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Properties of Selected Rickettsiae of the Spotted Fever Group

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Eight strains of spotted fever group rickettsiae were studied to gain insight into the extent of variation of their properties. Two standard strains of *Rickettsia rickettsii* and one strain of *Rickettsia conorii* were included among the eight for comparison. The molar percentage of guanine plus cytosine for each strain did not differ significantly from that for *R. rickettsii*, 32.6 ± 0.7%. Two strains caused extended fever in guinea pigs, one strain caused fever of short duration, and the other strains induced little or no fever. Polyacrylamide gel electrophoresis of the detergent-solubilized rickettsial proteins indicated that the protein content of all strains, except the two strains of *R. rickettsii*, were different, particularly in the molecular weight range of 40,000 to 60,000. Virulent strains produced large clear plaques in Vero cell monolayers; the strains of low virulence generally produced smaller or more turbid, or both, plaques. On the basis of agglutination reactions with rabbit antisera, the eight strains were placed into five serotypes. These results indicate considerable heterogeneity in properties of spotted fever group rickettsiae in the United States.

Rickettsiae of the spotted fever group have been isolated in this laboratory from several species of ticks and animals collected in various regions of the United States and from humans. These rickettsiae differed, in addition to host and geographical source, in (i) virulence for laboratory animals and (ii) affinity for fluorescent-labeled antibody to a standard laboratory strain of *Rickettsia rickettsii*. It was unclear whether these isolates were merely varieties of *R. rickettsii* or whether the relationships were more distant. To gain some insight into the nature of their relationships, some of the above strains were subjected to certain of the tests used to differentiate rickettsiae.

Various criteria have been used for the classification of spotted fever group rickettsiae. They may be classified as to group on the basis of their reaction with guinea pig antisera in the complement fixation test (26), their ability to grow within the nuclei of host cells (35), and their molar percentage of guanine plus cytosine (G+C) (29, 30, 33). Other properties serve to group strains at species or subspecies levels. Among these latter properties are the ability of antisera to neutralize the lethal effects of rickettsial toxins of other strains (5), the presence of antigens detectable by complement fixation tests with mouse antisera (16, 25), and the ability of live and Formalin-killed rickettsiae to stimulate production of antibodies which protect guinea pigs against infection by other rickettsiae (16). In addition, several investigators have shown that plaques produced in monolayers of several cell lines may differ morphologically from agent to agent (8, 19, 36).

Rickettsiae may also be compared on the basis of virulence for certain laboratory animals such as the guinea pig. However, considerable variation in virulence of spotted fever strains has been noted. Not only does virulence of strains recently isolated from nature differ (4, 22, 27), but virulence of a given strain has been found to vary with the physiological state of the organisms (12, 15, 31). Finally, virulence of a rickettsial strain may change after a number of passages in the laboratory (28, 35).

Spotted fever group rickettsiae have been differentiated on the basis of their protein profiles determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Rickettsial species within the spotted fever and typhus groups were distinguished from other species of rickettsiae in the same group as well as from rickettsiae belonging to the heterologous groups (10, 21, 23). Rickettsial strains of the same species appeared to have identical patterns (9).

With the microimmunofluorescence test, 72 strains of spotted fever group rickettsiae have been placed into a minimum of 12 serotypes according to their patterns of reaction with a battery of typing sera raised in mice (24). Because of this demonstrated antigenic heterogeneity, these workers suggested that the spotted fever group rickettsiae may be as antigenically

diverse as the scrub typhus rickettsiae.

Of the above numerous methods for characterization of rickettsiae, we chose to use relatively few tests for several reasons. Some of the tests are not universally applicable. For example, not all rickettsiae produce readily demonstrable disease in guinea pigs, so reciprocal cross-protection tests are not always possible. Also, the toxin neutralization test cannot be performed in some instances, because some rickettsiae do not produce toxin. Finally, complement fixation tests with mouse antibody are sometimes impractical because of the great difficulty in obtaining a complement fixation antibody response in mice to some strains (16). Therefore, for our comparisons, we used methods which promised to be both informative and feasible for all isolates. The results of these tests, which verified our preliminary impressions of heterogeneity among the strains, are presented in the following report.

MATERIALS AND METHODS

Strains of rickettsiae. The eight strains used in this study are listed in Table 1. They include, for comparison, two well-known strains of *R. rickettsii* and one strain of *Rickettsia conorii*. Species rank has been proposed for two of the other isolates but, thus far, only the first three listed belong to species recognized in *Bergey's Manual of Determinative Bacteriology* (35). The species name *R. rhipicephali* (7) has been proposed for the fourth strain listed, and *R. montana* (16) has been suggested for certain nonpathogenic isolates represented by the sixth strain mentioned. The Sheila Smith strain of *R. rickettsii* was not compared in all tests, and the WB-8-2 strain was not compared in the plaque and virulence experiments because it did not grow sufficiently well in the embryonated chicken egg.

Cultivation of rickettsiae. The rickettsiae were grown either in the yolk sacs of chicken embryos (32) or in monolayers of L or Vero cells (2).

Guinea pigs. Male Hartley strain guinea pigs were obtained from a local supplier.

DNA base composition. Deoxyribonucleic acid (DNA) was obtained from rickettsiae purified by centrifugation in Renografin density gradients (34) and lysed with 1% SDS in the presence of 8 M urea and 1 mM ethylenediaminetetraacetic acid (6). The lysate was treated with Proteinase K (Beckman Instruments Inc., Palo Alto, Calif.), 250 μg/ml of lysate, held at 56°C for 10 min and then at 25°C for 30 min, and chromatographed on a hydroxyapatite (DNA grade Bio-Gel HTP, Bio-Rad Laboratories, Richmond, Calif.) column (20). Fractions containing the DNA were pooled and dialyzed against saline-citrate (0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Thermal denaturation (T_m) profiles for at least four samples of DNA from each of two lots of rickettsiae of each strain were determined in a Beckman Acta M-VI spectrophotometer equipped with an Acta M automatic sampling system. Molar percentages of G+C were calculated from the T_m temperatures by the formula $T_m = 69.3 + 0.41$ (G+C) (18).

Escherichia coli DNA was included in each run as an internal control. The average T_m value for *E. coli* DNA of 90.5 ± 0.2 calculated from our data agreed exactly with that reported in the literature (18).

Virulence assay. Virulence, for the purposes of this study, is measured solely by the extent of fever induced in guinea pigs by a standard dose of rickettsiae. One thousand plaque-forming units of each strain grown in chicken embryos and diluted in Snyder I solution (14) were inoculated intraperitoneally into guinea pigs, which weighed approximately 450 g, in groups of six. Rectal temperatures were determined daily for 12 days with a telethermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio). Areas under the fever curves were determined with a model 9100A Hewlett-Packard calculator coupled to a 9125B Hewlett-Packard calculator plotter (3).

SDS-PAGE. Rickettsiae purified by centrifugation in Renografin density gradients (34) were suspended in 0.05-ml quantities of 10 mM potassium phosphate buffer, pH 7.1, and held at −65°C until needed. Before use the samples were thawed and digested in Laemmli

TABLE 1. *Rickettsial isolates studied*

Rickettsia strain	Host	Geographical source	Year	Maximum no. of passages
R. rickettsii R	Tick (*Dermacentor andersoni*)	Western Mont.	1945	>58 EP[a]
R. rickettsii Sheila Smith	Human	Western Mont.	1946	8 EP, 1 TC, 1 GP, 10 EP
R. conorii Kenya tick typhus	Tick (*Haemaphysalis leachii*)	Kenya	1953	18 EP, 2 TC
R. rhipicephali 3-7-♀6	Tick (*Rhipicephalus sanguineus*)	Miss.	1973	1 M, 23 EP
Rickettsia sp. 275-F	Tick (*Dermacentor andersoni*)	Eastern Mont.	1962	1 M, 3 EP, 18 TC
Rickettsia sp. M-5/6	Meadow vole (*Microtus* sp.)	Eastern Mont.	1963	2 M, 9 EP, 13 TC
Rickettsia sp. TVA-7E1-♀2	Tick (*Dermacentor variabilis*)	Tenn.	1970	1 M, 14 EP, 6 TC
Rickettsia sp. WB-8-2	Tick (*Amblyomma americanum*)	Tenn.	1970	1 TC, 14 EP, 6 TC

[a] EP, Egg passage; M, *Microtus*; TC, tissue culture; GP, guinea pig.

solubilizer (17) for 4 min in a boiling water bath; 50 μg of protein (13) in 50 μl was electrophoresed (8 mA/tube for 10 h) on 7.5% polyacrylamide gel rods (0.6 by 11.0 cm) containing 0.2% SDS in 0.2 M sodium phosphate buffer, pH 7.1, in a Pharmacia GE-4 apparatus (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.). The electrode buffer contained 0.1 M sodium phosphate buffer, pH 7.1, and 0.1% SDS. Myosin, β-galactosidase, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (Bio-Rad Laboratories) were electrophoresed as molecular weight standards with the rickettsial proteins.

The gels were then stained with 0.25% Coomassie brilliant blue R-250 in methanol-water-acetic acid (5:5:1) for 2 h, destained overnight in a Pharmacia Gel Destainer GD-4 containing methanol-water-acetic acid (5:5:1), and stored in 7% acetic acid.

Densitometric scans of the gels were made at 550 nm and 1.5 cm/min in a Beckman UV 5260 spectrophotometer.

Plaque assays. The number of plaque-forming units in the various rickettsial preparations was determined in Vero cells (8). Plaque diameters were determined from photographic enlargements (×2) of the original plaques.

Serotyping. Sera were raised in New Zealand white rabbits (White's Wabbit Wanch, Kooskia, Idaho) inoculated with Formalin-inactivated rickettsiae purified by sucrose density gradient centrifugation in a zonal rotor (2). The rabbits were bled from the marginal ear veins 2 weeks after the second of two weekly intravenous injections of 10-μg doses of six of the strains of rickettsiae suspended in 67 mM phosphate-buffered saline at pH 7.4, and the sera were stored at −20°C. Three additional 10-μg doses of *R. rhipicephali* and WB-8-2 were given to the appropriate rabbits (intravenously on day 35, subcutaneously on day 48, intravenously on day 49, bleeding on day 59) to obtain suitable levels of antibody. Equal volumes of each serum in a group were pooled before use.

Suspensions of each rickettsia (250 μg/ml of phosphate-buffered saline) were tested against dilutions of each of the above sera by the microagglutination technique (11). Acridine orange was added to each well to render the rickettsiae more readily visible.

RESULTS

DNA composition. None of the G+C values differed significantly ($P > 0.05$) from that of 32.6% determined for the R strain (Table 2). These results indicated that all of the rickettsiae belonged to the spotted fever group (33).

Virulence. Average temperatures for the guinea pigs in each group inoculated with 1,000 plaque-forming units of the appropriate rickettsiae are presented in Fig. 1. From these results each strain was assigned to one of three virulence classes. The most virulent strains, TVA-7E1-♀2 and R, induced the highest temperatures over the longest period of time. (The Sheila Smith strain was shown earlier to be at least as virulent as the R strain [1].) Temperatures of animals inoculated with the single representative of the intermediate group, 275-F, rose sharply, but almost as rapidly dropped back toward normal. The least virulent strains, KTT, 3-7-♀6, and M-5/6, induced more moderate changes in temperature. Interclass differences in areas under the fever curves were significant at the 0.05 level.

TABLE 2. *Molar percentages of G+C of rickettsial DNA determined from thermal denaturation temperatures*

Rickettsia strain	Absorbance ratios of purified DNA		G+C (mol%)
	260 nm/280 nm	260 nm/230 nm	
R. rickettsii R	1.8, 1.9[a]	2.1, 2.4	32.6 ± 0.7[b]
R. conorii Kenya tick typhus	1.8, 1.9	2.4, 2.5	32.6 ± 0.3
R. rhipicephali 3-7-♀6	1.8, 1.8	1.8, 2.1	32.2 ± 0.5
Rickettsia sp. WB-8-2	1.8, 1.8	2.4, 2.2	32.3 ± 0.6
Rickettsia sp. 275-F	1.8, 1.8	2.0, 2.1	32.9 ± 0.7
Rickettsia sp. M-5/6	1.8, 1.9	2.2, 2.3	33.1 ± 0.3
Rickettsia sp. TVA-7E1-♀2	1.7, 1.8	1.7, 2.1	33.0 ± 0.4

[a] Values for each of two batches of DNA analyzed.
[b] Mean of eight or more determinations ± standard deviation.

SDS-PAGE. Comparisons of solubilized proteins from whole purified rickettsiae were made by SDS-PAGE. Gel scans of the patterns of proteins from all eight strains electrophoresed in the same run are shown in Fig. 2. The patterns for the R and Sheila Smith strains were basically similar. Although occasionally there were quantitative differences in the areas under corresponding peaks of these two tracings, such as the seventh and eighth from the right, generally the position and size of the peaks conformed closely.

Differences in patterns of the other isolates were more extensive. The majority of differences occurred in the 40,000- to 60,000-molecular-weight range, but differences also were noted both above and below this range (see patterns for WB-8-2 and M-5/6). These patterns indicated significant differences in protein composition of the rickettsiae.

Plaque morphology. Several kinds of plaques were produced by the various rickettsiae in Vero cells (Table 3). Strains R, TVA-7E1-♀2, and 275-F produced clear plaques averaging from 1.7 to 2.0 mm in diameter. *R. conorii* produced a target-like plaque with a central portion of intact cells surrounded by a clear peripheral area. Other strains (3-7-♀6 and M-5/6) had turbid plaques which differed considerably in size. The observed differences were distinct enough so that many of the strains could be readily distinguished from one another.

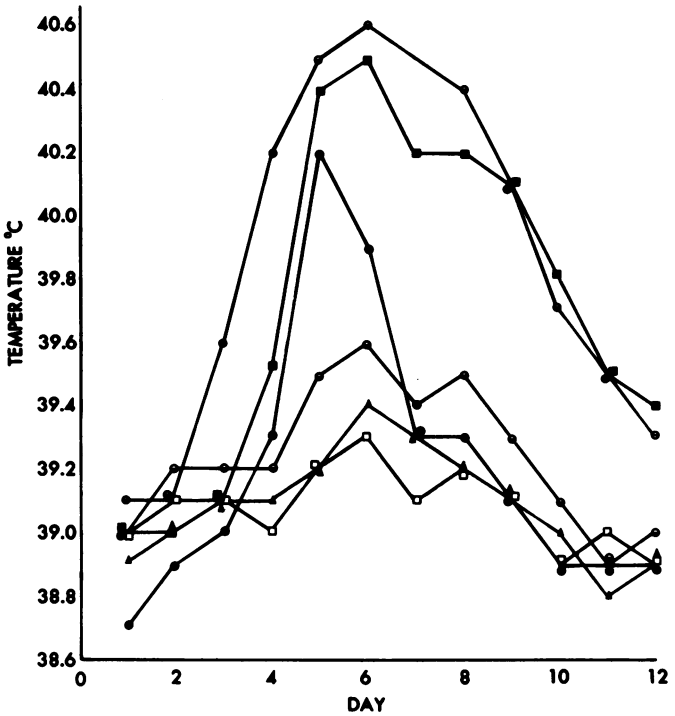

FIG. 1. *Mean temperature of male Hartley strain guinea pigs inoculated intraperitoneally with 1,000 plaque-forming units of the appropriate rickettsial strain. Each point represents the mean temperature of six animals.* ■, *R. rickettsii R;* ⊖, *R. conorii KTT;* ▲, *R. rhipicephali 3-7-♀6;* □, *Rickettsia sp. M-5/6;* ●, *Rickettsia sp. 275-F;* ⊖, *Rickettsia sp. TVA-7E1-♀2.*

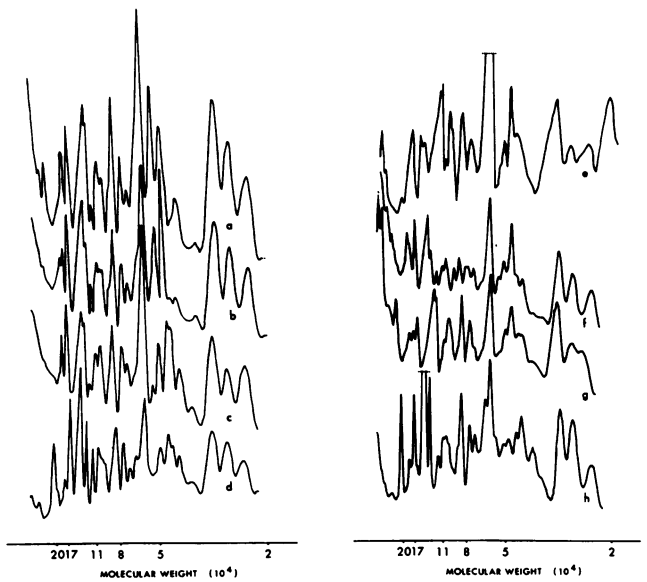

FIG. 2. *Gel scans of SDS-PAGE patterns of solubilized proteins of rickettsial strains. (a) R. rickettsii R; (b) R. rickettsii Sheila Smith; (c) R. rhipicephali 3-7-♀6; (d) 275-F; (e) WB-8-2; (f) TVA-7E1-♀2; (g) M-5/6; (h) R. conorii Kenya tick typhus. Some of the taller peaks were truncated to permit better juxtaposition of the tracings.*

Serotyping of strains. Microagglutination patterns of reactivity of each rickettsia with the various rabbit antisera are presented in Fig. 3. The four strains seen at the top (R, Sheila Smith, TVA-7E1-♀2, and 275-F) reacted with the test sera in a nearly identical manner, whereas the pattern for each of the other four strains was unlike that for any of the other seven strains.

DISCUSSION

Results of our tests demonstrated both similarities and differences among the selected strains. The analysis of the G+C ratios indicated that either all strains were indeed representatives of the spotted fever group (33) or the strains were unrelated to these rickettsiae but by mere coincidence had the appropriate G+C content. However, because of the history of these strains and the antigenic relationships initially demonstrated by fluorescent-antibody staining, this latter possibility was judged to be extremely unlikely. Therefore, our remaining tests were designed to examine more specific properties of the rickettsiae.

TABLE 3. *Comparison of plaques produced in Vero cells by rickettsial isolates*

Rickettsia strain[a]	Diameter (mm)	Appearance
R. rickettsii R	1.9 ± 0.2[b]	Clear
R. conorii Kenya tick typhus	1.7 ± 0.2	Target-like
R. rhipicephali 3-7-♀6	1.2 ± 0.2	Turbid
Rickettsia sp. 275-F	1.7 ± 0.3	Clear
Rickettsia sp. M-5/6	1.7 ± 0.3	Turbid
Rickettsia sp. TVA-7E1-♀2	2.0 ± 0.4	Clear

[a] Vero cell monolayers were infected with rickettsiae grown in chick yolk sacs.

[b] Mean diameter of 25 plaques ± standard deviation.

Results from the other tests revealed a uniformity among the virulent strains and considerable heterogeneity among the other strains. The strains of little or no virulence (M-5/6, 3-7-♀6, KTT, and presumably WB-8-2) differed from the virulent strains and from each other in SDS-PAGE pattern, serotype, and plaque morphology. Strains M-5/6, 3-7-♀6, and WB-8-2 apparently were no more closely related to *R. rickettsii* R than they were to *R. conorii* KTT, a strain isolated from a different continent.

Of possible significance is the fact that the virulent strains (R, Sheila Smith, TVA-7E1-♀2, and 275-F) were similar in a number of properties. These strains produced fever in guinea pigs, clear plaques about 2.0 mm in diameter after 6 days in Vero cells, and gave essentially identical agglutination patterns with the rabbit typing sera. It is unknown whether this linkage of traits is merely coincidental or whether one trait may be causally related to another. Conceivably, there may be some common factor which potentiates (i) infection of guinea pig cells in vivo and (ii) relatively rapid invasion and destruction of tissue culture cells in vitro. Since virulence, in the few strains we have tested, occurs in those strains which appear to have the same surface antigens, it is tempting to speculate that a surface antigen(s) is responsible, at least in part, for virulence.

To the extent we were able to compare strains, results from our serotyping experiments with rabbit antisera agree with those of Philip et al. (24) who used mouse antisera and the microim-

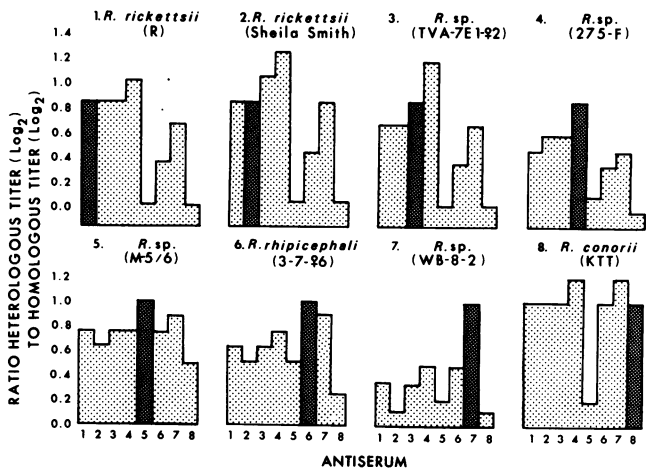

FIG. 3. *Antigenic relationships among rickettsial strains determined by microagglutination tests with rabbit antisera. Each bar graph illustrates the agglutination pattern of one strain (shown at the top of each graph with its corresponding antiserum number) with each of the antisera listed at the bottom. The homologous reactions are depicted with fine cross-hatching.*

munofluorescence test for typing. Both laboratories placed the R, Sheila Smith, and TVA-7E1-♀2 strains in the same serotype. We also placed the 275-F strain in this group, whereas Philip et al. put 275-F with the closely related Hlp-like strains of *R. rickettsii*. Each of the other four strains was determined to be antigenically distinct by both serological procedures.

Of considerable importance to the value of the criteria employed here for strain characterization is the stability of the particular traits analyzed. Unfortunately, we do not have evidence that these properties are reasonably stable and diagnostic. As mentioned above, virulence of rickettsiae is perhaps one of the less stable properties and depends upon the level of innate virulence of the wild strain, occurrence of mutations during laboratory cultivation, and the physiological state of the organisms. Less is known about the permanence of the other characteristics, except possibly that of the proteins demonstrated by SDS-PAGE. Dasch et al. (9) showed that the PAGE patterns of all strains in each of the three species of the typhus group, regardless of the source, were identical and distinct from the patterns for the other species. Our data show that the patterns for the R and Sheila Smith strains of *R. rickettsii* are also essentially identical, even though these strains have been maintained for more than 30 years. It seems probable that either rickettsial proteins do not easily change under laboratory conditions or that the proteins do change but to another specific and stable array of proteins.

Current results, as well as those cited by Philip et al. (24), indicate that ticks and their hosts in the United States harbor rickettsiae that differ widely in properties. Whereas some of these isolates show a number of features in common and probably are closely related, others exhibit marked diversity in antigenic and protein composition. It is presently unclear whether these differences are great enough to warrant elevation of some of these strains to species rank or whether these differences represent normal deviations from the properties of classical strains of *R. rickettsii*. Based on our data, one may speculate that the spotted fever group rickettsiae in the United States have diverged from a common ancestor in several lines by a series of mutations.

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