Production of Monoclonal Antibodies against *Rickettsia massiliae* and Their Use in Antigenic and Epidemiological Studies

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Rickettsiae are gram-negative, obligate intracellular bacteria which have historically been divided into three groups: the typhus group, the scrub typhus group, and the spotted fever group (SFG). Recently, several new SFG rickettsiae have been characterized, and most of these species are associated with ticks and have, as yet, no known pathogenicity toward humans. *Rickettsia massiliae*, which is widely distributed in Europe and Africa, is one such rickettsia. In order to investigate the antigenic relationships between *R. massiliae* and other rickettsial species and to develop a more convenient methodology for identifying *R. massiliae*, we produced monoclonal antibodies against the type strain (Mtu1) of *R. massiliae* by fusing immunized splenocytes with SP2/0-Ag14 myeloma cells. A panel of 16 representatives were selected from the 163 positive hybridomas identified on initial screening, and their secreted monoclonal antibodies were further characterized. The reactivities of these 16 monoclonal antibodies with a large panel of rickettsial species were assessed by the microimmunofluorescence assay. All species of the SFG rickettsiae reacted with the monoclonal antibodies directed against epitopes on lipopolysaccharide, which is the common antigen among the SFG rickettsiae.

Some closely related species of the SFG, such as Bar29, “*R. aeschlimannii*,” and *R. rhicephali*, showed strong cross-reactivities with the monoclonal antibodies directed against epitopes on the two major high-molecular-mass heat-labile proteins (106 and 120 kDa). In addition, species-specific monoclonal antibodies demonstrated that *R. massiliae* is antigenically different from other rickettsial species. Moreover, these species-specific monoclonal antibodies were successfully used for identifying *R. massiliae* in the ticks collected from southern France, and are therefore potentially useful tools in the identification and investigation of *R. massiliae* in ticks in large-scale field work.

In recent years, with the introduction of improved techniques for their isolation and identification (22, 36, 44, 46–48), numerous novel SFG rickettsiae have been identified, but their pathogenicities toward humans remain unclear (5, 11, 12). *R. massiliae* is such a species which was originally isolated from *Rhipicephalus turanicus* ticks collected from regions near Marseille, France (11, 13). Subsequently, an isolate of this species had also been found in *Rhipicephalus sanguineus* ticks in Greece (5). *R. turanicus* ticks collected in Portugal (6) and *Rhipicephalus musmarum*, *Rhipicephalus longipes*, and *Rhipi cephalus sulcatus* ticks collected in the Central African Republic (51) have also now been shown to be infected with *R. massiliae*, indicating that the species is widely distributed. Because shell vial centrifugation isolation methods (36) and molecular biology-based identification schemes (22, 44, 46, 47) for *R. massiliae* are laborious in large-scale field work, we aimed to develop a more convenient, monoclonal antibody-based methodology to facilitate the detection of *R. massiliae* in ticks. Additionally, phenotypic and genotypic characterization of *R. massiliae* has suggested that this species is closely related to the other rickettsial species, such as Bar29, “*R. aeschlimannii*,” and *R. rhicephali* (12, 47, 48). Therefore, we also aimed to investigate the antigenic relationships between *R. massiliae* and other rickettsial species, using monoclonal antibodies produced against Mtu1, the type strain of *R. massiliae* (13).

**MATERIALS AND METHODS**

**Rickettsiae.** The sources of all strains used in this study are presented in Table 1. The following strains were obtained from the American Type Culture Collection (ATCC; Rockville, Md.): *R. akari*, *R. conori*, *R. rickettsii*, *R. prowazekii*, *R. typhi*, *O. tsutsugamushi*, and *Coxiella burnetii*. *R. africae*, *R. massiliae* Mtu1 and

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<th>Strain</th>
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*a* The unrecognized and unnamed species are marked with asterisks.

*b* SFG, spotted fever group; TG, typhus group; STG, scrub typhus group.
GS, “R. aesculainni,” Bar29 strain, and Astakhan fever rickettsia were isolated in our laboratory. R. australis, Israeli tick typhus rickettsia, *R. rickettsii,* “R. slowace,” and Thai tick typhus rickettsia were kindly provided by G. A. Duch (Naval Medical Research Institute, Bethesda, Md.). *R. bellii, R. honiei, R. japonica, R. montana,* and *R. parkeri* were kindly provided by D. H. Walker (University of Texas, Galveston). *R. honei* was kindly supplied by W. Burgdorf (Rocky Mountain Laboratory, Hamilton, Mont.). *R. sibirica* and *R. canadensis* were obtained from the Gamaleya Research Institute (Moscow, Russia).

**Production of monoclonal antibodies.** Typhus group rickettsiae, SFG rickettsiae, and other rickettsiae were prepared for immunization or sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) by further treating harvested cells by sonication, followed by purification by passage through a 25% (wt/vol) sacrose and a 28% (wt/vol) sucrose, followed by purification by passage through a 25% (wt/vol) sacrose and a 28% (wt/vol) sucrose density gradient as described previously (59, 60).

**Production of monoclonal antibodies.** Purified *R. massiliae* were used as the immunogen, and 6-week-old female BALB/c mice were used to provide immunized splenocytes for hybridoma formation (28, 60). After three intraperitoneal injections with ~2 × 10^3 organisms at 1-week intervals, mice were boosted with ~4 × 10^4 organisms by tail vein injection. Three days later, the mice were sacrificed and their splenocytes were fused with SP2/0.Ag14 myeloma cells by using 50% (wt/vol) polyethylene glycol (molecular weight, 1,500 to 1,600; Sigma Chemical Co., St. Louis, Mo.) (28). The fused hybridoma cells were distributed into 96-well plates (Nunc, Roskilde, Denmark) and were grown with 1% hypo-xanthine, amphotericin B, and hypoxanthine selection medium (Sigma). Ten days after fusion, supernatants were recovered from wells containing cloned cells. The presence of antibodies was determined by a micro-IF assay incorporating the set of antigens of *R. massiliae, R. australis, R. conorii, R. rickettsii,* and *R. typhi* (see below). Positive hybridomas exhibiting different reactivities with screening antigen were selected and subcloned by limiting dilution (28). The immunoglobulin class and subclass of each monoclonal antibody derived from hybridomas were determined by using an immunotype mouse monoclonal antibody typing kit (stock no. ISO-1, Sigma). Amino acid sequence was determined by in situ hybridization into BALB/c mice by standard methods (28).

The total number of monoclonal antibodies showing cross-reactivity with screening antigen.

**Micro-IF assay.** Antigens were applied into wells of 24-well microscope slides with a pen nib and were then fixed in acetone for 20 min at room temperature. The micro-IF assay was performed as described previously (60). Briefly, antigen slides with hybridoma culture supernatants and ascitic fluids (undiluted) by the micro-IF assay. The nospecific binding sites were blocked by using ImmunoBlock (underlined) by the micro-IF assay.

**Preparation of antigen.** Typhus group rickettsiae, SFG rickettsiae, and *R. massiliae* were tested for the presence of antibodies was determined by a micro-IF assay incorporating the set of antigens of *R. massiliae, R. australis, R. conorii, R. rickettsii,* and *R. typhi* (see below). Positive hybridomas exhibiting different reactivities with screening antigen were selected and subcloned by limiting dilution (28).

**Western immunoblotting.** Purified *R. massiliae* were transferred onto a nitrocellulose membrane (0.45-μm pore size; Trans-Blot; Bio-Rad) (60). Briefly, nonspecific binding sites were blocked by incubating hybridoma culture supernatants and ascitic fluids diluted 1:1,000 in PBS for 2 h at room temperature and washed as described above. The stained slides were then incubated with dichlorotriazinyl amino fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG) and IgM (heavy and light chains; AffiniPure; Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa.) diluted 1:200 in PBS in a humidified chamber at 37°C for 30 min, followed by three washes in PBS for 30 min each time. The dried slides were then incubated with diaminobenzidine (DAB) solution (BioMérieux, Marcy l’Etoile, France), and examined by epifluorescence microscopy (Axioskop 20; Carl Zeiss, Göttingen, Germany) at ×400 magnification. Pooled polyclonal antiserum from immunized mice used for hybridoma formation and pooled sera from uninfected mice were used as positive and negative controls, respectively.

**SDS-PAGE and Western immunoblotting.** Purified organisms were subjected to SDS-PAGE (33) in a 3.0% stacking gel and a 7.5% resolving gel. Prestained SDS-PAGE standards (low- and high-range; Bio-Rad, Richmond, Calif.) were included in each run for estimating protein band size.
in acetone for 20 min at room temperature. Monoclonal antibody MA6-F3, diluted 1:500 in PBS, was incorporated into a micro-IF assay described above to detect R. massiliae in the smears. L929 cells infected with R. massiliae Mtu1T were used as a positive control, and monoclonal antibody AF1-D12, specific for only R. afeie and R. parkeri (60), was used as a negative control.

PCR-RFLP analysis. Template DNA was extracted from the remainder of the ticks by using the QIAamp tissue kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. PCRs incorporating two previously described primer pairs (Rp CS.877p-1258n and Re 190.70p-602n) (22, 44) were used to amplify rickettsial DNA from these templates. In each amplification, DNAs extracted from R. massiliae Mtu1-infected L929 cells and noninfected L929 cells were used as positive and negative controls, respectively. The PCR amplification products were monitored by rapid electrophoresis (22, 47). DNA standard size marker V (Eurogentec, Seraing, Belgium) was included in each run to estimate the molecular masses of the resolved products.

The PCR amplicons were subsequently identified by restriction fragment length polymorphism (RFLP) analysis (22, 44). Restriction endonuclease Alul (10 U/μl; Boehringer Mannheim, Mannheim, Germany) was used for digestion of the Rp CS.877p-1258n amplification products (22), and PstI (10 U/μl; Boehringer Mannheim) and Rsal (10 U/μl; Boehringer Mannheim) were used for digestion of the Re 190.70p-602n amplification products (44). The amplified product from R. massiliae Mtu1-infected L929 cells was included in these digests. The restriction fragments were separated electrophoretically and were visualized under a UV transilluminator (22, 47). DNA standard size marker V (Boehringer Mannheim) was used to estimate the fragment size.

RESULTS

Production and characterization of monoclonal antibodies against R. massiliae. Fourteen days after fusion, a total of 163 hybridomas producing monoclonal antibodies against immunogen were obtained. Of these hybridomas, 16 were selected for limiting dilution. The corresponding monoclonal antibodies designated by the prefix MA were further characterized.

By the micro-IF assay, three monoclonal antibodies were species specific; three demonstrated extensive cross-reactivities with the large rickettsial panel: MA1-D2 reacted with all the rickettsial strains except O. tsutsugamushi and C. burnetii, MA1-B8 and MA7-B12 reacted with all species of the SFG, and the others showed reactivities with only a few SFG rickettsiae, including Bar29, “R. aeschlimanni,” “R. rhipicephali,” R. japonica, and Israeli tick typhus rickettsia (Table 2).

Except for MA1-D2, the monoclonal antibodies could be characterized as follows: two belonged to the IgM class, two belonged to the IgG1 class, six belonged to the IgG2a class, three belonged to the IgG2b class, and two belonged to the IgG3 class (Table 2).

The specificity of this monoclonal antibody panel except for that of MA1-D2 was determined by Western immunoblotting (Fig. 1). Two monoclonal antibodies, MA1-B8 and MA7-B12, which demonstrated broad cross-reactivities with the SFG rickettsial species, were found to be directed against the lipopolysaccharide (LPS)-like antigen which exhibited a ladder-like pattern after the whole-cell lysate had been treated with protease K (Fig. 2). Monoclonal antibodies MA2-A1 and MA2-E2 are directed against epitopes located on the 106-kDa heat-labile protein. The others are directed against epitopes located on the 120-kDa heat-labile protein (Fig. 1 and 3).

The further analysis by isotyping indicated that all monoclonal antibodies which reacted with protein epitopes belonged to the IgG class, including the IgG1, IgG2a, IgG2b, and IgG3 subclasses, and that monoclonal antibodies directed against the LPS-like antigen belonged to the IgM class (Table 2).

Antigenic relationships revealed by monoclonal antibodies. All rickettsial species of the typhus group and the SFG rickettsiae showed antigenic relationships, as demonstrated by monoclonal antibody MA1-D2, which exhibited extensive reactivities among rickettsial species. All species of the SFG exhibited clear antigenic relationships on LPS, as shown by anti-LPS monoclonal antibodies MA1-B8 and MA7-B12.

The 13 monoclonal antibodies directed against epitopes of surface proteins showed cross-reactivity with only a few SFG rickettsial species. Among these SFG rickettsial species, the GS strain (5) reacted with all 13 monoclonal antibodies; Bar29, “R. aeschlimanni,” and R. rhipicephali reacted with six, five, and three monoclonal antibodies, respectively. Additionally, R. japonica and Israeli tick typhus rickettsia showed cross-reactivities with a few monoclonal antibodies (Table 2).

Antigenic relationships and the epitope distribution on proteins among the species of SFG rickettsia, which reacted with anti-R. massiliae monoclonal antibodies, were determined by Western immunoblotting (Fig. 4). Two major high-molecular-mass proteins, such as the 120- and 135-kDa proteins of Bar29, the 104- and 124-kDa proteins of “R. aeschlimanni,” and the...
30, 57, 58). However, usually, only a few species like R. rickettsii, R. conorii, R. sibirica, R. akari, and R. australis were included in those studies. In our study, we included all the reference rickettsial species and C. burnetii in an analysis of their antigenic relationships using monoclonal antibodies raised against R. massiliae, an SFG rickettsia isolated only from ticks (13).

Most of the SFG rickettsiae (R. africae, R. akari, Astrakhan fever rickettsia, R. australis, R. conorii, R. honei, R. helvetica, R. montana, R. parkeri, R. rickettsii, R. sibirica, “R. slovaca,” and Thai tick typhus rickettsia) shared only the common LPS antigen with R. massiliae, whereas the other species of the SFG rickettsiae (Bar29, “R. aeschlimannii,” R. rhipicephali, R. japonica, and Israeli tick typhus rickettsia) shared epitopes on surface proteins as well as on LPS. These shared protein epitopes, which were located on both immunodominant proteins of R. massiliae (106- and 120-kDa proteins), were also demonstrated to exist on the surface proteins of Bar29, “R. aeschlimannii,” R. rhipicephali, and R. japonica by Western immunoblotting. Among these species, Bar29 isolated from R. sanguineus ticks from the Catalan region (Spain) had the most epitopes in common with R. massiliae, concurring with previous studies on their phenotypic and genotypic characteristics (12, 47, 48).

Further analysis showed that many of the protein epitopes recognized were located on different-sized proteins in different species. The epitopes on the 106-kDa protein of R. massiliae were only found on the corresponding surface proteins of Bar29, “R. aeschlimannii,” and R. rhipicephali, although epitopes on the 120-kDa protein were distributed more widely. These results suggested that some of the epitopes on the high-molecular-mass proteins of R. massiliae are shared among closely related rickettsial species. Furthermore, we found that some proteins (i.e., the 120-kDa protein of R. massiliae) which appear as predominant bands in protein profiles are also the principal immunogens in the humoral response of mice. However, some proteins (i.e., the 106-kDa protein of R. massiliae) which appear only as weak bands in protein profiles are probably strong immunogens in mouse immunization studies. A previous study of monoclonal antibodies against R. africae also demonstrated this phenomenon (60). Thus, the more promi-

![Image](http://jcm.asm.org/)

**FIG. 3.** Western immunoblotting of mouse polyclonal antisera and monoclonal antibodies with R. massiliae Mtu1<sup>1</sup> proteins treated at 100°C for 5 min. Lane 1, mouse polyclonal antiserum; lane 2, noninfected mouse serum; lane 3, MA1-D2; lane 4, MA1-B8; lane 5, MA1-B12; lane 6, MA2-A1; lane 7, MA2-E2; lane 8, MA4-C11; lane 9, MA6-F3; lane 10, MA7-A11; lane 11, MA7-B12; lane 12, MA8-G2; lane 13, MA8-F6; lane 14, MA10-D4; lane 15, MA10-E5; lane 16, MA10-A12; lane 17, MA12-G4; lane 18, MA12-G12. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.

![Image](http://jcm.asm.org/)

**FIG. 4.** Western immunoblotting of monoclonal antibodies with R. massiliae Mtu1<sup>1</sup> and other SFG rickettsial strain. Lane 1, R. massiliae Mtu1; lane 2, Bar29; lane 3, “R. aeschlimannii”; lane 4, R. rhipicephali; lane 5, R. japonica. Molecular mass markers of 205, 116.5, 106, 80, 49.5, and 32.5 kDa (from top to bottom, respectively) were loaded on the left.
nent bands in the SDS-PAGE profile do not always correspond to the principal immunogens of rickettsial species. In the present study, Bar29, “R. aeschlimannii,” and R. rhipicephali also showed more antigenic similarities to R. massiliae than to other species of SFG rickettsia. These four SFG rickettsial species, which also exhibit close phylogenetic relationships (47, 48), could be considered one subgroup of the SFG rickettsiae.

R. massiliae has a unique epitope which stimulates the production of species-specific monoclonal antibodies. These species-specific monoclonal antibodies revealed the antigenic diversity between R. massiliae and other closely related rickettsial species. Furthermore, they also provide an alternative tool for identifying this species in ticks (60). In the past, the identification of R. massiliae has relied on its isolation (5, 11) or on PCR-RFLP techniques (6, 52). These two methodologies are both time- and material-consuming. For example, the identification of rickettsiae in 20 ticks by the PCR-RFLP technique requires 48 to 72 h and costs $3 to $4 per sample (51). By comparison, the detection of R. massiliae in the same number of ticks by a micro-IF assay incorporating a species-specific monoclonal antibody takes only 3 h and costs $0.30 per sample. The species-specific monoclonal antibodies raised from R. massiliae therefore appear to be a powerful and more acceptable tool for identifying R. massiliae in ticks in large-scale field and epidemiological work. Similarly, the use of a subgroup-specific monoclonal antibody can also be extended to identifying the species of the subgroup including R. massiliae, Bar29, “R. aeschlimannii,” and R. rhipicephali.

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REFERENCES


\[\text{TABLE 3. Antigenic relationships of surface protein of SFG rickettsiae exhibiting cross-reactivity with monoclonal antibody to } R. \text{ massiliae Mu1}^{16}\]

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<td>MA12-G12</td>
<td>IgG2b</td>
<td>120</td>
</tr>
<tr>
<td>MA7-A11</td>
<td>IgG2a</td>
<td>120</td>
</tr>
<tr>
<td>MA8-F6</td>
<td>IgG2a</td>
<td>120</td>
</tr>
</tbody>
</table>

\({}^{16}\) The cross-reactivity of R. japonica and Israeli tick typhus rickettsia with monoclonal antibodies MA10-E5 and MA10-A12 cannot be detected.

\(\cdots\) no reactivity; ND, not detected.


