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Plaque Formation in Tissue Cultures by *Rickettsia rickettsi* Isolated Directly from Whole Blood and Tick Hemolymph

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A simple technique is described for isolation of *Rickettsia rickettsi* directly from tick hemolymph and whole blood of rickettsemic guinea pigs by means of the plaque assay technique in primary chicken embryo tissue cultures. Plaque-forming units per drop of hemolymph were almost 100-fold greater for partially engorged ticks than for unengorged ticks. Rickettsemia in guinea pigs fed upon by infected ticks was detected as early as 24 hr before fever. No morphological differences were noted between plaques formed by rickettsiae isolated from tick hemolymph or from whole guinea pig blood.

Isolation of rickettsial agents from ticks or from tissues of animals with known or suspected infection is usually made by injection of suspensions of these tissues into laboratory animals such as adult male guinea pigs, meadow voles, and mice. After development of clinical signs (elevated temperature, scrotal swelling, etc.) or upon microscope detection of rickettsiae in certain tissues, such tissues are triturated and injected into embryonated hen eggs for isolation and characterization of rickettsiae. Direct injection of field material into embryonated chicken eggs is often unsuccessful because of the high incidence of bacterial contamination, even in the presence of antibiotics.

Although different types of cell cultures have been developed and are used for studying biological and physiological properties of rickettsial agents, to the best of our knowledge, use of such cultures for isolation of rickettsiae from either arthropods or infected animals has not been reported.

The present study is an evaluation of the primary chicken embryo (CE) plaque assay technique (6) for direct isolation of the spotted fever agent, *Rickettsia rickettsi*, from hemolymph and blood of infected *Dermacentor andersoni* and guinea pigs, respectively.

MATERIALS AND METHODS

Isolation of R. rickettsi from tick hemolymph. Ticks from the 10th filial generation of a naturally infected *D. andersoni* female were used. This specimen was collected from vegetation in Sawtooth Canyon on the west side of the Bitter Root Valley in 1961 and was found to be infected with a highly virulent strain of *R. rickettsi*. Male guinea pigs fed upon by ticks derived from this female developed fever for 5 to 12 days, and approximately 85% of them died with signs typical of virulent spotted fever.

Before acquisition of hemolymph, the unengorged and partially engorged *D. andersoni* males and females were immersed for 1 hr in aqueous Merthiolate (1:1,000) and then washed twice for 30 min in sucrose-phosphate-glutamate solution (1) containing 1,000 units of penicillin per ml. The ticks were dried under ultraviolet light, and a drop of hemolymph was obtained under sterile conditions by amputating the distal portion of a leg with dissecting scissors. The hemolymph drop was drawn into 0.1 ml of ice-cold brain heart infusion broth (Difco) in a 1.0-ml tuberculin syringe fitted with a 25-gauge needle. This suspension, either undiluted or diluted 10-fold in ice-cold brain heart infusion broth, was inoculated into CE tissue cultures. As controls, hemolymph from partially engorged *D. andersoni* from our normal colony was used. Presence and absence of *R. rickettsi* in infected and normal ticks, respectively, was confirmed by the hemolymph test (2). This test consists of obtaining hemolymph from a wound produced by amputating the distal portion of one or more legs. The hemolymph is collected on a slide, heat fixed, stained by the method of Giménez (3), and examined microscopically.

Isolation of R. rickettsi from guinea pig blood. Each 500-g male Hartley guinea pig was exposed to feeding of one or two of the infected adult *D. andersoni*. On days 1 to 4, 6, and 8 after tick attachment, 0.5 ml of whole blood was drawn from each guinea pig by intracardiac puncture with a 3.0-ml heparinized syringe fitted with a 23-gauge needle. The blood,

either undiluted or diluted 10-fold in ice-cold brain heart infusion broth, was inoculated into CE tissue cultures. Rectal temperatures of the guinea pigs were recorded daily; serological conversions were determined by complement-fixation (4) and microagglutination (5) tests. Microagglutination and complement-fixation titers $\geq$1:8 were considered significant. As controls, blood samples from guinea pigs fed upon by normal *D. andersoni* were similarly cultured and tested.

Cell cultures. Preparation of CE tissue cultures, inoculation with tick hemolymph or guinea pig blood, and determination of plaque-forming units (PFU) per milliliter were as previously described (7). To confirm the fact that plaque formation was by *R. rickettsi*, whole CE cell sheets were stained by the method of Gimenéz, or smears of plaques were stained by direct fluorescent antibody (6) for microscope examination.

RESULTS

Isolation of R. rickettsi from tick hemolymph. Hemolymph of all nine unengorged and six partially engorged, infected ticks contained rickettsiae that formed plaques (Table 1) which were characteristic of those described earlier for *R. rickettsi* (7). The PFU per drop of hemolymph for partially engorged ticks was almost 100-fold greater than that for unengorged ticks, even though in most cases the estimate of infection by the hemolymph test revealed only slight differences. No differences in plaque morphology were noted between the rickettsiae isolated from these two groups of ticks.

No plaques developed in cultures inoculated with the hemolymph of four partially engorged, uninfected ticks that were used as controls.

Isolation of R. rickettsi from whole blood of infected guinea pigs. Rickettsemia initiated by feeding of one or two infected *D. andersoni* was readily detected by plaque formation in CE tissue cultures (Table 2). There were no morphological differences between plaques formed by rickettsiae from whole guinea pig blood and those from tick hemolymph (Fig. 1). All plaques examined with a microscope were

TABLE 1. *Plaque-forming units (PFU) per drop of hemolymph for unengorged and partially engorged Dermacentor andersoni infected with Rickettsia rickettsi*

Unengorged ticks		Partially engorged ticks	
Tick no.	PFU/drop of hemolymph	Tick no.	PFU/drop of hemolymph
1	5	8928A	34×10^2
2	11	8931A	72×10^2
3	10	8931B	10×10^2
4	216	8932A	110×10^2
5	29	8934A	37×10^2
6	4	8934B	14×10^2
7	19		
8	41		
9	187		
Avg PFU	58	Avg PFU	46×10^2

TABLE 2. *Plaque-forming units (PFU) per milliliter of whole blood of guinea pigs fed upon by Dermacentor andersoni infected with Rickettsia rickettsi*

Guinea pig no.[a]	PFU/ml				
	Prebleed	Day 1-3	Day 4	Day 6	Day 8
8928	0	0	0	6.0×10^1	9.6×10^2
8931	0	0	6.0×10^1	4.2×10^2	3.0×10^3
8932	0	0	0	6.0×10^1	1.6×10^3
8934	0	0	0	1.8×10^2	7.0×10^3

[a] Guinea pigs were fed upon by one or two ticks of corresponding number(s), as listed in Table 1. Sustained fever, 40 C or higher, began on day 5 for guinea pigs no. 8931 and 8934, and on day 6 for guinea pigs no. 8928 and 8932.

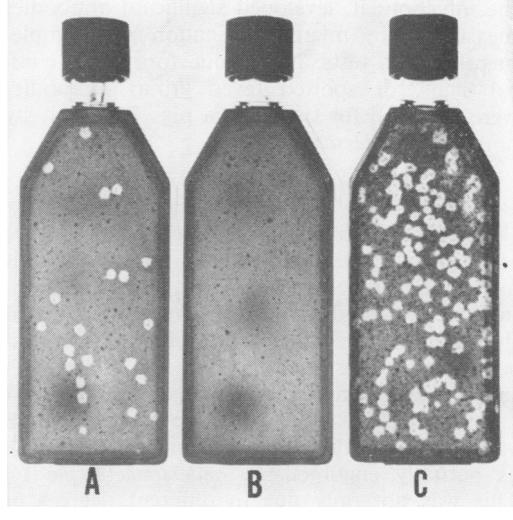

FIG. 1. *Typical plaques of Rickettsia rickettsi 9 days after inoculation of primary chicken embryo tissue cultures with whole guinea pig blood or Dermacentor andersoni hemolymph. A, Whole blood of guinea pig fed upon by infected ticks; B, whole blood of guinea pig fed upon by normal ticks; C, hemolymph of infected tick.*

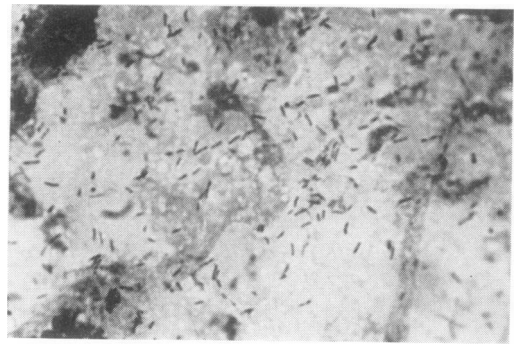

FIG. 2. *Giménez-stained Rickettsia rickettsi in plaques in primary chicken embryo cell sheets 9 days after inoculation of hemolymph of infected Dermacentor andersoni. R. rickettsi isolated from guinea pig blood was identical morphologically.*

found to have *R. rickettsi* only within plaques and areas immediately adjacent to plaque borders (Fig. 2). Guinea pig no. 8931, fed upon by two infected ticks (Table 1, ticks no. 8931A and B), had rickettsiae circulating as early as 4 days after tick attachment and as early as 24 hr before fever of 40 C or higher (Table 2). The other three host animals were positive 6 days after attachment. All four guinea pigs developed clinical signs typical of virulent spotted fever. Only guinea pig no. 8932 survived the infection; it developed significant antibodies measurable by microagglutination and complement-fixation tests. No plaque formation, clinical signs, or spotted fever group antibodies were recorded for two guinea pigs fed upon by normal *D. andersoni*.

DISCUSSION

Sterilization of the tick integument before acquisition of hemolymph was important. Following the procedure described above, contamination presented only a minor problem, and there was no need to add antibiotics to the tissue cultures. Less than 5% of the hemolymph samples were contaminated with bacteria.

The rickettsial PFU per drop of hemolymph varied considerably among unengorged as well as partially engorged *D. andersoni* (Table 1). This was not only due to different degrees of infection in ticks, but also to the fact that the volume of hemolymph drops measured by micropipette varied from tick to tick. Drops of hemolymph were approximately $5 \pm 3 \times 10^{-4}$ ml, with the largest drop sixfold greater than the smallest one. Such variation in drop size depends on a variety of factors, including size and sex of ticks, degree of engorgement, time necessary for the wound to close (by clotting), as well as the efficiency by which amputation of the distal leg portion was performed. However, despite these considerations, there is no doubt that there was a considerable increase in PFU per drop of hemolymph in partially engorged *D. andersoni*, as compared to those which were unengorged. This increase probably was due to rickettsial multiplication during tick feeding.

It has previously been shown (7) that centrifugation of CE tissue cultures inoculated with *R. rickettsi* may increase the average PFU by a factor of 7.2. Because this was not done in this study, numbers of rickettsiae in hemolymph and in blood, as measured by PFU, might have been higher than those presented in Tables 1 and 2, and rickettsemia in guinea pigs might have been detected earlier.

The results of these studies indicate that the rickettsial plaque assay technique is a valuable tool for isolation and cultivation of rickettsial agents directly from ticks or blood of animal hosts. It appears to be of particular value in conjunction with the hemolymph test, since infected ticks can be bled repeatedly for rickettsial isolation and cultivation. The rickettsial plaque assay technique may be applicable to studies concerned with the role of animal hosts as sources of rickettsial infections for ticks and other hematophagous arthropods. The rickettsial plaque assay system is presently being used in our laboratory in an attempt to isolate spotted fever group rickettsiae from ticks which previously have been shown to be nonpathogenic for laboratory animals.

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