Analysis of the Cross-Reactivity of Various 56 kDa Recombinant Protein Antigens with Serum Samples Collected after Orientia tsutsugamushi Infection by ELISA

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Abstract. Orientia tsutsugamushi, the etiologic agent of scrub typhus, has a highly expressed and immunodominant 56-kD outer membrane protein. This protein is one of the leading candidates for diagnosis and vaccine development for scrub typhus. Previous studies using recombinant 56-kD protein (r56s) derived from Karp strain (Kpr56) in a mouse model have shown good homologous protection but only moderate to poor heterologous protection. We evaluated the cross-reactivity of recombinant 56-kD proteins from Karp, Kato, Gilliam, TA763, and three chimeric 56-kD proteins. Not all r56s are equally reactive with strain-specific serum samples. These data provide a first glance of how reactive these r56s are toward the antisera of different strains and which r56 exhibits the broadest reactivity. A formulation of this combination has the potential to provide broad protection against the heterologous challenge and to be used in a highly sensitive diagnostic assay.

INTRODUCTION

Orientia tsutsugamushi, an obligate intracellular bacterium, is the causative agent of scrub typhus. The disease is characterized by fever, rash, eschar, pneumonia, meningitis, and in some cases, disseminated intravascular coagulation that may lead to circulatory failure. It accounts for up to 23% of all febrile illnesses in the Asia-Pacific disease-endemic region. If left untreated, Orientia can cause up to 35% mortality. The disease has been re-emerging and occurring in new areas in many countries including Japan, South Korea, Sri Lanka, China, Maldives, India, Palau, Malaysia, Taiwan, and Australia, and evidence of antibiotic resistance has been shown. At the present time, no vaccine is available for protection against scrub typhus.

Orientia exhibits considerable strain variation. Homologous protection developed from natural infection persists for at least one year, but heterologous protection may remain for less than six months. Both humoral and cell-mediated immune responses are important in protective immunity against scrub typhus. Prior vaccine development efforts using the whole organism have suggested that a scrub typhus vaccine is possible. Although a recent report suggested that long-term adaptation in egg-yolk sac has increased the yield of Orientia, considerable difficulties still exist in mass production of pure Orientia and in retaining its stability upon storage. Thus, a subunit vaccine composed of genetically engineered antigens capable of inducing protective immunity in human subjects may be a good alternative.

Western blot analysis of whole cell lysates with scrub typhus patient serum samples has identified at least four protein antigens of Orientia with molecular weights of 22 kD, 47 kD, 56 kD, and 110 kD. Among them, the 56-kD antigen accounts for 10–15% of the total cell protein. Almost every clinically diagnosed patient serum recognizes the 56-kD antigen, but not every patient serum reacts with the 22-kD, 47-kD, or 110-kD antigens. Recombinant 56-kD protein has been shown to be protective in mice against homologous challenge. High titers of antibodies to Orientia were also detected in mouse serum samples. A dose-dependent pattern of lymphocyte proliferation and levels of interferon-γ and interleukin-2 production (cytokine profile of Th1 cells) were observed in spleen mononuclear cells from the immunized mice. The 56-kD protein also plays a role in the adhesion and internalization of Orientia into host cells, and antibodies against this antigen can block Orientia infection of fibroblasts. These results suggest that the 56-kD protein is an ideal candidate for vaccine development.

A recombinant protein (Kpr56) has been shown to provide 60–100% protection against homologous challenge in an outbred mouse model and it was safe and immunogenic in the cynomolgus monkey model. This vaccine candidate has recently been evaluated for protection against heterologous challenge with five non-Karp strains of Orientia. Partial protection (11–56%) was observed in CD-1-challenged mice. Administration of a binary vaccine composed of Kpr56 and Ktr56 resulted in similar partial protection (33–67%). A trivalent vaccine (KpKtGm r56) has also been evaluated and showed no significant improvement in heterologous protection. These results demonstrated that the addition of Ktr56 and Gmr56 in vaccine formulation did not provide broader heterologous protection.

Recently, we produced another r56 from the TA763 strain. It has been shown that mouse antibodies against TA763 reacted with many heterologous strains, and the recombinant TAR56 was recognized by serum samples of many serotypes, demonstrating its broad reactivity. Thus, inclusion of TAR56 may provide additional heterologous protection. To improve the broadness of seroreactivity and to minimize numbers of recombinant proteins included in vaccine, we designed chimeric 1, 2, and 3 proteins by shuffling the epitopes in the variable domains of the Kpr56 and TAR56. We evaluated the seroreactivity of these proteins with serum samples from mice infected with various strains of Orientia.

MATERIALS AND METHODS

Chimeric proteins. The 56-kD protein sequences from Karp and TA763 strains of Orientia were used as the building blocks for the chimeric proteins. Any substitution of amino
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acids was determined based on an epitope prediction program. Chimeric 1 (C1) was designed manually by using the Karp 56-kD protein sequence as the backbone and replacing the variable domain 1 with the TA763 sequence with modifications. The final sequence of C1 is shown in Figure 1A. Similar criteria were used for the design of chimeric 2 (C2), but the sequence of the 56-kD protein from the TA763 strain was used as the backbone, and variable domain 3 was replaced with the sequence of that region from the Karp strain. The sequence is shown in Figure 1B. Chimeric 3 was designed by modifying variable domains 1, 2, and 3, and to ensure the presence of epitopes throughout the sequence (Figure 1C). The DNA was synthesized by Bioclone (San Diego, CA) and cloned into a pET29a vector (Novagen, Madison, WI) with built-in Ndel and XhoI restriction sites. The synthesized DNA sequences were confirmed.

**Cloning of 56-kD protein genes from Karp, Kato, Gilliam, and TA763 strains of *O. tsutsugamushi*:** The primers with built-in BamH1 and Ndel restriction sites for the 56-kD protein gene from amino acids 80 to 456 (according to the sequence of Karp strain) of the open reading frame were designed based on the available DNA sequences of all four strains in the National Center for Biotechnology Information (Bethesda, MD) database. Genomic DNA extracted from renograin gradient–purified *Orientia* was used as template in combination with appropriate primers for each specific strain in a polymerase chain reaction. The amplicons were cloned into a pET24a vector (Novagen). Colonies were selected and sequences of the amplicons were verified. The plasmid was transformed into BL21(DE3) cells (Invitrogen, Carlsbad, CA) for protein expression.

**Expression of recombinant 56-kD proteins from BL21(DE3) transformants.** BL21(DE3) transformants containing correct amplicons were selected, grown in Luria-Bertani medium in the presence of 50 µg/mL of kanamycin (Invitrogen) in a 37°C shaker and agitated at 200 rpm. The cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (Sigma, St. Louis, MO) when the optical density at 600 nm reached 0.8–1.0. After induction for 19 hours, the cells were centrifuged at 4,000 rpm for 30 minutes in a SS34 rotor to separate cells from medium. The cell pellet was stored at −20°C until use.

**Extraction and solubilization of inclusion bodies containing recombinant 56-kD (r56) proteins.** The cell pellet was thoroughly resuspended in 2% deoxycholate (Sigma) in 20 mM Tris-HCl, pH 7.5 (buffer A) and sonicated (Sonicator Ultrasonic Liquid Processor Model XL2020; Misonix Inc., Farmingdale, NY) with a standard tapered microtip) on ice. Disrupted cell extract was centrifuged at 8,000 × g for 30 minutes at 4°C. The pellet was resuspended in 2 M urea (Arcos Organics USA, Morris Plains, NJ) in buffer A, incubated with gentle rocking for 30 minutes, and centrifuged again as described. The entire process was then repeated with 4 M urea in buffer A and then with 6 M urea in buffer A. Finally, the pellet was dissolved and incubated with gentle rocking for 30 minutes in 8 M urea in buffer A. The supernatant (approximately 10 mL) containing most r56 proteins was in 6 M urea.

**Chromatographic purification of recombinant 56-kD proteins in 6 M urea.** The 6 M urea supernatant containing r56 proteins was purified by diethylaminoethane (DEAE) anion-exchange chromatography with a 50-minute linear gradient of 0.30–0.70 M NaCl (Sigma) in 6 M urea, 20 mM Tris-HCl, pH 8.0 (buffer B). The purity of protein in each fraction was accessed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and fractions with > 95% purity were pooled. If the purity of protein was not satisfactory, a second run of DEAE chromatography was carried out after dialysis of the pooled fractions to remove NaCl. The procedure for the second DEAE purification was similar to that for the first DEAE purification but the linear gradient was 0.30–0.60 M NaCl for 50 minutes. The final fractions containing r56 proteins were evaluated for protein purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

**Refolding of pure recombinant 56-kD proteins by dialysis.** The purified r56 in 6 M urea was refolded by sequential dialysis in decreasing concentrations of urea as described by Ching and others. The purified polypeptides at a concentration of approximately 0.5 mg/mL in buffer B were transferred into a dialysis bag (24 mm, molecular weight cutoff value = 12,000 daltons) and dialyzed sequentially against 4 M, 2 M, and 0 M urea in buffer A. The final dialysis was carried out without urea in large excess of buffer A at 4°C to remove traces of urea.

**Analysis of purified recombinant 56-kD protein and verification of reactivity with antibody by Western blotting.** N-terminal sequencing and Western blotting were used to confirm that purified r56 was reactive with antibody. The protein was transferred from the gel onto a 0.45-µm polyvinylidene difluoride (Invitrogen) membrane and stained with CodeBlue staining solution to visualize protein bands. The single band between 37 kD and 50 kD was cut out of the membrane and analyzed...
formed as described. 40  Briefly, coated plates were first rinsed individual protein overnight at 4°C. The experiment was per-
method. The plates were coated with 0.3 µg/well/100 µL of 
ous strains of 
teins and serum samples from mice challenged by vari-
protocol # D01-06.
Center Institutional Animal Care and Use Committee under 
by the Walter Reed Army Institute/Naval Medical Research 
weeks after challenge and their blood was collected by cardiac 
mice that survived the infection after 21 days were killed three 
for an additional 21 days to assess morbidity and mortality. 
ology challenged with different strains of live Orientia as 
and, The challenged mice were monitored once a day 
for an additional 21 days to assess morbidity and mortality. Mice that survived the infection after 21 days were killed three 
weeks after challenge and their blood was collected by cardiac puncture. The animal protocol used in this study was approved 
by the Walter Reed Army Institute/Naval Medical Research 
Center Institutional Animal Care and Use Committee under 
protocol # D01-06.

Mouse immunization. Female CD-1 mice were intraperi-
toneally challenged with different strains of live Orientia as 
described. 39  The challenged mice were monitored once a day 
of the minimum number of r56 proteins to provide the 
for the development of a broadly protective vaccine. With the exception of TA763, they 
were human isolates from a wide range of geographic locations within the Orientia triangle. The restriction fragment length polymorphism of GroEL and Western blot analysis of

by a protein N-terminal 491 sequencer (Applied Biosystems, 
Forster City, CA) to confirm that the expressed protein was 
correct. In addition, another gel was run and transferred onto 
nitrocellulose membrane. The membrane was blocked with 
10% milk in Tris-buffered saline (TBS)–Tween20 (TBST; Bio-
Rad, Hercules, CA) for one hour with gentle agitation, washed three times with TBST (five minutes/wash), and incubated 
with anti-Kpr56 mouse serum in TBST containing 5% milk 
for one hour at room temperature. The membrane was washed 
three times (five minutes/wash) and incubated with horserad-
ish peroxidase (HRP)–conjugated anti-mouse IgG secondary 
 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 
TBST with 5% milk for one hour. The membrane was then 
washed and incubated with substrate as described by the man-
ufacturer (Bio-Rad).

Analysis of reactivity of various recombinant 56-kD proteins 
and serum samples from mice challenged by vari-
ous strains of O. tsutsugamushi. The concentration of each 
r56 protein antigen was determined by using the Bradford 
method. The plates were coated with 0.3 µg/well/100 µL of 
individual protein overnight at 4°C. The experiment was per-
formed as described. 41 Briefly, coated plates were first rinsed 
three times with 1× phosphate-buffered saline (PBS) contain-
ing 0.1% Tween 20 (1× PBST), blocked with 200 µL/well of 
10% milk in 1× PBST, and incubated for one hour at room 
temperature. Primary antibody (mouse serum samples for 
12 strains and human serum samples for 2 strains) was serial 
diluted by a factor of 4 (1:100, 1:400, 1:1,600) in 5% milk in 
1× PBST. Plates were washed after blocking and incubated 
with diluted primary antibody. The plates were then washed 
again and incubated with HRP-conjugated goat anti-mouse 
IgG or HRP-conjugated rabbit anti-human IgG (Santa Cruz 
Biotechnology) at 1:4,000 dilution for one hour at room 
temperature. At the end of incubation, plates were washed and 
substrate (Kirkegaard and Perry Laboratories, Gaithersburg, 
MD) was added. The plates were incubated at room tempera-
ture for 15–30 minutes in the dark and read at 405–650 nm 
on a UVmax kinetic microplate reader (Molecular Devices, 
Sunnyvale, CA). The mean of the negative controls plus two 
times the standard deviation was used as the cutoff value for 
determining a positive result. The cutoff value was 0.1 for 
mouse serum samples, 0.1 for MAK119 serum samples, and 
0.04 for MAK243 serum samples.

RESULTS

Protein purification and Western blotting. All r56 proteins 
were purified to achieve > 98% purity as shown in Figure 2A. 
In most cases, protein was overloaded in each gel lane to 
ensure purity. The same amount of each r56 was loaded 
onto a gel for Western blotting. Figure 2B shows that all r56 
proteins reacted with the specific antibody against the 56-kD 
protein from the Karp strain. Although they are all reactive to 
the same antibody, the intensity of the band differed, indicat-
ning that there are differences among different r56. Ktr56 and 
Gmr56 appeared to be the least reactive with the antibody.

Evaluation of cross-reactivity of r56s with strain-specific 
mouse serum by enzyme-linked immunosorbent assay. We 
have repetitively evaluated the protective efficacy of r56 as a 
vaccine candidate by challenging immunized mice with homol-
ogous and heterologous strains of Orientia. From our previous 
work, it is evident that r56 from several different strains when 
used alone or in combination was unable to provide substan-
tial protection from heterologous challenges. To better under-
stand whether inclusion of certain r56s would be beneficial as 
a vaccine candidate, we designed three additional chimeric r56 
proteins and evaluated their cross-reactivity.

Comparison of cross-reactivity was also performed with r56 
protein from Karp, Kato, Gilliam and TA763 strains to deter-
mine which r56 appeared to be the most reactive with dif-
ferent strain-specific mouse serum samples. Because mouse 
serum samples were obtained from mice that survived the 
lethal challenge by individual strains of Orientia, the antibod-
ies generated by each strain should exhibit the strongest reac-
tivity with its respective r56 protein. This feature enabled us 
to find the minimum number of r56 proteins to provide the 
broadest and strongest cross-reactivity.

We obtained serum samples from mice that survived chal-
lenge with 12 different strains and serum samples from two 
human patients who were infected with two additional strains. 
These strains represented a wide range of strain diversity 
(Table 1) and were carefully selected for the development of a 
broadly protective vaccine. With the exception of TA763, they 
were human isolates from a wide range of geographic loca-
tions within the Orientia triangle. The restriction fragment length polymorphism of GroEL and Western blot analysis of

![Figure 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting of all r56 recombinant proteins of Orientia tsutsugamushi. A, SDS-PAGE of all 7 r56s with purity > 98%. B, Western blot results with anti-Kpr56 mouse serum. All r56s were loaded in equal amounts. This figure appears in color at www.ajtmh.org.](image-url)
whole cell lysate by monoclonal antibody are distinctly different. Their growth rates in tissue culture are also different. Immunization using r56 was never protective to subsequent challenged with MAK119 and MAK243 in our mouse model. Thus, human patient serum samples were used for these two strains.

All serum samples tested diluted to 1:100 reacted with every r56. However, the titers of each serum sample against all seven r56 proteins were different (Tables 2 and 3). Kpr56, Ktr56, Gmr56, and TA763r56 had the highest titers with respective serum samples from homologous challenge, confirming that these antigens were most reactive towards their own specific antigen. It was observed (Table 2) that 1) Kpr56 was the most reactive antigen for serum samples from TH1812, 18032460, 18032404, Woods, and Citrano; 2) TA763 was the most reactive antigen for TH1811, TH1812, TH1814, AFC-27, 18032404, Woods, Citrano, and MAK243; 3) Gmr56 was the least reactive antigen for serum samples from Karp, Kato, TA763, TH1811, TH1812, AFC-27, Citrano, MAK 119, and MAK243; and 4) Ktr56 was the least reactive antigen for serum samples from Karp, Gilliam, TA763, TH1811, TH1814, 18032460, 18032404, Woods, Citrano, MAK119, and MAK243. The results that Ktr56 and Gmr56 were the least reactive antigen with Karp-challenged serum are consistent with Western blot results.

All chimeric r56s reacted similarly for any given serum sample. Ktr56 or Gmr56 was not more reactive than any of the chimeric r56 toward any heterologous strains (Tables 2 and 3). Furthermore, for most tested serum samples with the exception of Karp, TA763, TH1811 and TH1812, all chimeric r56s were as reactive as either Kpr56 or TAr56. These results suggested that although the variable domain sequence was modified on the basis of the parent 56-pkd protein, these chimerics still retained similar seroreactivity with most serum samples and could substitute both Kpr56 and TAr56. When comparing the chimerics, C1 appeared to be the best antigen because 14 serum samples had higher titers against C1 than those against C2 and C3. Titers against C1 were as high as those against C2 and C3. Titers against C1 were as high as those for Kpr56 or TAr56 for seven different serum samples (Table 3). Although it is difficult to correlate titer against a specific antigen with the overall stimulation of protective immune responses, which consists of humoral and cellular responses, the measurement of titers provides information about the reactivity of certain antigens to antibodies. Thus, it is plausible that these chimerics can be used as reagents for diagnostic purposes and as vaccine candidates. Careful examination of data presented in Tables 2 and 3 shows that inclusion of C1 and Ktr56 is the best combination that may provide the most reactive mixture of r56s with high titers (minimum = 1,600) against all strains evaluated. This combination may provide vaccine candidates that cover the broadest strain variation and have high reactivity for future vaccine candidate evaluation.

**Discussion**

Cross-reactivity of chimeric protein antigens with serum samples from mice challenged individually with various strains of *Orientia* were analyzed. Titers of these serum samples against the recombinant 56-kD protein of Karp, Kato, Gilliam, and TA763 strains were also compared. These newly made chimeric proteins exhibited similar reactivity with different strain-specific serum samples as the parent proteins.
Furthermore, these chimeric antigens were recognized readily by 14 disparate strains of Orientia and they appeared cross-reactive with more strains than Ktr56 and Gmr56. Therefore, one of the three chimerics, particularly C1, can be used as a substitute for the parent proteins for use in diagnosis of Orientia infection and as a vaccine candidate to increase the broad protective efficacy. A similar approach can be used to generate more chimerics based on 56-kD protein sequences from different strains of Orientia to minimize the number of proteins included in the final vaccine formula and still provide a broad protection against a wide range of strains. 

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