JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1993, p. 598–605
0095-1137/93/030598-08$02.00/0

Vol. 31, No. 3

High-Level Expression of a 56-Kilodalton Protein Gene (*bor*56) of *Rickettsia tsutsugamushi* Boryong and Its Application to Enzyme-Linked Immunosorbent Assays

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Received 2 July 1992/Accepted 15 December 1992

The 56-kDa protein of *Rickettsia tsutsugamushi*, which is located on the rickettsial surface, has been shown to be an immunodominant antigen. The gene that encodes the 56-kDa protein of *R. tsutsugamushi* Boryong (*bor*56) was cloned. Sequencing revealed an open reading frame of 1,602 bp encoding 534 amino acids with a molecular weight of 56,803. The 56-kDa protein of *R. tsutsugamushi* Boryong (Bor56) was expressed as a fusion protein with the maltose-binding protein of *Escherichia coli* by deleting 252 bp from the 5′ end of the open reading frame and subcloning it into the *Stu*I site of pIH821. The recombinant fusion protein was purified by amylose column chromatography for application in an enzyme-linked immunosorbent assay to evaluate the ability of the method to detect the antibody to *R. tsutsugamushi* in human patient sera. By using sera from 100 patients with scrub typhus and 70 patients with other febrile diseases, a high diagnostic sensitivity (95%) and a high diagnostic specificity (100%) were demonstrated, suggesting the suitability of the recombinant antigen for use as an immunodiagnostic tool.

Scrub typhus is a mite-transmitted rickettsiosis (8). The disease, as well as the rickettsial diseases tick typhus and murine typhus, is endemic in the Asia-Pacific region including the Republic of Korea (4, 7, 18). For poorly understood reasons, the incidence of disease in humans has increased sharply in the Republic of Korea during the past 5 years (7). Nevertheless, underdiagnosis or misdiagnosis of scrub typhus is common and may result in delayed or inappropriate treatment. Confirmatory diagnosis of scrub typhus is generally based on serologic procedures, such as the Weil-Felix test, the immunoperoxidase test, and the indirect immunofluorescent-antibody (IF) test (3–6, 11). However, these serodiagnostic tests have shortcomings or requirements which limit their usefulness. The Weil-Felix test, although very simple to use, has been shown to be neither sensitive nor specific for the diagnosis of scrub typhus (1, 6). The IF test is highly sensitive and specific, but it requires an immunofluorescence microscope which may not be available in developing countries, rural hospitals, and field situations. In the recently developed immunoperoxidase test, a fluorescence microscope can be replaced by an ordinary light microscope or a microscope is not necessary, but there is still the need to culture and purify *Rickettsia tsutsugamushi*, which require expensive tissue culture facilities (11, 20, 26). A more practical approach to the development of new serodiagnostic methods which possess all of the advantages of other tests is to clone the immunodominant genes of *R. tsutsugamushi* and express their structural genes in *Escherichia coli*. These recombinant products could then be produced and purified in adequate amounts for use as antigens in developing a convenient and inexpensive diagnostic method.

R. tsutsugamushi, the causative agent of scrub typhus, is an antigenically diverse microorganism. Several antigenic variants, such as representative strains Gilliam, Karp, and Kato, and other isolates have been reported (8, 14, 16, 23, 25). We also isolated strain Boryong (previously strain B119) from a patient in Boryong Prefecture (7).

The major polypeptides of *R. tsutsugamushi* are proteins of 70, 60, 56, 47, and 25 kDa (22). Of these proteins, the 56-kDa protein, which is present on the surface of *R. tsutsugamushi* at a high concentration, is an immunodominant antigen, and antigenic diversity depends on variation in this molecule (9, 13). The 56-kDa protein is reactive with group-specific and strain-specific monoclonal antibodies, suggesting the existence of group-specific and strain-specific epitopes in this molecule (22). Also, it is known that sera from most patients with scrub typhus react with this 56-kDa protein (17). The immunogenicity of this protein suggests that it is, a priori, a diagnostic antigen candidate.

Here we report the molecular cloning, nucleotide sequencing, and expression of the 56-kDa protein and an evaluation of the diagnostic potential of the recombinant protein by comparing the results obtained by the recombinant enzyme-linked immunosorbent assay (ELISA) with those obtained by the standard IF test.

MATERIALS AND METHODS

Bacterial strains and vectors. *E. coli* Y1090 and Y1089 were used as host strains for bacteriophage lambda *gt*11 (27). *E. coli* XL-1 Blue (Stratagene, La Jolla, Calif.) was used for the preparations of all plasmids. A plaque-purified *R. tsutsugamushi* Boryong strain isolated in Boryong Prefecture, Republic of Korea, was used in molecular cloning experiments (7). *R. tsutsugamushi* Gilliam, Karp, Kato, and Boryong were used in the IF test. The *E. coli* vectors used in the present study were lambda *gt*11 (Promega, Madison, Wis.), pBluescript SK(−) (Stratagene), and pIH821 (New England Biolabs, Beverly, Mass.).

Media and growth conditions. Luria-Bertani medium was

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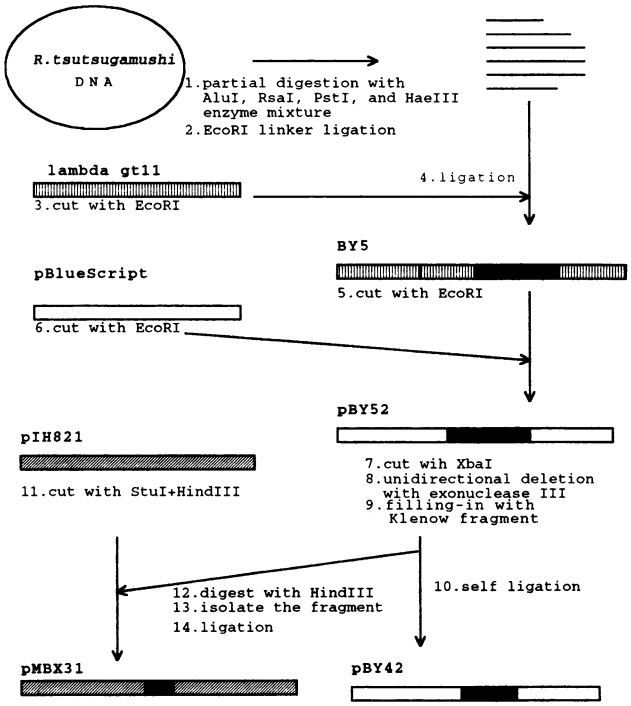

FIG. 1. Strategy for cloning and construction of 56-kDa *mbp-bor* fusion proteins.

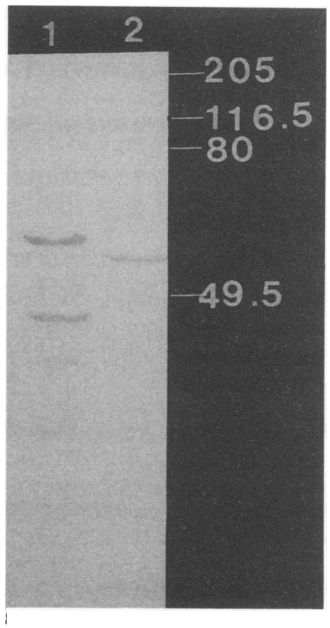

FIG. 2. Expression of 56-kDa protein in *E. coli*. The proteins of *E. coli* XL-1 Blue carrying plasmids were separated on an SDS–10% polyacrylamide gel and transferred to nitrocellulose filter paper. Rickettsial proteins were stained immunologically with monoclonal antibody KI58. Lane 1, polypeptides of *R. tsutsugamushi* Boryong; lane 2, protein of *E. coli* carrying pBY52. Monoclonal antibody KI58 is specific to protein Bor56, but in lane 1, another 48-kDa polypeptide that was reactive with monoclonal antibody KI58 was observed; it might have been a degradation product of protein Bor56. The molecular mass of the Bor56 protein derived from clone pBY52 was somewhat less than that of the native one. Sizes on the right are in kilodaltons.

used for routine maintenance of bacterial strains and for transformation experiments (12). For all strains harboring recombinant plasmids, ampicillin (50 μg/ml) was added to the culture medium. Broth cultures were started from a single bacterial colony, and 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 strain XL-1 Blue harboring plasmids to induce the *lac* or *tac* promoter. Rickettsiae were inoculated into mouse fibroblasts (L-929 cells), and the cells were incubated at 34°C in a humidified atmosphere of 5% CO_2 and 95% air. The rickettsiae were harvested 7 to 9 days postinfection and were purified by Percoll density gradient centrifugation as described by Tamura et al. (24).

Genomic library construction and screening. *R. tsutsugamushi* genomic DNA was isolated from purified rickettsiae as described by Maniatis et al. (12). All restriction enzymes were purchased from New England Biolabs, T4 DNA ligase was from Industrial Biological Laboratories Inc. (Rockville, Md.), and the Klenow fragment was from Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Total DNA from *R. tsutsugamushi* was partially digested with a mixture of *Sau*3AI, *Alu*I, *Hae*III, and *Rsa*I and was fractionated by 0.8% agarose gel electrophoresis as described by Maniatis et al. (12). Fragments ranging in size from 2 to 5 kb were isolated from the gel, and the ends were blunted by digestion with S1 nuclease and filling in with the Klenow fragment. The lambda *gt*11 library was constructed in *E. coli* Y1090 by using a mixture of *Eco*RI linkers (8-mer, 10-mer, and 12-mer) as described by Maniatis et al. (12). The genomic library was screened with monoclonal antibody KI58, which is specific to the 56-kDa protein of *R. tsutsugamushi* Boryong, as described by Maniatis et al. (12), and one clone (BY5) which expresses the whole 56-kDa protein was isolated. Inserts of BY5 were excised with *Eco*RI and recloned in the bacterial expression vector pBlueScript SK(−) (pBY52).

DNA preparation and sequencing. Plasmid DNA from *E. coli* was isolated by using the alkaline lysis method (2). pBY52 was digested with *Xba*I, and unidirectional progressive deletion was done by using the Erase-a-Base kit (Promega). The extent of exonuclease digestion was varied by stopping the reaction at different times. Ends were blunted by using S1 nuclease and Klenow fragments, and then DNA ligase was added to recircularize the molecules and the molecules were transformed into *E. coli* XL-1 Blue. Expression was monitored by immunoblot assay, pBY42, which contained the 1.8-kb insert, was isolated, and the protein was expressed by using its own promoter (Fig. 1). DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (19) by using a Sequenase kit (United States Biochemical Corporation) according to the manufacturer's instructions. M13 universal and reversal primers were used where appropriate.

Mbp-Bor protein fusion and high-level expression. pBY52 was digested with *Xba*I, and unidirectional progressive deletion was done as described above. The Klenow fragment was added to blunt the ends. The DNA fragments with different deletion endpoints were digested with *Hin*dIII, ligated with *Stu*I-*Hin*dIII-predigested pIH821, and transformed into *E. coli* XL-1 Blue (Fig. 1). The expression was monitored by immunoblot assays, and pMBX31, which expressed the Mbp (maltose-binding protein) fusion protein,

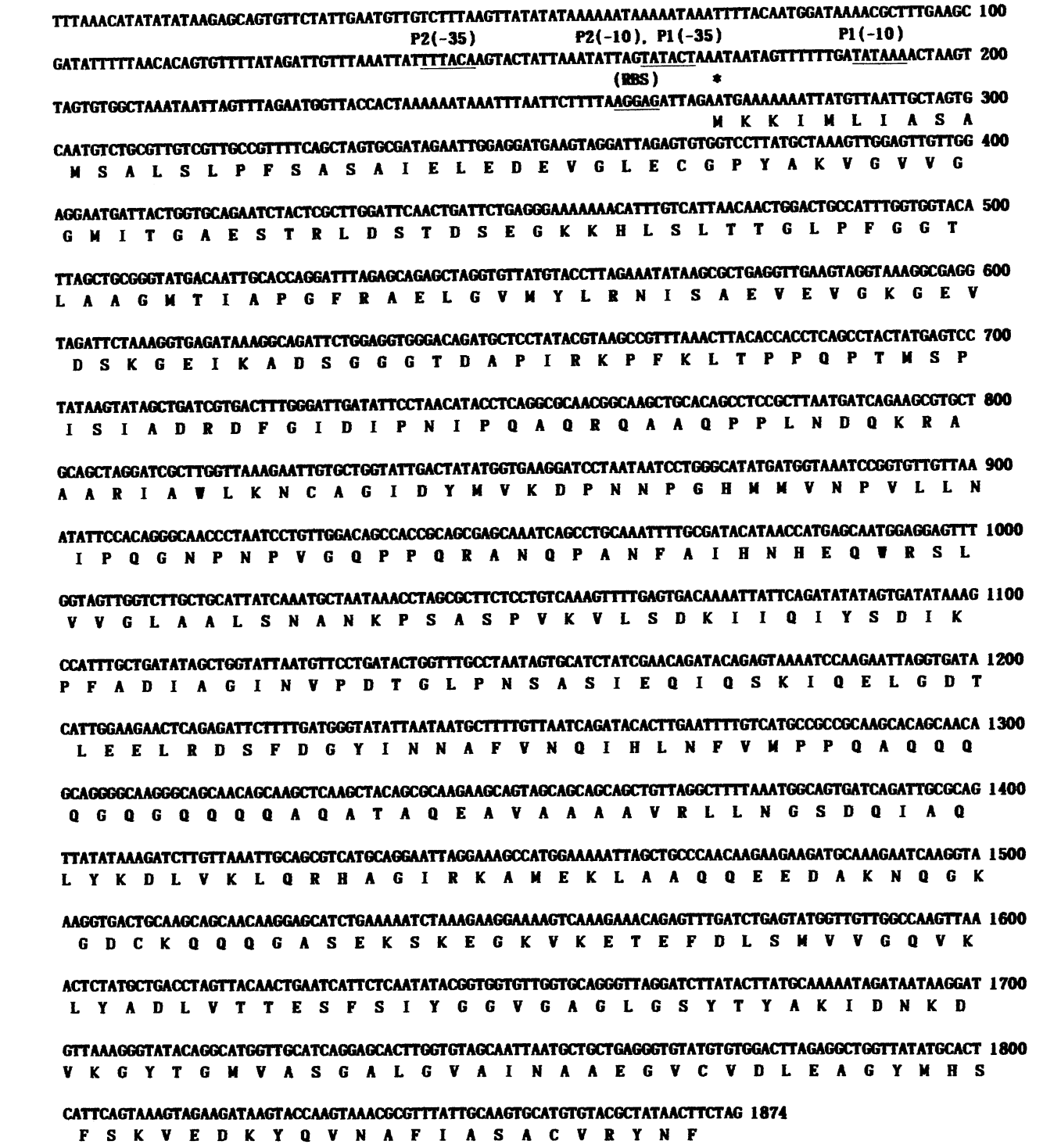

FIG. 3. Nucleotide sequence and deduced amino acid sequence of the *bor56* gene and the flanking region. Nucleotides are numbered beginning with the first nucleotide of the insert of pBY42. The presumed initiation codon for the Bor56 protein is denoted by an asterisk. Sequences resembling the consensus sequences for ribosomal binding site (RBS) and promoter −10 and −35 regions are underlined. The GenBank accession number is L04956.

was isolated. The deletion point was analyzed by nucleotide sequencing by using the primer MalE (New England Biolab).

The recombinant protein was purified by using an amylose column purchased from New England Biolab. Its use as an affinity chromatography matrix and digestion of the fusion protein with factor Xa were done by using the manufacturer's specifications.

SDS-PAGE and immunoblot analysis. Sodium dodecyl

sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were done as described by Tamura et al. (22).

Sera. Sera were obtained from the autumn of 1988 through 1991 from individuals in the Republic of Korea. A total of 100 scrub typhus cases were selected among patients with febrile exanthems compatible with scrub typhus. All of these patients had at least two of the three symptoms of scrub typhus (fever, rash, and eschar), and all exhibited seroconversion or fourfold rises in antibody titers to *R. tsutsugamushi* by the IF test. The titers of immunoglobulin M (IgM) and IgG determined in patient sera by the IF test varied from 1:20 to 1:20,480. A total of 70 serum specimens were collected from normal blood donors and were tested for the presence of antibody to *R. tsutsugamushi*. None of the serum specimens contained antibody to *R. tsutsugamushi*. Rheumatoid factor-positive sera were not included in the present study. Those sera were used in the analysis of the sensitivity and specificity of the recombinant ELISA. Three follow-up specimens were available from nine patients infected with *R. tsutsugamushi* Boryong and were used in the follow-up studies. Three follow-up serum specimens were obtained from four patients infected with *R. tsutsugamushi* Gilliam and were used in the analysis of the cross-reactivity of the recombinant antigen. All sera from patients with scrub typhus were obtained between 3 and 20 days after the onset of illness. The time at which follow-up serum specimens were collected are specified in Tables 1 and 2.

IF test. Serological diagnosis of scrub typhus was performed by the IF test as described by Bozeman and Elisberg (3). *R. tsutsugamushi* Gilliam, Karp, Kato, and Boryong propagated in L-929 cell cultures were used as antigens. The IF titers presented are the highest IF titers among the four separate tests (titers to Kato, Karp, Gilliam, and Boryong) unless stated otherwise. Fluorescein isothiocyanate-conjugated goat anti-human IgG and anti-human IgM were purchased from Cappel (Turnhout, Belgium).

ELISA procedure. Polystyrene microdilution plates (96-well; Costar) were coated with purified antigens at a concentration of 0.3 μg/ml in 0.05 M carbonate buffer (pH 9.6). After an overnight adsorption at 4°C, the contents of the plates were dumped and the wells were filled with 3% skim milk solution and incubated for 1 h at room temperature for blocking. The contents were again dumped and the wells were rinsed three times with phosphate-buffered saline–Tween 20. Identical plates were used to test for IgG and IgM. Sera were diluted 1:80 and were allowed to react with the antigen at 37°C for 30 min. This was followed by incubation with secondary antibodies at 37°C for 30 min and reaction with the substrates (1,2-phenylenediamine) at room temperature for 30 min. The secondary antibodies used were goat anti-human IgG (Cappel) and goat anti-human IgM (Cappel), both of which were conjugated with horseradish peroxidase. All reagents were used in a standard volume of 100 μl. After the reaction was stopped by adding 1 M H_2SO_4, the optical densities (ODs) at 492 nm were measured. Sera were always assayed in duplicate; and an air blank, negative control as well as a positive control were included on each plate.

Nucleotide sequence accession number. The sequence data reported here have been submitted to the GenBank Data Bank under accession number L04956.

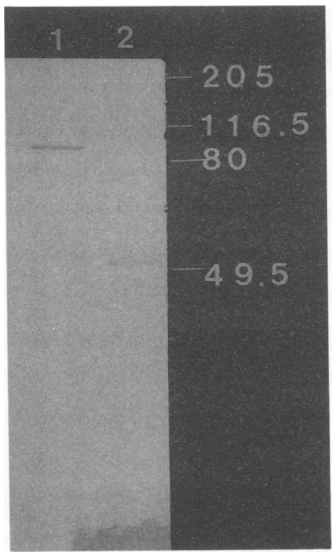

FIG. 4. SDS-PAGE pattern of the polypeptides of lysates from the bacterial clone. The proteins were affinity purified from IPTG-induced *E. coli* XL-1 Blue carrying plasmid pMBX31 (lane 1). The purified proteins were digested with factor Xa and were repurified by amylose affinity chromatography to remove the maltose-binding protein moiety (lane 2). The purified proteins were separated on an SDS–10% polyacrylamide gel and were transferred to a nitrocellulose membrane. Rickettsial proteins were visualized by immunostaining with monoclonal antibody KI58 specific to the Bor56 protein. Sizes on the right are in kilodaltons.

RESULTS

Molecular cloning of *bor56*. Screening of recombinant lambda *gt*11 plaques from a *R. tsutsugamushi* genomic library with monoclonal antibody KI58 resulted in the identification of a recombinant phage lambda BY5 clone. Lambda BY5 contains a 3.0-kb insert derived from *R. tsutsugamushi*. A 3.0-kb *Eco*RI fragment was recloned into the *Eco*RI site of pBluescript SK(−) and was identified by screening with monoclonal antibody KI58. The resulting plasmid, designated pBY52, was found by SDS-PAGE analysis to express a protein with an apparent molecular mass of 56 kDa, and expression of the 56-kDa polypeptide was not found to be dependent on IPTG induction (Fig. 2).

Nucleotide sequence of pBY42. Analysis of the nucleotide sequence of the 1.8-kb insert DNA revealed a single open reading frame of 1,602 bp preceded by a putative ribosome-binding site. The complete nucleotide sequences of the structural gene and the flanking regions are shown in Fig. 3. The open reading frame is composed of 534 amino acids with a calculated molecular weight of 56,803 (Fig. 3). An analysis of the deduced amino acids indicated that 28.8% were hydrophobic, 33.3% were semipolar, 10.7% were acidic, and 9.4% were basic.

Hyperexpression and purification of recombinant Bor56 protein. Nucleotide sequencing of the fusion site of pMBX31 revealed that 252 bp from the 5′ end of the open reading frame was deleted. Expression of Bor56 as a fusion protein was achieved by induction of the *tac* promoter with IPTG. Analysis by SDS-PAGE and immunoblotting of the soluble cellular proteins revealed the expression of a novel protein of approximately 90 kDa (Fig. 4). This is identical to the molecular mass that would be predicted for the recombinant protein.

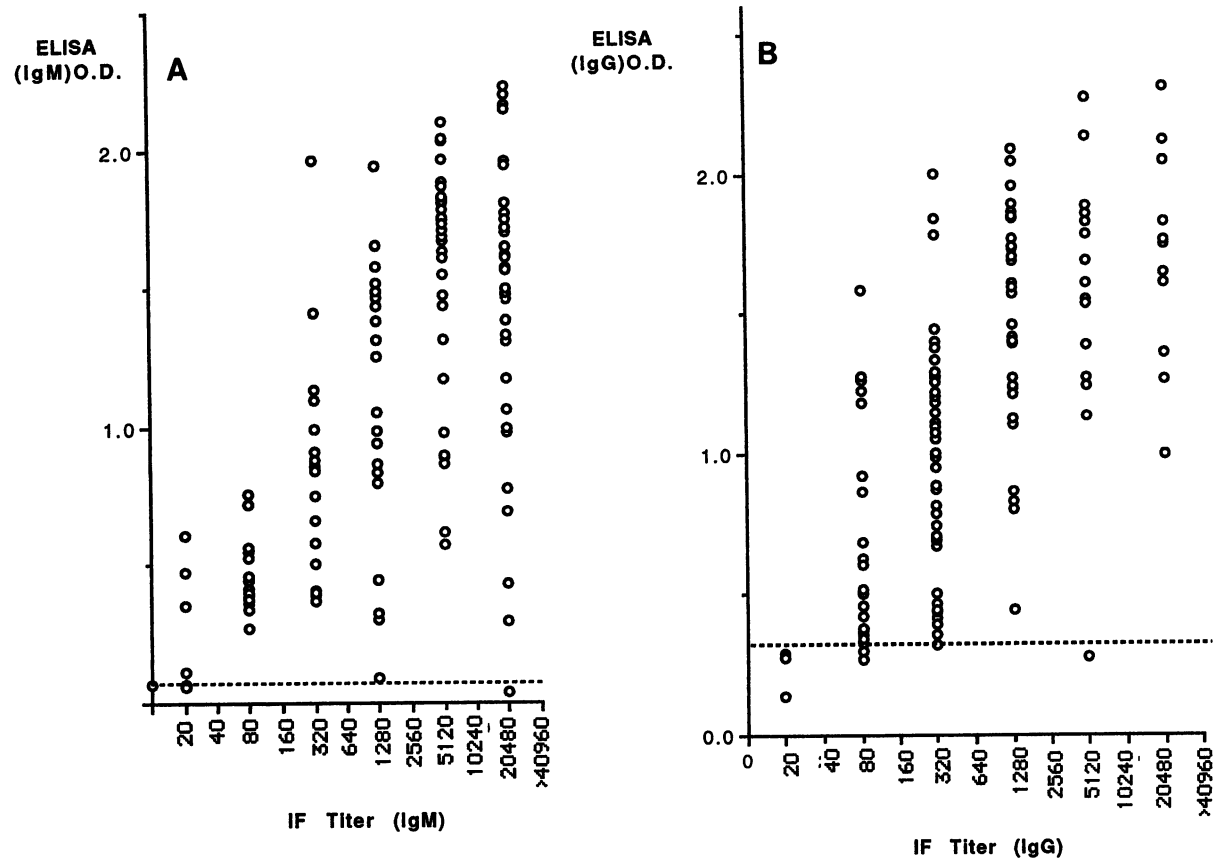

FIG. 5. Absorbances of recombinant IgM determined by ELISA plotted against the IgM titer to *R. tsutsugamushi* determined by the IF test (A) and recombinant IgG determined by ELISA plotted against the IgG titer to *R. tsutsugamushi* determined by the IF test (B) in patients with scrub typhus. The IF test titers presented are the highest titer among the four separate tests (titers to Karp, Kato, Gilliam, and Boryong). The dashed lines represent the cutoff values of the ELISA.

Taking advantage of the binding of Mbp to amylose and the factor Xa cleavage site in the fusion site of the recombinant protein, we affinity purified the Mbp-Bor fusion protein under nondenaturating conditions. The purified fusion protein was digested with factor Xa and was repurified by amylose affinity chromatography. This resulted in the removal of almost all other *E. coli* proteins. The 15 mg of fusion protein was purified from a 1-liter culture and 5 mg of Mbp-free Bor56.

Cutoff values for recombinant ELISAs. Background reactivity and possible cross-reactivity were assessed by analyzing 70 serum specimens from healthy Korean blood donors which proved to be negative to four strains of *R. tsutsugamushi* by IF tests. The cutoff values were set at OD_n + 3 standard deviations, where OD_n is the mean of the ODs recorded by use of the control serum specimens. The ODs of these control serum specimens varied from 0.008 to 0.254 for the IgG assay and from 0.002 to 0.135 for the IgM assay. In this way, for all investigations described below, sera with ODs greater than the calculated threshold ODs were regarded as positive and all others were regarded as negative.

Reactivity of the recombinant protein in ELISA. In order to test the applicability of the recombinant protein for use as an antigen in the recombinant ELISA, a panel of 100 serum specimens from patients with scrub typhus and 70 serum specimens from normal blood donors were used. Normal serum specimens were pretested by the IF test, and no sample was found to be positive for IgM or IgG antibodies to *R. tsutsugamushi*. The distributions of ODs and IF test titers of the serum specimens obtained from patients with scrub typhus are shown in Fig. 5. Of the 100 serum specimens from patients with scrub typhus, 3 serum specimens had ODs below the cutoff value by the recombinant ELISA for IgM. ODs and IF test titers of the serum specimens negative for IgM were 0.039 and 1:1,280 (to strain Gilliam), 0.093 and 1:1,280 (to strain Boryong), and 0.066 and 1:20 (to strain Kato), respectively. Of the 100 serum samples from patients with scrub typhus, 7 samples showed ODs below the cutoff value for the IgG assay and their ODs and IF titers for IgG were 0.325 and 1:80 (to strain Gilliam or Karp), 0.270 and 1:80 (to strain Gilliam), 0.326 and 1:80 (to strain Gilliam), 0.322 and 1:320 (to strain Kato), 0.339 and 1:80 (to strain Karp or Boryong), 0.358 and 1:80 (to strain Karp), and 0.275 and 1:1,280 (to strain Gilliam), respectively. No serum specimens from normal patients had ODs greater than the cutoff values for the IgM or IgG assay (data not shown).

Follow-up studies. To gain more insight into the sensitivity of the recombinant ELISA for the detection of IgM or IgG, 27 serial serum specimens from nine patients from whom *R. tsutsugamushi* was isolated were selected for determination of ODs by the recombinant ELISA (Table 1). The serotype of the causative agent in these patients was identified as the Boryong strain. Titers were determined by the IF test by using *R. tsutsugamushi* Boryong as an antigen. As time

TABLE 1. Time course of serological parameters in patients with scrub typhus determined by the IF test and recombinant ELISA

Patient no. and serum sample no.	Date of collection (day.mo.yr)	Titer by the IF test[a]		OD by ELISA[b]	
		IgM	IgG	IgM	IgG
31					
1	5.11.90	40	10	0.43	0.65
2	9.11.90	>2,560	>2,560	1.49	1.76
3	11.11.90	>2,560	>2,560	1.50	1.58
32					
1	18.10.90	160	80	0.40	0.63
2	22.10.90	1,280	1,280	1.39	1.28
3	28.10.90	1,280	1,280	1.14	1.09
33					
1	11.11.90	>2,560	160	1.27	0.66
2	13.11.90	>2,560	>2,560	1.67	1.85
3	15.11.90	>2,560	>2,560	1.66	1.74
34					
1	22.10.90	640	20	0.59	0.59
2	26.10.90	1,280	>2,560	1.04	1.54
3	31.10.90	1,280	>2,560	0.90	1.77
35					
1	29.10.90	640	10	0.62	0.57
2	31.10.90	640	80	0.92	0.89
3	2.11.90	>2,560	>2,560	1.28	1.36
36					
1	27.10.90	320	80	0.40	0.65
2	2.11.90	1,280	1,280	1.38	0.93
3	7.11.90	>2,560	>2,560	1.51	1.23
37					
1	22.10.90	10	80	0.29	0.47
2	27.10.90	320	>2,560	0.98	0.98
3	4.11.90	1,280	>2,560	0.88	0.93
38					
1	10.10.90	10	80	0.11	0.27
2	13.10.90	40	80	0.87	0.57
3	17.10.90	>2,560	>2,560	0.74	1.60
39					
1	21.10.90	40	40	0.62	0.71
2	24.10.90	>2,560	>2,560	1.48	1.68
3	28.10.90	>2,560	>2,560	1.42	1.49

[a] The Boryong strain was used as the antigen for the IF test. IgM or IgG antibodies were detected by using FITC-conjugated secondary antibody.

[b] The recombinant 56-kDa peptide was used for coating antigen, and IgM or IgG antibodies were detected by using horseradish peroxidase-conjugated secondary antibody from serum specimens.

elapsed, IgM and IgG titers by the IF test increased and were in full agreement with ODs by the recombinant ELISA. The initial serum specimen from patient 38, however, had ODs below the cutoff values for both IgM and IgG detection.

Cross-reactivity of the recombinant antigen. *R. tsutsugamushi* has several serotypes, and the reactivity of serum from humans to each strain of *R. tsutsugamushi* differs according to the serotype of the causative agent. It is also known that the 56-kDa polypeptide of *R. tsutsugamushi* is a major polypeptide which determines serotype specificity, because it has serotype-specific epitopes as well as species-specific epitopes. To analyze the cross-reactivity of the recombinant antigen to the sera obtained from patients who were infected with *R. tsutsugamushi* strains other than the Boryong strain, three serial serum specimens from four patients who were infected with *R. tsutsugamushi* Gilliam strain (Table 2) were selected. As infection times elapsed, both IgM and IgG titers determined by the IF test and ODs determined by the recombinant ELISA increased. The ODs of the initial serum specimens from patients 40, 41, and 43 for IgM detection, however, were below the cutoff value. For IgG detection by the recombinant ELISA, initial serum specimens from patients 42 and 44 and the ODs of the first and second serum specimens from patients 40 and 41 were below the cutoff values.

DISCUSSION

Scrub typhus, which is transmitted by trombiculid mites, is prevalent in the Asia-Pacific area (18). Diagnostic methodologies in rural areas where scrub typhus mainly occurs, however, are cumbersome, and in developing countries where expensive equipment is not available, the diagnosis of scrub typhus is difficult. The availability of an assay that could detect specific antibodies without expensive equipment and that could be adapted for testing large numbers of specimens would be an invaluable aid in the diagnosis and treatment of scrub typhus (13). The most formidable obstacle to the development of a convenient and sensitive diagnostic method is the production of adequate amounts of the diagnostic antigen. We initiated a study to improve the practicality and applicability of scrub typhus serology by producing the 56-kDa protein, a major outer membrane protein of *R. tsutsugamushi*, using gene cloning techniques. The 56-kDa polypeptide is a major outer membrane protein. This antigen appears to express strong antigenicity, and when *R. tsutsugamushi* is inoculated into mice, the antibody against this protein is predominantly produced (13). This antigen is known to be strain specific (22), but Hanson (9) reported that both group-specific and type-specific monoclonal antibodies react with this protein. Ohashi et al. (17) reported that most human patient sera react with the 56-kDa polypeptides of multiple strains, although some sera react predominantly with a 56-kDa polypeptide from a single strain. Therefore, the 56-kDa polypeptide could be the first recombinant antigen candidate for use in the diagnosis of scrub typhus.

The applicability of the recombinant antigen to the serological diagnosis of scrub typhus was studied by ELISA with 100 serum specimens from patients with scrub typhus and 70 serum specimens from normal blood donors. The study of 170 serum specimens demonstrated that the sensitivity and specificity of the recombinant ELISA were 97 and 100%, respectively, for IgM detection and 93 and 95%, respectively, for IgG detection, indicating that the recombinant ELISA developed in the present study is significantly sensitive and specific. However, it must be emphasized that most serum specimens available for use in the present study were collected from patients with scrub typhus in the Republic of Korea, where the *R. tsutsugamushi* Boryong strain is prevalent and the reactivities of patient sera to homologous strains are usually stronger than those to heterologous strains.

In the present study, signal levels determined by recombinant ELISA did not always correlate with the titers determined by the IF test, especially for IgM detection. This might be due to a relatively low cross-reactivity of IgM antibody to the heterologous 56-kDa protein.

Three serum specimens from patients with scrub typhus

TABLE 2. Serological parameters in patients infected with *R. tsutsugamushi* Gilliam determined by the IF test and recombinant ELISA

Patient no. and serum sample no.	Date of collection (day.mo.yr)	IF test titer								OD by ELISA[a]	
		Karp		Kato		Gilliam		Boryong			
		IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG	IgM	IgG
40											
1	22.10.90	—[b]	—	—	—	40	10	—	—	0.00	0.11
2	24.10.90	—	—	—	—	80	80	—	—	0.55	0.15
3	15.11.90	320	640	1,280	640	1,280	640	320	40	0.87	0.54
41											
1	18.10.90	—	—	—	—	20	40	—	—	0.06	0.14
2	21.10.90	—	—	—	—	40	40	—	—	0.20	0.23
3	24.10.90	320	640	160	640	640	1,280	320	640	0.78	0.67
42											
1	26.10.90	—	—	—	—	80	10	10	10	0.53	0.23
2	29.10.90	—	320	—	80	1,280	1,280	40	1,280	1.32	0.54
3	1.11.90	—	>2,560	—	160	1,280	1,280	160	>2,560	1.26	0.94
43											
1	22.10.90	—	—	—	—	20	10	10	—	0.16	0.27
2	24.10.90	10	10	10	20	80	40	20	20	0.47	0.55
3	1.11.90	40	320	640	1,280	1,280	>2,560	640	80	1.28	0.92

[a] See footnote *b* of Table 1.
[b] —, not reactive.

had a negative reaction for IgM detection and seven serum specimens from patients with scrub typhus had a negative reaction for IgG detection by the recombinant ELISA. There are three possible reasons for these results. The first possibility is that there are several strains of *R. tsutsugamushi* which cause scrub typhus in the Republic of Korea. Some patients were infected with *R. tsutsugamushi* strains other than the Boryong strain, and their sera may not have had enough cross-reacting antibodies to the 56-kDa protein of the Boryong strain. This possibility could be supported by the results that most initial serum specimens collected from the patients infected with the Gilliam strain showed weak or negative reactions for IgM and IgG antibody detection by the recombinant ELISA. The second possibility is that there were variations in the individual concentrations of monospecific antibody to the 56-kDa protein in the serum specimens. Ohashi et al. (17) reported that sera from their patients showed great variations in their reactivities to the antigenic peptides of *R. tsutsugamushi*. Most sera reacted only with the 56-kDa protein, but some sera also reacted with 35-, 46- to 47-, and 60-kDa proteins. Among the 100 serum specimens from patients with scrub typhus, some serum specimens may have been more reactive with other antigenic components of *R. tsutsugamushi*. The third possibility for the discordance between titers determined by the IF test and ODs determined by the recombinant ELISA is that the N-terminal portion of the 56-kDa protein which was absent from the recombinant protein (84 amino acids) is highly immunogenic and, in some serum specimens from patients with scrub typhus, antibody to the N-terminal portion is predominant. Ohashi et al. (15) reported that this portion is highly conserved among different strains of *R. tsutsugamushi*.

The true diagnostic value of the recombinant ELISA developed in the study described here would be better examined by using serum specimens from patients with proven, isolate-positive scrub typhus. Several improvements were made in the present study, however, to facilitate the development of serodiagnostic methods for scrub typhus. (i) We demonstrated that the recombinant 56-kDa protein of *R. tsutsugamushi* instead of whole *R. tsutsugamushi* could be used in the diagnosis of scrub typhus. The sensitivity of the recombinant ELISA developed in the present study was slightly lower than those of standard IF tests, but it seems that the sensitivity of the recombinant ELISA could be increased if the 56-kDa proteins of other strains were also included as diagnostic antigens. (ii) High yields of recombinant 56-kDa protein (>15 mg/liter) could be produced by growing transformed *E. coli* in Luria-Bertani broth and purified in large quantities to near homogeneity within 48 h at a low cost. The culture and purification of *R. tsutsugamushi* is time-consuming and expensive. Use of the recombinant 56-kDa protein would be helpful in developing inexpensive diagnostic tools. (iii) The recombinant antigen could be used in the development of various kinds of diagnostic methods for scrub typhus. The application of whole *R. tsutsugamushi* in the development of new serological tests, such as ELISA or passive hemagglutination, is limited. Many components of *R. tsutsugamushi* are not immunogenic to humans, and these components may interfere with the attachment or adhesion of immunogenic components of *R. tsutsugamushi* to ELISA plates, sheep erythrocytes, or other components. Because the recombinant 56-kDa protein can be purified to near homogeneity, it can easily be attached to ELISA plates or sheep erythrocytes in adequate amounts. The recombinant ELISA developed in the present study showed a high degree of sensitivity and specificity, and the passive hemagglutination test developed by using recombinant protein showed satisfactory results (11a).

The surface components of *R. tsutsugamushi* are presumed to modulate the essential biological events of attachment and to induce phagocytosis and the host immune response that contribute to immunity. The immunodominant 56-kDa protein has been implicated in some of these important functions, primarily by the association of many of these

processes with a strain-specific antigen (21, 22). The 56-kDa recombinant protein produced in the present study could be used to study the pathogenesis of *R. tsutsugamushi* and protective immunity to *R. tsutsugamushi*.

On the basis of the results of the present study, the following conclusions can be drawn. The recombinant 56-kDa protein used in our experiments could be used in the development of new diagnostic methods for scrub typhus, such as passive hemagglutination or ELISA. To increase the sensitivity of the test, especially in the early stage of the disease, the 56-kDa polypeptide of other serotypes could be included. There is a strong possibility that a rapid, reliable, and convenient method for the diagnosis of scrub typhus can be established by using recombinant 56-kDa polypeptides of *R. tsutsugamushi*, and this method will be tested under routine conditions in the near future.

ACKNOWLEDGMENT

This work was supported by a Basic Medical Science Research Fund from the Ministry of Education of the Republic of Korea, 1990.

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