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Mapping of Antigenic Determinant Regions of the Bor56 Protein of *Orientia tsutsugamushi*

SEUNG-YONG SEONG,[1] SAE-GWANG PARK,[2] MYUNG-SUK HUH,[2] WON-JONG JANG,[2] HANG-RAE KIM,[2] TAE-HEE HAN,[3] MYUNG-SIK CHOI,[2] WOO-HYUN CHANG,[4] AND IK-SANG KIM[2]*

Biomedical Research Center, Korea Institute of Science and Technology, Seoul 136-791,[1] Department of Microbiology, Seoul National University College of Medicine, 28 Yongon-dong, Seoul 110-799,[2] Department of Microbiology, Sungkyunkwan University College of Medicine, Suwon 440-746,[3] and Department of Microbiology, College of Medicine, Hallym University, Chunchon 200-702,[4] Republic of Korea

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The 56-kDa protein (Bor56) of *Orientia tsutsugamushi* is an immunoprotective antigen and is the target molecule of neutralizing antibodies. This antigen is recognized by almost all of the serum antibodies produced by patients in the convalescence phase of scrub typhus. We expressed the Bor56 open reading frame in *Escherichia coli* and generated from it a series of deletion constructs as MalE fusion proteins. Antibody-binding domains were characterized by using patient sera, mouse monoclonal antibodies (MAbs), and Bor56-immunized-mouse sera. None of the antibodies bound to a fusion protein containing the carboxy-terminal 140 amino acids (aa) of the Bor56 protein, suggesting that the carboxy-terminal domain of Bor56 is not exposed on the surface of the molecule. Human immunoglobulin M (IgM) antibodies predominantly bound to antigenic domain I (AD I; amino acids [aa] 19 to 113) and AD III (aa 243 to 328). Human IgG antibodies also showed preferential binding to AD I. The epitope recognized by strain-specific MAb (KI4) or group-specific MAb (KI57) was mapped to AD II (aa 142 to 203). Mouse serum antibodies, elicited by immunization with deletion mutants, consistently bound to AD III. Moreover, the carboxy-terminal 140 aa of the Bor56 protein did not elicit an antibody response in C3H/HeDub mice. A model of the antigenic structure of Bor56 is presented and discussed. These results suggest that antigenic fragments from AD I and AD III are useful in the induction of humoral immunity against *O. tsutsugamushi*. These antigenic analyses provide an important foundation for further analyses of the neutralizing-antibody responses generated during rickettsial infections. They also provide potential peptide substrates for diagnostic assays and vaccine strategies.

Scrub typhus is one of the most common vector-borne diseases in the Republic of Korea and in the Asia-Pacific region (5). It is caused by *Orientia tsutsugamushi*, which belongs to the family *Rickettsiaceae* (42). This disease is characterized by fever, rash, eschar, pneumonitis, meningitis, and disseminated intravascular coagulation, which in some cases leads to circulatory collapse (4, 23). It is thought to be transmitted to humans when chigger mites infected by *O. tsutsugamushi* bite them (44). *Leptotrombidium* (Acari; Trombiculidae) mites are the known vector and reservoir host for *O. tsutsugamushi*. In their larval stage, these mites must feed on the blood of vertebrates for their metamorphosis into eight-legged nymphs and subsequently into adults (44). As the transient parasitism of mites on wild rodents becomes established, the transmission of *O. tsutsugamushi* to humans can occur (44).

The natural immune response to *O. tsutsugamushi* is characterized by both prominent humoral responses (17, 27, 34) and TH1-mediated cellular responses (12, 19, 34). The mechanisms responsible for protective immunity of humans to *O. tsutsugamushi* may involve both humoral immunity and cell-mediated immunity (2, 3, 11, 12, 14, 19, 31, 34, 35, 38). The cell-mediated acquired immune response to *O. tsutsugamushi* infection in both humans and mice is a complex event that is believed to involve a variety of T-cell subsets, which manifest themselves in numerous functions, including protection (15, 19, 28), delayed-type hypersensitivity (13), and the establishment of a state of memory immunity (19). Bor56 protein stimulates a potent proliferative response and cytokine production in mouse lymphocytes (34), suggesting that this protein plays an important role in cell-mediated immunity.

The role of antibody-mediated immunity after infection is also important in protective immunity (11, 31, 33, 35). The protection of mice from acute infection was experimentally accomplished by the transfer of immune serum (1, 33). The in vivo neutralizing capacity of immune serum from a convalescing or hyperimmunized animal was used in the analysis of antigenic variants of *O. tsutsugamushi* (1, 30). The antigenic heterogeneity of *O. tsutsugamushi* strains isolated from infected humans, animals, and chigger sources has previously been well documented (1, 5, 30). A series of such studies has previously demonstrated the protection of mice from an otherwise lethal dose of *O. tsutsugamushi* by strain-specific immune serum. Furthermore, a strain-specific neutralizing activity of immune serum was shown in in vitro culture systems (11). Mice that have recovered from infection with one serotype of *O. tsutsugamushi* develop antibodies that react with homologous and heterologous serotypes, but the protective immunity is largely serotype specific. A recent study has shown that antibodies to Bor56 neutralize rickettsial infection in vitro (35).

Several vaccines for the prevention of scrub typhus have previously been studied (3, 12, 34, 38). However, vaccine use is limited because of high production costs and low protectivity against heterotypic strains. Therefore, to develop effective recombinant-subunit vaccines, protective antigens and their T-

* Corresponding author. Mailing address: Department of Microbiology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Korea. Phone: 82-2-740-8304. Fax: 82-2-743-0881.

TABLE 1. Oligonucleotide primers used in this study

Primer	Description	Sequence[a]
Pmbrf1	*bor56* gene forward PCR primer at positions 606 to 630	5′-GAT GGT ACC TCC GGT GTT GTT AAA T-3′
Pmbrf2	*bor56* gene forward PCR primer at positions 984 to 1007	5′-TCA GGT ACC CTT GAA TTT TGT CAT-3′
Pmbrf3	*bor56* gene forward PCR primer at positions 1179 to 1202	5′-CAT GGT ACC ATT AGC TGC CCA ACA-3′
Pmbrf4	*bor56* gene forward PCR primer at positions 1446 to 1469	5′-CAT GGT ACC ATC AGG AGC ACT TGG-3′
P1613r	*malE* gene reverse PCR primer at positions 2707 to 2684 (pIH821 reverse complementary sequence)	5′-TAG AGG ATC CAT CGA GGG TTT TAC-3′

[a] The *Kpn*I restriction site is underlined.

and B-cell epitopes must be identified. The immune responses to Bor56 are important in preventing infection (34). The Bor56 protein, which is the type-specific antigen (Tsa) of *O. tsutsugamushi*, is a major outer membrane protein of 54 to 56 kDa. This protein induces both humoral immunity and cellular immunity in mice (34, 35); mice immunized with recombinant Bor56 are protected from challenge with the homologous strain of *O. tsutsugamushi* (34). As a result, Bor56 has become the primary candidate for a scrub typhus recombinant vaccine. Mice immunized with Bor56 develop antibodies reactive with *O. tsutsugamushi* Boryong shortly after immunization. Furthermore, humans infected with *O. tsutsugamushi* also develop antibodies that are reactive with Bor56 (17, 18). Hence, characterization of T- and B-cell epitopes of the Bor56 protein is directly relevant to the development of a novel rickettsial vaccine. The potential importance of the Bor56 protein as an immunogen led us to examine the antibody responses of patients to this antigen. Because the antigenic determinants on this molecule could form the basis of a recombinant-subunit vaccine, determinations of the antigenicity and immunoaccessibility of epitopes should permit the rational selection of candidate domains. In an effort to identify antibody-binding domains of Bor56, we generated a family of deletion mutants of Bor56. Using these constructs, we identified mouse antibody-binding domains and regions of the protein that are immunogenic in mice. We also used these constructs to identify a region of the molecule that may be preferentially recognized by the sera of patients recuperating from scrub typhus.

MATERIALS AND METHODS

Construction of Bor56 expression vectors. The Bor56 open reading frame of *O. tsutsugamushi* Boryong was retrieved from a genomic expression library in λgt11 by screening with a mouse monoclonal antibody (MAb) (17). This clone was inserted into pBluescript (Stratagene, La Jolla, Calif.) and modified as described previously (17). The *Sma*I-*Bst*EII fragment of pBY52 (17) was removed by digestion with *Sma*I-*Bst*EII, end refilled with Klenow fragment, and self-ligated with T4 DNA ligase to generate clone pMS89. This plasmid was digested with *Sac*I-*Not*I and unidirectionally deleted by using an Erase-a-Base kit (Promega, Madison, Wis.). The variable length of DNA was obtained by exonuclease digestion for various reaction times. Ends were blunted by using S1 nuclease and Klenow fragment. DNA fragments with various deletion endpoints were digested with *Hin*dIII, ligated with *Stu*I-*Hin*dIII-predigested pIH821 (New England Biolabs, Beverly, Mass.), and then used to transform *Escherichia coli* XL1-Blue (Stratagene). Expression was monitored by immunoblot assays as previously described (17).

Some deletion mutants (ΔBor56) were generated by PCR. Plasmid pMS89, containing the *bor56* gene cloned from *O. tsutsugamushi* genomic DNA, was used as the template for PCR. Oligonucleotide primers flanked by restriction sites were designed on the basis of the *bor56* sequence (GenBank accession no. L04956) and are shown in Table 1. Each primer was used at a concentration of 0.4 mM. Twenty-five cycles of PCR were performed, with each cycle consisting of denaturation at 94°C for 15 s and annealing-extension at 60°C for 1 min. Amplified ΔBor56 fragments were electrophoretically separated on 2% agarose gels and purified by using a Qiaex gel elution kit (Qiagen Inc., Catsworth, Calif.). The fragments were digested with *Kpn*I-*Eco*RI, purified, and directionally cloned into the pIH821 expression vector. *E. coli* XL1-Blue was transformed by electroporation. Recombinant colonies were grown on Luria-Bertani plates containing 100 μg of ampicillin per ml. Recombinant clones were identified by colony hybridization with the insert DNA of pMBR56 (18) labeled with digoxigenin as the probe as described by the manufacturer (DIG labeling kit; Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

The nucleotide sequences of the 5′ ends of deletion plasmids were determined with a *malE* primer (New England Biolabs). Purified double-stranded DNA was used as a substrate for sequencing. DNA sequencing was performed by the dideoxy nucleotide chain termination method with α-^{35}S-dATP and Sequenase (U.S. Biochemical Corp., Cleveland, Ohio).

Purification of deletion mutants and immunoblot analysis. Recombinant proteins were purified by amylose column chromatography (New England Biolabs) as described previously (17, 18). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as described elsewhere (17, 18).

Preparation of anti-ΔBor56 mouse serum. Ten female C3H/HeDub mice were immunized with ΔBor56 as follows. ΔBor56 (1 μM) in 0.2 ml of phosphate-buffered saline was emulsified with aluminum hydroxide and injected subcutaneously. Mice were immunized three times over a 4-week interval. Blood samples were collected from each mouse to monitor antibody titers. Three weeks after the last immunization, mice were bled and their sera were prepared as described previously (5). Antibody titers to *O. tsutsugamushi* Boryong and MalE were examined by indirect immunofluorescent antibody (IFA) test (17, 18) and passive hemagglutination assay (18), respectively.

MAbs. The isolation of MAbs KI4 and KI57 has been described previously (5). These MAbs are specific for the Tsa of *O. tsutsugamushi*. Hybridoma cells secreting MAbs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco/BRL, Grand Island, N.Y.). MAbs were prepared as culture supernatants in spent tissue culture medium. They were used without further purification. The specificity and titer of each MAb to *O. tsutsugamushi* Boryong was determined by IFA test (5).

Patients. Sera were obtained from individuals as described previously (21, 23). A total of 20 cases were selected among patients with immunoglobulin G (IgG) antibody titers of more than 1:1,280 and IgM antibody titers of more than 1:320 to the Boryong antigen by IFA test.

Competition ELISA. Antibody binding to the amino-terminal regions of ΔBor56 was determined by a competition enzyme-linked immunosorbent assay (ELISA). In order to detect antibodies bound to the amino terminus of each ΔBor56, sera were preabsorbed in the presence of competitive ΔBor56, which was smaller than the antigen coated in solid phase.

A preliminary titration was conducted to determine the optimal concentration of competitive ΔBor56 needed to block antibodies completely when 5 nM ΔBor56 was used in the solid phase. Polystyrene 96-well plates (Nalge Nunc International, Naperville, Ill.) were coated with purified ΔBor56 at a concentration of 5 nM in 0.05 M carbonate buffer (pH 9.6). After overnight adsorption at 4°C, the contents of the plate were discarded and wells were filled with a 5% skim milk solution and incubated for 1 h at room temperature for blocking. Pooled patient sera were preabsorbed with variable concentrations of competitive ΔBor56 for 1 h at room temperature during blocking. The same deletion construct was used to coat and compete for preliminary titration. The contents were again discarded, and preabsorbed sera were allowed to react with the antigen in the solid phase. This reaction was followed by incubation with secondary antibodies and reaction with the substrate (1,2-phenylenediamine). All reagents were used in a standard volume of 100 μl, and the reaction continued for 1 h at room temperature. After stopping the reaction by adding 1 M H_2SO_4, the optical density at 492 nm (OD_{492}) was measured.

Because no significant decrease in OD_{492} was observed at a concentration of competitive ΔBor56 of more than 50 nM, competitive ΔBor56 was used at 50 nM throughout this study. To validate the complete absorption of antibodies to competitive ΔBor56, sera from mice immunized with each ΔBor56 were analyzed for reactivities to a ΔBor56 that was larger than the antigen used in immunization. Wells were coated with this larger ΔBor56, and sera from mice immunized with a smaller ΔBor56 were preabsorbed to a ΔBor56 of the same size as that used in immunization before being reacted with a larger ΔBor56 in the solid phase. The average OD_{492} and standard deviation were 0.16 and 0.13, respectively. When the cutoff value of 0.41 (average plus 2 standard deviations) was considered, the specific binding of serum to the larger ΔBor56 was not observed after absorption. MAbs showed specific binding to only one region of ΔBor56 when the cutoff value was considered.

To detect whether antibodies bound to the amino terminus of ΔBor56, patient

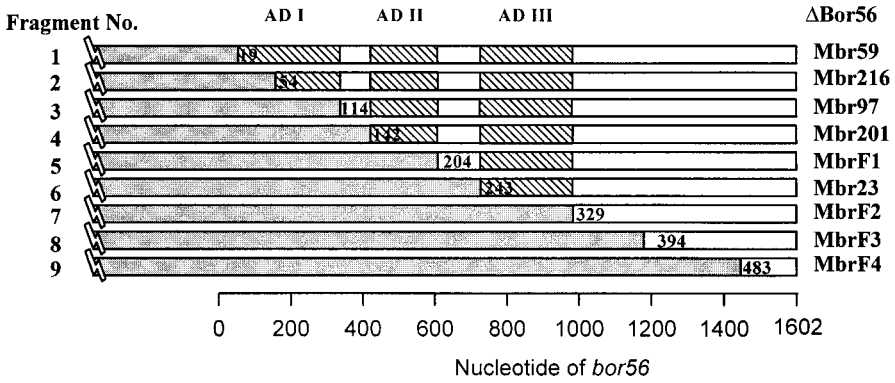

FIG. 1. Deletion of the Bor56 protein from its amino terminus. The Bor56 open reading frame was fused to the *malE* gene of expression vector pIH821 at the *Stu*I-*Hin*dIII site. Deletion constructs were made by fusing the vector with fragments of the *bor56* gene, generated by unidirectional deletion or PCR-mediated deletion of amino-terminal sequences. Filled and open bars represent MalE and deletion fragments of Bor56 protein, respectively. A hatched region denotes an AD suggested in this study. Nucleotide residues are indicated at the bottom. The number immediately after a filled bar denotes the first amino acid of the deletion mutant fused to MalE.

sera were diluted 1:50 with phosphate-buffered saline containing 0.05% Tween 20 and allowed to react with an equal volume of competitive ΔBor56 in 50 nM at room temperature for 1 h. Preabsorbed sera were reacted in the solid phase with an antigen that was larger than the competitive ΔBor56. For example, to detect antibodies directed to the amino terminus of fragment 8 (amino acids [aa] 394 to 482), sera were preabsorbed to fragment 9 (aa 483 to 533) and were reacted with fragment 8 (aa 394 to 533) in the solid phase. To detect antibodies directed to the carboxy terminus of Bor56 (aa 483 to 533), sera were preabsorbed with MalE.

The secondary antibodies used were γ-chain-specific goat anti-human IgG, μ-chain-specific goat anti-human IgM, and goat anti-mouse $F(ab')_2$ (Organon Teknika Corp., Durham, N.C.). All of the secondary antibodies were conjugated with horseradish peroxidase. Sera were always assayed in duplicate, and substrate-blank, negative controls that lacked primary antibody were included on each plate. Serum-negative controls were also included for each ΔBor56, and their values were subtracted from the sample ODs obtained from the corresponding ΔBor56. The reactivities of antibodies to the amino terminus of each fragment were calculated from the ODs obtained by competition ELISA as follows. Because the molecular masses and altered structures of recombinant antigens could determine the binding efficiency to a 96-well plate, the ODs of serum specimens to each ΔBor56 were normalized by the binding efficiency factor of the recombinant antigen. The binding efficiency of the Mbr59 protein was arbitrarily assigned a value of 1. The binding efficiency of each ΔBor56 was derived by dividing the OD of the anti-MalE antibody to ΔBor56 by the OD of the anti-MalE antibody to Mbr59 protein. In the OD range from 0.2 to 1.8, the OD correlated well with the amount of antigen tested. The corrected ODs of serum specimens for each fragment were obtained by dividing the ODs by the binding efficiency of each ΔBor56.

RESULTS

Generation of deletion mutants of Bor56 protein. Prokaryotic expression plasmids encoding either the full-length protein (without its signal peptide) or truncated forms of Bor56 were expressed in *E. coli*. Deletions of the Bor56 protein were constructed by unidirectional cloning or PCR-mediated deletion (Fig. 1) to create a family of proteins that contain various lengths of coding sequence. Five of the clones (pMBR59, pMBR216, pMBR97, pMBR201, and pMBR23) were constructed by unidirectional deletion; four clones (pMBRF1, pMBRF2, pMBRF3, and pMBRF4) were constructed by PCR because these smaller proteins could not be cloned from the deletion library by immunologic screening with mouse anti-Boryong serum. Southern blot analysis showed that each recombinant plasmid contained the truncated *bor56* gene. The nucleotide sequences of the 5′ ends of deletion mutants were determined by DNA sequencing. The nucleotide of the 5′ end of each deletion clone and its inferred first amino acid residue are shown in Fig. 1. Each of these expression clones was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma, St. Louis, Mo.). Each construct encoded a fusion product that was clearly distinguishable on a Coomassie-stained gel. Truncated proteins were purified from *E. coli* lysates by amylose affinity chromatography. MalE was also purified from the lysate of *E. coli* transformed by vector pIH821. Figure 2 shows the results of SDS-PAGE analysis of

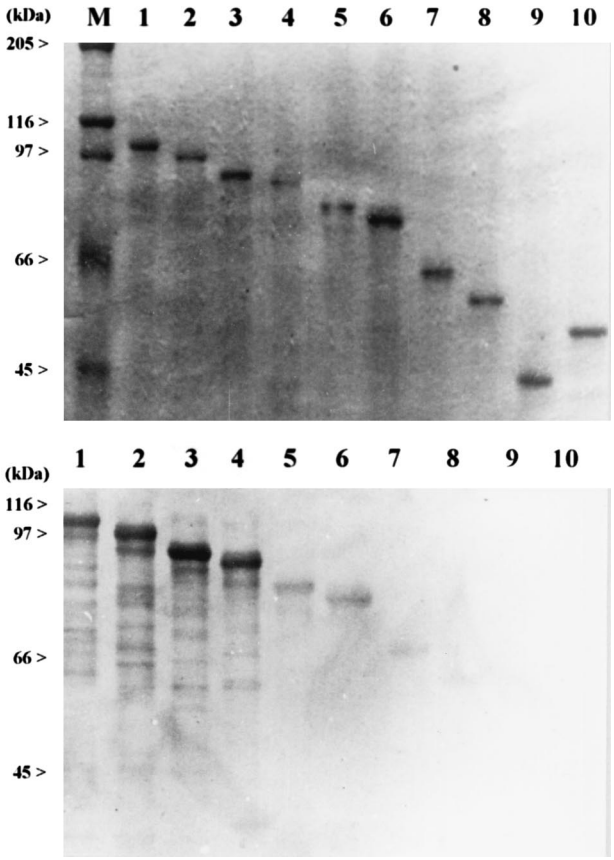

FIG. 2. Coomassie brilliant blue staining (top) and immunoblot analysis (bottom) of purified ΔBor56 proteins. Recombinant proteins were purified by affinity chromatography and separated by SDS–10% PAGE. Immunoblots were performed with mouse anti-Boryong serum. Lane M, molecular mass markers; lanes 1, Mbr59; lanes 2, Mbr216; lanes 3, Mbr97; lanes 4, Mbr201; lanes 5, MBRF1; lanes 6, Mbr23; lanes 7, MBRF2; lanes 8, MBRF3; lanes 9, MBRF4; lanes 10, MalE.

TABLE 2. Binding of Bor56 recombinant fragments by sera from scrub typhus patients and hyperimmunized mice and by MAbs

Serum group or MAb	Ig class(es) with highest level(s) of binding to fragment[a]								
	1 (19)	2 (54)	3 (114)	4 (142)	5 (204)	6 (243)	7 (329)	8 (394)	9 (483)
Human[b]									
1	G,M	−	−	−	−	−	−	−	−
2	G,M	−	−	−	−	−	−	−	−
3	G	M	−	−	−	−	−	−	−
4	G	M	−	−	−	−	−	−	−
5	G	M	−	−	−	−	−	−	−
6	G	−	−	M	−	−	−	−	−
7	−	G,M	−	−	−	−	−	−	−
8	−	G	−	−	M	−	−	−	−
9	−	G	−	−	M	−	−	−	−
10	−	G	−	−	−	M	−	−	−
11	−	G	−	−	−	M	−	−	−
12	−	−	G,M	−	−	−	−	−	−
13	M	−	−	−	−	G	−	−	−
14	−	M	−	−	−	G	−	−	−
15	−	M	−	−	−	G	−	−	−
16	−	−	M	−	−	G	−	−	−
17	−	−	−	−	M	G	−	−	−
18	M	−	−	−	−	−	G	−	−
19	−	M	−	−	−	−	G	−	−
20	−	−	−	−	−	M	G	−	−
NHS[c]	−	−	−	−	−	−	−	−	−
Mouse[d]									
1	−	−	+	−	+	−	−	−	−
2	−	−	+	+	−	+	−	−	−
3	−	−	−	+	−	+	−	−	−
4	−	−	−	+	−	+	+	−	−
5	−	−	−	−	+	−	−	−	−
6	−	−	−	−	−	+	+	−	−
7	−	−	−	−	−	−	+	−	−
8	−	−	−	−	−	−	−	−	−
9	−	−	−	−	−	−	−	−	−
MalE	−	−	−	−	−	−	−	−	−
MAbs									
KI4	−	−	−	+	−	−	−	−	−
KI57	−	−	−	+	−	−	−	−	−

[a] Parenthetical data are the first amino acids of the corresponding Bor56 recombinant fragments.

[b] Human sera are listed according to patient number. −, other binding levels.

[c] NHS, normal human serum pooled from 20 healthy human donors. It showed an antibody titer to Boryong of less than 1:10 by IFA test.

[d] The number of the fragment used to immunize each mouse is indicated. Pooled serum from 10 C3H/HeDub mice, each immunized with a different ΔBor56 mutant, was used in the competition ELISA. +, binding levels above the cutoff value; −, binding levels below the cutoff value.

several of the constructs illustrated in Fig. 1 after induced overexpression and purification. SDS-PAGE analysis of purified ΔBor56 proteins demonstrated that the fusion proteins contained an additional expected component, MalE (Fig. 2). Western blot analysis of the mutant proteins with mouse anti-Boryong serum showed that mouse antibodies bound preferentially to the amino-terminal region of Bor56 and were only weakly reactive with the carboxyl end.

Mapping antibody-binding domains of patient sera. Serum samples were obtained from 20 scrub typhus patients and used in a competition ELISA. The highest levels of binding by serum antibodies from individual patients to the defined region of ΔBor56 are shown in Table 2. IgM antibodies from patients showed the highest binding to aa 19 to 113 (antigenic domain I [AD I]) of Bor56. The fragment containing aa 243 to 328 (AD III) showed the highest binding of human IgM from three patients (15%). The highest levels of human IgG binding were also associated with AD I (11 cases) and AD III (5 cases) (Table 2). Eighty percent of sera showed preferential IgG binding to these two domains. None of the sera showed high IgG binding to aa 142 to 242 or to the carboxy-terminal 140 aa. As shown in Table 2, although the human IgM response to the Bor56 protein was localized to the amino-terminal 328 aa, the human IgG response to Bor56 localized predominantly to AD I and AD III. Seventy percent (14 of 20) of sera showed binding of the IgM antibody to AD I, and 65% (13 of 20) showed IgM binding to AD III (Fig. 3). Sixty-five percent of

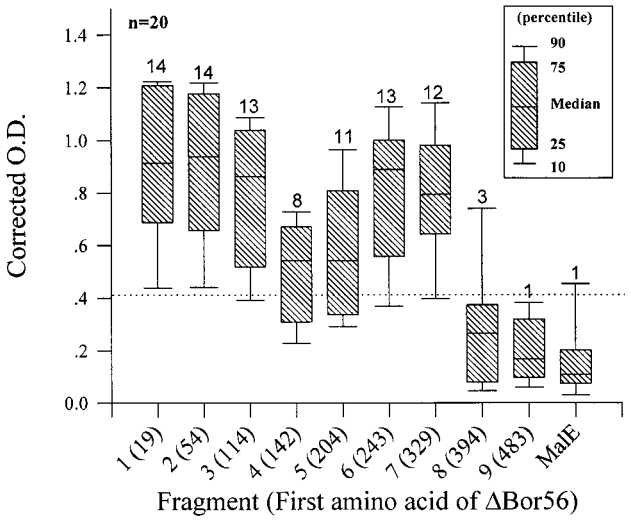

FIG. 3. Human IgM responses to deletion mutants, as determined by a competition ELISA. Human IgM was predominantly reactive with the amino-terminal part (aa 330 to 394) of Bor56. The lower boundary of each box indicates the 25th percentile, the line within each box marks the median, and the upper boundary of each box indicates the 75th percentile. Error bars above and below each box indicate the 90th and 10th percentiles, respectively. Twenty human serum samples with IgM titers of over 1:320 and IgG titers of over 1:1,280 by IFA test were analyzed. A number above an error bar represents the number of patient sera that showed an OD that was higher than the cutoff value of 0.41.

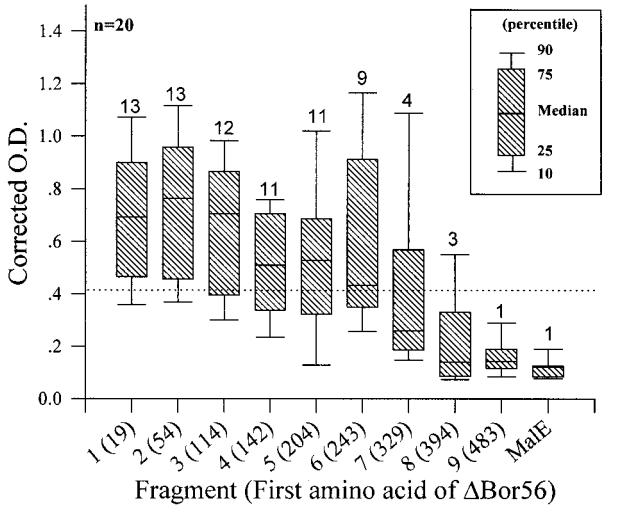

FIG. 4. Human IgG responses to deletion mutants, as determined by a competition ELISA. For an explanation of the presentation of data, see the legend to Fig. 3.

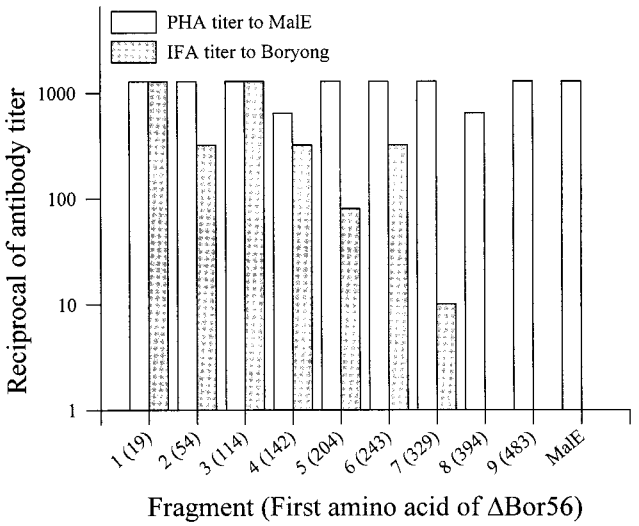

FIG. 5. Antibody production of mice immunized with Bor56 deletion mutants. Sera were serially diluted twofold and reacted with Boryong cultured in L929 cells for IFA tests. Murine IgG was detected by fluorescein isothiocyanate-conjugated goat anti-mouse Ig. Passive hemagglutination assays (PHA) (18) were also performed. The carboxyl fragment that extends from aa 395 to 533 of the native Bor56 protein did not elicit an antibody response in C3H/HeDub mice.

sera showed IgG binding to AD I (Fig. 4). Serum IgM and IgG responses to the carboxy-terminal 215 aa were consistently weak.

Mapping antibody-binding domains of mouse hyperimmune sera. We obtained sera from mice immunized with each ΔBor56 mutant to determine whether the anti-ΔBor56 antibody could recognize antigenic determinants on native Bor56 and to analyze the immunogenic domains of recombinant ΔBor56. C3H/HeDub mice were immunized with each ΔBor56 mutant. Antibody titers were determined by IFA test to detect antibody reactivity with the authentic protein of Boryong. Sera were also analyzed by passive hemagglutination assay to monitor the immunization procedure through the detection of anti-MalE antibodies. All of the fragments induced anti-MalE antibody production in mice. The anti-MalE antibody titers were consistently higher than 1:640 (Fig. 5). Deletions beyond aa 394 abrogated the immunogenicity of Bor56 protein. Immunizations of C3H/HeDub mice with fragment 7 (aa 329 to 533) induced weak antibody responses to the amino-terminal region of the antigen used for immunization. MalE-Bor56 protein, which encodes aa 85 to 533 of Bor56 protein, induces neutralizing antibodies and provides protective immunity in C3H mice (34, 35). The polyclonal humoral responses of mice elicited by immunization with MalE-Bor56 protein were also evaluated. This protein induced the production of antibodies directed to both AD II and AD III, producing the same immunogenic profile as that of fragment 3 (aa 114 to 533) (Table 2).

A competition ELISA was used to identify the immunogenic regions of each ΔBor56 mutant (Table 2). Fragments 2, 3, and 4 elicited antibody responses that were directed to AD II and AD III. However, fragment 1 induced antibody responses that were directed to aa 114 to 141 and 204 to 242. C3H/HeDub mice immunized with fragments 5 and 7 produced antibodies that were directed to the amino-terminal portions of immunizing antigens. Immunizations with fragments 8 and 9, however, did not produce any antibodies that were directed to rickettsial proteins or recombinant proteins.

MAb binding site. We produced strain-specific MAb KI4 and group-specific MAb KI57 (5). The present study was undertaken to identify the domains of Bor56 that determine antibody binding by these MAbs. To map the binding sites of these MAbs in Bor56, a competition ELISA was performed with the ΔBor56 expression clones depicted in Fig. 1. Table 2 shows the results of this analysis with both MAbs. When ΔBor56 mutants were probed with MAbs KI4 and KI57, only the fragment from aa 142 to 203 (AD II) was recognized.

DISCUSSION

The Tsa of *O. tsutsugamushi* is a surface-exposed (43), integral membrane protein (8, 24–26, 43). The strong immune responses of humans to this protein reflect its abundance on the cell surface and its potent immunogenicity (8, 10, 17, 18, 27). Animals inoculated with a crude preparation of *O. tsutsugamushi* develop prominent immune responses to the Tsa (34, 35, 39). Previous studies have shown that mice immunized with recombinant Bor56, the Tsa derived from *O. tsutsugamushi* Boryong, elicit T cells and antibodies that react with *O. tsutsugamushi* (39). Furthermore, the significance of the antibody response to Bor56 protein in neutralizing infection has previously been elucidated (35).

Hyperimmune antirickettsial serum has previously been shown to prevent *O. tsutsugamushi* infection (1, 2, 11, 22, 30–33). In those studies, serum neutralization assays were used to identify serovariants (1). The results of such studies suggest that antibodies play important roles in protective immunity as well as in cell-mediated immunity.

As a basis for rational vaccine design, we analyzed the immunogenic and antibody-binding domains of Bor56. The amino-terminal 113 aa (AD I) of Bor56 showed the highest level of binding with both human IgG and IgM. However, the ΔBor56 immunogen did not elicit antibody recognition of this region in mice. This difference in reactivities between humans and mice indicates that these two groups are exposed to different antigenic determinants on the protein. Human anti-Bor56 serum was generated by epitopes exposed on the surface of an organism. In contrast, mouse anti-ΔBor56 serum was generated in response to soluble protein, which may have had different epitopes exposed on it. Therefore, the dissimilar results may simply reflect the availability of certain antigenic determinants to react with antibodies. In the case of recombinant proteins, the N-terminal region of Bor56 protein could be sterically hindered because of MalE, which is fused to the N terminus of Bor56 protein. The soluble form of ΔBor56 used in immunization probably has numerous antigenic determinants that differ from those of membrane-bound Bor56. Membrane-bound Bor56 protein likely exposes only limited determinants on the surface of the outer membrane of the organism. Furthermore, the major histocompatibility complex background of humans is so heterogeneous that T-cell help is probably directed to a variety of determinants. The humoral response of a mouse to this protein, however, is relatively confined to specific regions, because the *H-2* background of the mice used in this study is specific ($H\text{-}2^k$). Although the antigen is processed and presented to helper T cells, the selection of a B-cell clone by the antigen depends primarily on the initial contact between the antigen and B-cell receptor (29). For this reason, the structural differences between the two forms of the molecule may explain the discrepancy between the immune responses to native membrane-bound Bor56 protein and soluble recombinant Bor56 protein. Although the N terminus of the ΔBor56 fusion protein was not able to activate a B-cell clone(s), such a clone(s) could be activated by authentic membrane-bound Bor56 protein.

This result suggests that the removal of MalE from the N terminus of Bor56 protein could enhance the antibody response against membrane-bound Bor56 protein.

A previous comparative DNA analysis of genes from six strains revealed that the Tsa has four regions of amino acid variability interspersed among five regions of amino acid conservation (25). Previous studies have shown that a type-specific B-cell determinant for the Karp strain is present in the VD I region (aa 105 to 133) (41); however, in this study, strain-specific MAb KI4 bound to the VD II region (aa 151 to 174), which partially overlaps with AD II. Moreover, we obtained similar results with group-specific MAb KI57. We found that KI4 bound only to Boryong among Gilliam, Karp, Kato, Boryong, Kuroki, Kawasaki, Shimokoshi, TA678, TA686, TA716, TA763, and TH1817. However, KI57 was reactive with all of the strains listed above by IFA test (5). The finding that these MAbs bound to the same region and showed different patterns of binding to various strains indicates that the strain-specific and group-specific epitopes are located close together in this region. The murine anti-ΔBor56 serum generated by immunization with fragments 2, 3, and 4 bound to this region, suggesting that it has strong immunogenic potential. However, only one serum specimen from a patient showed high IgM binding to this region. Since the MAbs used may recognize epitopes distinct from those recognized by human serum, it should be possible to develop a capture ELISA that is specific for anti-Bor56 antibodies as well as for Bor56 protein itself.

In addition to the VD II region, we were able to map epitopes between VD III (aa 200 to 233) and VD IV (aa 397 to 427), an area that shows a moderate degree of sequence variation with a typical coiled-coil structure. In humans, IgG and IgM antibodies recognized epitopes in the AD III region. In addition, fragments 2, 3, 4, and 6 induced mice to produce antibodies that were reactive with AD III. Because AD III is immunodominant in both mice and humans, this region may be important in peptide-based vaccine development.

Although the VD IV region is highly variable, our results showed that a truncated protein containing this region (fragment 8) did not elicit Bor56-reactive antibodies and that human IgG and IgM antibodies did not bind to this region. We can, therefore, assume that this variable region is buried inside the molecule or inside the membrane. The regions from aa 454 to 470 and from aa 481 to 501 were predicted to be transmembranous by a method that predicts the secondary structures and topologies of integral membrane proteins based on the recognition of topological models (16). This finding suggests that the carboxy-terminal portion of Bor56 protein is embedded in the membrane, which would explain its low immunogenicity (Fig. 6).

The individual epitopes on an antigen that are reactive with antibody tend to be conformational rather than sequential, although sequential epitopes are also reactive (29). Because the structural features of a protein are important determinants of its immunogenicity (29), we based our model structure of Bor56 (Fig. 6) on its antibody binding patterns, secondary structure (as predicted by the Chou-Fasman algorithm [6]), and transmembranous region, as suggested by Jones et al. (16). The antibody molecule within the B-cell plasma membrane can react with epitopes on soluble antigen molecules and on whole cells. The binding sites of an antibody embedded within the plasma membrane of a B cell can noncovalently associate with an epitope expressed on an antigen and form a binary complex. The types of epitopes usually recognized are molecule accessible; that is, they are not usually hidden within the folds of the protein but are available on the surface of the antigen (45). Consequently, in a natural aqueous environment, such epitopes tend to be more hydrophilic than hydrophobic (20). In addition, epitopes tend to be mobile; the best epitopes are discrete antigen regions that exhibit a fair amount of molecular movement (the statistical probability of an epitope assuming an appropriate site-fitting shape increases when the region is dynamic rather than static) (40, 46). Based on this reasoning, the amino-terminal region of Bor56 protein should be highly immunogenic. When the X-ray crystallographic and antigenic structures of a protein are known, as is the case with influenza virus neuraminidase, MAbs bind to that protein in an overlapping pattern of sites at the exposed head of the protein (7). The stalk of the neuraminidase protein is not immunogenic, apparently because it is almost entirely covered by carbohydrate, which may sterically interfere with antibody binding to the protein. Clearly, accessibility on the surface is a sine qua non for an antigenic determinant for B cells. The patterns of antibody binding to Bor56 protein suggest that all of the ADs (AD I through AD III) are exposed on the surface of the molecule and that the regions between the ADs, as well as the carboxy part of the molecule, are buried inside the molecule (Fig. 6). When the β-turn sites were predicted by the Chou-Fasman algorithm, AD I, AD II, and AD III were localized to the loop region; the regions between the ADs were buried inside the molecule.

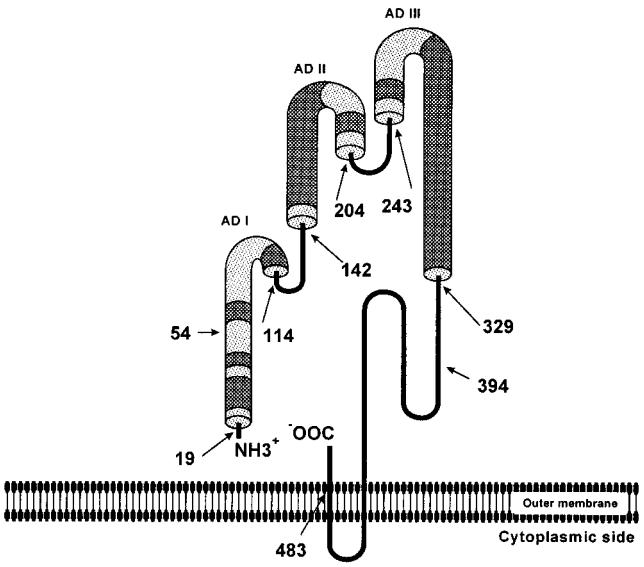

FIG. 6. Locations of three potential antigenic determinants on a model of the Bor56 protein. Numbers correspond to amino acid positions in the mature Bor56 protein. Columns represent ADs (AD I through AD III), and lines represent intervening amino acid sequences. Hydrophilic regions that are thought to contribute to the antigenicities of ADs were calculated by using the Kyte-Doolittle algorithm (20) and are depicted as cross-hatched columns.

We believe that the findings presented here are also relevant to the diagnosis of scrub typhus. This protein is strain specific with respect to its antigenic properties (9, 10, 21, 24, 25, 36, 37, 43), which is particularly important in considering diagnostic tools, because various proteins from various strains would be required and the diagnostic assay system would therefore be independent of the infecting strain. The specificities of the serologic tests currently available for the diagnosis of scrub typhus could be improved by using recombinant proteins that include the N-terminal region of Bor56.

In this study, we found that the immunogenicity of Bor56 is mainly located within the amino-terminal 113 aa (AD I) and the regions from aa 142 to 203 (AD II) and from aa 243 to 328

(AD III). Further elucidation of the protective immunity induced by antibodies against these domains of Bor56 could provide the rationale for a more efficacious vaccine.

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