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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INTRODUCTION

Orientia tsutsugamushi, the etiologic agent of scrub typhus, 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.

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 acids is designed to create chimeric proteins that share the maximum sequence homology with the 56-kD protein from TA763. The chimeric proteins are constructed by shuffling the epitopes in the variable domains of the Karp and TA763 proteins. We evaluated the seroreactivity of these proteins with serum samples from mice infected with various strains of Orientia.

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.
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 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 NdeI 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 NdeI 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 renografin 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 the DNA sequences of chimeric 1 and 2, respectively. Amino acids in VD II and VD III of Kpr56 were replaced with corresponding amino acids in TAr56.

Similar criteria were used for the design of chimeric 2 (C2), which was used as the backbone, and variable domain 3 was replaced with the sequence of chimeric 3. Amino acids in VD I and VD III were the same as those in chimeric 1 and 2, respectively. Amino acids in VD II of TA56 were replaced with corresponding amino acids in Kpr56.

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 Ultrasound 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. Usually 3 dialysis bags, with 10 or 20 mL each in a 500-mL or 1,000-mL beaker, respectively, were dialyzed against buffer at a ratio of 1:15. The dialysis was continued overnight without ourea 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.

Figure 1. Protein sequences of three chimeric proteins of Orientia tsutsugamushi. Variable domains (VD) containing changed amino acids are underlined. A, sequence of chimeric 1. Amino acids in red within VD I of TA56 were replaced with corresponding amino acids in Kpr56. B, sequence of chimeric 2. Amino acids in red within VD III of Kpr56 were replaced with corresponding amino acids in TA56. C, sequence of chimeric 3. Amino acids in VD I and VD III were the same as those in chimeric 1 and 2, respectively. Amino acids in VD II of TA56 were replaced with corresponding amino acids in Kpr56.
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 horseradish 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 manufacturer (Bio-Rad).

**Mouse immunization.** Female CD-1 mice were intraperitoneally challenged with different strains of live *Orientia* as described.\(^{39}\) 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.

**Analysis of reactivity of various recombinant 56-kD proteins and serum samples from mice challenged by various 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 performed as described.\(^{40}\) Briefly, coated plates were first rinsed three times with 1× phosphate-buffered saline (PBS) containing 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 a 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 temperature 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, indicating 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 homologous 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 substantial protection from heterologous challenges. To better understand 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 determine which r56 appeared to be the most reactive with different strain-specific mouse serum samples. Because mouse serum samples were obtained from mice that survived the lethal challenge by individual strains of *Orientia*, the antibodies generated by each strain should exhibit the strongest reactivity 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 challenge 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 locations within the *Orientia* triangle. The restriction fragment length polymorphism of *GroEL* and Western blot analysis of...
ELISA titers of 14 strain-specific serum samples against chimeric r56 proteins of *Orientia tsutsugamushi*.

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* Enzyme-linked immunosorbent assay (ELISA) was performed as described in the Materials and Methods. All serum samples were from mice with the exception of MAK119 and MAK243, which were from humans. The positive cutoff value was at least the average optical density value at 405 nm for negative serum + 2 SD. For mouse serum samples, the cutoff was 0.1. For MAK119 and MAK243, the cutoff values were 0.1 and 0.04, respectively.

† Strains in bold are those with the same titers for at least one of chimerics and the parent Kpr56 and/or TAr56.

‡ Showed highest titers with C1.

§ Showed highest titers with C2.

¶ Showed highest titers with C3.

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 *Orienteria* 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 *Orienteria* 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 *Orienteria* 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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Disclosure: Chien-Chung Chao and Wei-Mei Ching are employees of the U.S. Government. This work was prepared as part of their official duties.

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