INDUCTION OF PROTECTIVE IMMUNITY AGAINST SCRUB TYPHUS WITH A 56-KILODALTON RECOMBINANT ANTIGEN FUSED WITH A 47-KILODALTON ANTIGEN OF ORIENTIA TSUTSUGAMUSHI KARP

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Abstract. A partial gene sequence encoding the 56-kD scrub typhus antigen (Sta56) was amplified from genomic DNA of the Orientia tsutsugamushi Karp strain by a polymerase chain reaction (PCR). The PCR product was ligated with the 47-kD scrub typhus antigen (Sta47) gene in the pQE30/47 expression vector, and the resulting recombinant expression vector was designated pQE30/56-47. A fusion antigen (Sta56-47) was expressed in Escherichia coli cells transformed with pQE30/56-47 after induction with isopropyl-β-D-thiogalactopyranoside. The Sta56-47 antigen was recognized by both Sta47 and Sta56 immune sera and by immune serum to Sta56-47 in an immunoblot assay. This antigen was purified and used to immunize BALB/c mice. The animals immunized with Sta56-47 exhibited profound humoral and cellular immune responses, as well as increased resistance to O. tsutsugamushi Karp compared with mice immunized with Sta56 or Sta47. These results strongly suggest that Sta56-47 contains antigenic epitopes of the Sta56 and Sta47 antigens of O. tsutsugamushi Karp, and is a more suitable candidate for replacing whole-cell antigen of O. tsutsugamushi Karp to induce protective immunity against scrub typhus.

INTRODUCTION

Scrub typhus is an acute vector-borne disease characterized by fever, rash, and eschar. It is endemic in China and the Asia-Pacific region and its incidence has increased in some countries during the past several years.1,2 Scrub typhus is caused by Orientia tsutsugamushi, an obligate, intracellular, gram-negative bacterium, and is transmitted by Leptotrombidium mites.3,4 Orientia tsutsugamushi is an antigenically diverse microorganism and is divided into Gilliam, Karp, Kato, and other serotypes.5−8 Most of the O. tsutsugamushi isolates in China are of the Karp or Gilliam serotypes.

Although scrub typhus is efficaciously treated with tetracycline-derived antibiotics,9 an effective vaccine against O. tsutsugamushi is more desirable for the control of scrub typhus. To date, considerable efforts have been invested in developing a vaccine for scrub typhus, but no vaccines for humans are available at the present time. Previous studies reported that protective immunity against scrub typhus was induced with irradiated whole cells of O. tsutsugamushi.10 However, the use of the irradiated vaccine in humans seems to be impractical because the large-scale culture and purification of this obligate intracellular microorganism is time-consuming and expensive. Therefore, the recombinant proteins produced in Escherichia coli or other organisms will most likely be the future vaccines against scrub typhus.

The major antigens of O. tsutsugamushi are proteins with molecular masses of 70, 58, 56, 47, and 22 kD. Of these proteins, both the 47-kD and 56-kD proteins are the major surface antigens of O. tsutsugamushi (scrub typhus antigen [Sta]). The 47-kD protein (Sta47) is found in outer membrane of O. tsutsugamushi and contains both scrub typhus group-reactive and strain-specific B cell epitopes.11 Previous studies have shown that a recombinant Sta47 induced vigorous proliferation in a polyclonal helper T cell line derived from O. tsutsugamushi-immune mice, indicating that Sta47 possesses potential T cell epitopes.11−13 Since cell-mediated immunity (CMI) is a major determinant in acquired resistance to O. tsutsugamushi, Sta47 has a potential importance in the development of subunit vaccine against scrub typhus.

The 56-kD protein (Sta56) is expressed on the outer mem-

brane at a high concentration14−17 and is recognized by almost all sera from patients in the convalescent phase of scrub typhus. This antigen is reactive with group-specific and strain-specific monoclonal antibodies, implying the existence of group-specific and strain-specific epitopes in this molecule.18 Previous studies demonstrated that mice immunized with Sta56 could generate neutralizing antibodies and showed an increased resistance to infection caused by homologous strain of O. tsutsugamushi.19,20 The splenic mononuclear cells from mice immunized with Sta56 proliferated and secreted significant levels of interleukin-2 (IL-2) and interferon-γ (IFN-γ) when stimulated with homotypic O. tsutsugamushi in vitro, suggesting that this protein has a capability to induce CMI against O. tsutsugamushi. These findings strongly suggest that Sta56 is frequently recognized by host immune systems and plays an important role in generating protective immunity against scrub typhus.19,20

In the present study, the gene encoding Sta56 was ligated with the gene encoding Sta47 and inserted into a prokaryotic expression vector (pQE30) as in our previous study.21 A fusion protein (Sta56-47) containing the antigenic determinants of Sta47 and Sta56 was expressed in E. coli cells transformed with the expression vector recombinated with a fusion gene encoding Sta47 and Sta56 antigen. The protective immunity to scrub typhus induced with the fusion protein was evaluated in mice, and the results obtained in the present study demonstrate that the fusion protein is capable of inducing stronger immune protection against scrub typhus compared with the recombinant Sta47 or Sta56 antigens individually.

MATERIALS AND METHODS

Bacterial strains and vectors. Escherichia coli M15 was used as host strain for prokaryotic expression vectors. The prokaryotic expression vector used was pQE30 (Qiagen GmbH, Hilden, Germany), and the recombinant vector pQE30/47 was constructed with the gene encoding Sta47 as previously described.21 Orientia tsutsugamushi Karp was provided by Dr. Ik-sang Kim (Medicine College, Seoul National University, Seoul, Korea).

Luria-Bertani (LB) medium was used for routine culture of
**E. coli** strains. For all strains harboring the recombinant vectors, ampicillin (50 μg/mL) and kanamycin (50 μg/mL) was added to the culture medium. Broth cultures were grown at 37°C with vigorous shaking (200 rpm) until the mid-logarithmic phase was attained. When noted, isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma, St. Louis, MO) was added to the culture of *E. coli* M15 harboring vectors to induce expression of the target genes.

*Orientia tsutsugamushi* Karp was grown in L929 cells as previously described.²² The L929 cells were propagated as monolayer cultures in RPMI 1640 medium (Perbio Science Co., Logan, UT) that was supplemented with 10% (v/v) fetal calf serum (FCS) (Perbio Science Co.). The cells infected with *O. tsutsugamushi* were maintained with RPMI 1640 medium supplemented with 5% (v/v) FCS and 0.4 mg/mL of daunomycin hydrochloride (Sigma) at 35°C in an atmosphere of 5% CO₂. When the cells started to detach from the culture substrate, the infected cells were harvested. The infected cells were homogenized on ice for 20 seconds using a homogenizer, the cellular debris was removed by brief centrifugation for 5 minutes at 4°C, and the supernatant containing rickettsial cells was used for *in vivo* and *in vitro* titration.

**Mice and immune sera.** Male BALB/c (6–8 weeks of age) were obtained from the Laboratory Animal Center of Beijing. The animal usage was approved by the Beijing administrative committee for laboratory animals and the animal care met the standard of the committee.

Five mice were immunized with each purified recombinant protein or *O. tsutsugamushi* Karp whole-cell lysate. Each mouse was subcutaneously injected with 20 μg of antigen emulsified in Freund’s incomplete adjuvant and was given an intraperitoneal booster injection of 10 μg of antigen emulsified in Freund’s incomplete adjuvant four weeks after the primary immunization. Two weeks after the booster immunization, mice were bled and sera were obtained and stored at −20°C.

**Constructions of recombinant expression vectors.** A primer pair, 56F292–247 (5’-CCGGATCCGGAGTATGACAATC GCTC-3’) and 56R107–1386 (5’-CGGTTGAATTTTCCAG AAGTATAAGCTA-3’), was designed based the nucleotide sequence of the 56-kDa protein gene (sta56) of the *O. tsutsugamushi* Karp strain.¹⁷ The restriction sites for *Bam* HI and *Kpn* I are underlined. The genomic DNA was extracted from *O. tsutsugamushi* Karp cells by a phenol-chloroform extraction method.²³

The truncated sta56 was amplified from genomic DNA of *O. tsutsugamushi* Karp in a mixture of 200 μM (each) deoxy-nucleoside triphosphate, 0.3 μM (each) primer, and 0.6 units of *Taq* polymerase (Promega, Madison, WI) in 10 mM Tris-HCl buffer, pH 8.3, supplemented with 2.0 mM MgCl₂ and 50 mM KCl by a polymerase chain reaction. The amplification was conducted at 95°C for 5 minutes, followed by 30 cycles at 94°C for 45 seconds, 57°C for 30 seconds, and 72°C for 90 seconds. A final step at 72°C for 7 minutes was added to the last cycle. The amplified fragment (1.18 kb) was digested with *Bam* HI and *Kpn* I (TaKaRa Biotechnology Co., Dalian, People’s Republic of China) and ligated with vectors pQE30 and pQE30/47 that were digested with *Bam* HI and *Kpn* I to construct the recombinant vectors pQE30/56 and pQE30/47-56, respectively, according to standard methods (Figure 1).²⁴

*Escherichia coli* M15 was transformed with the resulting ligation mixture, and the transformed colonies were screened on medium containing ampicillin and kanamycin.

**Expression of genes of *O. tsutsugamushi* in *E. coli* cells.** *Escherichia coli* cells transformed with pQE30/47, pQE30/56, or pQE30/56-47 were propagated overnight in LB media containing ampicillin (50 mg/L) and kanamycin (50 mg/L), respectively, at 37°C with shaking. The next day, 1 mL of the overnight culture was inoculated into 100 mL of fresh medium containing the antibiotics and the culture was allowed to grow to an absorbance at 600 nm of 0.6. The culture was induced with 1 mM IPTG and grown for an additional four hours at 37°C. The cells were harvested by centrifugation at 2,000 × g for 10 minutes.

The bacterial proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (5% stacking gel and 10% separation gel), and the recombinant protein expressed in *E. coli* cells were identified by immunoblott assays according to standard methods.²⁴

**Purification of recombinant proteins.** Cell pellets from 100-mL cultures were resuspended in 3 mL of 10 mM Tris-HCl, pH 7.0, containing 1 mM EDTA. Ultrasonic disruption of the cell was performed by using an Ultrasonic Liquid Processor (VCX750) with a standard tapered microtip (Sonics and Materials, Inc., Newtown, CT) at 150 W for 30 minutes with six-second cooling on ice per six-second sonication. The recombinant proteins, which had been tagged with six consecutive histidine residues, were purified from the cellular debris by affinity chromatography with nickel-nitrilotriacetic resin
(Qiagen GmbH) under native conditions according to the manufacturer’s protocol.

**Endotoxin content determination.** Endotoxin content was estimated by a chromogenic *Limulus* amebocyte lysate (LAL) endpoint assay according to the manufacturer’s instructions (Associates of Cape Cod, Falmouth, MA). Dilutions of all protein samples and the LAL standard were prepared in pyrogen-free vials. Positive control solutions prepared for the standard curves ranged from 1 to 0.06 endotoxin units/mL in two-fold serial dilutions in a 96-well plate. After incubation at 37°C for 20 minutes, the plates were read at 405 nm on a microplate reader (SPECTRAMax® Plus, Molecular Devices Corp., Sunnyvale, CA).

**Immunization.** For each mouse, 20 μg of antigen (recombinant proteins or lysate of *O. tsutsugamushi* Karp) emulsified in Freund’s incomplete adjuvant were injected by the subcutaneous route. After four weeks, each mouse was given intraperitoneal booster injections twice at two-week intervals with 10 μg of homologous antigen emulsified in Freund’s incomplete adjuvant. Phosphate-buffered saline (PBS) emulsified in Freund’s incomplete adjuvant was used to inject mice as a mock immunization.

**Detections of IgG, IL-2, and IFN-γ in sera.** Thirty mice were divided into five groups of six mice per group and each group was immunized with Sta47, Sta56, Sta56-47, a lysate of *O. tsutsugamushi* Karp, or PBS. For monitoring of the humoral immune responses, mice were bled from the tails on days 14, 42, 56, 70, and 84 after primary immunization, the blood samples of each group were mixed, and sera were analyzed by an indirect immunofluorescence assay (IFA) with antigen slides of *O. tsutsugamushi* Karp propagated in the yolk sacs of embryonated chicken eggs according to established protocols. Fluorescein isothiocyanate–conjugated goat antimouse IgG was obtained from the Sihuan Sci-Technic Company (Beijing, People’s Republic of China).

Production of IFN-γ and IL-2 was determined in each mixed serum collected from six mice per group on day 7 after first booster immunization by murine IFN-γ and IL-2 double-sandwich enzyme-linked immunosorbent assay kits (Boster Biologic Technology Ltd., Wuhan, People’s Republic of China), respectively, according to the manufacturer’s instructions. Briefly, 10 μL of mouse sera or 10 μL of standards, 50 μL of anti-mouse cytokine biotin, and 50 μL of anti-mouse cytokine peroxidase were added to each well (coated with polyclonal antibody specific for the mouse cytokine) of a 96-well plate for 45 minutes at room temperature. Plates were washed five times with 350 μL of washing buffer prior to the addition of tetramethylbenzidine substrate. Each group serum was analyzed in triplicate and the plates were read at 450 nm on microplate reader.

**Spleenic proliferation assay.** Ten days after the last booster injection, three mice in each group were killed and spleens were ground into single cell suspensions in RPMI 1640 medium supplemented with 10% FCS for a cell proliferation assay. They were seeded in triplicate in flat-bottomed 96-well plates at a concentration of 1 × 10^6 cells in a 100-μL volume per well. After the addition of various concentrations of the recombinant protein (0.3–10 μg/mL) as a stimulating antigen, the plates were incubated at 37°C for 48 hours in a humidified atmosphere of 5% CO₂. Ten microliters of tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution was then added to each culture well, and the plates were incubated for four hours at 37°C. One hundred microliters of lysing buffer containing 10% Triton, 50% isopropanol alcohol, and 0.01 M HCl was then added to each well, and the plates were incubated overnight. The absorbances at 570 nm and 630 nm were measured on microplate reader. Statistical analysis was carried out using the Student’s t-test.

**Delayed-type hypersensitivity (DTH) assay.** Two weeks after the second booster immunization with whole-cell lysate of *O. tsutsugamushi* Karp, mice were challenged in both hind footpads by subcutaneous injection of 5 μg of recombinant antigens in 25 μL of saline. The irrelevant protein (a 27-kDa recombinant protein of *Coxiella burnetii* prepared in our laboratory) and PBS were used as challenge antigens in the control groups. The footpad thickness was measured at 0, 24, and 48 hours with a microdial thickness gauge (Ozaki, Tokyo, Japan). The increase in footpad thickness at 48 hours was used to assess the DTH.

**Protective immunity.** Sixty mice were divided into six groups of 10 each and each group was immunized with Sta47, Sta56, Sta56-47, Sta47 and Sta56, a lysate of *O. tsutsugamushi* Karp, or PBS. Two weeks after the second booster immunization, each mouse was challenged with a 10-fold 50% lethal dose of *O. tsutsugamushi* Karp stock suspension calculated by probit analysis. The level of protection afforded by each antigen was expressed as the number of mice surviving three weeks after the challenge. Statistical analysis was performed using SPSS version 11.5 for Windows (SPSS, Inc., Chicago, IL). A P value < 0.05 was considered statistically significant.

**RESULTS**

Expression of *O. tsutsugamushi* 56 gene and 56-47 fusion gene in *E. coli*. Analysis by SDS-PAGE showed that proteins with apparent sizes of approximately 45 kD (Sta47), 50 kD (Sta56), and 90-kD (Sta56-47) were observed in *E. coli* cells transformed with pQE30/47, pQE30/56, and pQE30/56-47, respectively (Figure 2). Immunoblot assays showed that Sta47, Sta56, and Sta56-47 were recognized by *O. tsutsugamushi* Karp-immunized mouse serum and sera from mice immunized with homologous antigens; Sta47 and Sta56 were both

**Figure 2.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the expression products of pQE30/56, pQE30/47, and pQE30/56-47. Lane M, protein molecular mass markers; lane 1, *Escherichia coli* cells harboring pQE30; lanes 2 and 3, *E. coli* cells harboring pQE30/56; lane 4, inclusion bodies extracted from *E. coli* cells harboring pQE30/56; lanes 5 and 6, *E. coli* cells harboring pQE30/47; lane 7, inclusion bodies extracted from *E. coli* cells harboring pQE30/47; lane 8, *E. coli* cells harboring pQE30/56-47; lane 9, *E. coli* cells harboring pQE30; lane 10, inclusion bodies extracted from *E. coli* cells harboring pQE30/56-47; lane 11, purified 56–47 kDa protein; lane 12, purified 47-kDa protein; lane 13, purified 56-kDa protein. kDa = kilodaltons.
recognized by serum to Sta56-47, and Sta56-47 was recognized by both Sta47- and Sta56-immunized sera (Figure 3).

**Low endotoxin content of purified recombinant proteins.** The final purified recombinant proteins were examined by the *Limulus* amebocyte lysate assay for the presence of endotoxin. The final preparation of recombinant proteins contained between 10 and 30 endotoxin units per 50 µg of protein.

**Humoral responses.** The titers of IgG to *O. tsutsugamushi* Karp of Sta47-immunized mice was significantly lower than that of mice immunized with Sta56-47, Sta56, or *O. tsutsugamushi* Karp, with its maximum titer detectable two weeks after the second booster injection and a rapid decrease in the titer two weeks later (Figure 4). The high titers of IgG to *O. tsutsugamushi* Karp were determined in mice immunized with *O. tsutsugamushi* Karp, Sta56-47, or Sta56 two weeks after the first booster injection (Figure 4). However, the high titers were not determined in mice immunized with Sta47 until six weeks after the second booster injection (the final time in this test). The specific antibodies to *O. tsutsugamushi* Karp were not detected in the sera from controls injected with PBS (Figure 4).

**Cell-mediated immune responses.** Splenic cells were obtained from mice immunized with the recombinant antigens of *O. tsutsugamushi* Karp. The proliferative responses of splenic cells from mice immunized with Sta56-47 or whole cells of *O. tsutsugamushi* Karp were significantly greater than those from Sta47- or Sta56-immunized mice after stimulation with 1 µg/mL of homologous antigens (*P* < 0.05) (Figure 5). The IFN-γ and IL-2 levels in the sera from mice immunized with Sta56-47 or Sta47 were much higher than those in Sta56-immunized mice (*P* < 0.05), but not significantly different from that of *O. tsutsugamushi* Karp-immunized mice (*P* > 0.05) (Figure 6). Significant differences in levels of IFN-γ and IL-2 were observed between the recombinant antigen-immunized mice and the negative controls (*P* < 0.05) (Figure 6). The footpad thickness of mice immunized with whole-cell lysate of *O. tsutsugamushi* Karp was measured 24 and 48 hours after challenge with the recombinant antigens. The challenge with Sta56-47 induced the high level of DTH similar to that challenged with lysate of *O. tsutsugamushi* Karp.

**Protection induced with recombinant proteins.** The level of protection afforded by Sta56-47 was 50%, which was similar to that afforded by Sta56 and Sta47 and significantly higher than that of negative controls (*P* < 0.05) (Figure 7). However, the protection level of Sta56-47 was lower than that afforded by the lysate of *O. tsutsugamushi* Karp. Although the level of protection afforded by Sta47 or Sta56 was higher than that of negative controls (Figure 7), the difference was not statistically significant (*P* > 0.05).

**DISCUSSION**

Scrub typhus transmitted by trombiculid mites is prevalent in the Asia-Pacific area. Although the disease can be effectively treated with antibiotics, infection and relapses of scrub typhus occur frequently. In addition, there is a possibility that antibiotic-resistant strains will emerge. As a result of such potential problems, the development of scrub typhus vaccines...
should be pursued. Until now, the development of a scrub typhus vaccine had been directed toward the elucidation of immunogenic antigens and their roles in inducing both humoral immunity and cellular immunity in response to *O. tsutsugamushi* challenge infections.20 Previous studies have indicated that the antigenic diversity of *O. tsutsugamushi* strains should be considered in vaccine construction because the need for heterologous protection.28–30 Even though the protection against the heterologous strain is not as long-lasting as that against the homologous strain, there is some degree of resistance to heterologous challenge, indicating that the cross-reactive antigens of scrub typhus strains may play some roles in protective immunity against heterologous strains of *O. tsutsugamushi*.28,30 Both the 56-kD and 47-kD surface proteins of *O. tsutsugamushi* are recognized as major protective antigens with group- and strain-specific epitopes, which may induce both homologous and heterologous protective immunity against scrub typhus. Based on this reasoning, a fusion protein of Sta56 and Sta47 should be a rational candidate for the development of a more efficacious subunit vaccine.

On the basis of group- and strain-specific epitopes identified in Sta56 and Sta47,11,13 we constructed the recombinant prokaryotic expression vector pQE30/56-47 and prepared the fusion protein Sta56-47 in this study. Although the expressed Sta56 does not contain the N-terminal 76 residues and the C-terminal 65 residues of the intact 56-kD protein as deduced from the open reading frame of its encoding gene, it may present epitopes similar to those found in the full-length native protein. Both regions deleted from the N- and C-termini were predicted to be rather hydrophobic and responsible for association of the intact 56-kD protein with the rickettsial outer membrane.31 Therefore, the truncation of these termini may favor the expression of fusion sta56-47 gene, but should not reduce the immune activity of Sta56-47 fusion protein.

In an immunoblot assay, Sta56-47 reacted with immunoserum to Sta56, Sta47, or *O. tsutsugamushi* Karp, and the immunoserum to Sta56-47 also reacted with Sta56 and Sta47. This strongly demonstrates that the fusion antigen contains epitopes of both Sta56 and Sta47. The humoral immune responses of mice immunized with the recombinant proteins of *O. tsutsugamushi* Karp were monitored by IFA, and the IgG antibodies to *O. tsutsugamushi* Karp in mice immunized with Sta56-47 appeared as early as and decreased as slowly as those in mice immunized with *O. tsutsugamushi* Karp whole-cell lysates. This result suggests that Sta56-47 may induce a high level of humoral immune responses against scrub typhus similar to whole-cell antigen of *O. tsutsugamushi*. Antibody titer of Sta56-immunized mice is much higher and last much longer than that in Sta47-immunized mice, which strongly suggests that the high level of long-lived antibodies to Sta56 play a key role in humoral immune responses against scrub typhus. Although it is not as efficient as Sta56 in eliciting the humoral immune responses, Sta47 was recognized as an efficient antigen in eliciting cellular immune responses to scrub typhus in a previous study11 and the present study.

Many reports suggest that protective immunity to scrub typhus is due to the development of cell-mediated immunity.32 This has been demonstrated by passive transfer of resistance with T cells,33 development of DTH,34 and *in vitro* cytokine secretion of T cells in response to *O. tsutsugamushi* antigens.10,11,13,35 Th1 cells have been shown to be responsible for the DTH response, which has been found to correlate with resistance to lethal challenge in mice infected with scrub typhus.34,36 The proliferative level of splenic cells from mice immunized with Sta56-47 antigen was much higher than that in Sta56- or Sta47-immunized mice in response to homologous antigens. Furthermore, the DTH of *O. tsutsugamushi* Karp-immunized mice challenged with Sta56-47 was

### Table 1

Comparison of recombinant proteins for induction of delayed-type hypersensitivity※

<table>
<thead>
<tr>
<th>Group†</th>
<th>Increase in footpad thickness (SEM)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24 hours</td>
</tr>
<tr>
<td>PBS</td>
<td>0.19 (0.02)</td>
</tr>
<tr>
<td>Irrelevant protein</td>
<td>0.26 (0.03)</td>
</tr>
<tr>
<td>Sta 47</td>
<td>0.27 (0.01)</td>
</tr>
<tr>
<td>Sta 56</td>
<td>0.29 (0.02)</td>
</tr>
<tr>
<td>Sta 56-47</td>
<td>0.37 (0.02)</td>
</tr>
<tr>
<td>Ot Karp</td>
<td>0.38 (0.02)</td>
</tr>
</tbody>
</table>

※ PBS = phosphate-buffered saline; Sta = scrub typhus antigen; Ot = *Orientia tsutsugamushi*.

† There were six BALB/c mice per group.

‡ Footpad measurements are in millimeters.

§ P < 0.05 versus PBS control.

¶ P < 0.05 versus irrelevant protein.
significantly increased compared with that in mice challenged with Sta56 or Sta47. These findings demonstrate that the Sta56-47 fusion antigen has a much stronger capability of eliciting the cell-mediated immunity than Sta56 or Sta47. In addition, the levels of IFN-γ and IL-2 of mice immunized with Sta56-47 was similar to that of Sta47-immunized mice and markedly higher than that of Sta56-immunized mice, indicating that Sta56 subunit of Sta56-47 fusion antigen play a more important role in eliciting of the cell-mediated immunity than Sta56 subunit. Interferon-γ and IL-2 are both the products of CD4+ T (Th1) cells, and IFN-γ has been shown to inhibit rickettsial growth in vitro and is believed to play an important role in resistance to rickettsial infection in vivo.10,37,38 Moreover, Th1 cells are capable of inducing DTH and activating macrophages, making these cells particularly suited to deal with intracellular organisms.

Immunization with Sta56-47 elicited stronger protection against scrub typhus compared with that of Sta56 or Sta47 in the present study. The mortality of mice immunized with Sta56-47 was lower than that of Sta47 or Sta56 after challenge with O. tsutsugamushi Karp. The stronger protection afforded by Sta56-47 may contribute to the rational combination of antigenic epitopes of Sta56 and Sta47, by which the immune system was efficiently stimulated to generate a high level of humoral and cellular immune responses against scrub typhus. For this reason, Sta56-47 may be a suitable candidate for a polyvalent recombinant vaccine against scrub typhus. However, the mortality of mice immunized with the lysate of O. tsutsugamushi Karp was lower than that of those immunized with Sta56-47, suggesting that other components of O. tsutsugamushi Karp afforded protection against scrub typhus. Therefore, a superantigen harboring more than the two antigens of O. tsutsugamushi should be constructed.

Although the protection level afforded by Sta56-47 was equal to that of Sta56 and Sta47, the cost and labor of preparing Sta56-47 was only half that of preparing both Sta56 and Sta47. Therefore, construction of the fusion antigen is a reasonable endeavor.

Although Sta56-47 was shown to induce homologous protection in mice in the present study, it is not certain whether this fusion antigen could induce heterologous protection as well. However, the group- and strain-specific epitopes of Sta56 and Sta47 were well recognized in previous studies.11,20,28 Whether Sta56-47 has an ability provide efficient protection against scrub typhus remains to be demonstrated.

In conclusion, the data presented in this paper suggest that the Sta56-47 fusion protein containing the antigenic properties of the 56-kD and 47-kD major surface antigen of O. tsutsugamushi is a more efficient recombinant antigen in eliciting humoral and cellular immunity against scrub typhus. It may be a good candidate for preparing of subunit vaccine of scrub typhus.

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