Species-Specific Monoclonal Antibodies to *Rickettsia japonica*, a Newly Identified Spotted Fever Group Rickettsia

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A total of 192 hybridomas were developed from mice immunized with *Rickettsia japonica*, a newly identified spotted fever group rickettsia pathogenic for humans. Of these hybridomas, 101 were species specific, 37 were spotted fever group reactive, and the other 54 were also reactive with one or more of the other pathogenic species of spotted fever group rickettsiae, *Rickettsia akari*, *Rickettsia australis*, *Rickettsia conorii*, *Rickettsia rickettsii*, and *Rickettsia sibirica*. Seven of the species-specific monoclonal antibodies were characterized. These monoclonal antibodies all belong to the immunoglobulin G class and react with all five strains of *R. japonica* at the same immunofluorescence titers, indicating that the five strains all belong to a single species. The species-specific epitopes reactive with these monoclonal antibodies are located on the surface proteins of the organisms demonstrated as 145- and 120-kilodalton bands on Western immunoblots. These two antigenic bands were shown to be proteins, because treatment with protease K completely destroyed the reactivity of the bands with the monoclonal antibodies.

The spotted fever group (SFG) of the genus *Rickettsia* has been shown to comprise the following five species of pathogenic intracellular bacteria: *Rickettsia rickettsii* (Rocky Mountain spotted fever), *Rickettsia conorii* (boutonneuse fever), *Rickettsia sibirica* (north Asian tick typhus), *Rickettsia australis* (Queensland tick typhus), and *Rickettsia akari* (rickettsialpox) (18, 19). These species of SFG rickettsiae occur in geographically distinct regions separated for the most part by natural boundaries of the earth (8).

Until 1984, there had been no report of SFG rickettiosis in Japan, where tsutsugamushi disease (or scrub typhus) had been recognized as a major rickettsial infection (12). The causative agents of a newly recognized disease were isolated from patients with febrile exanthematous illness on Shikoku, one of the main islands of Japan (13, 14). The organisms had ultrastructures characteristic of SFG rickettsiae (16). Recently, we identified these organisms as a new species of SFG rickettsia (15). The analysis was carried out by reciprocal cross-reactions of mouse monoclonal antibodies (PAbs) to strains of the Japanese isolates and SFG rickettsiae by the method of Philip et al. (11). The results demonstrated that five Japanese strains isolated from different patients belong to a single species distinct from any SFG rickettsiae known to be pathogenic to humans. In addition, we demonstrated that none of the Japanese isolates reacted with mouse monoclonal antibodies (MAbs) that are species specific for *R. conorii*, *R. rickettsii*, *R. sibirica*, and *R. akari* (7, 9, 15). We proposed the taxonomic name *Rickettsia japonica* sp. nov. for the new species that differs from other previously known pathogenic rickettsiae of the SFG (15).

In this report, we describe that MAbS directed to epitopes on the surface proteins of strain YH of *R. japonica* reacted with all Japanese strains tested but not with standard strains of other pathogenic SFG rickettsial species, supporting the conclusion that *R. japonica* is a new species of SFG rickettsia.

**Materials and Methods**

**Rickettsia strains.** Five Japanese strains of SFG rickettsiae, YH, NT, NK, YKI, and TKN, established persistent infections of Vero cells (VC) (13, 14). A total of 6 to 15 passages were performed before the strains were used. As reference strains of SFG rickettsiae pathogenic to humans, the following strains were employed: *R. akari*, Kaplan strain (149 passages in VC and 2 passages in chicken embryo [CE]); *R. australis*, Cutlass strain (2 passages in VC and 3 passages in CE); *R. conorii*, Malish 7 strain (1 passage in guinea pig, 613 passages in VC, and 113 passages in CE); *R. rickettsii*, Sheila Smith strain (105 passages in CE); and *R. sibirica*, strain 232 (21 passages in CE).

**Production of MAbs.** MAbs to *R. japonica* (strain YH) were prepared essentially according to the procedure described by Li et al. (7). Two 8-week-old female BALB/c mice were inoculated intraperitoneally on days 0 and 14 with Renografin (E. R. Squibb & Sons, Princeton, N.J.)-purified rickettsiae grown in BHK-21 (C-13) cells. Three days after the second injection, spleen cells from two mice were subjected to fusion with SP2/0-Ag14 myeloma cells (5:1) by using 50% polyethylene glycol (molecular weight, 3,500; Sigma Chemical Co., St. Louis, Mo.). Hybridomas were grown in 96-well microdilution plates in selective medium containing hypoxanthine and aminopterin. Supernatant fluids were screened for antibodies to *R. japonica* (strain YH) by microimmunofluorescence (micro-IF) assay with fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin M (lgM), IgG, and IgA antibodies (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.). Culture supernatants from positive clones were tested for reactivities to standard strains of pathogenic SFG rickettsiae to select species-specific MAbs. Seven species-specific hybridomas were subcloned twice by limiting dilutions. Isotypes of MAbs were determined by the method of Ouchterloney (10) with antisera to mouse IgM, IgA, IgG1, IgG2a, IgG2b, and IgG3.

**Micro-IF assay.** Micro-IF was performed by the method described previously (15). The culture supernatant fluids of the hybridomas were tested for antibody titers with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody.

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Antigens used for micro-IF were prepared from VC persistently infected with *R. japonica* YH, NT, NK, YKI, or TKN and VC heavily infected with other pathogenic SFG rickettsiae. In order to detect surface antigens of *R. japonica*, unfixed rickettsiae purified by Renografin density gradient centrifugation were used as antigens.

**Immunoelectron microscopy.** Immunoelectron microscopy was performed by using colloidal gold-conjugated protein A according to the procedure described previously (7). Renografin-purified *R. japonica* (strain YH) cells placed on Formvar-coated nickel grids were reacted with MAbs followed by reaction with colloidal gold-labeled protein A (Janssen Life Sciences Products, West Chester, Pa.). The grids were examined under a Philips 201 transmission electron microscope.

**SDS-polyacrylamide gel electrophoresis and Western immunoblotting.** Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western immunoblotting were performed essentially according to the methods described previously (6, 7). Embryonated eggs from hens (SPAFAS, Inc., Norwich, Conn.) were inoculated with VC persistently infected with *R. japonica* (strain YH). Infected yolk sacs were harvested, and rickettsiae were purified by Renografin density gradient centrifugation. Renografin-purified *R. japonica* cells were dissolved in sample buffer (0.0625 M Tris hydrochloride [pH 8.0], 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue) directly or, on the other hand, were treated or mock treated with 28 U of proteinase K (type XXVIII; Sigma Chemical Co.) per ml at 37°C for 90 min before solubilization. Electrophoresis was performed on a 7.5% polyacrylamide slab gel in the presence of 0.5% SDS at 8 mA per gel at 4°C overnight. The gel was stained with Coomassie brilliant blue, or the antigens on the gel were transferred to a nitrocellulose sheet at 195 mA for 5 h at 4°C. After the antigens were transferred, the nitrocellulose sheet was cut into strips and incubated at room temperature for 1 h in phosphate-buffered saline (PBS) containing 5% nonfat dry milk to block nonspecific binding of antibodies. Strips were washed twice for 10 min each time with PBS containing 0.05% Tween 20; they were then incubated with MAbs or with mouse PAb to *R. japonica* diluted 1:10 and 1:100, respectively, in PBS containing 1% milk at room temperature for 30 min. The strips were washed as described above and incubated for 30 min with horseradish peroxidase-conjugated goat antibody to mouse IgG (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:1,000 in PBS containing milk. After two washes for 10 min each time in PBS, color was developed with 3,3′-diaminobenzidine (Eastman Kodak Co., Rochester, N.Y.).

**RESULTS**

**Micro-IF titers and isotypes of MAbs.** Spleen cells obtained from BALB/c mice immunized with strain YH of *R. japonica* were fused with myeloma cells. A total of 192 of the hybridomas secreted antibodies reactive with the homologous antigen. Of these hybridomas, 101 were species specific; they reacted with *R. japonica* but not with *R. akari*, *R. australis*, *R. conorii*, *R. rickettsii*, or *R. sibirica*. Thirty-seven hybridomas were SFG reactive, while the other 54 hybridomas also reacted with one or more of the previously established species of pathogenic SFG rickettsiae (data not shown). Seven species-specific MAbs produced from subcloned hybridomas were examined for their reactivity with the other strains of *R. japonica*. These MAbs reacted with all strains of *R. japonica* at the same immunofluorescent-antibody titers as the homologous strain (Table 1). On the other hand, these MAbs showed no reaction with the other SFG rickettsiae pathogenic to humans. The MAbs all belong to either the IgG2a or the IgG2b subclass (Table 1).

**Surface localization of species-specific epitopes.** When *R. japonica* was reacted with MAb 2Y1-D4, the presence of gold particles was demonstrated on the surfaces of the organisms (Fig. 1A). Reactions with the other MAbs (2Y3-A3, 3Y5-B5, 3Y6-C1, 3Y7-C9, 3Y7-H6, and 3Y10-D1) showed similar features (data not shown). On the other hand, a species-specific MAb to *R. rickettsii* (F3-30) showed no surface gold binding when reacted with *R. japonica* (Fig. 1B). Immunofluorescence of unfixed *R. japonica* also showed surface fluorescence with these MAbs but not with F3-30 (data not shown).

**SDS-polyacrylamide gel electrophoresis.** Figure 2 shows the Coomassie brilliant blue-staining profile of *R. japonica* electrophoresed on an SDS-polyacrylamide gel. Three major protein bands (120, 135, and 145 kilodaltons [kDa]) with molecular sizes greater than 100 kDa were demonstrated. Among them, the molecule which migrated at 120 kDa was the most prominent one. The 200-kDa band was a protein derived from yolk sac (data not shown).

**Western immunoblotting.** Western immunoblotting revealed that the species-specific MAbs were reactive with the

### Table 1. Immunoglobulin subclasses and titers of species-specific MAbs to *R. japonica* strains

<table>
<thead>
<tr>
<th>MAb</th>
<th>IgG subclass</th>
<th>YH</th>
<th>NT</th>
<th>NK</th>
<th>YKI</th>
<th>TKN</th>
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<tr>
<td>2Y1-D4</td>
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<tr>
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</table>

* Antibody titers are expressed as reciprocals of the highest dilutions that gave a positive reaction. Undiluted fluids of the cultures of the hybridomas to pathogenic SFG rickettsial species (*R. akari*, *R. australis*, *R. conorii*, *R. rickettsii*, and *R. sibirica*) all showed negative reactions.

FIG. 1. Electron micrographs of *R. japonica* reacted with various MAbs and colloidal gold-conjugated protein A. (A) MAb to *R. japonica*, 2Y1-D4, shows surface location of reactivity by colloidal gold; bar = 0.3 μm. (B) Species-specific MAb to *R. rickettsii*, F3-30 (7), shows no reactivity with surface antigens of *R. japonica*; bar = 0.3 μm.
antigens of the 145-kDa band (Fig. 3, lane 1) and the 120-kDa band (Fig. 3, lanes 2 through 7). These two bands were also revealed to be predominant by using PAbs to R. japonica (Fig. 3, lane 8). Some minor bands were also observed to react with MAbs. Furthermore, when the antigens were treated with proteinase K, no bands were observed by using the MAbs in Western immunoblots (Fig. 3, lanes 9 through 15). PAbs also demonstrated a ladder of antigens with molecular sizes smaller than 42 kDa which remained reactive after protease treatment (Fig. 3, lane 16).

DISCUSSION

MAbs to rickettsial antigens have been utilized to analyze epitopes for species specificity and cross-reactivity (2, 3, 7, 9). It has been shown that a number of shared and unique epitopes are present on surface proteins of rickettsiae (1, 3, 4, 7) and that lipopolysaccharidelike antigens contain the common epitopes which define the SFG (1, 3). The MAbs react with all strains of R. japonica at the same immunofluorescent-antibody titers as does the homologous strain but do not react with other pathogenic species of SFG rickettsiae. Thus, these MAbs recognize species-specific epitopes of R. japonica which are present on all five Japanese isolates. These results confirm that these Japanese strains belong to a single species, as was demonstrated by reciprocal cross-reactions with mouse PAbs (15). All of these species-specific MAbs recognize epitopes present on the surface of R. japonica. The species-specific epitopes corresponding to these MAbs were shown to be located predomi-

FIG. 2. Coomassie brilliant blue-stained SDS-polyacrylamide gel electrophoresis of the protein profile of R. japonica. Arrowheads show 145-, 135-, and 120-kDa bands, respectively, from top to bottom.

FIG. 3. Immunoblots of antigens of R. japonica. Lanes 1 through 8, Mock treated with proteinase K; lanes 9 through 16, treated with proteinase K. Lanes 1 and 9, MAb 2Y1-D4; lanes 2 and 10, MAb 3Y3-A8; lanes 3 and 11, MAb 3Y3-B5; lanes 4 and 12, MAb 3Y6-C1; lanes 5 and 13, MAb 3Y7-C9; lanes 6 and 14, MAb 3Y7-H6; lanes 7 and 15, MAb 3Y10-D1; lanes 8 and 16, murine PAbs to R. japonica.

nantley on the antigens of the 145- and 120-kDa bands on Western immunoblots. The antigen migrating at a molecular size of 120 kDa was the major band among polypeptides with a molecular size greater than 100 kDa. Some minor bands also reacted weakly with the MAbs. These antigens may share amino acid sequences with the major polypeptides. This issue is currently being investigated. Furthermore, the two major antigenic bands were shown to be proteins, because treatment with proteinase K completely destroyed the reactivity of these bands with the MAbs. This result corresponds to the findings with species-specific epitopes of R. conorii and R. rickettsii (7). As was reported by Anacker et al. (1) and Feng et al. (4), the ladderlike profile of antigens demonstrated by Western immunoblotting with PAbs to R. japonica after treatment with proteinase K is suggestive of lipopolysaccharide molecules.

SFG rickettsial infections in Japan have occurred mainly along the Pacific coast of the islands of Shikoku, Kyushu, and Honshu (5, 12–14, 20). Until now, there had been no reported isolation of R. japonica outside Japan. The recent isolates of SFG rickettsiae in northwestern and northeastern China, the nearest regions where SFG rickettsial organisms have been isolated from humans, were identified as R. sibirica (17). Surveys of SFG rickettsiosis should be performed in southeastern Asia, since no human isolates of SFG rickettsiae have been reported from the biogeographic Oriental region, except from Japan (5, 8, 13, 14, 20). Also, the vector of R. japonica has yet to be identified. A set of MAbs to R. japonica will provide a powerful tool to identify etiologic agents isolated in ecologic studies.

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LITERATURE CITED


