VIRULENCE OF MALAYSIAN ISOLATES OF *ORIENTIA TSUTSUGAMUSHI* IN MICE

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Abstract. The pathogenicity of Malaysian isolates of *Orientia tsutsugamushi* was investigated by a mouse virulence assay. The isolates could be differentiated as low (4 isolates), moderately (3 isolates) and highly virulent (2 isolates) based on the different responses in infected mice. No direct correlation between severity of human scrub typhus infections and virulence of the *O. tsutsugamushi* in mice was observed. Mice infected with virulent strains of *O. tsutsugamushi* showed splenomegaly, ascitis accumulation and enlargement of kidneys and livers whereas avirulent *O. tsutsugamushi* strains were asymptomatic and exhibited ruffled fur for a short period after infection. There was low antibody response in mice infected with isolates of low pathogenicity as compared with those of highly virulent isolates. Upon dissection of the infected mice, enlargement of mouse organs such as spleen, kidney and liver was noted. Presence of rickettsemia in mice was confirmed by the growth of *O. tsutsugamushi* in the L929 cells when inoculated with blood from infected mice. *O. tsutsugamushi* was also cultured from the peritoneal exudates of the infected mice. However, DNA of *O. tsutsugamushi* was only detected in the peritoneal exudates (by PCR) and blood (by cell culture) and not from other tissue samples.

INTRODUCTION

Scrub typhus infection, caused by *Orientia tsutsugamushi*, is a major cause of febrile illness throughout the Asia-Pacific region (Rapmund, 1984). A high prevalence of antibody in some rural areas of Malaysia was noted in previous serological surveys, indicating high rates of transmission among the population (Cadigan *et al*, 1972; Tay *et al*, 2000). Patients may suffer from symptoms such as fever, rash, eschar, pneumonias, endocarditis, encephalitis, and disseminated intravascular coagulation and death if not treated (Oaks *et al*, 1983). The organism is transmitted to humans through the bites of infected *Leptotrombidium* mites.

The finding that mice are highly sensitive to *O. tsutsugamushi* (Ogata *et al*, 1932) contributes greatly to the progress of research on this organism. Many investigators use mice not only for the passage of rickettsiae but also for the isolation of rickettsiae from patients or from the natural animal hosts. Some investigators have suggested that the virulence of *O. tsutsugamushi* for mice is related to its virulence for man (Kawamura *et al*, 1939; Irons, 1946); however, others have not found this to be true (Smadel *et al*, 1950; Jackson and Smadel, 1951). In scrub typhus infections, the mechanisms causing the deaths of infected animals or patients is obscure. The basic pathology of scrub typhus is reported to be focal vasculitis and perivasculitis of the small blood vessels, involving a number of organs. The objective of this study was to determine the susceptibilities and various pathogenic responses of mice to Malaysian isolates of *O. tsutsugamushi*.

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MATERIALS AND METHODS

O. tsutsugamushi isolates

Nine Malaysian isolates including 7 human isolates collected during 1984-1985 that

had been passaged 3-4 times in mice (labeled as R1, R2, R4, R5, R6, R7 and R9) and kept at -70ºC in mice spleen/liver homogenates, one isolate obtained from the whole blood specimen of a patient in 1996 (labeled as R3) and 1 chigger isolate (labeled as R8) were include in this study.

Mouse virulence assay

For each *O. tsutsugamushi* isolate, 2 adult ICR mice were infected intraperitoneally with 0.2 ml of the samples and the clinical manifestation in mice was observed daily. At day 10, the mice were sacrificed and internal organs such as spleen, liver and kidneys were harvested and a 20% (w/v) tissue homogenate was prepared in Snyders' I solution (0.22 M sucrose, 0.1 M potassium phosphate, 5 mM potassium glutamate, pH 7.4). To compare the virulence of this organism in mice, 0.2 ml of the tissue homogenates was inoculated intraperitonealy into another 10 mice. The clinical manifestations of the mice were observed for a period of 28 days. Mice were challenged with a lethal dose of Karp strain and mortalities were recorded for a period of 28 days.

Infectivity of *O. tsutsugamushi* in mice

Mice were dissected 10 days post-infection when general clinical symptoms were apparent such as ruffled fur, inactivity and enlargement of abdomen. The mice were dissected and representative samples of various abdominal organs (spleen, liver and kidney) were promptly fixed in 10% buffered neutral formalin and sectioned for hematoxylin and eosin (H&E) staining. Sections were also examined for the presence of *O. tsutsugamushi* organisms by direct immunofluorescence (DFA) assay using fluorescent-isothyocyanate (FITC)-labeled antisera against *O. tsutsugamushi* and Giemsa staining. For detection of *O. tsutsugamushi* in the organ samples, the tissues were homogenized in phosphate buffered saline (PBS) and 0.2 ml was used for DNA extraction and polymerase chain reaction (PCR) as described by Furuya *et al* (1993). 0.2 ml of blood and peritoneal exudates were also plated onto L929 cells monolayers to establish *O. tsutsugamushi* infections.

RESULTS

The Malaysian *O. tsutsugamushi* isolates could be differentiated as low, moderately and highly virulent strains based on the different responses in infected mice. Four isolates (R3, R4, R5 and R9) were considered as low virulent strains (Table 1). Mice infected with these isolates were asymptomatic and none demonstrated any clinical illness or deaths upon infection for 28 days. Antibody titers ranging from 1:50 to 1:200 were detected in these mice on day 21 (Table 1). Upon dissection, there was only slight or no splenomegaly; no ascitis accumulation was observed.

Three isolates (R1, R2 and R7) were considered as moderately virulent. Mice infected with these isolates appeared sick after day 10 and deaths of less than 50% of the infected mice were observed after 28 days. High antibody titers ranging from 1:400 to 1:1,600 were detected on day 21 (Table 1). Upon dissection of the dead mice, splenomegaly and ascitis accumulation were observed.

Two isolates were considered as highly virulent (R6 and R8). Mice infected with these isolates demonstrated typical clinical signs as early as day 5 and more than 50% of mice died before day 28. Among the surviving mice, high antibody titers ranging from 1:400 to 1:1,600 were obtained (Table 1). Upon dissection, splenomegaly and ascitis accumulation was observed.

Upon dissection of mice infected with *O. tsutsugamushi*, isolates that were considered moderately or highly virulent, enlargement of mouse organs such as spleen, kidney and liver were noted. Accumulation of peritoneal exudates was obvious and could be withdrawn by a syringe from the peritoneal cavity of infected mouse. The presence of rickettsemia was confirmed by growth of *O. tsutsugamushi* organisms in L929 cells infected with blood (confirmed by Giemsa staining and PCR). *O. tsutsugamushi* organisms were also cultured from the peritoneal exudates of the infected mice. However, DNA of *O. tsutsugamushi* was only

Table 1
Different responses of ICR mice infected with Malaysian *O. tsutsugamushi* isolates.

O. tsutsugamushi isolates	Sources	Clinical appearance at day 10th	Spleno-megaly	Ascitis accumu-lation	No. mice died at day		Antibody titers by IIP assay
					10th	28th	
Low virulent							
R3	Human	Asymptomatic	-	-	0	0	1:50-1:200
R4	Human	Asymptomatic	±	-	0	0	1:50-1:200
R5	Human	Asymptomatic	±	-	0	0	1:50-1:200
R9	Human	Asymptomatic	-	-	0	0	nd
Moderately virulent							
R1	Human	Sick	+	+	2	0	1:400-1:1,600
R2	Human	Sick	+	+	1	3	1:400-1:1,600
R7	Human	Sick	+	+	0	2	nd
Highly virulent							
R6	Human	Sick	+	+	0	5	1:400-1:1,600
R8	Chigger	Sick	+	+	2	9	1:400-1:1,600

Note: The sickness of mice is indicated by mice having ruffled fur, loss of appetite and being inactive.
nd = not determined.

amplified from the peritoneal exudates and not from any other tissue samples. Sections by H&E staining revealed histological changes in spleen such as enlargement of red pulps and increased numbers of lymphocytes. Giant cells were large and vesicular were observed in the sections of liver and kidney. However, no *O. tsutsugamushi* organisms were observed in all the tissue sections by Giemsa staining, DFA and H & E staining.

DISCUSSION

The various responses of *O. tsutsugamushi* infection in mice showed that there was a difference in the degree of virulence of these isolates. It is generally noted in this study that mice infected with highly virulent rickettsial isolates showed manifestations of disease such as ruffled fur and enlargement of the abdomen (probably due to enlarged spleen/liver or accumulation of ascitis) as compared to mice infected with low virulent *O. tsutsugamushi* isolates.

Different responses of mice infected with *O. tsutsugamushi* have been observed among the *O. tsutsugamushi* isolates in this and other studies (Tamura *et al*, 1991; Moree and Hanson, 1992). When these less pathogenic isolates were infected into mice, they survived the infections without manifestation of symptoms, with only some mice exhibiting ruffled fur for a short period.

The virulence of 9 Malaysian *O. tsutsugamushi* isolates could be categorized into three types: 1) highly virulent strains (R6 and R8) where all mice were severely sick 10 days post-infection with > 50% deaths after 28 days and the isolates eliciting a high antibody titer (>1:400) in mice that survived; 2) moderately virulent strains (R1, R2 and R7) where all mice were sick but there was less than 50% deaths 28 days post-infection with these isolates also eliciting high antibody titers (>1:400) in mice that survived; and 3) low virulent strains (R3, R4, R5 and R9) where all mice were asymptomatic with no lethal infection in mice and where weak antibody responses (IIP antibody titer of 1:50 to 1: 200) were observed in the surviving mice. The low virulence of some *O. tsutsugamushi* isolates in this study may imply the low immunogenicity of these organisms or

enhanced host resistance towards *O. tsutsugamushi* infections.

Many researchers have empirically acknowledged the existence of *O. tsutsugamushi* strains that have low virulence in mice (Groves and Osterman, 1978; Groves *et al*, 1980; Jerrells and Osterman, 1981). It has been shown that inapparent infections in areas endemic for scrub typhus were due to *O. tsutsugamushi* strains with low virulence in humans (Shishido, 1962; Kawamura *et al*, 1980; Fan *et al*, 1987). In this study, *O. tsutsugamushi* isolated from patients with clinical symptoms are mostly pathogenic to humans but they showed different degrees of virulence in mice. Therefore no direct correlation between severity of human scrub typhus infections and virulence of the *O. tsutsugamushi* strains in mice was observed in this study. The virulence of *O. tsutsugamushi* strains in the laboratory mice is known to be influenced by at least 3 factors, *ie*, route of inoculation, antigenic strain and natural resistance of the host. Investigators have recognized that the route of inoculation (Groves and Osterman, 1978; Kelly and Rees, 1986) and isolates of organisms (Irons and Armstrong, 1947; Jackson and Smadel, 1951; Kelly and Rees, 1986) can alter observed pathogenicity. Catanzaro *et al* (1976) suggested that the survival of mice infected with *O. tsutsugamushi* depended on a delicate balance between the proliferation of the organism and the intensity of the immune response generated by the host. This may influence the success of initial isolation of the rickettsial agents as well as obscuring the results of challenge tests.

The examination of *O. tsutsugamushi* organisms by light microscopy and PCR in this study did not show any organisms in the tissue sections of spleen, kidney and liver of the infected mice. The finding suggests that the *O. tsutsugamushi* organism may have been cleared off by the immune system, or the rickettsiae were present at below detectable levels. The Karp strains of *O. tsutsugamushi* had been recovered from the blood and tissues of mice 610 days post-infection (Kelly and Rees, 1986). In addition, mice infected with Gilliam and Karp strains, consistently maintained a detectable rickettsemia over a 1-year period. Rickettsiae were recovered from the spleens of 95% of these mice 52 weeks postinfection. However, mice with infections of reduced pathogenicity did not have detectable rickettsemia from week 20 onwards (Groves and Kelly, 1989). It was suggested that the pathogenicity differences observed result from the more rapid growth of the rickettsiae resulting from infections with more pathogenic strains. However, more studies are needed to verify these properties.

The presence of *O. tsutsugamushi* organisms was however detected in the blood (by cell culture) and peritoneal exudates (by cell culture and PCR) in this study. The intraperitoneal inoculation of susceptible mice to *O. tsutsugamushi* with pathologic manifestation essentially restricted to the peritoneal cavity has been observed by others (Kundin *et al*, 1964; Catanzaro *et al*, 1976). The peritoneal cavity is rich in mesothelial cells, a cell type that readily supports growth of rickettsial organisms (Kundin *et al*, 1964; Kokorin *et al*, 1976) and it is also constantly bathed in fluid that readily serves to transmit infectious particles from cell to cell. The plasma membrane of the mesothelial cells has been shown to be intimately involved in the infection cycle of scrub typhus rickettsiae during the course of an established infection in the mouse peritoneal cavity (Ewing *et al*, 1978). It is therefore not surprising to be able to detect and culture *O. tsutsugamushi* organisms in the peritoneal exudates of infected mice. This study shows that peritoneal exudates may be used for the isolation of *O. tsutsugamushi* organism if the fluid could be withdrawn aseptically from the peritoneal cavity of infected mice.

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