Causative Agent of Spotted Fever Group Rickettsiosis in Japan

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Received 26 October 1989/Accepted 13 January 1990

Since 1984, it has been known that spotted fever group rickettsiosis exists in Japan. We isolated three strains of the causative rickettsiae, designated Katayama, Misaka, and Abe, from patients with the disease and studied the characteristics of the isolates. Nude mice and cyclophosphamide-treated mice died after infection with the isolates. However, infected normal mice recovered and acquired immunity. Infected adult male guinea pigs had fever, a scrotal reaction, and seroconversion. The isolates propagated well in tissue-cultured vero cells. Analysis by the cross-immunofluorescence antibody method showed that these isolates were closely related serologically. To reveal their immunological properties in detail, we produced 21 anti-Katayama monoclonal antibodies. Seven of these antibodies reacted with all representative strains of spotted fever group rickettsiae used in this study, and five others reacted only with the homologous strain, revealing that the Katayama strain has a strain-specific antigen(s) different from those of other spotted fever group rickettsiae. Moreover, these strain-specific antibodies also reacted with the Misaka and Abe strains. These results demonstrate that the causative agent of spotted fever group rickettsiosis in Japan is a new serotype of spotted fever group rickettsiae.

Spotted fever group (SFG) rickettsiosis is widely endemic throughout the world, being known as Rocky Mountain spotted fever, Siberian tick typhus, boutonneuse fever, rickettsialpox, Australian tick typhus, and so on. In Japan, Mahara et al. (10) first reported three cases of SFG rickettsiosis that occurred in Anan City, Tokushima, in 1984. In 1986, the causative rickettsia was isolated by Uchida et al. from a patient in Kochi (13).

We isolated the Katayama (8) and Abe strains in Tokushima in 1987 and 1988, respectively, and the Misaka strain in Hyogo in 1988 from patients with SFG rickettsiosis in Japan. In the present paper, we report the characteristics of these strains from both biological and immunological viewpoints.

MATERIALS AND METHODS

Rickettsiae. The SFG rickettsiae used are shown in Table 1. The Katayama strain was isolated from a 70-year-old woman, a farmer, in Anan City, Tokushima, in 1987 (8). The Misaka strain was isolated from a 68-year-old man, a forester, on Awaji Island, Hyogo, in 1988. The Abe strain was isolated from a 77-year-old woman, also a farmer, in Anan City, Tokushima, in 1988. These strains were isolated by inoculation of peripheral blood into nude mice. Rickettsia rickettsii Smith, R. conorii Moroccan, R. akari MK (Kaplan), and R. montana tick strain were obtained from the American Type Culture Collection, Rockville, Md. R. sibirica 246 and Rickettsia sp. strain Thai TT-118 were obtained from N. Tachibana (Miyazaki Medical college, Miyazaki, Japan). R. australis Phillips was obtained from N. D. Stallman (Laboratory of Microbiology and Pathology, Brisbane, Queensland, Australia). All rickettsial strains were passaged in mice or cultured cells (BSC-1 cells, vero cells, or L cells) in our laboratory.

Animals. Congenitally athymic nude mice of BALB/c background and BALB/c mice were obtained from our breeding colony. Cyclophosphamide-treated BALB/c mice were inoculated subcutaneously with 2.5 to 5.0 mg of the drug at the same time as they were inoculated with rickettsiae (7). Adult male guinea pigs weighing 400 to 650 g were obtained commercially.

Preparation of immune mouse sera. BALB/c mice (8 to 12 weeks old) were inoculated intraperitoneally with 0.2 ml of pH 7.2 phosphate-buffered saline (PBS) containing a 10% spleen suspension from nude mice infected with each strain. About 6 weeks later, the mice were exsanguinated, and the sera were pooled and stored frozen at −20°C (5).

Production of monoclonal antibodies. Anti-Katayama monoclonal antibodies were produced by the method described previously (6). Briefly, 6-week-old female BALB/c mice were inoculated intraperitoneally with 0.2 ml of PBS containing a 10% spleen suspension from a nude mouse infected with the Katayama strain. The mice received a booster immunization in the same way about 6 to 8 weeks later. Three days later, spleen cells were harvested and fused with P3X63Ag8.635 mouse myeloma cells by use of 45% polyethylene glycol 4000 (Wako Pure Chemical Industries, Osaka, Japan) at a spleen cell/myeloma cell ratio of 10:1. The cells were distributed into 96-well microplates in HAT selective medium; HAT medium consisted of RPMI 1640 with 20% fetal calf serum, 2 mM l-glutamine, 10−4 M hypoxanthine, 4 × 10−7 M aminopterin, 1.6 × 10−5 M thymidine, streptomycin (100 μg/ml), and penicillin G (100 U/ml). When clones appeared macroscopically between 10 and 20 days, the supernatant fluids were screened by an indirect immunofluorescence assay (IFA). Anti-Katayama antibody-secreting hybridomas were cloned twice or more by limiting dilution. After the cloning, the antibody-producing hybridomas were inoculated into BALB/c mice previously treated with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.). After about 10 to 14 days, ascitic fluid was harvested and sedimented by centrifugation to remove cells and fibrin clots, and the clear ascitic fluid was stored frozen at −20°C.

Determination of antibody class and subclass. Cell culture supernatants containing monoclonal antibodies were con-
centrated to about 1:50 by the 50% saturated ammonium sulfate precipitation method and analyzed by the micro-Ouchterlony method against rabbit antisera reactive with mouse-specific immunoglobulin G (IgG), IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA (kappa and lambda chains; Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.).

Reactivity of monoclonal antibodies and immune sera. The reactivity of monoclonal antibodies was examined by the IFA by use of a previously described method (4). Briefly, antigens for the IFA were prepared from infected BSC-1 cells, Vero cells, and L cells in tissue culture. The cells infected with the rickettsiae were harvested, destroyed with a Dounce homogenizer, and centrifuged at 150 × g for 5 min. The supernatant fluid was aspirated and centrifuged at 13,000 × g for 30 min. The pellet was suspended in PBS, applied to microscope slides, air dried, and fixed in acetone. Ascitic fluids containing monoclonal antibodies were used as antibody samples. The initial antibody dilution was 1:10 in PBS, and subsequent serial twofold dilutions were prepared. Each antibody dilution was overlaid on the rickettsial antigens. The slides were incubated in a moist chamber for 30 min at 37°C, washed in PBS, and air dried. Fluorescein-conjugated goat anti-mouse immunoglobulins (IgA, IgG, and IgM [heavy- and light-chain specific; Organon Teknika, Malvern, Pa.] were overlaid on the rickettsial antigens, and the slides were incubated, washed, and air dried as described above. The slides were finally examined with a UV microscope (Universal type; Zeiss, Oberkochen, Federal Republic of Germany). Fluorescence was graded from 4+ to negative according to its intensity. The antibody titer was considered positive if the intensity was 3+ or more. The endpoint of the antibody reaction was defined as the highest positive dilution.

The reactivity of immune mouse sera was examined in the same way. In the case of immune guinea pig sera, fluorescein-conjugated goat anti-guinea pig IgG (heavy- and light-chain specific; Organon Teknika) was used as the second serum.

Smears prepared from the peritoneum of infected mice were also used as antigens.

RESULTS

Biological characteristics of the causative agent of SFG rickettsiosis in Japan. The pathogenicity of the isolates was examined to reveal their biological characteristics in experimental animals. Most of the nude mice inoculated intraperitoneally with 0.2 to 0.5 ml of a 10% spleen suspension from a nude mouse infected with the Katayama, Misaka, or Abe strain showed weakness and splenomegaly and died within 2 or 3 weeks after infection. The causative rickettsiae were demonstrated in smears prepared from the peritoneum of the mice by Giemsa staining and the IFA. Normal BALB/c mice showed ruffled fur between 5 and 10 days after infection and then recovered from the disease and acquired immunity, as proved by elevation of the antibody to the homologous strain and other SFG rickettsiae revealed by the IFA. All of the cyclophosphamide-treated mice inoculated with the Kata-
ymama, Misaka, and Abe strains died between 4 and 7 days after infection, and the causative rickettsiae were demonstrated as in infected nude mice.

Adult male guinea pigs inoculated intraperitoneally with 3 ml of a 10% spleen suspension from a nude mouse or a cyclophosphamide-treated mouse infected with the Kata-
ymama or Misaka strain had fever and showed swelling and redness of the scrotum between 2 and 8 days after infection. However, no necrosis of the scrotum, ears, or footpads was observed. They then recovered from the disease and acquired immunity, as proved by elevation of the antibody to the corresponding strain revealed by the IFA. The temperature curve of a guinea pig infected with the Katayama strain is shown in Fig. 1. In this case, fever was defined as 39.6°C or more from measurement of the rectal temperature with an electronic clinical thermometer (Terumo, Tokyo, Japan). The serum obtained from this guinea pig on day 44 after infection showed elevation of the antibody to the homologous strain. The scrotal reaction of this guinea pig at day 7 after infection is shown in Fig. 2 alongside a normal control.

The Katayama, Misaka, and Abe strains propagated well in Vero cells cultured in Eagle minimal essential medium (Nissui Pharmaceutical Co., Tokyo, Japan) containing 2% fetal calf serum without antibiotics when a 10% spleen suspension from an infected nude mouse or a cyclophosphamide-treated mouse was used as the inoculum. The rickettsial particles appeared as diplobacillary and diplococcal forms, similar to other SFG rickettsiae.

Serological characteristics of the causative agent of SFG rickettsiosis in Japan. The serological characteristics of the Katayama and Misaka strains were analyzed by the cross-immunofluorescence antibody method. Strains Katayama, Misaka, R. rickettsii Smith, R. sibirica 246, R. conorii Moroccan, R. akari MK (Kaplan), and R. australis Phillips, R. montana tick strain, and strain Thai TT-118 were used as antigens. Samples of immune mouse serum against strains Katayama, Misaka, R. sibirica 246, R. australis Phillips, and

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**TABLE 1. Strains of spotted fever group rickettsiae used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Geographical source</th>
<th>Yr</th>
<th>Passage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Katayama</td>
<td>Human</td>
<td>Japan</td>
<td>1987</td>
<td></td>
</tr>
<tr>
<td>Misaka</td>
<td>Human</td>
<td>Japan</td>
<td>1988</td>
<td></td>
</tr>
<tr>
<td>Abe</td>
<td>Human</td>
<td>Japan</td>
<td>1988</td>
<td></td>
</tr>
<tr>
<td>R. rickettsi Smith</td>
<td>Human</td>
<td>Montana</td>
<td>1946</td>
<td>GP/2, CE(YS)/18</td>
</tr>
<tr>
<td>R. sibirica 246</td>
<td>Dermacltor nutalli</td>
<td>USSR</td>
<td>1945</td>
<td>Unknown</td>
</tr>
<tr>
<td>R. conorii Moroccan</td>
<td>Unknown</td>
<td>Morocco</td>
<td>1953</td>
<td>GP/numerous, CE/292, Vero/5, CE(YS)/4</td>
</tr>
<tr>
<td>R. akari MK (Kaplan)</td>
<td>Human</td>
<td>New York</td>
<td>1946</td>
<td>M/2, CE/12, GP1, CE/3</td>
</tr>
<tr>
<td>R. australis Phillips</td>
<td>Human</td>
<td>Australia</td>
<td>1944</td>
<td>Unknown, BGM/1</td>
</tr>
<tr>
<td>R. montana tick</td>
<td>D. andersoni and D. variabilis</td>
<td>Montana</td>
<td>1963</td>
<td>CE/20</td>
</tr>
<tr>
<td>Thai TT-118</td>
<td>Ixodes and Rhipecephalus larval ticks</td>
<td>Thailand</td>
<td>1962</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

* GP, Guinea pig; CE, chicken embryo; YS, yolk sac; Vero, Vero cells; M, mouse; BGM, BGM cells. Numerals (or description) after the shifts indicate the passage numbers.
Thai TT-118 were used as antisera (Table 2). The antiserum against the Katayama strain reacted at a high titer with the homologous antigen and the Misaka antigen. This antiserum also reacted with other SFG rickettsiae at relatively low titers. The antiserum against the Misaka strain reacted at a higher titer with the homologous antigen and the Katayama antigen than with the other SFG rickettsiae. The antisera against *R. sibirica* 246 and strain Thai TT-118 reacted at a relatively low titer with the respective homologous strains. From these results, it was difficult to draw a definite conclusion about the serological characteristics of the isolates.

To reveal the immunological properties of the isolates in detail, we established 21 hybridomas secreting monoclonal antibodies against the Katayama strain. These were designated KMA 1 to 21. The isotypes of the monoclonal antibodies were determined by the micro-Ouchterlony method. They were classified into 3 clones of IgG1, 11 clones of IgG2a, and 7 clones of IgG3 (Table 3). Light chains of all clones were of the kappa type.

The reactivities of the anti-Katayama monoclonal antibodies with the homologous strain and representative strains of SFG rickettsiae determined by the IFA are shown in Table 4. KMA 1 to 5 reacted with the Katayama strain specifically. It was ascertained that the Katayama strain has a strain-specific antigen(s) which is different from those of the other SFG rickettsiae. KMA 15 to 21 reacted with all strains of SFG rickettsiae. It was thus shown that the Katayama strain shares a common antigen(s) with the strains of the other SFG rickettsiae. KMA 6 to 14 reacted with one or two strains of SFG rickettsiae besides the Katayama strain and at an intermediate level. KMA 6, 7, 8, 9, 13, and 14 reacted with strain Thai TT-118. KMA 10 reacted with the *R. montana* tick strain. KMA 11 and 13 reacted with *R. rickettsii* Smith, and KMA 12 and 14 reacted with *R. sibirica* 246. On the other hand, none of the monoclonal antibodies reacted with *R. conorii* Moroccan, *R. akari* MK (Kaplan), or *R. australis* Phillips, except for those antibodies that reacted with all strains of SFG rickettsiae. Furthermore, the serological characteristics of the Misaka and Abe strains were analyzed with the strain-specific antibodies to the Katayama strain, KMA 1 to 5. All of these antibodies reacted with the Misaka and Abe strains (Table 5). Thus, it was apparent that the Misaka and Abe strains belong to the Katayama strain serotype.

**DISCUSSION**

It is well known that SFG rickettsiosis is widely endemic throughout the world. However, until recently, only scrub typhus was thought to be an important rickettsial disease in Japan. In 1984 and thereafter, patients with SFG rickettsiosis were reported in various parts of Japan, including Tokushima, Kochi, Miyazaki, and Hyogo (10, 12, 14, 19). The causative rickettsia, showing cross-reactivity with *R. montana*, was isolated by Uchida et al. from a patient in Kochi in 1986 (13), and subsequently four strains were isolated from patients who were ill in 1985 and 1986 (15). In the present study, we attempted to reveal the biological and immunological characteristics of the causative agents of SFG rickettsiosis in Japan which we had isolated from patients in Tokushima and Hyogo.

It has been shown that SFG rickettsiae, especially *R. akari* and *R. australis*, are pathogenic for adult white mice (17). In the present study, it was revealed that the causative agents of SFG rickettsiosis in Japan were also moderately pathogenic for normal BALB/c mice. In addition, their pathogenicity for nude mice and cyclophosphamide-treated mice was striking. This result suggested that a sound immune response is important for recovery from the disease.

The isolates caused fever, a scrotal reaction, and seroconversion in infected guinea pigs. This finding corresponds to
the pathogenicity of other SFG rickettsiae for guinea pigs (18); thus, the present isolates are not distinguishable from other SFG rickettsiae from this viewpoint. However, it seems that the causative agents of Japanese SFG rickettsiosis are less virulent for guinea pigs than are \textit{R. rickettsii} R-type strains, which evoke necrotic lesions of the scrotum, footpads, and ears and invariably kill the animals (1).

From a serological analysis with immune mouse sera by the cross-immunofluorescence antibody method, it became apparent that the isolates may be closely related to each other serologically. However, it was difficult to reveal their immunological relationships in detail because of wide cross-reactivities among SFG rickettsiae. In the present study, we produced 21 monoclonal antibodies against a new isolate, the Katayama strain, from a patient with SFG rickettsiosis in Japan and analyzed their serological properties.

Seven anti-Katayama monoclonal antibodies, KMA 15 to 21, reacted with all representative strains of SFG rickettsiae used in the present study, and it was revealed that the Katayama strain has an antigen(s) in common with those of SFG rickettsiae. Five anti-Katayama monoclonal antibodies, KMA 1 to 5, reacted specifically with the Katayama strain, and it was proved directly that the Katayama strain has a serotype-specific antigen(s) different from those of the other SFG rickettsiae used in this study. Moreover, these serotype-specific monoclonal antibodies reacted with two other new isolates in Japan, the Misaka and Abe strains. Therefore, it appears that these two strains belong to a new serotype, Katayama, of the spotted fever group and that Japanese SFG rickettsiosis is a new, independent disease. In this regard, Uchida et al. also characterized Japanese strains by using mouse antisera and monoclonal antibodies to \textit{R. conorii}, \textit{R. rickettsii}, \textit{R. sibirica}, and \textit{R. akari} (16).

Lackman et al. (9) and Robertson and Wiseman (11) did a classical subgrouping of SFG rickettsiae by using the complement fixation test and the mouse toxin neutralization test, respectively. Subgroup A consists of \textit{R. rickettsii} and \textit{R. sibirica}. \textit{R. conorii} and \textit{R. parkeri} are in subgroup B. \textit{R. akari} and \textit{R. australis} are in subgroup C, and \textit{R. montana} and Western Montana U are in subgroup D. Pakistan JC-880

\begin{table}[h]
\centering
\caption{Cross-immunofluorescence antibody method results for spotted fever group rickettsiae tested with immune mouse sera}
\begin{tabular}{|c|c|c|c|c|}
\hline
Antigen & Katayama & Misaka & \textit{R. sibirica} & \textit{R. australis} & Thai TT-118 \\
\hline
Katayama & 2,560 & 1,280 & 160 & 80 & 160 \\
Misaka & 1,280 & 2,560 & 160 & 80 & 160 \\
\textit{R. rickettsii} Smith & 160 & 320 & 80 & 80 & 160 \\
\textit{R. sibirica} 246 & 80 & 320 & 320 & 40 & 80 \\
\textit{R. conorii} Moroccan & 160 & 320 & 160 & 40 & 160 \\
\textit{R. akari} MK (Kaplan) & 160 & 160 & 80 & 160 & 80 \\
\textit{R. australis} Phillips & 80 & 80 & 80 & 1,280 & 80 \\
\textit{R. montana} tick & 160 & 160 & 80 & 80 & 80 \\
Thai TT-118 & 80 & 320 & 80 & 40 & 160 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Isotypes of anti-Katayama monoclonal antibodies}
\begin{tabular}{|c|c|c|}
\hline
KMA & Isotype \textsuperscript{a} & KMA & Isotype \textsuperscript{a} \\
\hline
1 & IgG2a & 12 & IgG2a \\
2 & IgG1 & 13 & IgG2a \\
3 & IgG1 & 14 & IgG2a \\
4 & IgG2a & 15 & IgG3 \\
5 & IgG1 & 16 & IgG3 \\
6 & IgG2a & 17 & IgG3 \\
7 & IgG2a & 18 & IgG3 \\
8 & IgG2a & 19 & IgG3 \\
9 & IgG2a & 20 & IgG3 \\
10 & IgG2a & 21 & IgG3 \\
11 & IgG2a & & \\
\hline
\end{tabular}
\end{table}

\textsuperscript{a} KMA, Anti-Katayama monoclonal antibody.

\textsuperscript{b} Light chains of all clones were of the kappa type.
and Thai TT-118 belong to subgroup E. In the present study, we compared the serological characteristics of the isolates in Japan and representative strains of these SFG rickettsia subgroups. Six of nine monoclonal antibodies with intermediate reactivity reacted with strain Thai TT-118. Moreover, two of these antibodies reacted with \( R. \) \textit{rickettsii} and \( R. \) \textit{sibirica}. KMA 13 showed cross-reactivities with Katayama, Thai TT-118, and \( R. \) \textit{rickettsii} Smith. KMA 14 showed cross-reactivities with Katayama, Thai TT-118, and \( R. \) \textit{sibirica} 246. On the other hand, the nine antibodies did not react with \( R. \) \textit{conorii}, \( R. \) \textit{akari}, or \( R. \) \textit{australis}. These findings suggested that the Katayama strain may be closely related to the Thai TT-118 strain, i.e., subgroup E, serologically, a possibility which is of interest because these strains are both endemic in east Asia. It was also suggested that the Katayama strain may be more closely related to \( R. \) \textit{rickettsii} and \( R. \) \textit{sibirica} (subgroup A) than to \( R. \) \textit{conorii} (subgroup B), \( R. \) \textit{akari} (subgroup C), and \( R. \) \textit{australis} (subgroup C).

In east Asia, it has been reported that \( R. \) \textit{akari} exists in the Republic of Korea (3) and that \( R. \) \textit{sibirica} is distributed in northern China (2). Thus, the distribution of SFG rickettsiae is complicated and diversified. Hereafter, it will be important to reveal the distribution of this new rickettsia serotype of the spotted fever group for etiological, epidemiological, and clinical studies of the disease. For this purpose, Katayama serotype-specific monoclonal antibodies KMA 1 to 5 will be extremely useful for identifying the causative agent.

**ACKNOWLEDGMENTS**

We thank F. Mahara and K. Kodama for giving us information about clinical cases. N. Tachibana for supplying \( R. \) \textit{sibirica} and Thai TT-118, and N. D. Stallman for supplying \( R. \) \textit{australis}.

**LITERATURE CITED**


