Virulence in Mice of *Orientia tsutsugamushi* Isolated from Patients in a New Endemic Area in Japan

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Received May 20, 1996. Accepted July 17, 1996

Abstract: Four strains of *Orientia tsutsugamushi* (KN-1, KN-2, KN-3 and GJ-1) isolated from patients in an area of Gifu Prefecture, Japan, in which tsutsugamushi disease is newly endemic, were examined for their virulence in mice. Among these, KN-1 (identified as Kawasaki type), GJ-1 (identified as Kuroki type) and KN-2 strains were found to be non-lethal for BALB/c mice as well as CH3/HeJ mice, even with high doses (10^6 being the 50% mouse infectious dose). On the other hand, the KN-3 strain was found to be sufficiently virulent to kill BALB/c mice. Among the prototype strains (Gilliam, Karp and Kato), the Karp and Kato strains exhibited high virulence in mice, while the Gilliam strain killed only a susceptible strain of mouse. BALB/c mice infected with KN-1 and KN-2 strains showed significant splenomegaly and moderate ascites accumulation in the first week of infection, while these symptoms became prominent during the second week of infection using KN-3, Karp and Kato strains. After infection with the GJ-1 strain, these symptoms were not observed. Antibody responses induced by infections with highly virulent strains were lower than that with low or intermediate virulent strains.

Key words: *Orientia tsutsugamushi*, Virulence in mice, Scrub typhus

The scrub typhus, or tsutsugamushi disease, has become an important febrile illness in Japan, Korea, China and other countries in Southeast Asia in recent years (3, 12). The rate of incidence in Japan increased for about 10 years until 1984. In Gifu Prefecture, located in the center of Honshu island, the first case of tsutsugamushi disease was diagnosed in 1982, and since then 13-56 patients a year have been reported in the region (7). We elucidated endemic strains of *Orientia tsutsugamushi* isolated from patients, wild rodents and trombiculid mites in Gifu Prefecture (6, 8). Four of six new strains have been isolated from patients, i.e., KN-1 (identified as Kawasaki type), GJ-1 (identified as Kuroki type), KN-2 and KN-3, all of which can be distinguished antigenically from each other (17, 18). Groves and Osterman (4) reported that *O. tsutsugamushi* strains also differ in virulence in mice. Their study emphasized the importance of genetic differences among mouse strains in assessing the virulence of *O. tsutsugamushi*. In our study, we examined the susceptibilities and various pathogenic responses of mice to *O. tsutsugamushi* strains isolated from patients, and compared the virulence of each strain in mice.

Materials and Methods

Animals. Male ddy, BALB/c and C3H/HeJ haired mice were obtained from Clea Japan (Tokyo) and male Crj:CD-1(ICR) nude mice from Charles River (Tokyo). The mice, aged 4-5 weeks, were used for all experiments.

Strains. Four newly isolated strains, i.e., KN-1, KN-2, KN-3 and GJ-1 strains (17, 18), and three prototype strains, i.e., Karp, Kato and Gilliam strains, were used. Each strain was maintained by passing from mouse to mouse, and the mouse spleens were stored in a liquid nitrogen stocker. Before use, the stocked strains were passed several times through the mice. Hairless ddy mice and ICR nude mice were used for highly virulent (Karp, Kato and KN-3) and low virulent strains (Gilliam, KN-1, KN-2 and GJ-1), respectively. For titration of antibodies, the antigens were prepared in BS-C-1 cells as described previously (17).

Evaluation of virulence of *O. tsutsugamushi* in mice. The 50% mouse lethal dose (MLD50) was determined by intraperitoneal (i.p.) inoculation into a group of five

Abbreviations: IFA, indirect immunofluorescent assay; IFN, interferon; i.p., intraperitoneal; MID50, 50% mouse infectious dose; MLD50, 50% mouse lethal dose; SPG, sucrose phosphate glutamine.
mice with 0.25 ml each of 10-fold dilutions of spleen homogenate from infected mice in sterile SPG solution (1). The 50% mouse infectious dose (MID_{50}) was determined by the method of Groves and Osterman (4). Briefly, mice surviving 28 days after the original challenge were rechallenged i.p. with 1,000 MLD_{50} of Karp strain. Mice dying during the original challenge and mice surviving the rechallenge were considered infected. MLD_{50} and MID_{50} values were calculated using the method of Reed and Muench (13). The resistance index was defined as (log_{10} MID_{50} - log_{10} MLD_{50}).

**Infection of BALB/c mice with O. tsutsugamushi.**

BALB/c mice were inoculated i.p. with 0.25 ml of Karp, Kato and KN-3 strains diluted to contain 1,000 MID_{50}, and with 0.25 ml of Gilliam, KN-1, KN-2 and GJ-1 strains diluted to contain 10^4 × MID_{50} in the spleen homogenate from mice infected with each strain. Control mice were injected with the same amount of non-infected spleen homogenate. At one-week intervals after infection, 3~4 mice were sacrificed, and their sera were collected and stored frozen at -20°C. The wet-weights of their spleens and ascites were measured, and the ratios to body weight were determined. The weight of ascites fluid was measured by infiltrating the fluid into a small piece of filter paper, of which the weight had been predetermined.

**Titration of antibodies.**

The reactivity of immune mouse sera was examined by indirect immunofluorescent assay (IFA) as described previously (17). Briefly, all strains propagated in BS-C-1 cells were collected, smeared on a glass slide, fixed with acetone and used for IFA antigens. Sample sera were reacted with each antigen, and fluorescein-labeled anti-mouse immunoglobulin G (heavy and light chains) goat sera was added. The maximum dilution of serum showing a positive reaction was considered as the titer.

**Statistics.**

Statistics were calculated using Student’s t-test for comparisons between infected and control groups or between strains.

**Results**

**Virulence of Each Strain of O. tsutsugamushi in BALB/c and C3H/HeJ Mice**

Karp, Kato and KN-3 strains were lethal not only for C3H/HeJ mice but also for BALB/c mice. On the other hand, the response of mouse strains to Gilliam infection varied, i.e., BALB/c mice were resistant and C3H/HeJ mice were susceptible. Three isolated strains (KN-1, KN-2 and GJ-1) produced a resistant response pattern in both C3H/HeJ and BALB/c mice (Table 1). Therefore, O. tsutsugamushi strains were divided into three classes according to their virulence in mice, such as the highly virulent strains of Karp, Kato, and KN-3, the low virulent strains of KN-1, KN-2, and GJ-1, and the intermediate virulent strain of Gilliam.

**Splenomegaly and Ascites Accumulation in BALB/c Mice Infected with Each Strain of O. tsutsugamushi**

As shown in Tables 2 and 3, splenomegaly and ascites accumulation in BALB/c mice infected with KN-3 exhibited a similar pattern to the cases of infection with Karp or Kato strains; i.e., enlargement of the spleen was not clear during the first week, but became significant (P < 0.01) in the second week. Ascites also increased significantly (P < 0.005) during the second week, and all mice died by the third week. On the other hand, mice infected with KN-1 and KN-2 strains showed about

<table>
<thead>
<tr>
<th>Rickettsial strain</th>
<th>Mouse</th>
<th>MID_{50} (log_{10})</th>
<th>MLD_{50} (log_{10})</th>
<th>Resistance index MID_{50} - MLD_{50} (log_{10})</th>
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<tbody>
<tr>
<td>Karp</td>
<td>BALB/c</td>
<td>7.6</td>
<td>6.8</td>
<td>0.8</td>
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<tr>
<td></td>
<td>C3H/HeJ</td>
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<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>Kato</td>
<td>BALB/c</td>
<td>6.2</td>
<td>3.8</td>
<td>2.4</td>
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<tr>
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<td>C3H/HeJ</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Gilliam</td>
<td>BALB/c</td>
<td>7.5</td>
<td>&lt; 1.0</td>
<td>&gt; 6.5</td>
</tr>
<tr>
<td></td>
<td>C3H/HeJ</td>
<td>7.6</td>
<td>2.0</td>
<td>5.6</td>
</tr>
<tr>
<td>KN-1</td>
<td>BALB/c</td>
<td>7.2</td>
<td>&lt; 1.0</td>
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<tr>
<td></td>
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<td>C3H/HeJ</td>
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<td>&lt; 1.0</td>
<td>&gt; 6.0</td>
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<tr>
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<td>N.D.</td>
</tr>
<tr>
<td>GJ-1</td>
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<td>6.7</td>
<td>&lt; 1.0</td>
<td>&gt; 5.7</td>
</tr>
<tr>
<td></td>
<td>C3H/HeJ</td>
<td>7.2</td>
<td>&lt; 1.0</td>
<td>&gt; 6.2</td>
</tr>
</tbody>
</table>

a) N.D.: not determined.
two-fold splenomegaly and three-fold ascites accumulation as compared to the values of normal control mice in the first week after infection. However, the weight of the spleen and ascites in the infected mice almost returned to the levels of normal mice by the third week.

In the case of infection with GJ-1 strain, splenomegaly and ascites accumulation displayed no significant change throughout the course of the infection. On the other hand, mice infected with Gilliam strain showed more splenomegaly and more ascites accumulation than in the cases of KN-1 and KN-2 ($P<0.005$). The peak of splenomegaly was observed in the second week and that of ascites accumulation in the first week after infection.

**Antibody Titers in BALB/c Mice after Infection with Each Strain of O. tsutsugamushi**

Antibody titers in mice infected with Gilliam strain increased with the passing of time, and exhibited the highest titer in the third week of infection (Fig. 1). However, infection with the highly virulent strains of KN-3, Kato or Karp induced lower levels of antibody response, especially in the Karp strain, and all mice died after the second week of infection. In infections from low virulent strains, i.e., KN-1, KN-2 and GM, the level of titers was found to lie somewhere between the Gilliam and the highly virulent strains in the second week, and then declined during the third week.
Discussion

In infections of *O. tsutsugamushi*, the mechanism causing the deaths of infected animals or patients is quite obscure. However, *O. tsutsugamushi* is known to show antigenic phenotypic diversity, and three antigenic prototypes are generally recognized, i.e., Karp, Gilliam and Kato. In recent years, several investigators have reported new isolates which were antigenically distinguished from the prototype strains (11, 15, 16). The strains we isolated from patients in Gifu Prefecture were classified into four groups according to their antigenicities (17, 18). The virulence of these four strains falls into two types: 1) a highly virulent strain, such as KN-3, Karp and Kato, in which a few infectious organisms, introduced intraperitoneally, are capable of killing mouse strains tested; and 2) a low virulent strain, such as KN-1, KN-2 and GJ-1, which even in relatively large doses, a non-lethal infection occurs regardless of the mouse strains tested. The Gilliam strain can be classified as being intermittently virulent, readily killing susceptible mouse strains (e.g., C3H/HeJ) but not those that are resistant (e.g., BALB/c). Groves and Osterman (4) have reported that nine inbred mouse strains including C3H/HeJ mice were susceptible to Gilliam infection, and six inbred mouse strains including BALB/c mice were resistant. They also emphasized the importance of genetic differences among mouse strains in assessing the virulence of *O. tsutsugamushi*, while other investigators (2, 9) suggested that resistant strains of mice effectively suppress Gilliam proliferation in the peritoneal cavity through the evolution of macrophages. However, the precise mechanism underlying susceptibility or resistance to lethal Gilliam infection was not determined. Among low virulent strains, GJ-1 is classified as the lowest because even a $10^5 \times \text{MID}_{50}$ challenge failed to result in any significant splenomegaly or ascites accumulation. KN-1 and KN-2 strains are similar in antigenicity (18) as well as in virulence to mice. In pathogenic and antibody responses of BALB/c mice, highly virulent strains were characterized as follows: 1) splenomegaly and ascites accumulation were not so prominent in the first week after infection but progressed in the second week, and all mice died by the third week; and 2) antibody responses were at relatively low or non-detectable levels. Mice infected with low virulent strains were characterized as follows: 1) peak responses of splenomegaly and ascites accumulation in the first week; and 2) higher antibody responses with the peak in the second week. In other words, the non-lethal strains caused earlier responses of spleen proliferation with ascites accumulation and then induced higher levels of antibody production. Recently, investigators have been observing different replication rates, plaque size variation and susceptibility to IFN-mediated inhibition of rickettsial growth among different strains (5, 10). The virulence of *O. tsutsugamushi* strains among humans is not clear at present. Although the level of virulence among mice and humans may differ, reports of deaths where strains of Kawasaki (KN-1) and Kuroki (GJ-1) types are endemic (14) have seldom been reported, while most deaths have been reported in northern Japan where KN-3 and strains other than the Kawasaki or Kuroki types are prominent.

References


