RAW 264.7 cells, as evident from the production of TNF- α and IL-6.

Production of IFN- γ and IL-2 in naive mouse splenocytes: The production of IFN- γ was higher in mouse splenocytes stimulated with rOMP28 as compared with

the control group and differences were observed in a time-dependent manner ($**P < 0.01$) (Fig. 3A). In addition, the level of IL-2 increased in cells treated with rOMP28 as compared with the control group at 24 h ($**P < 0.01$) (Fig. 3B). Therefore, rOMP28 induced high immune-stimulating activities through the production of IFN- γ and IL-2 in naive mouse splenocytes.

Analysis of antigen-secreting cells in splenocytes from mice immunized with rOMP10, rOMP19, and rOMP28: The number of antigen-specific IgG-secreting B cells from mice splenocytes was analyzed using ELISpot following 28 days of immunization with the recombinant proteins. The number of antigen-specific IgG-secreting B cells significantly increased in mice immunized with rOMP19 compared that in mice in the non-immunized group, while no increase was observed in response to rOMP10 and rOMP28. No difference was reported in the number of IFN- γ - and IL-4-secreting T cells involved in the production of Th1-related and Th2-related cytokines, respectively, for the groups (Table 1).

Production of antibodies in mice immunized with rOMP10, rOMP19, and rOMP28: The production of antibodies specific to each recombinant protein was confirmed from the sera obtained in the in vivo immunoreactivity test (Fig. 4). The recombinant protein-specific antigen was generated on day 7 after immunization (Fig. 4A, B). However, the level of antibody to rOMP28 was found to be lower than that for other recombinant proteins (Fig. 4C). Nevertheless, this observation suggests that the recombinant proteins used in this study may induce protein-specific immune responses in vivo. The level of IgM antibody against rOMP28 was significantly increased compared with that in the control group on day 3, while the level of antibody to rOMP19 increased on day 14 ($*P < 0.05$) (Fig. 5A). The level of IgM produced in groups immunized with each of the 3 recombinant pro-

Table 1. Number of antigen-secreting cells (ASC) from splenocytes of mice immunized with rOMP10, rOMP19, and rOMP28 of *Brucella abortus* at 28 days post immunization ($**P < 0.01$)

Immunized group	No. of IgG-secreting cells	No. of IFN- γ -secreting cells	No. of IL-4-secreting cells
PBS	36.17 $\pm$ 13.96	5.5 $\pm$ 1.11	27.5 $\pm$ 4.28
rOMP10	28.5 $\pm$ 7	3.75 $\pm$ 5.19	14.5 $\pm$ 3.87
rOMP19	210.75 $\pm$ 35.42**	8.25 $\pm$ 3.77	31.25 $\pm$ 7.63
rOMP28	30 $\pm$ 4.16	7.25 $\pm$ 2.87	18.75 $\pm$ 8.81

Fig. 1. Analysis of purified recombinant OMP10, OMP19, and OMP28 of *Brucella abortus*. SDS-PAGE (A) and Western blot (B) analysis of soluble protein fractions of pCold TF expressed with expected size. M: molecular weight markers, Lane 1: outer membrane protein 10 (OMP10) (65.86 kDa), Lane 2: outer membrane protein 19 (OMP19) (71 kDa), Lane 3: outer membrane protein 28 (OMP28) (80 kDa), Lane 4: TF protein (52 kDa).

Fig. 2. Production of nitric oxide (NO) and cytokines in RAW 264.7 cell after stimulation with recombinant proteins. (A) Production of NO was increased after stimulation with rOMP19 at 24 h. (B) Production of TNF- α was significantly increased after stimulation with rOMP10, rOMP19, and rOMP28 in a time dependent manner. (C) Production of IL-6 was increased after 8 h of stimulation with rOMP10 and rOMP19, whereas stimulation with rOMP28 induced production of IL-6 at 24 h. (** $P < 0.01$).

Fig. 3. Production of IFN- γ and IL-2 by naïve mice splenocytes. (A) Production of IFN- γ was increased after stimulation with purified rOMP28 at 12 h and 24 h post stimulation. (B) Production of IL-2 was increased after stimulation with purified rOMP28 at 24 h post stimulation. (** $P < 0.01$).

teins was similar to that observed in the control group on days 7 and 28, while the level of IgG in all 3 recombinant groups was significantly higher than that in the control group on day 14 after immunization (** $P < 0.01$) (Fig. 5B). Stimulation with OMP19 led to an increase in the production of IgG1 after 3 days, whereas OMP28 group showed an increase in IgG1 level after 14 days. The production of Th1-related antibody, IgG2a, gradually in-

creased in rOMP19 group after 7 days, but only after 28 days of immunization in rOMP10 and rOMP28 groups. On day 28, all 3 groups showed an increase in the level of IgG2a compared with the control group (Fig. 6A). The production of Th-2 related antibody, IgG1, in rOMP10 group was significantly increased on day 3, while that in rOMP19 group increased 7 days after immunization. All 3 groups showed an increase in IgG1 level on day 14 after

Fig. 4. Detection of antigen-specific antibodies in immunized mice after reaction with recombinant TF protein. Antibodies to the rOMP10 and rOMP19 recombinant proteins were increased at Day 14 after immunization except the rOMP28 immunized group. The production of antibodies the rOMP10 (A), rOMP19 (B) and rOMP28 (C) proteins were detected by ELISA using the sera from the 3 recombinant proteins immunized mice groups. The antibodies to the recombinant proteins were produced time-dependent manner. At 28 days after the immunization, the production of all antibodies were increased in the 3 immunized mice groups (rOMP10, rOMP19, and rOMP28) compared to control mice group. (* $P < 0.05$, ** $P < 0.01$).

immunization (Fig. 6B).

DISCUSSION

In this study, we cloned and expressed the 3 genes encoding immunogenic outer membrane proteins as recombinant proteins using pCold TF expression system (16). The recombinant proteins were identified with SDS-PAGE and western blot analysis at their expected sizes following purification using anti-histidine column. In addition, we

evaluated immunological characteristics of these recombinant proteins from *B. abortus*.

Many studies have focused on the investigation of immune responses, including evaluation of the production of proinflammatory cytokines such as TNF- α , IL-6, IFN- γ , and IL-4 following stimulation and/or immunization with various antigens of *Brucella* spp. in vitro (15,21,24). Selected recombinant proteins, rOMP10, rOMP19, and rOMP28, induced the production of TNF- α and IL-6 in

Fig. 5. Humoral immune responses induced in mice by immunization with rOMP10, rOMP19, and rOMP28 of *Brucella abortus* (IgM and IgG). (A) Different levels of IgM production were observed after immunization with rOMP28 protein at 3 days and rOMP19 protein at 14 days. (B) Production of IgG was increased after immunization with rOMP10, rOMP19, and rOMP28 in a time dependent manner. Stimulation of rOMP19 increased from 3 days after immunization. (* $P < 0.05$, ** $P < 0.01$).

Fig. 6. Humoral immune response induced IgG2a and IgG1. Production of IgG2a (A) was induced by immunization with rOMP19 after 7 days, while it was induced by rOMP10 and rOMP28 after 28 days. Production of IgG1 (B) was induced immunization with rOMP10 after 3 days, and rOMP19 after 7 days. IgG1 production increased 14 days after stimulation with the 3 recombinant proteins. (* $P < 0.05$, ** $P < 0.01$).

RAW 264.7 cells after 4 and 8 h of stimulation. The protein rOMP19 induced the production of exceptionally high levels of these cytokines than other recombinant proteins, consistent with the results of other studies (15,25,26). The production of NO significantly increased 24 h after stimulation of cells with rOMP19 as compared with control. The production of IFN- γ and IL-2 was high at 24 h after stimulation with rOMP28 in splenocytes from naive mice, whereas the stimulation with rOMP10 and rOMP19 induced the production of these cytokines at very low levels. IFN- γ is one of the major cytokines used to evaluate immune response both in vitro and in vivo after stimulation or immunization with antigens. These results imply that rOMP28 triggered Th1 cell-mediated immune response via secretion of IFN- γ and IL-2. These findings are in line with the observation of high level of IFN- γ produced in splenocytes by other antigen sources without LPS (15,27,28). The production of antigen-specific IgM and IgG was observed in mice immunized with these 3 recombinant proteins. The production of antigen-specific IgM was lower than that of antigen-specific IgG. All 3 recombinant proteins showed strong antigen-specific IgG production 28 days after stimulation. In particular, rOMP19 showed the strongest immunogenicity from day

3 to 28 after immunization. Moreover, IgG1 and IgG2a production increased in a time-dependent manner, indicative of the shift of immunological response from Th1- to Th2-mediated response. The production of antigen-specific IgG-secreting B cells and IFN- γ - or IL-4-secreting T cells in the spleen was observed in mice immunized with rOMP10, rOMP19, and rOMP28. However, no difference was observed in levels of IFN- γ - or IL-4-secreting T cells in mice immunized with the 3 recombinant proteins as compared with the control group. Therefore, results of this study contribute to the understanding of the immunogenicity of rOMP10 and rOMP19.

These OMPs have been generally studied to develop vaccines against diseases caused by various species, including *Anaplasma marginale* (29), *S. enterica* (30), *Haemophilus parasuis* (31), and *E. coli* (32). Pathogenic infections against host cells and the consequent cytokine production have been investigated thoroughly in the field of immunology and vaccine development (33–36). Thus, the evaluation of proinflammatory cytokine production is essential for the analysis of the protective immunity against pathogens. IFN- γ , IL-2, IL-4, IL-6, and TNF- α are some of the major target cytokines used to evaluate immunogenicity after stimulation and/or immunization with

recombinant proteins. In addition, IFN- γ and IL-2 are induced by spleen cells after immunization with live antigens (37). IgG response is another factor involved in imparting protection against antigens. Our results indicate that recombinant proteins drive a shift from Th1 to Th2 immune responses after immunization of mice. Some studies have reported that Th1/Th2 balance is disturbed in response to stress or infection by antigens, resulting in the shift toward Th2-dominant immunity (38,39). Furthermore, the levels of IgG1 and IgG2a were shown to increase after immunization with the recombinant outer membrane protein 25 in mice, although no significant increase in cytokine-secreting splenocyte population was observed (40).

In conclusion, this study evaluated the immunogenicities of 3 recombinant proteins (rOMP10, rOMP19, and rOMP28) of *B. abortus* by determining various immune responses. Of rOMPs, rOMP10 and rOMP19 showed strong immune responses in vitro and in vivo, although we failed to observe any significant increase in levels of IFN- γ and IL-2 in naive mice splenocytes. Thus, these proteins may serve as possible candidates for further investigation of the pathogenesis of *Brucella* infection.

Acknowledgments This study was supported by the NRF (No. 2014R1A2A2A01007291), a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: H116C2130), BK21 PLUS, and Research Institute of Veterinary Science, Seoul National University, Republic of Korea.

Conflict of interest None to declare.

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