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Rapid Diagnosis of Scrub Typhus by a Passive Hemagglutination Assay Using Recombinant 56-Kilodalton Polypeptides

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The genes encoding the 56-kDa polypeptides were amplified by polymerase chain reaction from the genomic DNAs of three serotypes of *Rickettsia tsutsugamushi*, Gilliam, Karp, and Boryong. The amplified products were cloned into expression vector pIH821, and the recombinant antigens were expressed in *Escherichia coli* as fusion proteins with maltose-binding protein. The recombinant 56-kDa polypeptides were purified by affinity chromatography for the sensitization of sheep erythrocytes. The recombinant 56-kDa polypeptides were evaluated with 89 serum specimens from healthy blood donors, 94 serum specimens from scrub typhus patients, and 31 serum specimens from patients with other febrile diseases by a passive hemagglutination assay (PHA). Among the scrub typhus patients diagnosed by indirect immunofluorescent-antibody testing, the antibodies to *R. tsutsugamushi* were detected in 93 patients (99%). One serum specimen from a healthy person showed a false-positive reaction by this method. The recombinant PHA showed no cross-reactions with sera obtained from other febrile patients with diseases such as murine typhus, hemorrhagic fever with renal syndrome, and leptospirosis. In conclusion, this recombinant PHA could be substituted for the conventional indirect immunofluorescent-antibody test and the immunoperoxidase test.

Scrub typhus, or tsutsugamushi disease, caused by *Rickettsia tsutsugamushi* is endemic in many areas of the world, especially in Asia and the Pacific region. Nonetheless, the disease is one of the most undiagnosed febrile illnesses. Because of the lack of definitive signs and symptoms combined with the limited availability of serological tests, the differentiating of scrub typhus from other common febrile illnesses such as hemorrhagic fever with renal syndrome, leptospirosis, and other rickettsial diseases has been difficult. Recently, an indirect immunofluorescent-antibody (IFA) test which is highly sensitive and specific has been developed (2, 3). However, the test requires fluorescence microscopes, trained personnel, and cell culture facilities capable of producing rickettsial antigens. Although the most widely used test for the diagnosis of scrub typhus is the indirect immunoperoxidase test, which does not require fluorescence microscopes (11, 28), it requires laborious procedures for preparing rickettsial antigens. Consequently, it has been proved to be impractical for use in local hospitals. Therefore, other simple, convenient, and accurate diagnostic methods are still needed (7, 12, 26). The rickettsial antigens for the serodiagnosis of scrub typhus have been produced by cell or yolk sac culture methods. However, these methods are quite expensive and cumbersome for meeting the need for routine serodiagnosis. The use of recombinant antigens offers a more practical alternative.

The 56-kDa polypeptide is a major antigen expressed on the surface of *R. tsutsugamushi* (6, 9, 23) and is one of the major proteins recognized by the immune systems of mice and humans; thus, the antibodies against the protein are present at a high titer in the sera of all scrub typhus patients (20). Because both strain-specific and group-specific epitopes are present within the protein (6, 9, 16, 17, 19, 23), it may be useful in the development of tools for serologic diagnosis.

The genes encoding the 56-kDa polypeptide of *R. tsutsugamushi* Gilliam (18), Karp (21), and Boryong (12) were cloned and expressed in *Escherichia coli*. The recombinant antigens could be purified efficiently as a fusion protein containing part of maltose-binding protein (MBP) by single-step amylose affinity chromatography (5, 14) and adsorbed to sheep erythrocytes (sRBCs). The passive hemagglutination assay (PHA) was evaluated for its sensitivity and specificity relative to those of the IFA test for the diagnosis of scrub typhus. Since no significant differences were observed when the fusion protein was used in place of the antigen free of the MBP moiety, the fusion protein was used in all procedures in the PHA.

The PHA offers advantages over the conventional diagnostic test in general availability, ease of handling, and accuracy in the diagnosis of scrub typhus.

MATERIALS AND METHODS

Bacterial strains and preparations of *R. tsutsugamushi*. Plaque-purified *R. tsutsugamushi* Gilliam, Karp, and Boryong (4, 12) strains were used throughout the study. Rickettsiae were grown in mouse fibroblasts (L929 cells), incubated at 34°C in a humidified atmosphere of 5% CO_2, and purified by Percoll density gradient centrifugation (24). Recombinant antigens were expressed in *E. coli* XL1-Blue (Stratagene, La Jolla, Calif.). *E. coli* was grown in Luria-Bertani broth or Luria-Bertani agar. Luria-Bertani broth was supplemented with ampicillin (100 μg/ml) for *E. coli* containing all plasmids.

Gene cloning, expression, and purification of recombinant antigens. All restriction enzymes were purchased from New

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England Biolabs (Beverly, Mass.). T4 DNA ligase and calf intestinal alkaline phosphatase were purchased from Industrial Biological Laboratories, Inc. (Rockville, Md.) and Boehringer Mannheim Biochemicals (Indianapolis, Ind.), respectively. When a heavy growth of rickettsiae was observed for many cells in smears of infected cells stained with Giemsa stain, cells were harvested in centrifugal pellets and the rickettsial DNA was extracted as described previously (12, 15). A pair of primers (forward primer, 5′-CCA GGA TTT AGA GCA GAG-3′; reverse primer, 5′-CTA GAA GTT ATA GCG TAC AC-3′) for polymerase chain reaction were synthesized by Oligo Etc., Inc. (Wilsonville, Oreg.). The first nucleotide of the forward primer corresponds to the 253rd nucleotide of the structural gene of the Gilliam and Boryong strains and the 250th nucleotide of that of the Karp strain. Target DNAs were amplified with GeneAmp PCR System 9600 (Perkin-Elmer Cetus) in 35 cycles; 94°C for 15 s and 60°C for 1 min. The reaction was continued for 5 min at 72°C following the last cycle. Ends of the amplified products were filled by treating with Klenow fragment. The end-repaired DNA fragment was cloned into the *Stu*I site of a bacterial expression vector, pIH821 (New England Biolabs). The *E. coli* was transformed and plated onto nitrocellulose paper (25). Colonies were screened with mouse anti-*R. tsutsugamushi* sera reactive to the 56-kDa polypeptide (29). The analysis of antigen-producing clones and the purification of the fusion protein were performed as described previously (12, 13).

PHA and IFA test. The sRBCs were coupled with the recombinant antigen as described by Hudson and Hay (10) with some modifications. In brief, sRBCs were treated with 0.002% tannic acid–0.4% gluteraldehyde. The sRBCs were suspended with phosphate-buffered saline (PBS) to 4% (vol/vol) and were coupled with the recombinant antigen (10 μg/ml). The sensitized sRBCs were suspended in a diluent buffer (PBS [pH 7.2], 0.5% rabbit sera, 0.002% Tween 20) to a final concentration of 0.6% (vol/vol). One part diluted serum was mixed with one part sensitized sRBC suspension in a U-shaped 96-well microtiter plate. In each plate, nonsensitized sRBCs and healthy human sera were used as negative controls for the antigen and sera, respectively.

The IFA test was performed as described by Bozeman and Elisberg (2). For the IFA test, fluorescein isothiocyanate-conjugated heavy-chain-specific goat anti-human immunoglobulin M (IgM) or IgG (Cappel, Cochranville, Pa.) was used. The slides were examined at ×400 magnification with the Zeiss UV microscope (Axioscope model).

In our previous report (4), we described the serotypes of 113 strains isolated in Korea. The Boryong, Gilliam, and Karp strains were isolated from 77, 11, and 7% of the patients studied, respectively. Thus, we used these three strains for all of the procedures in the present study. For each serum specimen, the highest titers of the IFA test were compared with the highest titers of the PHA among the three strains. In addition, sRBCs which were coupled with each antigen were mixed (1:1:1, vol/vol/vol) and were used in the PHA. The titers in the PHA in which the mixed antigen was used were compared with the highest titers in the IFA test. Specificity and sensitivity for the serodiagnosis of scrub typhus were calculated with 2 × 2 binary tables as described previously (8).

Sera. A total of 94 serum specimens were collected from scrub typhus patients diagnosed by the IFA test. All of these patients had at least two of the three symptoms of scrub typhus (fever, a rash, and eschars), and all exhibited seroconversion or fourfold rises in antibody titers to *R. tsutsugamushi* by the IFA test (3, 28). The IFA titers of the sera were between 1:20 and 1:5,120. A total of 89 serum specimens were obtained from healthy blood donors, and all of them did not react with *R. tsutsugamushi* by the IFA-IgM or -IgG test. In addition, 31 serum specimens from patients with other febrile diseases (hemorrhagic fever with renal syndrome, 12 specimens; murine typhus, 9 specimens; and leptospirosis, 10 specimens) were also obtained. Hemorrhagic fever with renal syndrome and murine typhus were identified by the IFA test (1, 27), and leptospirosis was identified by the microscopic agglutination test (22).

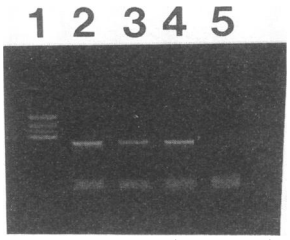

FIG. 1. Electrophoresis of amplified DNA by PCR from Gilliam (lane 2), Karp (lane 3), Boryong (lane 4), and L929 (lane 5) cells. The molecular size marker, *Hin*dIII-digested lambda phage DNA, is also shown (lane 1). The sizes of fragments are 23,130, 9,416, 6,557, 4,361, 2,322, 2,027, and 564 bp.

Nucleotide sequence accession number. The GenBank accession number of the gene for the 56-kDa polypeptide of the Boryong strain is L04956.

RESULTS

Expression and purification of the recombinant 56-kDa polypeptides. After the polymerase chain reaction, the amplified DNAs were electrophoresed and visualized by ethidium bromide staining. These primers amplified a 1,322-bp fragment in the Gilliam strain and 1,349-bp fragments in the Karp and Boryong strains (Fig. 1). The amplified products were then subcloned into the *Stu*I site of pIH821. The resulting plasmids expressed MBP fusion proteins visible in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The MBP fusion proteins were purified, and 15 mg of fusion protein was obtained from 1-liter cultures of all three strains as described previously (12). Western blot (immunoblot) analysis revealed that the purified proteins were reactive with the sera of scrub typhus patients (Fig. 2).

Comparison of PHA and IFA. Potential nonspecificities of the recombinant PHA were assessed by investigating the 89 serum samples obtained from the healthy blood donors. None of the 89 control serum specimens were reactive with rickettsial antigens by the IFA-IgM and IFA-IgG tests. In the assay with 89 control serum specimens, 8 serum specimens showed PHA titers of 1:40 and 1 serum specimen showed a titer of 1:80. Thus, for all analyses, the sera with PHA titers higher than 1:80 were considered PHA positive and all of the others were considered PHA negative. There were no cross-reactions with sera obtained from patients with other febrile diseases, such as murine typhus, hemorrhagic fever with renal syndrome, and leptospirosis. Overall, 98.9% specificity was obtained when results were compared with those of the IFA test. One serum specimen from one of the 94 scrub typhus patients was not reactive by the PHA. The titer of this serum specimen was 1:20 by IFA-IgM, and the specimen was reactive mainly with rickettsial antigens other than the 56-kDa protein by Western blotting (data not

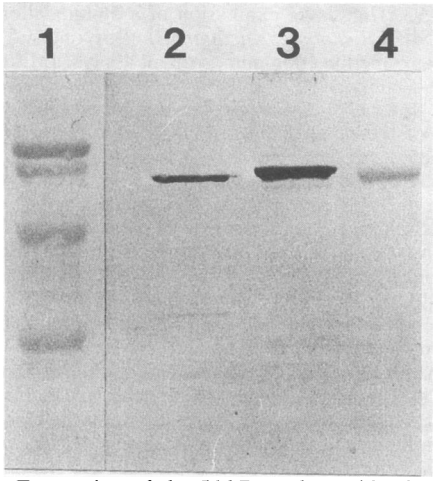

FIG. 2. Expression of the 56-kDa polypeptides in *E. coli*. The proteins were affinity purified from IPTG (isopropyl-β-D-thiogalactopyranoside)-induced *E. coli* XL1-Blue carrying plasmids pMBR56 (lane 2), pGI101 (lane 3), and pKP44 (lane 4). The plasmids pMBR56, pGI101, and pKP44 contain genes encoding the 56-kDa polypeptide of strains Boryong, Gilliam, and Karp, respectively. The purified proteins were separated on an SDS–10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. Rickettsial polypeptides were visualized by immunostaining with the scrub typhus patients' sera. The plasmids pMBR56 and pGI101 have inserts corresponding to polypeptides which span from the 85th amino acid to the C termini of the antigens of the Gilliam and Boryong strains; that from pKP44 spans from the 84th amino acid to the C terminus of the antigen of the Karp strain. The resulting expression products are fusion proteins with 45-kDa MBP. Sizes in lane 1 are 205, 116.5, 80, and 49.5 kDa.

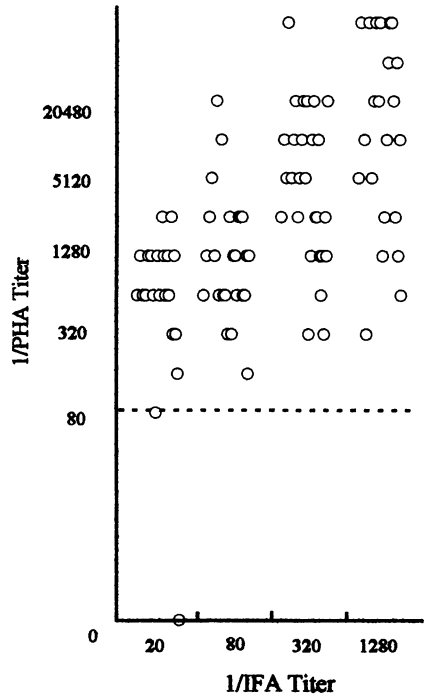

FIG. 3. Correlation between IFA-IgM titer and highest titer by PHA. Each circle represents one serum specimen ($n = 94$). The plot indicates the highest titer by the IFA test and the highest titer obtained by the PHA against one of the antigens of the Gilliam, Karp, and Boryong strains. The related coefficient was 0.75 ($P < 0.001$). The dashed line represents the cutoff titer of the PHA. To elucidate the data for each sample, the overlapping points were plotted separately by using the IFA titer.

shown). With the highest titer among the three strains, the related coefficient of the recombinant PHA and IFA-IgM test was 0.75 ($P < 0.001$) (Fig. 3). In the PHA with mixed antigens, the related coefficient with the IFA-IgM test was 0.79 ($P < 0.001$). When results were compared with those of the IFA-IgG test, no significant differences were obtained.

DISCUSSION

The cloning, expression, and purification of the recombinant antigen of *R. tsutsugamushi* have been described. For the serodiagnosis of scrub typhus, the IFA test or the immunoperoxidase test is widely used; however, microscopic observation is necessary in both procedures. In the PHA, antibody-positive sera can be distinguished from antibody-negative sera by observation by the naked eye, and if proper negative controls are provided, anyone can easily make the final evaluation. Furthermore, the antigen preparation by recombinant organisms is more simple and economical than that by cell or yolk sac culture.

Compared with the IFA test, the PHA with recombinant antigens allowed highly sensitive and specific detection of anti-*R. tsutsugamushi* antibodies in human patients' sera. The PHA titers correlated with those determined by the IFA-IgM and IFA-IgG tests. It has been shown previously that antibody titers determined by the PHA were parallel to IFA-IgM titers rather than IFA-IgG titers for other diseases. But in this study, no significant differences in the related coefficient of the PHA titer and the IFA-IgM or IFA-IgG titer were observed. This may be due to the antigens used in the test. Since the antigen used in the PHA is a unique polypeptide among the rickettsia antigens and since whole rickettsial polypeptides were used in the IFA test, the related coefficient of the IgM titer and that of the IgG titer could not be comparable. In the early phase of infection, the IgM antibody response is diverse against whole rickettsial antigens. However, the IgG response is more specific to major rickettsial immunogens in the late phase of infection. For this reason, the IgM antibody titers detected by the recombinant PHA may be less than real IgM antibody titers to whole rickettsial antigens. However, we have shown here that recombinant 56-kDa polypeptides can be used for the generation of a recombinant PHA to detect antibodies against *R. tsutsugamushi* and that a conventional IFA or immunoperoxidase test could be substituted for by the recombinant PHA.

In our study, although some sera reacted predominantly with the recombinant 56-kDa polypeptide of a single strain, most of the sera showed cross-reactions with the recombinant antigens of the three strains. But false-negative reactions were observed in two cases from 94 patients when a single recombinant antigen of the Gilliam strain was used and in three cases when only the Karp strain was used. These false-negative reactions were not observed in most cases when recombinant antigens of three strains were mixed and used in diagnosis. However, in one case, the antibody was not detected by the recombinant PHA. This false negativity may be due to the variety in the immune responses shown by individual patients, and this one serum specimen was reactive with a polypeptide other than a 56-kDa polypeptide.

Since there are several antigenic variants in *R. tsutsugamushi* and a type-specific 56-kDa antigen is responsible for the variation (6, 9, 16, 17, 19, 23), all of the type-specific antigens derived from endemic strains should be included for diagnosis. By using recombinant polypeptides representing type-specific antigens of endemic strains in a PHA, the sensitivity of a conventional IFA test can be achieved. The genes for the strain-specific antigens of the various strains which are prevalent in the local field could be cloned and expressed in *E. coli* by polymerase chain reaction as described in the present study, and more a detailed analysis with a combination of the recombinant antigens derived from various endemic strains will be necessary to design more-sensitive and more-specific diagnostic tools for worldwide usage.

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