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Longevity of Antibodies to Live *Orientia tsutsugamushi* Inoculated in Sprague Dawley Rats

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=Abstract=

In Sprague Dawley (SD) rats, antibodies against strains of *Orientia tsutsugamushi*, Kato, Karp and Gilliam, were produced in order to investigate their longevity and cross-reactivities to their corresponding homologous and heterologous antigens. By immunofluorescence assay (IFA) of IgG and IgM, it was shown that the immunity to the homologous strains persisted at a higher level (longevity of at least 34 weeks with higher IFA titers). On the other hand, the immunity to the heterologous strains persisted at a lower level (longevity of 10 to 34 weeks with lower IFA titers). Since infection with one strain of *O. tsutsugamushi* does not preclude reinfection with other strains, understanding of the antigenic diversity of *O. tsutsugamushi* and duration of the immunity to both homologous and heterologous strain is very important in diagnosis of scrub typhus.

Key Words: *Orientia tsutsugamushi*; Kato, Karp, Gilliam strain; rickettsemia; scrub typhus; IFA test

INTRODUCTION

Orientia tsutsugamushi has an extremely wide host range [1], and it is known to infect human beings [2,3,4,5,], mites (*Trombicula akamushi, T. scutellaris, T. pallida, T. deliensis,* etc.) [6,7, 8], many species of rodents [9], and occasionally goats and pigs. Various experimental animals [10] are susceptible to this rickettsia - monkey [11,12], mouse [13,14,15], rat [16], rabbit, guinea pig, cotton rat, hamster, Mongolian gerbil, dog, goat, chicken, pigeon, embryonated eggs etc. Most of cultured cells [17,18,19] are also susceptible. Rickettsia is obligatory intracellular parasite [20,21], and may cause persistent infection [22]. Orientiae remain viable somehow in host cells for months or years after recovery from infection. Thus the host-parasite relationship is preserved in balance for a long time.

Previous studies of *O. tsutsugamushi* infections in rodents suggest that persistent or dominant infections may be common. In endemic areas, a large number of wild rodents have yielded isolates from blood, tissue pools or both [1,23]. *O. tsutsugamushi* occurs in lymph nodes of a patient 1～2 years after in-

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fection. Little is known about the persistence of scrub typhus organisms in a host and its effect on resistance [24,25] to subsequent infection by *O. tsutsugamushi.* The immunity conferred by the first infection was inadequate to prevent the second. However, such an inadequacy might be attributed as easily to relative pathogenicity of strains as to antigenic differences. Although experimental evidence indicates that laboratory animals which serve infection with one strain are completely immune to infection with other strains [26,27,28, 29], this by itself is not a conclusive proof of the complete antigenic identity of such strains. In an earlier study, silvered leaf monkeys [30, 31,32], infected with different strains of scrub typhus organisms, were challenged with homologous and heterologous strains when the antibody titer fell to $\leq$1:10 (approximately one year post-infection).

Numerous studies of immune responses [33, 34] in scrub typhus have concentrated on the detection of humoral immunity. The circulating antibody [35,36] is produced in response to natural and experimental infections with *O. tsutsugamushi.* Although the role of antibody in acquired resistance to *O. tsutsugamushi* infection is unclear, a possible role of antibody in immunity is suggested by data demonstrating a close relationship between peak antibody titers and acquisition of resistance to reinfection [37]. Furthermore, animals immunized with viable *O. tsutsugamushi* do not develop a rickettsemia after challenge. More direct evidence for the role of antibody in resistance to infection comes from studies that immune sera could neutralize the infectivity of *O. tsutsugamushi* for animals [38] or tissue culture. In addition, administration of immune sera to naive [39] mice was showed to protect against challenge. Primary scrub typhus infection renders humans [40] solidly immune to reinfection [41] by the homologous strain of *O. tsutsugamushi* in at least 1 year but protection against the several known heterologous strains wanes rapidly with susceptibility to disease reappearing within months. A similar period of protection against homologous challenge is observed in rodent animal models but the duration of heterologous immunity is less clearly defined since most studies have tested heterologous resistance within 1 to 2 months after initial infection. Several authors have noted that strains of *O. tsutsugamushi* have different degree of virulence for laboratory animals. Many strains isolated from naturally infected chiggers [42,43,44] and rodents produce no signs of disease in laboratory mice [45,46] but our Karp and Gilliam strain are virulent to mice.

In our laboratory, Kato, Karp [47] and Gilliam strain [48] have been subcultured in the yolk sac of chicken embryonated egg for a long time. Thus, these strains were adapted well for mass growth in the yolk sac membrane of chicken embryonated egg. For the IFA test [49,50,51], fluorescein isothiocyanate conjugated heavy-chain-specific goat anti-rat immunoglobulin IgM or IgG (Cappel, Cochranville, Pa.) and acetone-fixed smears of these antigens were used. We now report the production of serum antibodies to live *O. tsutsugamushi,* immunization in Sprague Dawley (SD) rats, and their longevity and cross reactivities.

MATERIALS AND METHODS

Rats

Male SPF SD rats were obtained from Charles River Japan, Inc. and used at the age of 5 weeks.

Rickettsia

The Karp strain (56th egg passage), the Kato strain (147th egg passage) and the Gilliam strain (169th egg passage) were used exclusively in the present investigation. *O. tsutsugamushi* was prepared by inoculating eggs by the yolk sac route with 0.1ml of a 10^{-1} or 10^{-2} dilution folds of the seed which was stored as

a 20% yolk sac suspension (w/v) in Synder's buffer containing 0.25M sucrose, 0.0038M KH_2PO_4, 0.0086M Na_2HPO_4, and 0.0049M glutamic acid. Yolk sacs were harvested from the live eggs when about 20% of the inoculated eggs were dead. Following freezing and thawing, the yolk sacs were diluted to 20% (w/v) with 0.01M phosphate buffered saline (PBS) pH 7.6 but the seed for inoculation of SD rats was diluted with Synder's buffer and disrupted by treating the chilled suspension in an ice bath with Waring blender for 3 minutes. The solution was centrifuged at 400g for 15 minutes and the middle layer was collected and used as a *O. tsutsugamushi* seed for inoculation of SD rats. Serial 2-fold dilutions of this antigen preparation were examined by IFA test employing high titer rat sera. The suspension was diluted with PBS to yield approximately 1,000 cells per 500X microscopic field, dispensed into 0.1ml aliquots, and stored at -70℃.

Production of antisera

Rickettsial antisera were obtained at one week interval after inoculation of SD rats with a single intramuscular injection of 0.5ml dose of 20% (w/v) infected yolk sac suspension of live *O. tsutsugamushi*.

Slide preparations

Spots of the Karp, Kato, or Gilliam antigen were applied to each well of a slide by positioning the precleaned slide over the template and touching the spot with a pen nib filled with each antigen. Volume of the spot delivered was approximately 0.1μl. After completion of spotting, the slide was dried for at least 30 minutes at room temperature prior to 10 minutes fixation in acetone. All the fixed slides were then allowed to dry at room temperature and stored at -20℃ for not longer than 2 weeks.

IFA

Ten microliters of 2-fold serially diluted rat sera in PBS (the initial dilution fold of 32) was applied on each antigen spot of the slide glass, incubated for 30 min at 37℃, and washed off thoroughly in a staining vessel with 2 changes of PBS at 3min intervals. Ten microliters of goat anti-rat IgG or IgM antiserum labeled with fluorescein isothiocyanate (KPL, M.D., USA) (2 units of IF titer per 10μl in PBS) was layered on each spot of the slide glass. After 30 min incubation at 37℃, the slide glass was washed thoroughly with PBS as above, covered with glycerin buffer (0.05M carbonate buffer, pH 9.5 in fluorescence-free glycerin), and observed at 500X magnification by a Carl Zeiss incident light fluorescent microscope equipped with an OSRAM HBO 200W lamp with a barrier and exciter filters. Antibody titer was defined as reciprocal of the highest dilution fold at which fluorescence was recognized on the rickettsia.

RESULTS

The antisera against Karp, Gilliam, and Kato strains were developed in SD rats and used to observe their IgG or IgM longevity and cross-reactivity to their corresponding homologous and heterologous antigens (Fig. 1, 2, and 3, respectively). For each strain, antiserum was obtained from two rats and their responses to the homologous and heterologous antigens were determined by IFA test.

Firstly, the antisera against Karp strain showed an IF antibody response to the homologous antigen as in Fig. 1.(A). The IgG was detected about 3 weeks after inoculation and reached a maximum level at 10～12 weeks, and decreased slowly but still it was demonstrable at 34 weeks. The IgM was detected about 5～7 weeks after inoculation and reached a peak at about 10～14 weeks, and slowly declined to a level demonstrable at 34 weeks after inoculation. On the contrary, the heterologous IF antibody response of the antisera of different rats showed different profiles against Gilliam

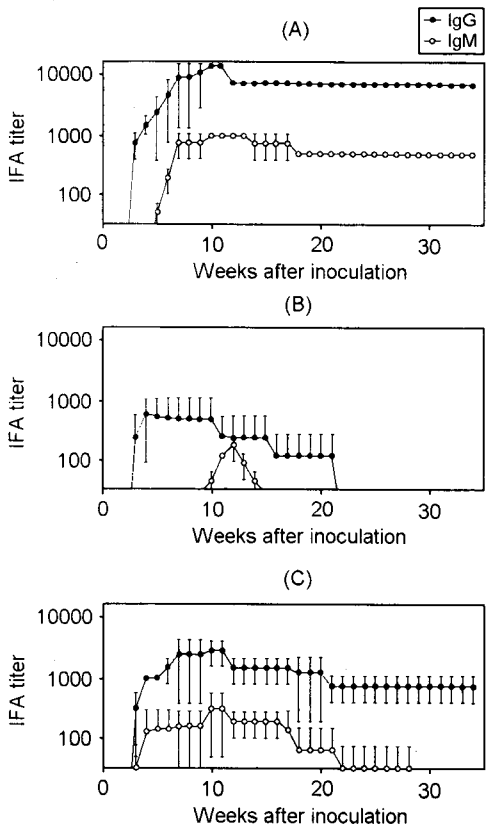

Fig. 1. Antibody response of the anti-Karp sera to the homologous and heterologous antigens, Karp strain **(A)**, Gilliam strain **(B)**, and Kato strain **(C)**. Open and closed symbols represent data of each serum obtained from different two rats of the group.

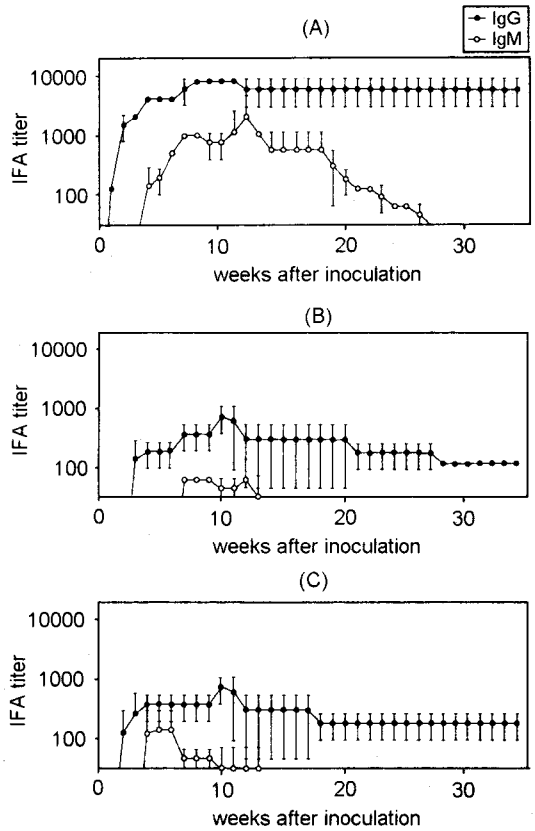

Fig. 2. Antibody response of the anti-Gilliam sera to the homologous and heterologous antigens, Gilliam strain **(A)**, Karp strain **(B)**, and Kato strain **(C)**. Open and closed symbols represent data of each serum obtained from different two rats of the group.

strain (Fig. 1(B)). The IgG was detected about 3 weeks after inoculation and reached a maximum level at 4~10 weeks, and decreased to a detectable level until 22 weeks after inoculation. However, the antisera from both rats showed little difference in their IgM production profiles. After 7~8 weeks, the IgM was detected and reached a peak at 12 weeks, and then declined to a detectable level until 18 weeks after inoculation. The heterologous antibody responses of IgG and IgM to another antigen, Kato strain, showed a very similar pattern as against Gilliam strain (Fig. 1.(C)) such that the IgG induction was detected at 3 weeks after inoculation with a maximum plateau at 7~11 weeks and it was demonstrable by 34 weeks after inoculation. The IgM was also detected about 3 weeks after inoculation with a peak at 10~11 weeks, and a demonstrable level of the antibodies remained at 34 weeks.

Secondly, the homologous IF antibody response of the antisera against Gilliam strain (Fig. 2(A)) showed that IgG was detected about 1 week after inoculation and reached a maximum level at 9~11 weeks, and remained over 34 weeks. IgM was detected about 3~4 weeks after inoculation and reached a peak at 10~13 weeks and then declined to a demonstrable level until 27 weeks after inoculation. The IF antibody response curves against a heterologous antigen, Karp strain, is

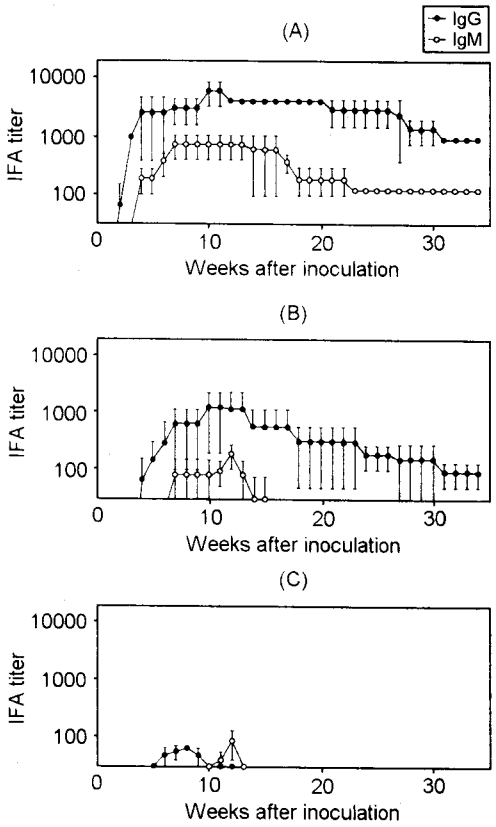

Fig. 3. Antibody response of the anti-Kato sera to the homologous and heterologous antigens, Kato strain (**A**), Karp strain (**B**), and Kato strain (**C**). Open and closed symbols represent data of each serum obtained from different two rats of the group.

shown in Fig. 2.(B). IgG was detected 3 weeks after inoculation and reached a maximum level on 10~11 weeks, and decreased slowly but it was still demonstrable at 34 weeks after inoculation. In comparison, IgM was detected at 7 weeks and reached a peak at 7 or 12 weeks, and showed a clearance of the antibodies after the peak level. The heterologous IF antibody response against Kato strain was also observed (Fig. 2.(C)). The IgG antibodies were detected at 3 weeks after inoculation showing a maximum level at 10~11 weeks, and then decreased slowly but still remained demonstrable at 34 weeks after inoculation. IgM was detected and reached a peak at 4~6 weeks and then declined to a demonstrable level until 14 weeks after inoculation.

Thirdly, the homologous IF antibody response of the sera against Kato strain was depicted in Fig. 3.(A). IgG antibodies were detected about 2~3 weeks after inoculation showing a maximum level reaching at 10~11 weeks, and declined slowly but still remained demonstrable at 34 weeks. The IgM induction was detected at 3~4 weeks after inoculation reaching a peak at 7~13 weeks, and declined to a demonstrable level until 34 weeks after inoculation. The heterologous IF antibody response profiles against heterologous antigens, Karp strain, are shown in Fig. 3.(B). The IgG antibody induction was detected at 4~5 weeks after inoculation and reached maximum on 10~13 weeks, showing a slow decrease to a demonstrable level at 34 weeks after inoculation. IgM antibodies were detected about 5~7 weeks after inoculation reaching a peak at 12 weeks followed by a clearance. A comparable profile of the IF antibody response was observed against the other heterologous antigen, Gilliam strain (Fig. 3(C)). The IgG antibodies were detected about 5 weeks after inoculation and reached maximum on 7~8 weeks. The IgM antibodies were detected 10 weeks and reached a peak at about 12 weeks. In both cases, the antibodies disappeared following the maximum level.

DISCUSSION

It is well known that *O. tsutsugamushi* shows a higher degree of internal phenotypic diversity [52,53] with respect to antigenic composition than any other rickettsial species. Whether antigenic differences occur among strains of scrub typhus is an important question from an epidemiological point of view [54]. In the IFA test, three antigenic types, Kato, Karp, and Gilliam [55], are usually demonstrated and other types were also detected in Thailand. In Korea, all the *Orientiae* isolated showed cross reactivity [56] with, at least,

one of the three prototype strains of Kato, Karp, Gilliam with no negative cross reactivities. Thus it is supposed that the IF test of *O. tsutsugamushi* may recognize not only type-specific antigen [57] but also group- or species-specific common antigen.

Rats have been used to a limited extent in the study of scrub typhus. Both white rats and cotton rats develop a fatal infection when i.p. injected. In earlier vaccine work, both types of rats were used for the preparation of experimental vaccines. Another study has shown that there are no fatalities in rats following s.c. inoculation of scrub typhus rickettsia. However, both spleens and lymph nodes are enlarged showing a persistent infection of the spleen for many weeks; the advantage for the maintenance of *O. tsutsugamushi* strains.

In this study, SD rats were used to observe the longevity of the immunity among the representative strains of Karp, Gilliam, and Kato. The immunity which developed in SD rats to the homologous strain of *Orientiae* persisted at a high level in most rats for at least 34 weeks. On the other hand, the immunity to the heterologous strain persisted at a low level (longevity of 10 to 34 weeks with lower IFA titers, Fig1. (B),(C), Fig.2. (B),(C), and Fig.3. (B),(C)). Hanson [37] described monoclonal antibodies which react not only with 58-60K of the homologous strain but also with the polypeptides of both homologous and heterologous strains, suggesting that these major polypeptides on the rickettsial surface might have both strain- and group-specific antigenic determinants. Since infection with one strain of *O. tsutsugamushi* does not preclude reinfection with other strains and immune sera react weakly with other strains of *O. tsutsugamushi*, understanding of the antigenic diversity of *O. tsutsugamushi* and duration of the immunity to both homologous and heterologous strain is very important in the diagnosis of scrub typhus.

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