Failure of Cyclophosphamide to Significantly Enhance Isolation of *Rickettsia tsutsugamushi* from Wild Philippine Rats¹

P. F. D. VAN PEENEN, C. M. HO, AND R. SEE

U.S. Naval Medical Research Unit No. 2, San Francisco, California 96263

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Treatment of wild rats from a known scrub typhus focus in the Philippines with cyclophosphamide did not significantly enhance the isolation rate of *Rickettsia tsutsugamushi*, using mouse inoculation techniques. Similarly, cyclophosphamide treatment of mice inoculated with organs of wild rats did not increase the recovery of rickettsiae, and isolation of rickettsiae was about equal from biopsies obtained before and after immunosuppression of wild rats.

Intraperitoneal (i.p.) inoculation of susceptible laboratory mice has long been the method usually used for isolation of *Rickettsia tsutsugamushi* from patients and, in epidemiological studies, from naturally infected chiggers and wild animals in Southeast Asia (5). The method is specific, since rickettsiae can be visualized by staining and examining the peritoneal exudates of moribund mice. However, sensitivity is often lacking, since in our own and other laboratories rickettsiae are often not recovered even though mice were inoculated with known infected material.

Immunosuppression of humans and animals enhances susceptibility to a wide range of latent infectious agents, including rickettsiae other than the scrub typhus agent (3, 6). We attempted enhancement with cyclophosphamide in the case of rodent *R. tsutsugamushi* infections at the suggestion of colleagues at the U.S. Army Research Unit, Kuala Lumpur, Malaysia. Our first experiments with standard strains were encouraging, since laboratory mice experimentally inoculated with the normally nonlethal Gilliam strain of *R. tsutsugamushi* died of infection and in the few survivors antibody production was unimpaired. However, the value of immunosuppression as a diagnostic aid necessarily depended upon tests with natural infections. The opportunity for such tests arose in examinations of wild rodents trapped in a scrub typhus focus in the Philippines. Results of these examinations are reported herein.

MATERIALS AND METHODS

Wild rats were trapped in Central Luzon (15°09'N, 120°27'E, 300-m elevation). Most of the rats were *Rattus mindanensis*, but a few specimens of *R. everetti*, *R. exulans*, and *R. panglima* were also collected. Rats were transported alive to Taipei, Taiwan, where all experimental work was performed. Animals were bled from the heart or orbital sinus, and their sera were tested by the indirect fluorescent antibody test (IFAT) of Bozeman and Elisberg (2). Antigen spots for IFAT were prepared from heavily infected yolk sacs of the Karp, Gilliam, and Kato strains of *R. tsutsugamushi* obtained from the Naval Medical Research Institute, Bethesda, Md. Locally prepared, fluorescein-conjugated, anti-*R. norvegicus* and anti-*Mus musculus* rabbit globulins were used in the IFAT for wild rats and for laboratory mice, respectively.

Wild rats were injected i.p. with cyclophosphamide (Cytosan, Mead Johnson) at dosages varying from 100 to 300 mg/kg and were sacrificed 4 to 7 days later. Spleens and kidneys were removed and ground in brain heart infusion broth to make a 20% suspension. Groups of four laboratory mice were injected i.p. with 0.2 ml of this suspension and observed for signs of illness. Sick animals were sacrificed and autopsied: peritoneal smears were stained with Giemsa and examined microscopically for the presence of rickettsiae. Although second mouse passages were made if animals appeared healthy after 14 days and survivors were challenged with 100 mouse mean lethal doses of a Taiwan strain *R. tsutsugamushi*, we did not isolate rickettsiae from mice that showed no illness during the first passage. Rickettsiae were subsequently identified as *R. tsutsugamushi* by the presence of antibodies in sera of mice surviving more than 14 days after inoculation. In all cases, there was a positive IFAT against Karp, Gilliam, or Kato strains and a negative IFAT against *R. mooseri*, the only other rickettsia known to occur in this area.

Wild rats were also examined without immunosuppressant treatment, and suspensions of spleen

¹ Address reprint requests to: Publications Office, U.S. Naval Medical Research Unit no. 2, Box 14, APO San Francisco, CA 96263, or 7-1 Kung Yuan Road, Taipei, Taiwan, Republic of China.

and kidney from either single or pooled rats were injected i.p. into six laboratory mice. Mice were divided into two groups of three each: one group of this first mouse passage received 8 mg of cyclophosphamide after 4 days. The other group did not receive cyclophosphamide. Criteria for isolation of rickettsiae from either group were as described above.

RESULTS

Treatment of wild rats with cyclophosphamide. A total of 64 wild rats were examined by mouse inoculation. Of these, nine lacked IFAT antibodies against *R. tsutsugamushi* at a titer of $\geq 1:20$ and were predictably negative when examined for rickettsiae. Rickettsiae were isolated from 3 of 13 (23%) IFAT antibody-positive wild rats treated with Cytoxan and from 12 of 42 (29%) that were not so treated.

Treatment of laboratory mice with cyclophosphamide. Groups of six mice each were inoculated with organs from 88 different wild rats. Results are outlined in Table 1. The IFAT antibody status of rats that served as donors of organs was known in 11 cases: rickettsiae were isolated from two of seven mouse groups inoculated with organs from rats with IFAT antibodies at $\geq 1:20$ and in none of four inoculated with organs from rats that lacked antibodies.

Handling of cyclophosphamide-treated mice was difficult since the drug itself resulted in the death of more than a third of passage 1 mice before 9 days. Results from two mouse groups were not considered, since all mice died over a weekend and could not be examined.

Isolation of rickettsiae from the same rat before and after Cytoxan treatment. Forty wild rats, all IFAT antibody positive at a titer $\geq 1:20$, were examined. Attempts were made to isolate rickettsiae from a kidney and, sometimes, a portion of the spleen was removed surgically. Animals were permitted to recover from surgery for 2 weeks and were then treated i.p. with 150 mg of cyclophosphamide per kg. After 4 days, the remaining kidney and spleen were examined for rickettsiae, with the following results. Rickettsiae were isolated both be-

fore and after treatment in 1 case and were not isolated either before or after treatment in 13 cases. Rickettsiae were isolated before, but not after, treatment in 4 cases, and the reverse was seen in 5 cases.

DISCUSSION

Cyclophosphamide treatment of wild Philippine rats with antibodies against *R. tsutsugamushi* did not result in higher isolation rates of rickettsiae than if animals were not immunosuppressed. In fact, in the first experiment, isolation rates from cyclophosphamide-treated wild rats (23%) were lower than from nontreated rats (29%), but the numbers of rats that were actually infected was not known for each group.

Better comparisons were possible when both cyclophosphamide-treated and untreated mice were injected with the same rat organs (Table 1). Although eight treated mouse groups were positive when nontreated mouse groups were negative, the reverse occurred twice and the higher recovery of rickettsiae from immunosuppressed mice was not significant ($P = 0.06$), according to the McNemar test (1). In addition, cyclophosphamide treatment was often lethal for rickettsia-negative mice, and in these experiments such treatment also suppressed production of antibodies, which are useful markers of infection. As noted in the introduction, antibody suppression did not occur when Cytoxan-treated mice were injected with Gilliam strain *R. tsutsugamushi*; therefore, the observed suppression may be related to antigenic differences inherent in the Philippine rickettsia strains.

There was essentially no difference in isolation rates when organs of the same rat were examined before and after immunosuppression. Surprisingly, of the 10 rats found to harbor rickettsiae in that experiment, rickettsiae were isolated both before and after immunosuppression in only one case. It appeared that the more times an infected wild rat was examined, the greater the opportunity for recovering rickettsiae, and that immunosuppression per se did not significantly increase the probability of isolations.

These results are somewhat at variance with those of Kazar et al., who administered cyclophosphamide to mice experimentally infected with the agents of epidemic typhus and Q fever and with *R. akari* (4). In all cases, there was a suppression of antibodies, as reported herein, but treatment also enhanced recovery of rickettsiae. These discrepancies may reflect not only basic differences between the kinds of rick-

TABLE 1. Isolation of rickettsiae from laboratory mice injected with organs of wild Philippine rats^a

Treated mice	Nontreated mice	No. of mouse groups
Positive	Positive	8
Positive	Negative	8
Negative	Positive	2
Negative	Negative	70

^a Half of the mice in each group of six were treated with 8 mg of cyclophosphamide.

ettsiae studied, but also differences inherent in the study of experimental versus natural infections.

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