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Epitope Mapping of the Sta58 Major Outer Membrane Protein of *Rickettsia tsutsugamushi*

RAMKUMAR LACHUMANAN,[1] SHAMALA DEVI,[2] YUET-MENG CHEONG,[3] STUART J. RODDA,[4] AND TIKKI PANG[1]*

Institute of Advanced Studies[1] and Department of Medical Microbiology,[2] University of Malaya, and Institute for Medical Research,[3] Kuala Lumpur, Malaysia, and Chiron Mimotopes, Clayton, Victoria, Australia[4]

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Binding studies of 160 overlapping, synthetic octapeptides from the hydrophilic regions of the Sta58 major outer membrane protein of *Rickettsia tsutsugamushi* with sera from patients with scrub typhus revealed 15 immunodominant peptides which are recognized by all the sera tested. Further analysis of the specificity of peptide binding with five of these peptides indicated that the peptides showed significantly stronger binding to scrub typhus patients' sera than they did to sera from patients with other febrile illnesses common in the region, i.e., malaria, dengue fever, typhoid fever, and leptospirosis. The main antibody class binding to these peptides appears to be immunoglobulin M, and there appears to be little correlation between reactivity with peptides and antibody titers measured by the indirect immunoperoxidase test.

Throughout the Asia-Pacific region scrub typhus is a major cause of febrile illness (15); it is caused by *Rickettsia tsutsugamushi*, which is transmitted to humans by the *Leptotrombidium* mite vector. In Malaysia, for example, scrub typhus is the most frequent diagnosis (19%) in patients admitted to a hospital with fever (1). Although the disease can be effectively treated with antibiotics such as doxycycline (14), relapses do occur and there is renewed concern of the possibility of emergence of antibiotic-resistant strains among pathogenic bacteria generally (2). In cognizance of these potential problems, it seems prudent that the development of vaccines against scrub typhus be pursued (6). In the past, the development of scrub typhus vaccines has been complicated by antigenic diversity or heterogeneity of rickettsial strains (6) and lack of knowledge of the major protein immunogens involved in protective immunity. More recently, however, information on the identity and characteristics of the major protein immunogens of *R. tsutsugamushi* has become available (5, 17). Molecular sequence data for some of these immunogens (16), together with the development of methods for the synthesis of multiple peptides on polyethylene pins (4), have also enabled epitope-mapping studies of these immunogens, which may be relevant to diagnostic tests and vaccine design. We report here the results of a study to identify and map the immunodominant epitopes within the hydrophilic regions of the Sta58 major outer membrane protein of *R. tsutsugamushi* by the multiple-pin peptide approach.

A total of 160 overlapping octapeptides (1 amino acid [aa] moved at a time) selected from the hydrophilic regions of the published sequence of Sta58 (16) were synthesized in duplicate by using the Epitope Scanning Kit (Chiron Mimotopes, Clayton, Victoria, Australia) according to the supplier's instructions. The software supplied with the kit arbitrarily designated 10 hydrophilic segment numbers based on the sequence of Sta58 and the hydrophobicity profile (16). The following list indicates the hydrophilic segment number, number of peptides synthesized, and starting amino acid residue: segment 1, 4 peptides, aa 40; segment 2, 4 peptides, aa 112; segment 3, 26 peptides, aa 128; segment 4, 36 peptides, aa 176; segment 5, 6 peptides, aa 278; segment 6, 40 peptides, aa 328; segment 7, 12 peptides, aa 386; segment 8, 6 peptides, aa 432; segment 9, 14 peptides, aa 472; and segment 10, 12 peptides, aa 524. The method used is based on Fmoc chemistry, and *N*-L-Fmoc-protected amino acids were purchased from Milligen/Biosearch (Burlington, Mass.). The success of the syntheses was monitored by the simultaneous synthesis of a positive (PLAQ) and a negative (GLAQ) control peptide and subsequently testing their binding to the supplied monoclonal antibody. The synthesized peptides, on pins configured to a 96-well microtiter plate format, were then tested for binding with sera from scrub typhus patients by a modified enzyme-linked immunosorbent assay (ELISA). A blocking step was first performed on the pins by incubating the pins for 1 h at room temperature with 2% bovine serum albumin–0.1% Tween 20 in phosphate-buffered saline (PBS) (pH 7.2). The blocks of pins were then incubated overnight at 4°C with 175 μl of a 1:1,000 dilution of serum per well. Blocks were then washed four times with 0.01 M PBS (pH 7.2) and incubated with 175 μl of peroxidase-labeled, affinity-purified anti-human immunoglobulin G (IgG)-IgM-IgA (heavy plus light chains), anti-human IgG, anti-human IgM, or anti-human IgA (1/1,000 dilution; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) per well for 60 min at room temperature. After four washes with 0.01 M PBS (pH 7.2), the pins were placed in wells containing substrate (2,2′-azino-di-3-ethyl-benzthiazoline sulfonate; Sigma Chemical Co., St. Louis, Mo.) and color development was allowed to proceed for approximately 45 min. The reaction was stopped by simply removing the blocks, and plates were then read at 414 nm in an ELISA plate reader (Titertek Multiskan II; Flow Laboratories). After testing with a particular serum, antibodies bound to the peptides were then removed by sonicating the blocks in 0.1 M PBS with 1% sodium dodecyl sulfate at 60°C in a

* Corresponding author.

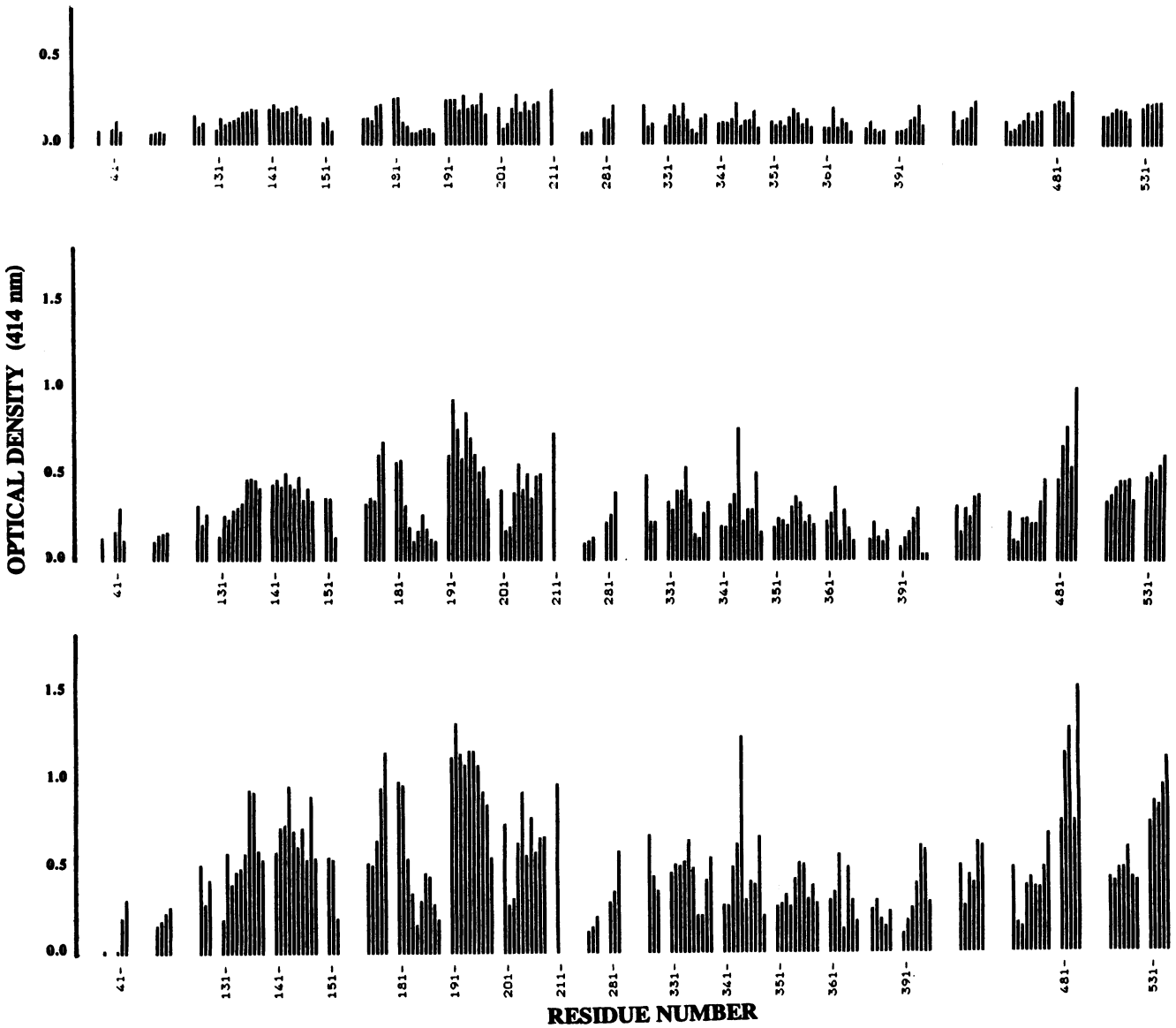

FIG. 1. Scan of antibody binding activity of two scrub typhus patients' sera (bottom two panels) and one normal serum sample (top panel) with overlapping Sta58 octapeptides from hydrophilic regions.

sonication bath (Branson model 3200). Pins were then ready for testing with the next serum sample. In order to identify which peptides give ELISA signals which are significantly above background values, the following algorithm was used: a mean was calculated for the lower half of all values, three times the standard deviation was added to this mean value, and all values above this cutoff are treated as significant. Sera from scrub typhus patients were obtained from patients admitted to the University and General Hospitals, Kuala Lumpur, Malaysia, and were positive both by the Weil-Felix test to *Proteus* strain OX-K antigens (18) and by the indirect immunoperoxidase technique (7). The majority of sera collected were acute-phase sera (in the first 2 to 3 weeks of illness). Patients had histories which were typical of scrub typhus as seen in Malaysia. Sera from patients with other febrile illnesses common in the region (malaria, dengue fever, leptospirosis, and typhoid fever) were also tested. Diagnosis in these cases was performed according to standard serological and/or isolation procedures.

On testing of the synthesized peptides with sera from scrub typhus patients, multiple, immunodominant regions which were not seen with normal sera were observed (Fig. 1). The plots presented in Fig. 1 were consistently seen with multiple sera from scrub typhus patients and normal, healthy individuals. The algorithm to determine ELISA values which are significant was then applied to ELISA results with nine scrub typhus patients' sera (Fig. 2). This analysis revealed that there were 15 octapeptides which gave significant ELISA values and which were recognized by all nine serum samples tested and 7 peptides which were recognized by eight of the nine serum samples (Fig. 2). There were no reactive peptides in hydrophilic segments 1 and 2 (Fig. 2).

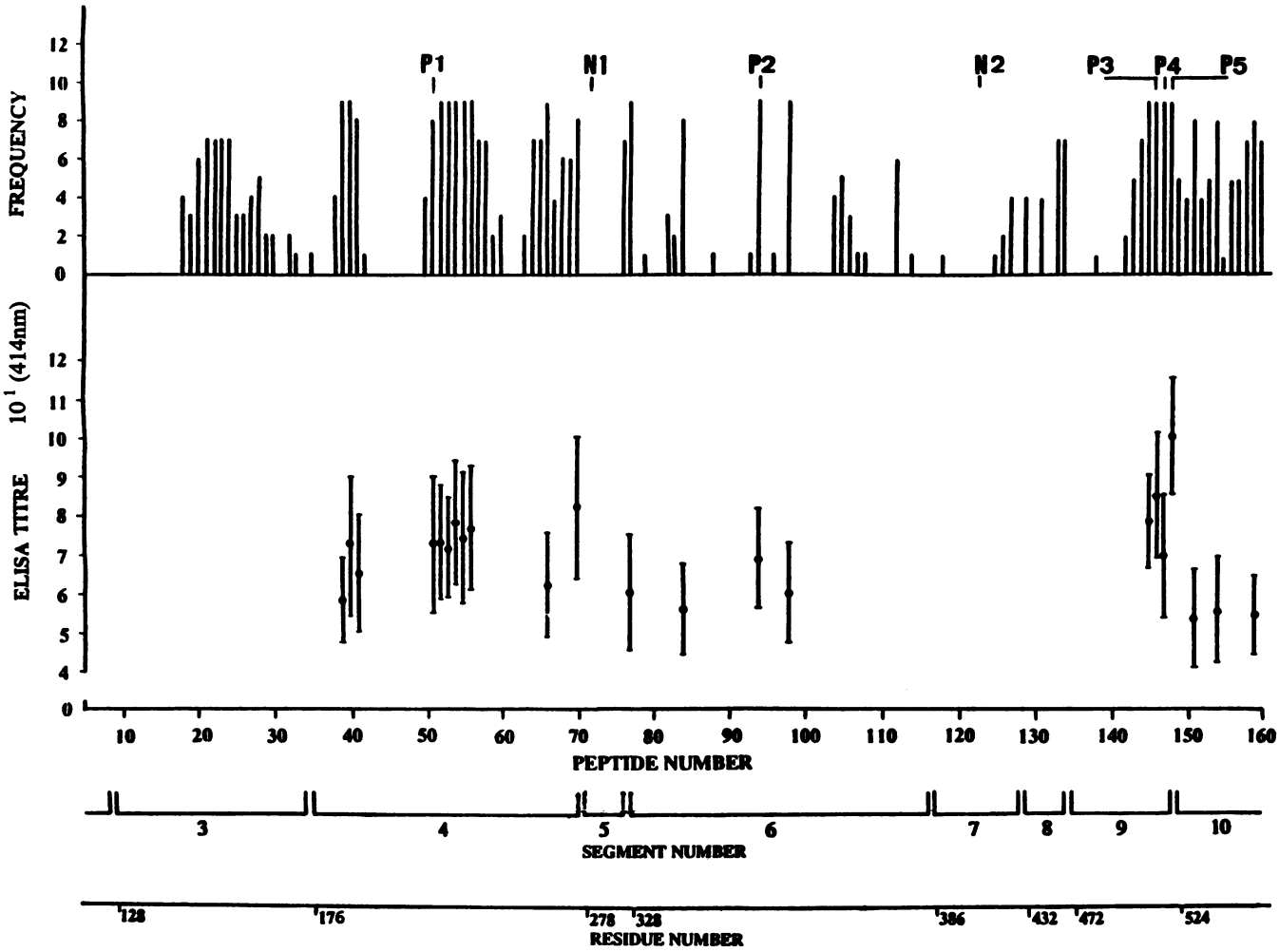

FIG. 2. Plot of frequency of binding seen with various peptides when tested with nine scrub typhus patients' sera (upper panel). The lower panel shows mean ELISA titers (A_{414}) for peptides which react with all nine or eight of nine serum samples.

Five peptides from the 15 reactive with all nine serum specimens (P1, MRFDRGYI; P2, VNSCREQI; P3, DART MQYV; P4, ARTMQYVD; and P5, RTMQYVDM; Fig. 2) were then selected for further study. These five peptides (P1 to P5), plus two negative peptides (N1, PGFGDRKK, and N2, EQKERKDR), were resynthesized on fresh pins and tested against a panel of sera. The results show strongest binding of these peptides to scrub typhus sera with significantly less binding to sera from patients with other febrile illnesses common in the region, i.e., dengue fever, leptospirosis, and typhoid fever (Fig. 3). Sera from patients with confirmed cases of malaria also showed significantly lower binding (data not shown). Analysis of antibody classes binding to P1 to P5 revealed that IgM is the predominant class (Fig. 4). There also appears to be little correlation between peptide binding and antibody titers evaluated by the indirect immunoperoxidase test in that sera with low IgM titers by the indirect immunoperoxidase test showed significant binding to P1 to P5 (left panel, Fig. 4) and sera with high IgG titers may or may not bind to P1 to P5 (center and right panels, Fig. 4).

The Sta58 outer membrane protein from *R. tsutsugamushi* is among the antigens most often recognized by the host immune response during infection in humans and animals (5, 17). This antigen is most probably identical to the 63-kDa protein described by Hanson (5) and the 60-kDa protein described by Tamura et al. (17). The present study shows that sera from scrub typhus patients are able to recognize multiple, continuous epitopes which were distributed across the hydrophilic regions of Sta58. Many immunogenic peaks were observed, and although variations occur between individual sera, 15 peptides were consistently recognized by all nine scrub typhus patients' sera tested. The presence of multiple reactivities is a common observation when human sera are tested by this approach and presumably means that no major population of antibodies directed to a linear epitope is present in the sera tested and that such antibodies are in a minority. This observation does not, however, diminish the potential value of such epitopes as peptide immunogens. In order to limit the number of peptides that need to be tested, we opted for those present on the hydrophilic regions of Sta58 on the assumption that most antigenic epitopes will be found on the protein surface and that relatively hydrophilic regions are more likely to be on the surface (11). This does not mean, of course, that immunogenic epitopes are not present in the hydrophobic regions, as was shown with

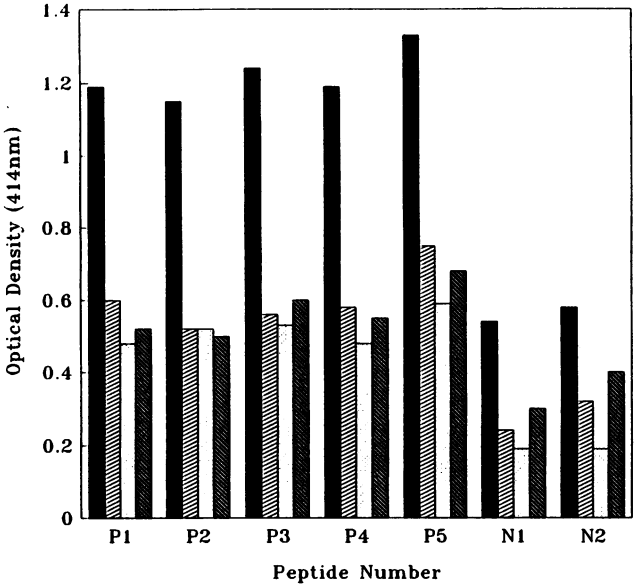

FIG. 3. Representative result of the reactivity of five selected peptides (P1 to P5) with various sera. Two negative, nonreactive peptides (N1 and N2) were also tested. P1, MRFDRGYI; P2, VNSCREQI; P3, DARTMQYV; P4, ARTMQYVD; P5, RTMQYV DM; N1, PGFGDRKK; N2, EQKERKDR. The standard single-letter code for amino acids is used. Sera were from patients with the following illnesses: scrub typhus (■), typhoid fever (▨), leptospirosis (▩), and dengue fever (▧).

foot-and-mouth disease virus (10). The multiple-peptide approach has also been used to define antigenic epitopes on other bacterial pathogens (3, 9, 13). The present study has shown that the systematic epitope-mapping approach used is useful for the identification of sequence-dependent linear epitopes that are recognized by host antibodies during infection. The data also suggest that the epitopes recognized by polyclonal human sera are widely distributed within hydrophilic regions of Sta58 and that sera from patients with other common febrile illnesses in the region do not bind to these epitopes. Of interest also is the finding that the reactive peptides bind primarily to IgM in the sera, perhaps reflecting recent infection with *R. tsutsugamushi*. Should some of these epitopes indeed prove to be scrub typhus specific, they may be valuable as pure diagnostic antigens in order to improve the sensitivity and specificity of serologic diagnosis (8). It should also be pointed out that the present study has not directly addressed the question of the absolute specificity of the peptides through absorption studies with native antigen or free peptides and that specificity is inferred from nonreactivity of normal sera and sera from patients with other illnesses common to the region.

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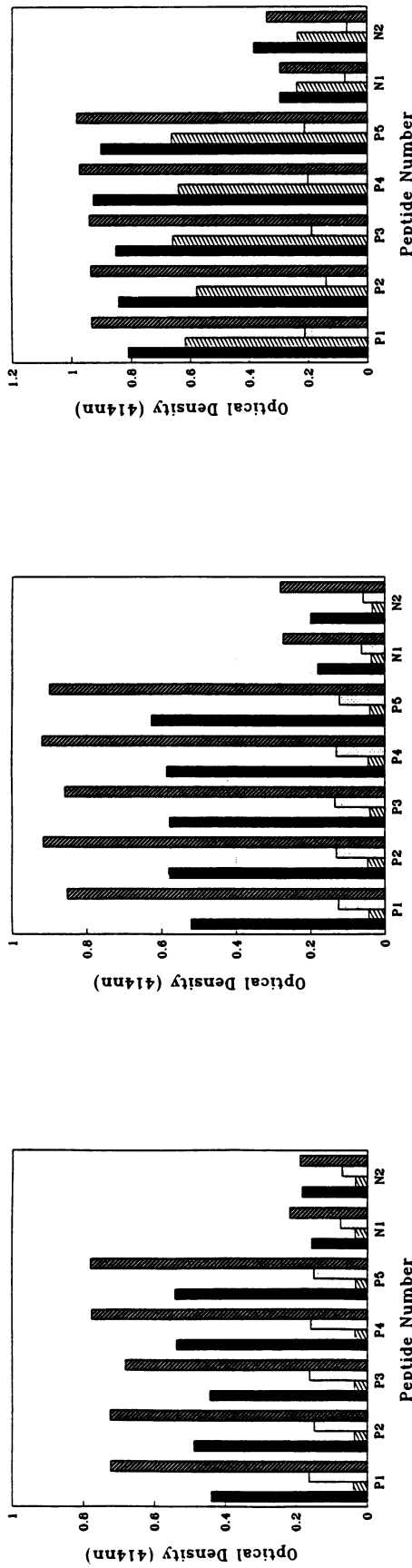

FIG. 4. Class of antibody binding to peptides P1 to P5 with three selected scrub typhus patients' sera. IgG titers of the three sera by the indirect immunoperoxidase test were all ≥1,600; IgM titers by that test were, from left to right, <50, ≥1,600, and ≥1,600. ■, IgG, IgA, and IgM; ▨, IgG; ▩, IgA; ▧, IgM.

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