Rhipicephalus sanguineus: Vector of a New Spotted Fever Group Rickettsia in the United States


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A rickettsia related to but distinct from the spotted fever agent, Rickettsia rickettsii, has been detected in 167 (18.9%) of 884 Rhipicephalus sanguineus taken off dogs in central and northern Mississippi. The organisms could readily be isolated in male meadow voles (Microtus pennsylvanicus), where it produced massive infections in the tissues of tunica vaginalis. It was practically nonpathogenic for male guinea pigs, although inoculation of these animals with infected tunica vaginalis of voles afforded in 30 of 38 instances solid immunity to challenge with virulent R. rickettsii. The Rhipicephalus rickettsia grew well in monolayers of chicken embryo fibroblast, Vero, mouse L, and HeLa cells. Cytopathogenic effects were minimal unless large concentrations of rickettsia were used as inocula. It also could be established in embryonated hen eggs but only after injection of massive doses of L cell-propagated organisms. Serological tests (complement fixation, microagglutination and/or micro immunofluorescence) indicated that the newly described rickettsia belongs to the spotted fever group but differs from R. rickettsii, R. akari, and R. conorii. Antigenic differences were also demonstrated by direct fluorescence microscopy as well as by vaccine potency and mouse-toxin neutralization tests.

The brown dog tick, Rhipicephalus sanguineus, is considered to be an important vector of the spotted fever group agent, Rickettsia rickettsii, in Mexico. In the United States, where it feeds only occasionally on man, it has never been found naturally infected with rickettsiae of the spotted fever group. Its efficiency as an experimental vector has long been recognized (9). This, together with its close association with the American dog tick, Dermacentor variabilis, on their common host, has caused several investigators to speculate that R. sanguineus could become a natural vector of R. rickettsii in the United States.

Anigstein and Bader (2) reported evidence of rickettsial infections in guinea pigs and rabbits inoculated with pools of R. sanguineus taken off dogs in Texas. Most guinea pigs, after incubation periods of 2 to 11 days, reacted with fever and developed an infection which could be passed to normal guinea pigs, some of which showed minor scrotal swellings. One of two rabbits became ill and hemorrhagic areas containing rickettsia-like organisms were found in the lungs. Similar organisms were detected in lung sections of one of four guinea pigs inoculated with tick suspensions. Unfortunately, the rickettsiae were not identified.

The present paper reports the isolation and identification of a rickettsia related to but distinct from R. rickettsii from R. sanguineus taken off dogs during a study of the clinical and epidemiological features of spotted fever in central and northern Mississippi (J. D. Sexton and W. Burgdorfer, South. Med. J., in press).

MATERIALS AND METHODS

Ticks collected from dogs were forwarded to the Rocky Mountain Laboratory, where they were examined for rickettsial agents by the hemolymph test (5). Ticks with positive hemolymph were dissected and smears of Malpighian tubules and hypodermis were examined by direct fluorescence microscopy after treatment with conjugated antisera to R. rickettsii, R. prowazekii, R. canadensis, and Coxiella burnettii (10).

For isolation of rickettsiae, the remaining tick tissues were triturated in 4 ml of cold brain heart infusion broth (Difco), and 0.25 ml of the suspension was injected intraperitoneally (i.p.) into each of four male meadow voles (Microtus pennsylvanicus). The remaining inoculum was frozen and stored at -70°C as reference material. Beginning day 3 after inoculation, voles were autopsied and smears prepared from the tunica vaginalis were stained by the Giménez method (8) as well as with the above-mentioned conjugates.

The behavior of the Rhipicephalus rickettsia was studied in guinea pigs, chicken embryos, and cell
cultures. Infected tunica vaginals and spleens of voles were triturated in brain heart infusion broth, and aliquots of 0.25 and 0.5 ml were injected i.p. into male guinea pigs or into the yolk sacs of 5-day-old embryos. In addition, monolayers of chicken embryo fibroblasts, Vero, mouse L, and HeLa cells were inoculated to compare growth patterns of the *Rhipicephalus* rickettsia with those described for *R. rickettsii* and other rickettsiae. The methods used for maintaining and inoculating these cultures were essentially those reported by Wike et al. (12), Cory et al. (6), or Anacker et al. (1). In some instances, inocula were centrifuged against the cell monolayers (1,700 × g for 30 min at 20°C) to enhance infection. Cultures were examined daily for cytopathogenic effects, and smears were prepared and evaluated for rickettsial growth.

Complement fixation (CF), microagglutination (7), and indirect microimmunofluorescence (micro-IF) (Philip et al., unpublished data) were used to determine the serological responses of guinea pigs, meadow voles, or mice. Test antigens included the *Rhipicephalus* rickettsia (strain 3-7-6) and one or more of the following: *R. rickettsii*, R and H lip strains, *R. conorii*, Simko strain, *R. akari*, *R. prowazekii*, Breil strain, *R. typhi*, Wilmington strain, and *R. canadensis*, strain 2678.

The *Rhipicephalus* rickettsial antigen used in the CF test was prepared by density gradient zonal centrifugation (1) from mouse L cell-grown rickettsiae. That used in the microagglutination test was prepared by an enzymatic digestion and sonication procedure developed by one of us (R.K.G.). In this procedure, 1 g of moist, packed L cells was suspended in 525 ml of 0.07 M phosphate-buffered saline (PBS), pH 7.0. Quantities of 105 ml each of the suspension were then exposed for 1 min to a 20-kc Biosonic II ultrasonic probe (Bronwill Scientific, Rochester, N.Y.) producing about 112 W of acoustic energy at the tip. After addition of 10.5 ml of 0.5% solution of Pronase (Grade B, Calbiochem, Los Angeles, Calif.) the suspension was incubated at 37°C for 2 h and centrifuged at 14,600 × g for 30 min. The pellet was suspended in one-half the original volume of PBS. Sonication, addition of Pronase to the same final concentration, incubation, and centrifugation were repeated. The pellet was suspended in 30 ml of PBS and centrifuged at 164 × g for 10 min in a horizontal rotor. Rickettsiae in the supernatant fluid were then recovered by centrifugation at 13,200 × g for 30 min, also in a horizontal rotor. Finally, the pellet was suspended in an appropriate quantity, usually 6 ml, of PBS.

Since details of the micro-IF test used at Rocky Mountain Laboratory have not yet been reported, a brief description of this procedure follows. Antigens from the *Rhipicephalus* rickettsia were prepared by centrifuging suspensions of infected mouse L cells, suspending the pellet in PBS, and sonicating. Other antigens were prepared by homogenizing yolk sacs containing rich growth of rickettsiae. All antigens were stored at −70°C until used, to maintain antigenic reactivity of the rickettsiae. Antisera were obtained from weaning Swiss mice (RML strain) intravenously inoculated with antigens on days 0 and 7; blood was drawn on day 10 and sera were harvested.

The micro-IF procedure used was that of Wang (11) for immunotyping isolates of *Chlamydia trachomatis*. Antigens were applied to slides with dip-pen points and then dried, fixed in acetone, and overlaid with serial two fold dilutions of mouse antisera in PBS containing 10% yolk sac to reduce background fluorescence. The slides were incubated in moist chambers at 37°C for 30 min, washed in PBS and overlaid with fluorescein isothiocyanate-labeled anti-mouse globulin (Antibodies Inc., Davis, Calif.). After incubation, washing, and mounting in buffered glycerine, the slides were read. End points against a particular antigen were the highest serum dilutions conferring definite fluorescence to rickettsiae.

Finally, vaccine potency (4) and mouse toxin neutralization (3) tests were used to study the immunological relationship of the *Rhipicephalus* rickettsia to *R. rickettsii*.

RESULTS

Of 884 *R. sanguineus* removed from 49 dogs, 167 (18.9%) were hemolymph test positive for rickettsia-like organisms (Fig. 1). Smears of tick tissues, particularly those of hypodermis and ovary, revealed massive intracellular, occasionally intranuclear growth, of rickettsiae that stained more intensely and appeared somewhat larger than *R. rickettsii* (Fig. 1B). The organisms reacted only with anti-*R. rickettsii* conjugate. The staining pattern was particular spotty, suggesting that only certain parts of the organisms reacted with the labeled antibodies (Fig. 1C).

Behavior of the *Rhipicephalus* rickettsia in laboratory animals. Male meadow voles injected i.p. with a 0.25-ml suspension of infected tick tissues had two- to fivefold enlargement of spleens and hemorrhagic inflammation of the tunica vaginals as early as 3 days after inoculation. Smears of tunica were negative until day 4, when, after prolonged microscopic examination, a few mesothelial cells packed with rickettsiae were detected. Continuous passage of the organism by inoculation of tunica vaginals into normal voles was at first unsuccessful. Later this was accomplished by injection of 6.25 mg of cortisone 24 h before injecting passage material.

The pattern of growth of the *Rhipicephalus* rickettsia in tunica vaginals of male voles differed greatly from that of *R. rickettsii*. It was characterized by massive development of organisms in localized mesothelial cells. The spotted fever agent, in comparison, shows considerably lower degree of development in these cells and appears to be more generalized even in the early phases of its infection of tunica. From 4 to 7 days after inoculation with the *Rhipicephalus* rickettsiae, most voles appeared hunched and exhibited scrotal swelling; deaths occurred occasionally.
There was little detectable pathogenicity for male guinea pigs. After i.p. injection of heavily infected suspensions of tunica vaginalis and spleens of voles, about 50% of guinea pigs responded with 2 to 5 days of fever and scrotal swelling. However, these reactions were rarely recorded in later experiments when suspensions of tunica vaginalis only or tissue culture material were used as inocula. In no instance was it possible to initiate infections by passing suspensions of blood or tunica vaginalis of febrile guinea pigs into normal guinea pigs.

Serological tests. All guinea pigs inoculated with suspensions of infected tunica vaginalis or tissue culture material developed CF antibodies in titers up to 1:32 against R. rickettsii (R and
Hlp) and R. conorii (Simko) antigens. Homologous titers were generally higher than heterologous titers but rarely by more than one twofold dilution. In comparison, sera from guinea pigs infected with R. rickettsii (Sawtooth 9-2) did not react with the Rhipicephalus rickettsiai antigen. Because they were anticomplementary, sera of meadow voles could not be tested for CF antibody.

In the microagglutination test, guinea pig and Microtus sera showed homologous titers as high as 1:256 and greater than 1:512, respectively, but were negative against the R. rickettsii antigens. On the other hand, guinea pigs immunized against R. rickettsii had titers exceeding 1:512 against homologous antigens but considerably lower titers (≤ 1:64) against antigens prepared from the Rhipicephalus rickettsiai.

Of particular interest were results of infection challenge tests. Of 38 guinea pigs inoculated with Microtus tissues infected with the Rhipicephalus rickettsiai, 30 (79%) were immune when challenged 50 days later with 1,000 egg mean infectious doses of the virulent Sawtooth 9-2 strain of R. rickettsii. This challenge dose killed 10 of 12 normal guinea pigs used as controls.

The extent of relationship of the Rhipicephalus rickettsiai to the spotted fever group rickettsiae became even more apparent in the micro-IF test of mouse sera. Mice immunized with this organism developed low-titered antibodies against the R and Hlp strains of R. rickettsii, and against R. akari and R. conorii, but a much higher titer against the homologous antigen (Table 1). There was no cross-reaction with the typhus group rickettsiae. Similarly, mice immunized with spotted fever group rickettsiae had higher titers against the homologous antigens than against the antigen prepared from the Rhipicephalus rickettsiai.

The antigenic relationship of the Rhipicephalus agent to the spotted fever group rickettsiae, as well as antigenic differences, were demonstrated also by direct IF. Fluorescein isothiocyanate labeled immuno globulins from recovered voles stained Rhipicephalus rickettsiae brightly and uniformly, resulting in clearly defined organisms (Fig. 1D). This conjugate, however, reacted very poorly with R. rickettsii and produced a highly particulate staining pattern. In contrast, the anti-R. rickettsii conjugate reacted strongly with Rhipicephalus rickettsiae although a particulate pattern of staining was again evident (Fig. 1C).

**Behavior of the Rhipicephalus rickettsiai in tissue cultures and embryonated hen eggs.**

The organism grew well in the tissue cultures used but HeLa cells appeared less susceptible than the others. Cytopathogenic effects were minimal in L cells unless very concentrated inocula were used. Monolayers sometimes appeared normal and yet showed massive growth of rickettsiae. This behavior was in sharp contrast to that of R. rickettsii (R). Infected by this organism in any part of a monolayer rapidly spread and resulted in destruction of all host cells. In chicken embryo fibroblast and Vero cells, plaques were detected as early as 7 days after inoculation, but these plaques, about 0.5 mm in diameter, were much smaller than those of R. rickettsii (Fig. 2).

Numerous attempts failed to establish the Rhipicephalus rickettsiai in embryonated hen eggs by injecting suspensions of infected tunica vaginalis of voles. When heavily infected L cell-

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<th>Antisera</th>
<th>Spotted fever group</th>
<th>Typhus group</th>
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<tr>
<td></td>
<td>R. rickettsii</td>
<td>R. akari</td>
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<td>Rhipicephalus rickettsiai (3-7-96)</td>
<td>1,024*</td>
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<td>R. akari</td>
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<td>R. canadai (2678)</td>
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* Median titers of four readings in duplicate tests.

* Trace.
suspensions were used as inoculum, eggs died between days 7 and 10 and revealed moderate numbers of rickettsiae in yolk sac smears. Serial passage of the rickettsia in yolk sac has been achieved but so far has not altered the degree of rickettsial infection.

Vaccine potency and mouse-toxin neutralization tests. Although only a limited number of tests has been performed thus far, vaccine doses as large as 2.05 μg, prepared by density gradient zonal centrifugation of L cell-propagated Rhipicephalus rickettsiae, did not protect against 2 mean lethal doses of R. rickettsii toxin. In contrast, the mean protection dose for the spotted fever vaccine ranged from 0.0020 to 0.0036 μg. Similarly, sera of guinea pigs infected previously with the Rhipicephalus rickettsia failed to neutralize R. rickettsii toxin.

DISCUSSION

Because R. sanguineus in the United States is said to feed on man only occasionally, its possible significance in the ecology of R. rickettsii has never been evaluated seriously despite the facts that this tick is recognized as the main vector of spotted fever in Mexico, and in other parts (Africa, Asia, Europe) of the world it is the vector of R. conorii, the etiological agent of boutonneuse fever. Patients with the latter disease exhibit in most instances an eschar (tache noire) at the site of the tick bite. Although boutonneuse fever has never been recorded in the western hemisphere, primary ulcers resembling the tache noire have been reported on patients in the United States (13; Sexton and Burgdorfer, unpublished data).

The rickettsial agent detected in and isolated from almost 19% of R. sanguineus from central and northern Mississippi represents the first agent of this nature from the brown dog tick in the United States. Based on its ecological and biological characteristics described above, it is definitely related to but distinct from any of the spotted fever group rickettsiae studied, and may indeed represent a hitherto undescribed member of this group.

If R. sanguineus in the United States parasitizes only dogs, it appears justified to incrimi-
nate dogs as the source of infection. On the other hand, one may also speculate that other species of ticks, including *D. variabilis*, may represent the primary vectors responsible for infecting dogs. Indeed, little work has been done on characterization of rickettsiae carried by *D. variabilis*, a species of tick which may be involved in the ecology of rickettsiae other than *R. rickettsii*.

Research pertaining to these questions is currently in progress at Rocky Mountain Laboratory in Hamilton and will be presented in future communications.

**LITERATURE CITED**