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Identification of an Antigen Specific to a Protective *Salmonella* Monoclonal Antibody

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Abstract

Previously, we developed the monoclonal antibody (mAb) 449 after immunizing BALB/c mice with a live attenuated mutant of *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), and it helped protect against *S.* Typhimurium infection. Although the immunogen specific for mAb449 has been found to be localized in the lipopolysaccharide (LPS) region, it is not clear which part of LPS is involved in the defense against *Salmonella* infection. The present study aimed to identify sites recognized by mAb449. Electron microscopy analysis showed that mAb449 binds to the surface of *Salmonella* serotypes that express the O4 antigen, such as serovar Abortusequi and Paratyphi B. In addition, mAb449 enhanced the uptake of only O4-positive *Salmonella* serotypes within mouse macrophage-like cells. The results demonstrated that mAb449 is specific for the O4 antigen. We further verified the protective potential of mAb449 against O4-positive *Salmonella* serotypes in mice infection model, and found that mAb449 exhibited protective effect against the O4-positive *Salmonella* serotypes such as Abortusequi and Paratyphi B.

Discipline: Animal health
Additional key words: infection, mAb449, O4 antigen, *Salmonella*

Introduction

Salmonellosis is one of the zoonoses, and currently over 2,600 serotypes of *Salmonella* have been identified. *Salmonella* has emerged as one of the leading causes of foodborne diseases worldwide (Byrne et al. 2014, Johnson et al. 2014). Besides the need for proper surveillance methods for a timely detection of *Salmonella* and thereby preventing *Salmonella* infections (Galanis et al. 2006, Issenhuth-Jeanjean et al. 2014), the identification of protective antigens that elicit an immune response in the host is also necessary for vaccine development.

Both cellular and humoral factors of the host are involved in the defense against *Salmonella*. Recently, we reported that humoral immunity protects against *Salmonella* infection, and this defense mechanism also involved cell-mediated immunity (Eguchi & Kikuchi 2010). Although most antigens involved in the defense against *Salmonella* infection have not been identified, we recently identified a B-cell antigen that is a lipopolysaccharide (LPS). In addition, we developed the monoclonal antibody (mAb)449, which protected BALB/c mice from *S. enterica* serovar Typhimurium infection, and found it to specifically recognize an LPS (Aribam et al. 2016). Although the LPS molecule has a tripartite structure comprising a lipid A, a core oligosaccharide, and an O antigen polysaccharide (Whitfield & Trent 2014), it is not clear which site is recognized by mAb449.

In the present study, we investigated the O4 antigen (LPS) specific for mAb449 by electron microscopy in vitro. The results showed that mAb449 recognized only one O4 antigen in the O4 serogroup. In addition, we investigated whether mAb449 has protective ability against other O4 serotype infections such as *S.* Abortusequi (O4 serogroup), *S.* Paratyphi B (O4 serogroup), *S.* Choleraesuis (O7 serogroup) and *S.* Dublin (O9 serogroup) using mice infection model. However, it did not provide protection against infection with *S.* Choleraesuis (O7 serogroup) and *S.* Dublin (O9 serogroup).

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Materials and Methods

1. Ethics statement

Six-week-old female BALB/c mice were purchased from SLC Japan Inc. (Hamamatsu, Japan) and maintained in accordance with the National Institute of Animal Health Research Animal Resource guidelines. Up to 5-8 mice were maintained in each cage and housed in a temperature-regulated room with free access to food and water. The handling of the animals in the study was performed under the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Research Institutions under the jurisdiction of the Ministry of Agriculture, Forestry and Fisheries, Japan. The specific experiments were approved by and conducted according to the guidelines of the experimental animal ethics committees of the National Institute of Animal Health (NIAH), Japan (Project license 15-019). All animal experiments were performed to ameliorate the suffering imposed to the animals according to the guidelines of the experimental animal ethics committees of the NIAH.

2. Bacterial strains

We used the following *Salmonella* serotypes: *S.* Abortusequi L-2508 (4,12:-:e,n,x) and *S.* Paratyphi B L-3615 (4,[5],12:b:1,2) from the O4 serogroup, *S.* Choleraesuis ATCC 7001 (6,7:c:1,5) from the O7 serogroup, and *S.* Dublin ATCC 15480 (1,9,12:g,p:-) from the O9 serogroup. All bacteria were grown overnight in Luria Bertani (LB) medium (Nacalai Tesque, Kyoto, Japan) at 37°C.

3. Electron microscopy analysis

For the electron microscopy analysis, the bacterial suspension was placed on collodion-coated copper grids (400 mesh; Nisshin EM, Tokyo, Japan) for 2 min. The grids were incubated with mAb449, followed by colloidal gold-conjugated goat anti-mouse immunoglobulin (10-nm diameter; BBI Solutions, Cardiff, UK) before imaging with a transmission electron microscope (H-7500; Hitachi, Tokyo, Japan).

4. Indirect enzyme-linked immunosorbent assay

Indirect enzyme-linked immunosorbent assay (ELISA) was carried out as previously described (Aribam et al. 2015). LPS was extracted from bacteria by the hot phenol-water method (Rezania et al. 2011). Ten micrograms of LPS-coated plates were incubated with 10 μg of mAb449. Normal mouse IgG was used as a negative control. The bound antibodies were detected using a HRP-conjugated goat anti-mouse IgG and visualized with 3,3′,5,5′-tetramethylbenzidine substrate (Pierce, Rockford, IL, USA).

5. In vivo studies

To determine the 50% lethal dose (LD_{50}), groups of four BALB/c mice were inoculated intraperitoneally with bacteria serially diluted in phosphate buffered saline (PBS). The mice were then scored for viability for 15 days.

For bacterial challenge, one day after the administration of 200 μg of mAb449, the mice were challenged with bacteria at a 10 times LD_{50} dose via intraperitoneal infection (Aribam et al. 2016). The mice that were administered PBS were used as controls. The challenged mice were monitored daily for their body weight loss and signs of sickness. The mice that were in a moribund condition or had lost more than 30% of their body weight were considered to have reached an experimental endpoint, and were humanely euthanized by carbon dioxide-bottled gas. The number of mice surviving two weeks after treatment was used to determine the relative degree of protection.

6. In vitro infection studies

The mouse macrophage-like cell line RAW264.7 was obtained from the American Type Culture Collection. RAW264.7 cells (1×10^5 cells/well) were infected with a multiplicity of infection (MOI) of 1 of the bacteria. In some experiments, the bacteria (MOI 1) were treated with 5 μg of mAb449 or control IgG for 30 min at 37°C before infecting to the cell culture (Aribam et al. 2016). One hour after infection with the pre-treated bacteria, the RAW264.7 cells were washed with PBS and incubated with medium containing 100 μg/mL gentamicin for 1 h at 37°C in a 5% CO_2 incubator. The medium was then replaced with fresh medium containing 30 μg/mL gentamicin. The cells were lysed with 1 mL of 0.2% Triton X-100 in PBS for 5 min to count the bacteria on LB plates.

7. Statistical analyses

Prism 6 (GraphPad, CA, USA) was used for the statistical analyses. The survival rate was analyzed by the Kaplan-Meier log-rank test. The statistical analyses were performed by the student's *t*-test to compare groups. Data are presented as mean ± standard deviation.

Results and Discussion

We investigated the O antigen recognized by mAb449. By electron microscopy analysis, we found that mAb449 binds to the surface of the *Salmonella* O4

serogroup *S.* Abortusequi and *S.* Paratyphi B (Fig. 1A). However, it did not bind the O4-negative serotypes *S.* Choleraesuis and *S.* Dublin (Fig. 1A). To determine the binding affinity of LPS from different serotypes to mAb449, the LPS bands were subjected to ELISA analysis (Fig. 1B). As shown in Figure 1B, the LPS of *S.* Abortusequi and *S.* Paratyphi B reacted with mAb449, whereas, the LPS of *S.* Choleraesuis and *S.* Dublin did not react. Therefore, it appears that the O4 antigen is essential for the binding of mAb449 to *Salmonella* LPS. The ability of mAb449 to bind to live *Salmonella* serotypes with the O4 antigen and the lack of cross-reactions with other serotypes makes it suitable for the detection of *Salmonella* pathogens with the O4 antigen from food, environmental, and clinical samples.

Furthermore, as mAb449 recognizes the O4 antigen, we evaluated the protective ability of mAb449 against *Salmonella* infection. BALB/c mice were challenged intraperitoneally with 10 LD_{50} of *S.* Abortusequi (1×10^4 CFU), *S.* Paratyphi B (1×10^6 CFU), *S.* Choleraesuis (1×10^3 CFU), or *S.* Dublin (1×10^5 CFU) one day after the intravenous administration of mAb449 or PBS as negative control. All the mAb449-treated mice challenged with *S.* Abortusequi or *S.* Paratyphi B survived (Fig. 2), whereas, most of those challenged with *S.* Choleraesuis and *S.* Dublin died within 7 days (Fig. 2). Most of the control mice died within 8 days of infection. These results suggest that mAb449 recognizes the immunogen in serotypes that express the O4 antigen (Ding et al. 1990). Additionally, the functional analysis of mAb449 RAW264.7 cells infection studies has revealed that the protective efficacy of mAb449 may be bacterial killing by macrophages (Aribam et al. 2016). Similarly, we found that the number of adherent and intracellular *S.* Abortusequi and *S.* Paratyphi B in the RAW264.7 cells increased after treatment with mAb449 (Fig. 3). However, the treatment of the *S.* Choleraesuis and *S.* Dublin serotypes with mAb449 did not enhance the bacterial uptake by the RAW264.7 cells (Fig. 3). This shows that mAb449 recognizes only the O4 antigen that functionally mediates macrophage activity (Aribam et al. 2016).

The present study was performed to determine the specific antigen recognized by mAb449. The results indicated the protective function of mAb449 against O4-positive *Salmonella* serotypes. Several monoclonal antibodies have been developed as an attempt to generate monoclonal antibodies specific for a particular serotype (Castillo et al. 2017). Given that mAb449 particularly recognizes the O4 serotype, it may be inferred that mAb449 will provide reproducible results in *Salmonella* detection assays. In addition, the identification of the protective O4 antigen may be important in using it as a vaccine component in developing vaccines against *Salmonella*.

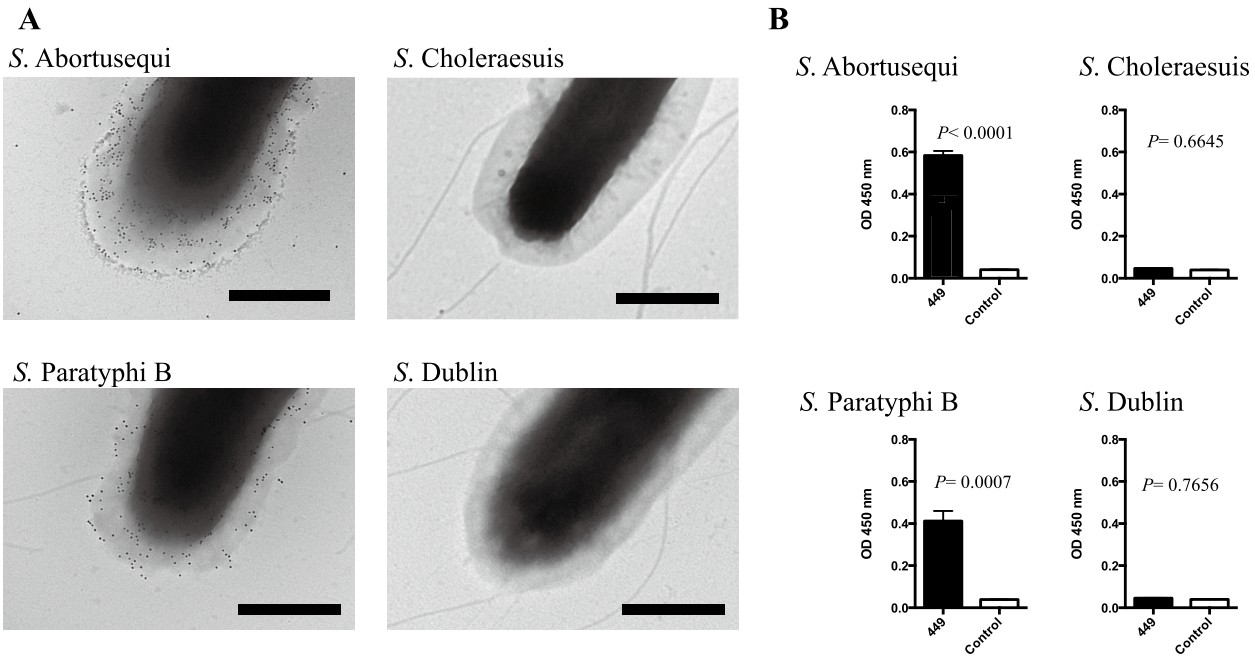

Fig. 1. Determination of the mAb449-specific O4 antigen

Electron microscopy images of *S.* Abortusequi, *S.* Paratyphi B, *S.* Choleraesuis, and *S.* Dublin. Black grains indicate the mAb449-binding regions on the bacterial cell surface (A). Bar = 500 nm. The ELISA with LPS from *S.* Abortusequi, *S.* Paratyphi B, *S.* Choleraesuis, and *S.* Dublin (B).

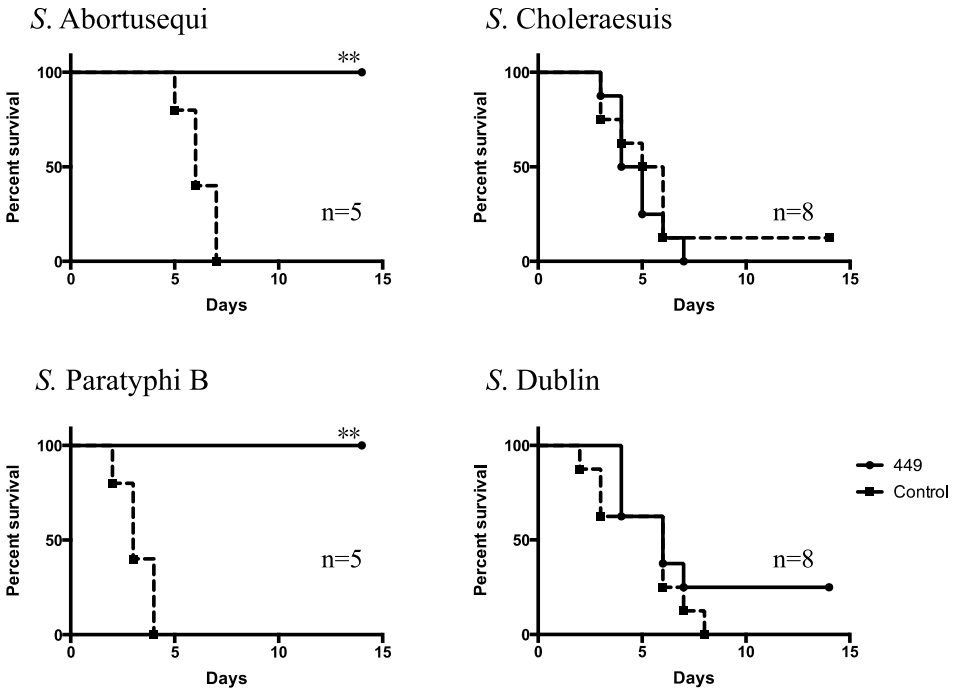

Fig. 2. Protection test against *Salmonella* infection by mAb449 in mouse

Mice were intravenously injected with mAb449 one day before the intraperitoneal challenge with 10-fold the LD_{50} of *S.* Abortusequi (1×10^4 CFU), *S.* Paratyphi B (1×10^6 CFU), *S.* Choleraesuis (1×10^3 CFU), and *S.* Dublin (1×10^5 CFU) (n = 5-8/group). Mice injected with PBS were used as control. The Kaplan-Meier log-rank test result stratified by regimen was significant at $^{**}p < 0.01$.

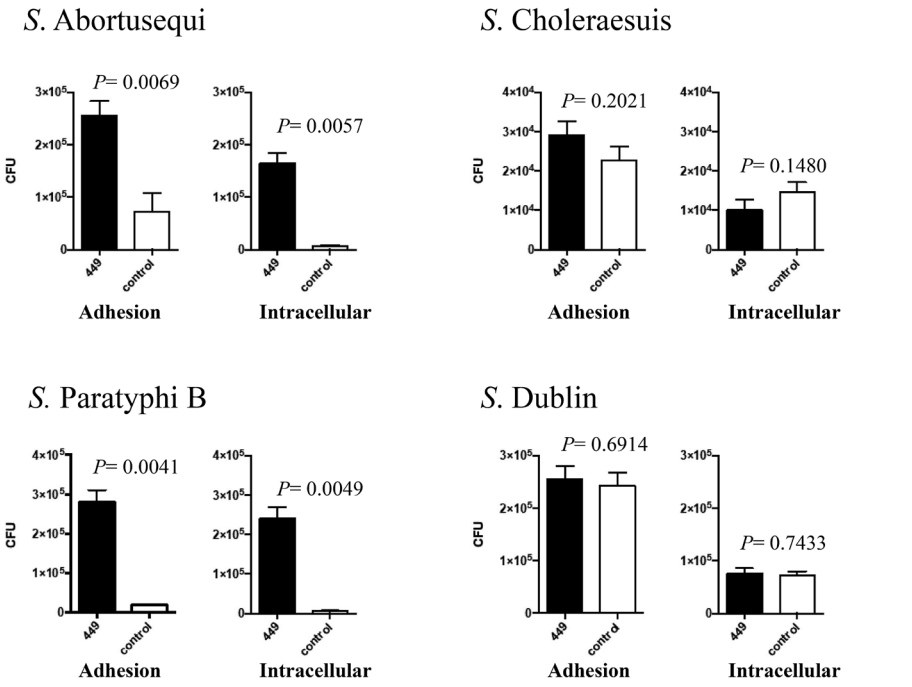

Fig. 3. Effect of mAb449 on bacterial uptake by RAW264.7 cells

The adherent (left) and intracellular (right) bacteria were quantified following infection at MOI 1 of *S.* Abortusequi, *S.* Paratyphi B, *S.* Choleraesuis, and *S.* Dublin pre-treated with mAb449 or control IgG. The significance was assessed by the student's *t*-test.

Conclusions

In the present study, we showed that the newly developed mAb449 recognizes the O4 antigen. Furthermore, mAb449 exhibited infection protection ability against *Salmonella* of the O4 group, such as *S.* Abortusequi and *S.* Paratyphi B, in a mouse infection experimental model. In addition, the results suggested that the O antigen is important for the defense against *Salmonella* infection.

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