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Plaque Assay and Cloning of Scrub Typhus Rickettsiae in Irradiated L-929 Cells

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It was demonstrated that gamma-irradiated L-929 cells support plaque formation by three strains of *Rickettsia tsutsugamushi* and representative species of the spotted fever and typhus group rickettsiae. Sensitivity of the plaque assay for detection of viable scrub typhus rickettsiae was similar to that achieved with intraperitoneal inoculation of random-bred mice. The concentration of irradiated cells and the temperature and length of incubation were all found to affect plaque size. A technique combining terminal dilution and plaque purification was used to obtain clones of three strains of scrub typhus rickettsiae.

Plaque formation by rickettsiae, particularly the typhus and spotted fever group organisms, has been described in primary chicken embryo cell cultures (5, 8) and in continuous cell lines (2, 7, 8). In addition, optimum cultural conditions and diluents have been carefully examined with the primary chicken embryo system (11, 12). However, there has been little refinement of the plaque assay technique for scrub typhus rickettsiae since the initial report of successful plaquing in primary chicken embryo cells (6). We investigated the ability of a continuous cell line to support plaque formation by *Rickettsia tsutsugamushi*, since this technique not only would provide a convenient plaquing procedure in urban laboratories, but would constitute a significant technical advance for overseas laboratories situated in remote geographical areas where pathogen-free eggs are not routinely available. Previous work from this laboratory (7) indicated that L-929 cells would support plaque formation by spotted fever group rickettsiae, and other investigators (9) noted that plaque formation occurred in irradiated L-929 cells infected with typhus or spotted fever group rickettsiae. Since *R. tsutsugamushi* had been reported to grow in irradiated L-929 cell monolayers (10), it seemed reasonable to expect that plaque formation would occur. The purposes of this study were to: (i) determine cultural conditions for infected, irradiated L-929 cells that resulted in well-defined rickettsial plaques; (ii) demonstrate the effectiveness of the plaquing system for classical scrub typhus strains and representative members of other rickettsial groups; and (iii) devise a method for plaque purification and cloning of scrub typhus rickettsiae.

(This work represents a portion of the research to be submitted by S.C.O. to the University of Maryland in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Rickettsiae. Scrub typhus organisms used were *R. tsutsugamushi* strains Karp (egg passage 52), Gilliam (egg passage 164), and Kato (egg passage 162). Spotted fever group organisms included *Rickettsia conorii* Casablanca (egg passage 41) and *Rickettsia rickettsii* Bitterroot (egg passage 61). Typhus group organisms were represented by *Rickettsia typhi* Wilmington (egg passage 16) and *R. prowazekii* Breinl (egg passage 156). All rickettsiae were prepared as 20% yolk sac suspensions (wt/vol) in sucrose-phosphate-glutamate buffer (1) and stored at −70°C.

Cell culture. L-929 cells (CCL 1, American Type Culture Collection, Rockville, Md.) were routinely propagated as monolayer cultures at 37°C, using medium 199 supplemented with 2 mM glutamine and 10% fetal bovine serum (all media were purchased from Microbiological Associates, Walkersville, Md.).

Irradiation procedure. Cells were trypsinized, counted, adjusted to 2×10^6 cells/ml in growth medium, and subjected to 3,000 rads of gamma radiation (Gamma Cell 220, Atomic Energy of Canada, Ltd., Ottawa, Canada). This dose produces nondividing, multinucleate giant cells capable of supporting rickettsial growth (9). Culture dishes (60 by 15 mm, Lux, Microbiological Associates) were seeded with 5 ml of growth medium containing various concentrations of irradiated L-929 cells, allowed to stand undisturbed for 20 min at 26°C on a level surface, and then were incubated overnight at 34°C in a humidified atmosphere of 5% CO_2 in air.

Plaque assay. The procedure was as previously described (7), except that medium 199 was employed as the growth medium. All dilutions of rickettsiae

were prepared in cold brain heart infusion broth. The neutral red staining overlays were applied at various times, depending on the organisms being plaqued and the experimental conditions. Due to the lengthy incubation period required for plaque formation, a 5-ml feeder overlay, identical to the initial overlay, was applied on day 9 in all experiments with *R. tsutsugamushi*. Unless otherwise specified, all plaque assays were conducted at 34°C in a humidified atmosphere of 5% CO_2 in air.

Mouse assay. The determination of mouse infective dose (MID_{50}) and mouse lethal dose (MLD_{50}) for strains of scrub typhus rickettsiae was performed in random-bred ICR mice (Walter Reed Army Institute of Research) in a manner similar to that described by Jackson and Smadel (4). Serial 10-fold dilutions of each strain of scrub typhus rickettsiae were prepared in sucrose-phosphate-glutamate buffer. Groups of five mice were injected intraperitoneally with 0.2-ml portions of each dilution. The animals were observed daily for 28 days and deaths were recorded. Mice surviving the entire observation period were back-challenged on day 28 with 1,000 MLD_{50} of a standardized suspension of the Karp strain. The MLD_{50} and MID_{50} were calculated by the Spearman-Karber method (3). For calculation of the MID_{50}, all mice that died in the original titration plus those that survived the back-challenge were considered to have been infected.

RESULTS

Effect of L cell concentration and incubation temperature on plaque formation. These two variables first were examined with *R. conorii*, a spotted fever group organism capable of rapidly producing large plaques, and then favorable conditions were tested with scrub typhus rickettsiae.

The density of L cell monolayers was controlled by adjusting the concentration of irradiated, nonreplicating cells placed in culture dishes. After overnight incubation at 34°C, each dish was infected with an appropriate dilution of *R. conorii*, overlaid, and reincubated at 34°C. Neutral red staining overlay was applied on day 7, and on day 8 the plaques were photographed and measured. Table 1 indicates that an increase in cell density from 0.25×10^6/ml to 2.0×10^6/ml was accompanied by a two-fold reduction in plaque diameter. In each instance, the plaques were symmetrical, with well-defined edges, although staining contrast was reduced at a cell density of 0.25×10^6/ml. The initial plaquing experiment with *R. tsutsugamushi* Gilliam was conducted at a cell concentration of 0.75×10^6/ml. Plaques were very small, usually less than 0.5 mm in diameter, and unsuitable for either plaque recovery or reliable enumeration. Only those cell concentrations favoring larger plaque diameter were suitable for further testing. Studies conducted with cell concentrations of 0.5×10^6/ml and 0.25×10^6/ml indicated that staining contrast was inadequate at the latter density. Plaques occurring in dishes seeded with 0.5×10^6 cells/ml were approximately 0.8 mm in diameter and clearly defined. This cell concentration was employed in all further experiments.

TABLE 1. *Effect of irradiated L-929 cell density on plaque size*

No. of L cells per ml ($\times 10^6$)	Plaque diam (mm)
0.25	1.41 (±0.03)[a]
0.50	1.37 (±0.04)
0.75	1.21 (±0.03)
1.00	0.95 (±0.02)
2.00	0.70 (±0.03)

[a] Standard error of the mean.

The effect of incubation temperature on plaque development also was studied first with *R. conorii* as the infecting organism. Adequate plaque formation was observed at 28, 30, 32, 34 and 36°C. Temperatures exceeding 37°C had a deleterious effect on the cell monolayers, and insufficient stain was incorporated to provide adequate contrast for plaque identification. Plaque development was rapid at 36°C, and well-defined spotted fever group plaques were visible 4 days after infection. Reduction in incubation temperature slowed plaque formation and, at 28°C, 13 days were required for formation of well-defined plaques. The initial experiment conducted with *R. tsutsugamushi* Gilliam indicated that 19 days were required for plaque formation when infected monolayers were incubated at 32°C. This lengthy incubation period suggested that higher temperatures, favoring more rapid plaque development, should be investigated. Figure 1 illustrates that plaques formed by scrub typhus organisms at 34°C were slightly larger and much more distinct than those observed at 32°C. Irradiated L cell monolayers did not remain viable after prolonged incubation at 36°C.

Cultural conditions optimal for plaque formation by *R. tsutsugamushi* and acceptable for *R. conorii* included a cell concentration of 5×10^5/ml and incubation at 34°C. These conditions were employed in all further studies.

Effect of length of incubation. The optimal time for plaque development and application of the neutral red overlay was determined for all three classical strains of scrub typhus rickettsiae and for representatives of the spotted fever and typhus group organisms. A large number of dishes containing monolayers of irradiated L cells were infected with appropriate dilutions of each of the rickettsial strains and were then

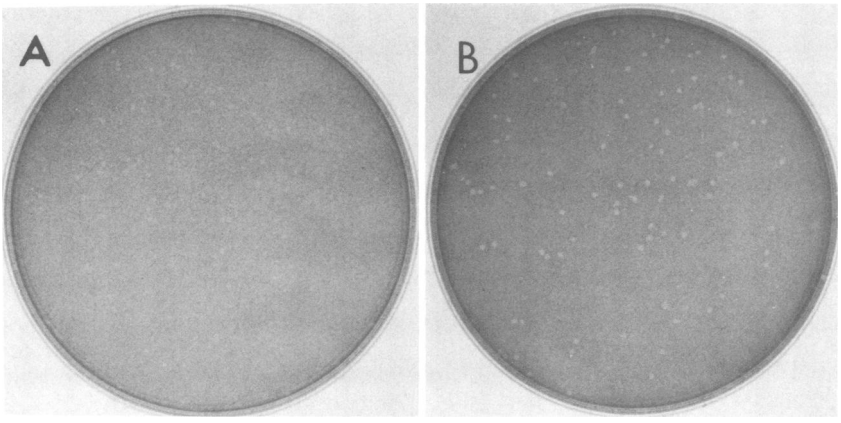

FIG. 1. *Effect of temperature on plaque formation by R. tsutsugamushi Gilliam. (A) Infected L cell monolayer incubated at 32°C for 19 days; (B) infected L cell monolayer incubated at 34°C for 16 days.*

overlaid and incubated at 34°C. The staining overlay was applied on successive days during the period when preliminary experiments had indicated that plaques were visible. The culture dishes were observed 24 h after application of the staining overlay to determine the presence and quality of plaque formation, as well as ease of enumeration. Table 2 indicates the first day that plaques were visible and also identifies the earliest day when plaque size and staining contrast were considered satisfactory for counting and plaque recovery. The scrub typhus group of rickettsiae required the longest incubation period, usually 19 days, although the Gilliam strain produced plaques in a noticeably shorter time than did the other two strains tested. The spotted fever group exhibited distinct, well-defined plaques after only 5 days of incubation. Plaques of the typhus group were small and indistinct after 12 days of incubation, but neither continued incubation nor application of an intermediate feeder overlay on day 7 improved plaque morphology or clarity of staining. Figure 2 illustrates typical plaque formation by each group of rickettsiae after the optimal length of incubation.

Quantitative comparison of plaque assay and mouse assay. The data in Table 3 suggest that the sensitivity of the two tests is similar. The plaque titer is similar to the MID_{50} and MLD_{50} values for strains Karp and Kato, which are lethal in small doses for this strain of mice. The Gilliam strain of *R. tsutsugamushi*, which is less virulent in ICR mice, evidences some disparity in titer between MLD_{50} and plaque-forming units, but the MID_{50} value is similar to the plaque titer.

Cloning of rickettsiae. Single plaques of scrub typhus rickettsiae were recovered with a sterile Pasteur pipette from monolayers evidencing only 1 to 10 plaques per dish and were triturated vigorously in tubes containing 0.3 ml of cold brain heart infusion broth. These suspensions were transferred to 35-mm dishes (35 by 10 mm, Lux, Microbiological Associates) containing monolayers of irradiated L cells at a concentration of 1.25×10^6 cells/dish, from which the growth medium had been removed. After adsorption for 1 h at room temperature, fresh growth medium was added, and the dishes were reincubated at 34°C. Monolayers were observed daily for signs of rickettsial infection (granulation, cytopathogenic effect, accumulation of detached cells in the medium), and a portion of cells was prepared for staining after 5 to 8 days of incubation. Slides were fixed in methanol for 1 min, rinsed, and stained with Giemsa stain (Harleco, Philadelphia, Pa.) for 5 min. When a majority of the cells observed were heavily infected, all cells were resuspended in growth medium with a sterile Teflon scraper, and the infected cell suspension was transferred to a microchamber assembly (Dupont Co., Instrument Products Division, Rock-

TABLE 2. *Length of incubation required for plaque formation by rickettsiae in irradiated L-929 cells*

Rickettsiae	Day staining overlay applied
R. tsutsugamushi Karp	17, 18, *19*,[a] 20
R. tsutsugamushi Gilliam	15, *16*, 17, 18
R. tsutsugamushi Kato	17, 18, *19*, 20
R. conorii	4, *5*, 6, 7, 8
R. rickettsii	4, *5*, 6, 7, 8
R. prowazekii	10, 11, *12*, 13
R. typhi	10, 11, *12*, 13

[a] Italic type indicates earliest day on which plaque development and staining contrast were satisfactory for enumeration and plaque recovery.

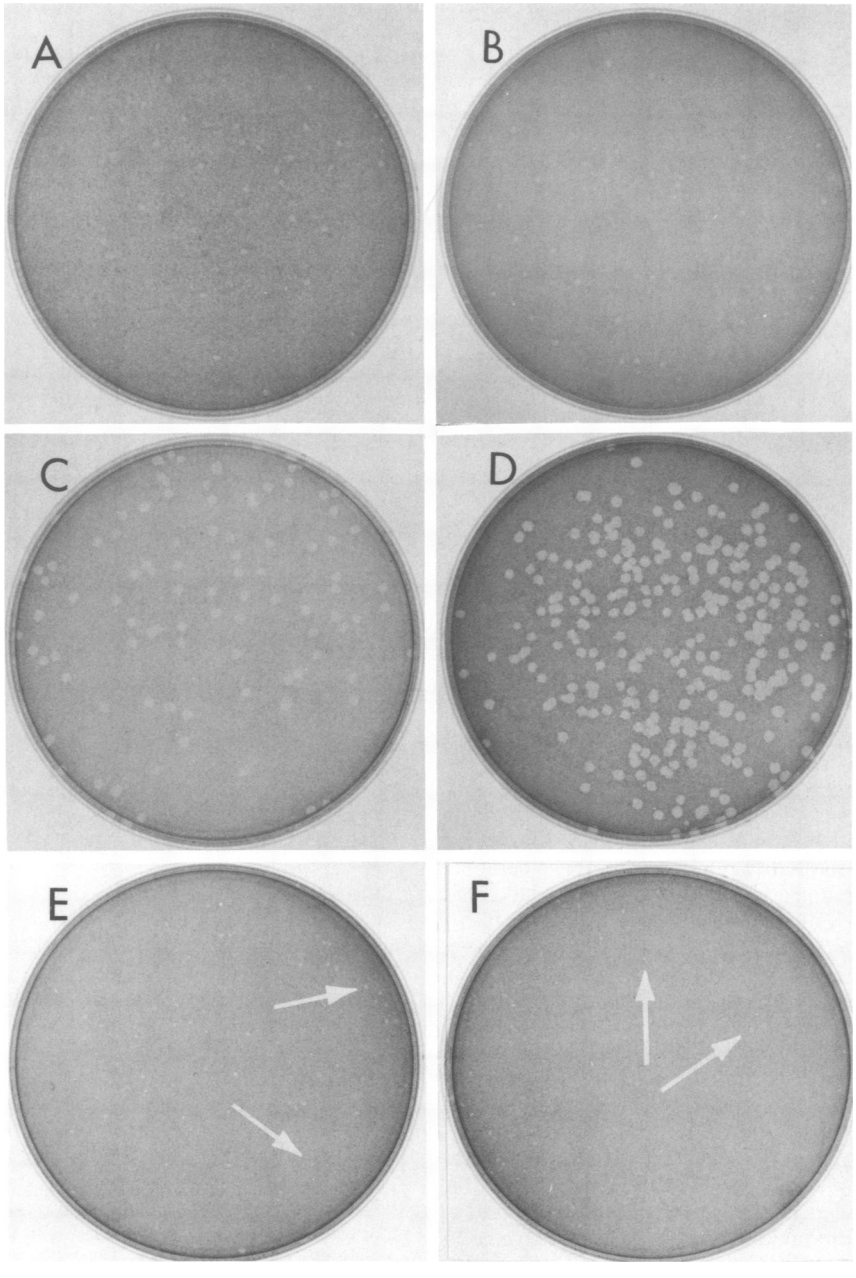

FIG. 2. *Plaque formation by scrub typhus group, spotted fever group, and typhus group rickettsiae in irradiated L-929 cells. (A) R. tsutsugamushi Karp; (B) R. tsutsugamushi Kato; (C) R. conorii; (D) R. rickettsii; (E) R. prowazekii, and (F) R. typhi. Arrows indicate representative plaques.*

ville, Md.) and blended three times at 45,000 rpm for 15 s. A portion of this suspension was immediately diluted in 10-fold increments and replaqued using the method described above, while the remainder of the suspension was frozen at −70°C. This procedure was repeated three times, with each plaque recovery made from monolayers containing only 1 to 10 plaques. The final yield of plaque-purified rickettsiae was propagated further in irradiated L

TABLE 3. *Comparison of the plaque titers of scrub typhus rickettsiae with the MLD_{50} and MID_{50} titers in ICR mice*

R. tsutsugamushi strain	Titer (log_{10} per ml)		
	MLD_{50}	MID_{50}	PFU[a]
Karp	7.3 (±0.3)[b]	7.3 (±0.3)	7.0 (±0.1)
Gilliam	7.5 (±0.5)	8.5 (±0.3)	8.3 (±0.1)
Kato	7.3 (±0.3)	7.3 (±0.3)	7.1 (±0.1)

[a] PFU, Plaque-forming units.
[b] Standard error of the mean.

cell monolayers to develop sufficient material for storage. This technique of plaque purification combined with terminal dilution was utilized to obtain clones of the three classical strains of scrub typhus.

DISCUSSION

We have clearly demonstrated the usefulness of an irradiated continuous cell line for plaque formation and cloning of scrub typhus rickettsiae. In addition, L-929 cells support large, well-defined plaque formation by representative members of the spotted fever group rickettsiae, but typhus group organisms produced small, indistinct plaques. The behavior of typhus group rickettsiae in irradiated L cells was interesting, because other investigators (2) have also indicated the failure of *R. prowazekii* to consistently form plaques in a mammalian cell line, although the organism is known to plaque reproducibly in chicken embryo cells (5, 12). The reciprocal relationship observed between L cell density and plaque diameter of *R. tsutsugamushi* and *R. conorii* has also been seen with chicken embryo cells infected with *R. typhi* or *R. rickettsii* (12).

Plaque assay of *R. tsutsugamushi* in chicken embryo cells (6) resulted in the formation of small, indistinct plaques by all three strains after 17 days of incubation. Irradiated, infected L cell monolayers required approximately the same incubation period for plaque development, but plaques were substantially more distinct. Previous studies comparing the sensitivity of the plaque assay in chicken embryo cells and the mouse assay (6) indicated that the plaque technique was considerably more sensitive than the MID_{50} procedure. However, the authors indicated, through quotation of a personal communication (B. Elisberg), that the strain of mice employed in animal testing or the physiological condition of the rickettsiae may have affected their results. Our studies, using irradiated L cells for plaque titrations and ICR mice for animal titrations, suggest that the two techniques are very similar in sensitivity for detecting viable scrub typhus rickettsiae.

This investigation is, to our knowledge, the first report of plaque selection to enhance homogeneity of populations of scrub typhus rickettsiae. Such procedures are a necessary prerequisite for antigenic characterization of these organisms and may increase the reliability of serological tests.

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