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Improved Plaque Assays for *Rickettsia prowazekii* in Vero76 Cells

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Typhus group rickettsiae, including *Rickettsia prowazekii* and *R. typhi*, produce visible plaques on primary chick embryo fibroblasts and low-passage mouse embryo fibroblasts but do not form reproducible plaques on continuous cell culture lines. We tested medium overlay modifications for plaque formation of typhus group rickettsiae on the continuous fibroblast cell line Vero76. A procedure involving primary overlay with medium at pH 6.8, which was followed 2 to 3 days later with secondary overlay at neutral pH containing 1 μg of emetine per ml and 20 μg of NaF per ml, resulted in visible plaques at 7 to 10 days postinfection. A single-step procedure involving overlay with medium containing 50 ng of dextran sulfate per ml also resulted in plaque formation within 8 days postinfection. These assays represent reproducible and inexpensive methods for evaluating the infectious titers of typhus group rickettsiae, cloning single plaque isolates, and testing the susceptibilities of rickettsiae to antibiotics.

The genus *Rickettsia* contains members of the typhus, spotted fever, and scrub typhus groups of organisms. These include arthropod-borne obligate intracellular parasites which are causative agents for debilitating and lethal vascular diseases in the human host, as well as many species which are nonpathogenic to humans (6, 19). Spotted fever group rickettsiae, including *R. rickettsii*, *R. conorii*, and *R. siberica*, can be readily enumerated and isolated by plaque formation on a variety of continuous cell lines (3, 11, 15, 20, 28). Typhus group and scrub typhus group organisms, however, form indistinct plaques on green monkey kidney (Vero) (3) and irradiated mouse fibroblast (L-929) cell lines (18). Another species, *R. canada*, that appears to occupy an intermediate phylogenetic position between typhus and spotted fever group rickettsiae (4, 21) also forms indistinct plaques on Vero cells (3).

There may be significant differences in host cell attachment, entry, and lysis between these groups (19, 30, 31). Spotted fever group rickettsiae demonstrate rapid cell-to-cell spread via actin-based intercellular motility (9, 27). This movement to culture medium and to uninfected cells seems to result in cytopathic effects in the absence of massive intracellular accumulation (23, 26, 33). Typhus group organisms, including *R. canada*, do not demonstrate this motility. As a result, *R. prowazekii* accumulates intracellularly during the primary round of chicken embryo fibroblast (CEF) infection and causes host cell lysis at 2 to 4 days postinfection (p.i.). Subsequent rounds of infection and proliferation result in asynchronous lysis of additional host cells (32, 33).

Typhus group organisms are capable of plaque formation on primary or low-passage cells. Rickettsia-infected CEFs (15, 29) or low-passage mouse embryo fibroblasts (MEFs) (8) overlaid with agarose-containing medium undergo consecutive rounds of lysis in a confined region of the monolayer, resulting in the formation of plaques. Typhus group plaques develop within 10 to 13 days of incubation at 32°C in CEFs and 7 to 11 days of incubation at 35°C in MEFs. Plaques develop more slowly and are typically one-half the diameter of the plaques of spotted fever group rickettsiae. The preparation of CEFs and MEFs is a time-consuming task that must be performed repeatedly in preparation for clonal isolation or evaluation of the titers of viable typhus group rickettsiae. Modification of the host-rickettsia interaction to allow for plaque assays of typhus group rickettsiae on continuous cell lines would eliminate the need for repetitive primary culture of host cell monolayers or the need for calibrated gamma irradiation sources to make continuous cell lines susceptible to plaque formation.

Vero cells are an African green monkey kidney fibroblast line commonly used for propagation (1, 7) and plaque assay (3, 11, 28) of spotted fever group rickettsiae. We analyzed treatments that can alter Vero cell metabolism and that might augment rickettsial growth rates and contribute to plaque formation. NaF has been used to enhance the growth of *R. typhi* isolates in embryonated eggs (5); NaF blocks glycolysis through inhibition of enolase phosphorylation (22). Rickettsiae do not possess the capacity for glycolytic conversion of glucose, but convert glutamate, pyruvate, and glutamic acid via the tricarboxylic acid cycle to CO_2 and NADH (16). Emetine, an inhibitor of eukaryotic protein synthesis (10), was also tested for its influence on plaque formation. Reduction of the overlay medium pH to 6.8 from a normal pH of 7.5 has been used to prolong monolayer survival and thereby allow *Coxiella burnetii* plaque formation on CEF monolayers (29). This pH reduction may inhibit oxidative phosphorylation, increasing the pools of metabolites that can be taken up by rickettsiae. In addition to these modifications, we tested *R. prowazekii* plaque formation on Vero76 cells in the presence of dextran sulfate. Both of these approaches resulted in improved plaque assay methods for typhus group rickettsiae.

MATERIALS AND METHODS

Strain history and passage in Vero76 cells. *R. prowazekii* E (human, 6 to 8 guinea pig passages [GPs], 272 egg passages [EPs], cloned in primary chick embryo culture [TCs], 2 EPs, and 8 to 11 Vero76 cell passages [VCs]) was treated at the seventh Vero cell passage with mycoplasma removal agent (ICN) for 7 days. *R. prowazekii* Breinl (human, 162 EPs, 1 TC, 3 EPs, and 2 VCs), *R. canada* McKiel (tick, 8 EPs, 1 TC, 2 EPs, and 3 VCs), and *R. rickettsii* R (tick, 53 EPs, 1 TC, 4 EPs, and 2 VCs) inocula were also used. An inoculum from infected Vero76 cells was prepared in brain heart infusion (BHI; Difco, Detroit, Mich.) and was stored at −70°C until use. Vero76 African green monkey kidney fibro-

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blast cells (ATCC CRL 1587) were maintained as recommended by the supplier (American Type Culture Collection, Rockville, Md.).

Immunofluorescent focus-forming assay. Vero76 cells were trypsinized from passage flasks, suspended at 2×10^5 cells per ml in RPMI (Life Technologies, Gaithersburg, Md.) containing 10% fetal calf serum (FCS; Life Technologies), and plated at 1 ml per well in 24-well trays (Corning, Inc., Corning, N.Y.). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. The medium was aspirated from the wells, and dilutions of rickettsial suspensions were added in 20 to 50 μl of BHI per well. The trays were placed at 37°C in a 5% CO_2 atmosphere for 30 min before the addition of 1 ml of medium 199 (Life Technologies) containing 2% FCS to each well. The trays were incubated at 34°C in a 5% CO_2 atmosphere for 3 or 4 days. The medium was aspirated, and the cells were fixed for 10 min in absolute methanol and air dried. The cells were washed twice with 66 mM NaCl–58 mM KH_2PO_4–8.4 mM Na_2HPO_4 (pH 7.2) (phosphate-buffered saline [PBS]), reacted for 1 h at 37°C with guinea pig or rabbit anti-*R. prowazekii* serum, washed three times with PBS, and reacted for 1 h at 37°C with fluorescein isothiocyanate-conjugated rabbit anti-guinea pig or goat anti-rabbit serum. The wells were again washed three times with PBS and overlaid with 200 μl of PBS, and fluorescent cell foci were enumerated with an inverted microscope (Fluovert FU, Leitz, Germany).

Plaque assay on Vero76 cell monolayers. (i) NaF-emetine method. Vero76 cells were trypsinized from passage flasks, suspended at 2.5×10^5 cells per ml in RPMI containing 10% FCS, and plated at 2.0 ml per well in six-well trays (Corning). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 24 h. Medium was aspirated from the wells, and dilutions of rickettsial suspensions were added in 200 μl of BHI per well. The trays were rocked for 30 min at room temperature before the addition of the primary overlay. The primary overlay consisted of medium 199 containing 0.88 g of $NaHCO_3$ per liter, 10% tryptose-phosphate broth (TPB; Difco), 5% FCS, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 6.8; Calbiochem, San Diego, Calif.). This mixture was equilibrated to 56°C and mixed with 5% SeaKem agarose (FMC, Rockland, Maine), which was autoclaved at 15 lb/in^2 for 20 min and cooled to 56°C before addition of 10 ml/100 ml of overlay medium. The overlay medium mixture was then cooled to 42°C before the addition of 2 ml to each well. The trays were incubated at 34°C in a 5% CO_2 atmosphere for 2 or 3 days. Secondary overlay consisted of L15 medium (Life Technologies) containing 2.2 g of $NaHCO_3$ per liter, 10% TPB, 5% FCS, 2 μg of emetine per ml, and 40 μg of NaF (Sigma Chemical Co., St. Louis, Mo.) per ml equilibrated to 56°C mixed with 10 ml of 5% SeaKem agarose per 100 ml of overlay medium, cooled to 42°C as described above, and added at 2 ml per well. The trays were incubated as described above for an additional 7 to 8 days. The overlays were removed with a spatula, and monolayers were fixed and stained for 10 min with phosphate-buffered formalin (3.7 to 4.0% formaldehyde, 45.7 mM Na_2HPO_4, and 28.9 mM NaH_2PO_4)–0.2% crystal violet. The stain was removed by aspiration, and the monolayers were washed several times in H_2O and air-dried before plaque enumeration.

(ii) Dextran sulfate method. Vero76 cells were plated, aspirated, and inoculated as described above before the addition of the overlay. The single overlay consisted of L15 medium containing 2.2 g of $NaHCO_3$ per liter, 10% TPB, 5% FCS, and 50 ng of dextran sulfate (sodium salt; molecular weight, 500,000; Sigma) per ml equilibrated to 56°C mixed with 10 ml of 5% SeaKem agarose per 100 ml of overlay medium, cooled to 42°C as described above, and added at 5 ml per well. The trays were incubated as described above for 7 to 8 days, and the monolayers were stained as described above.

Growth comparison in liquid media. Confluent Vero76 cells were inoculated with *R. prowazekii* E in 25-cm^2 flasks (Corning). The cells were fed medium 199 (pH 6.8) or standard L15 medium as described above, without agarose, and were incubated at 34°C for 2 days. At 2 days p.i., the flasks from each group were harvested, sonicated, and frozen in aliquots. An equal volume of NaF-emetine-L15 medium was then added to the remaining flasks containing medium 199 (pH 6.8), and an equal volume of L15 medium was added to the standard control flasks containing L15 medium without draining the initial medium. At 6, 8, 9, and 10 days p.i., sonicates of additional flasks were prepared as described above. The titers of the sonicates were then determined by the NaF-emetine overlay procedure.

RESULTS

Typhus group plaque formation under standard conditions. Monolayers inoculated with *R. prowazekii* E and Breinl or *R. canada* McKiel were compared at 7 days p.i. with monolayers inoculated with *R. rickettsii* R by using standard L15 overlay medium. The latter spotted fever group organism produced large (diameter, >4 mm) plaques by 7 days, while *R. prowazekii* Breinl and *R. canada* produced small areas of cytopathic change (diameter, < 0.1 mm) that were observed by microscopy at 10 days p.i. (data not shown). The areas of cytopathic change for *R. prowazekii* Breinl and *R. canada* were not visible after fixation and staining.

Screening of host cell metabolic inhibitors. Since only microscopic and nonreproducible plaque formations were obtained under standard conditions, we tested NaF and emetine separately and in combination to determine if these inhibitors of host cell metabolism could enhance plaque formation by *R. prowazekii* and *R. canada*. Emetine alone in L15 overlay medium was cytotoxic to infected or uninfected monolayers by 5 days p.i. NaF at concentrations of 10 to 100 μg/ml in the L15 overlay medium was not cytotoxic, but no increase in *R. prowazekii* E plaque size was detected at 10 days p.i. in stained monolayers. The combination of 1 μg of emetine per ml and 20 μg of NaF per ml added directly postadsorption also caused extensive damage to the monolayers by 5 days p.i. To attempt prolonged survival of the monolayer and thus a prolonged period for rickettsial infection, L15 overlay medium without additions was applied after rickettsial adsorption, and the cells were incubated at 34°C for 3 days. A secondary overlay of L15 overlay medium of equal volume to the first overlay containing 2 μg of emetine per ml and 40 μg of NaF per ml (final concentrations, 1 and 20 μg/ml, respectively) was then applied. At 10 days p.i., *R. prowazekii* E and Breinl and *R. canada* formed plaques of 0.1 to 0.15 mm in diameter in the monolayers, but damage to the monolayers caused by the NaF-emetine combination was still extensive. Delayed addition of the inhibitors therefore improved plaque formation, but the plaque size was small and detection on a depleted monolayer was unreliable.

Effect of reduced pH on plaque formation. A previous report indicated that medium 199 overlay medium at pH 6.8 permits CEF monolayer survival for up to 18 days p.i., compared with degradation after 13 days in medium 199 at pH 7.5 (29). Overlay with medium 199 at pH 6.8 directly after rickettsial adsorption did not result in *R. prowazekii* or *R. canada* plaque formation on Vero76 cells by 10 days p.i. (data not shown). However, when medium 199 at pH 6.8 was used postadsorption as a primary overlay and was followed at 3 days p.i. by the addition of L15 overlay medium containing NaF and emetine, we observed visible plaques of each of the rickettsiae examined (Fig. 1a to c). The plaque size at 10 days p.i. was ≥0.5 mm in diameter for both *R. prowazekii* and *R. canada* (Table 1, experiment 1). The cell monolayers remained largely intact under these conditions.

Time dependence of plaque development. Figure 2 demonstrates plaque development in a monolayer infected with *R. prowazekii* E. Inoculated cells received a primary overlay of medium 199 at pH 6.8; this was followed at 3 days p.i. by L15 medium containing NaF and emetine. Small foci of cell destruction were visible at 6 days p.i. (Fig. 2a), which increased in size from 7 to 9 days p.i. (Fig. 2b and c) with an accompanying accumulation of free rickettsial particles in the plaque center. Deterioration of the monolayer limited the period available for plaque development to 9 to 10 days (compare Fig. 2a to Fig. 2c and d). Average plaque size measurements at 7, 8, and 9 days p.i. for *R. prowazekii* E are presented in Table 1. No advantage was gained by maintaining the cultures past 8 days p.i., since the plaque size did not increase significantly between 8 and 9 days p.i. At 6 days p.i., foci of dead or damaged cells were four to five cells in diameter (Fig. 2a). At 7 days p.i., these plaque areas expanded to 0.21 mm in diameter and were 0.35 mm in diameter by 8 or 9 days p.i.

Comparison of NaF-emetine overlay and focus-forming assay. To assess the plaquing efficiency of the NaF-emetine overlay method, we did separate NaF-emetine overlay and focus-forming assays with infected monolayers. At 3 days p.i., Vero76 cells occupied by replicating *R. prowazekii* cells were detected in the focus-forming assay as a cytoplasmic immunofluores-

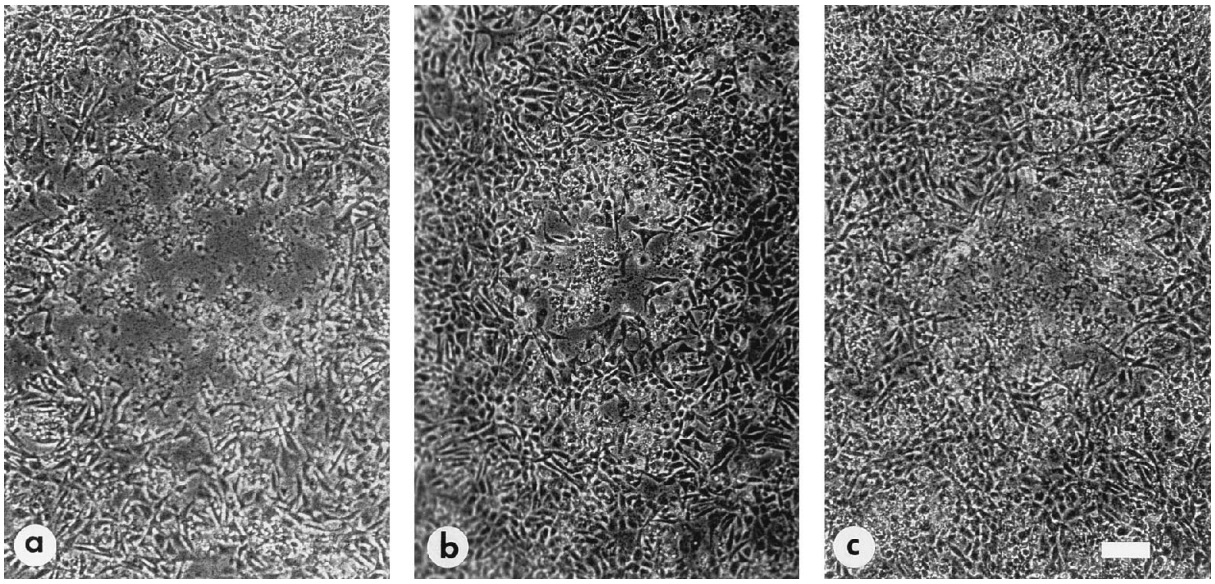

FIG. 1. *R. canada* McKiel (a), *R. prowazekii* Breinl (b), and *R. prowazekii* E (c) plaques at 9 days p.i. on Vero76 cells. Cell monolayers were overlaid with medium 199 at pH 6.8, 10% TPB, 5% FCS, and 0.5% agarose medium postadsorption, and the trays were incubated at 34°C. At 3 days p.i., the wells were overlaid with L15 medium, 10% TPB, 5% FCS, 40 μg of NaF per ml, 2 μg of emetine per ml, and 0.5% agarose medium, and the trays were incubated for 6 days at 34°C. Bar, 0.1 mm.

cence that contrasted with neighboring, uninfected cells (data not shown). The total numbers of *R. prowazekii* E cells in the inocula were quantified by acridine orange staining. Assays of three separate rickettsial inocula showed that different preparations differed in their percentages of viable rickettsiae (Table 2). Inoculum T79 contained approximately 1 PFU per 13 rickettsial particles, whereas inoculum T23 showed only 1 PFU per 140 rickettsial particles. More viable particles were detected in each inoculum by the NaF-emetine method than by the focus-forming assay.

Effects of NaF-emetine on titer in liquid media. The standard and two-step overlay media, minus agarose, were compared for their effects on the infectious titer of rickettsiae in free culture (Fig. 3). Incubation of *R. prowazekii* E-infected Vero76 cells in L15 medium results in a titer increase from 10^2 PFU/ml at day 2 to 10^8 PFU/ml at day 10. In cultures containing medium 199 at pH 6.8 and L15 with NaF-emetine, the titer increased from 1×10^5 PFU/ml on day 2 to 1×10^8 PFU/ml on day 5 and then declined to 3×10^5 PFU/ml on day 10. These data indicate that incubation in liquid medium equivalent to the two-step overlay medium does not increase the yield of rickettsiae but results in an early peak in the numbers of viable rickettsiae that is not sustained at 8 to 10 days p.i.

Dextran sulfate enhancement of plaque formation. The ability of charged dextran derivatives to enhance plaque formation was also examined. Dextran, DEAE-dextran, and dextran sulfate were added to L15 overlay medium at 50 ng/ml; the mixtures were then applied to monolayers directly after adsorption of the *R. prowazekii* E inoculum. Only those monolayers receiving dextran sulfate showed readily detectable plaques. Plaque development was adequate for visualization after monolayer fixation and staining at 8 days p.i., but incubation could be continued to 12 days p.i. prior to enumeration. Plaques were larger but more diffuse than those formed by the NaF-emetine procedure. Shown in Fig. 4 are formalin-fixed, crystal violet-stained monolayers of Vero76 cells, which were used to compare the NaF-emetine procedure (Fig. 4B) and the dextran sulfate procedure (Fig. 4D). Cells inoculated with *R. prowazekii* E and overlayed by the two methods displayed the differences in plaque morphologies consistently observed by the two methods. Control wells showed the absence of plaque formation if NaF and emetine (Fig. 4A) or dextran sulfate (Fig. 4C) was not included in the overlay medium.

TABLE 1. Comparison of plaque sizes of *R. prowazekii* and *R. canada* isolates with emetine-NaF overlay medium

Expt	Inoculum	Day p.i.	Diam (mm)[a]
1	*R. prowazekii* E	10	0.40 ± 0.07
	R. prowazekii Breinl	10	0.47 ± 0.07
	R. canada McKiel	10	0.50 ± 0.10
2	*R. prowazekii* E	7	0.21 ± 0.06
	R. prowazekii E	8	0.35 ± 0.06
	R. prowazekii E	9	0.35 ± 0.06

[a] Values are means ± standard deviations.

DISCUSSION

Successful titration of *R. prowazekii* plaques on CEFs requires 10 days of incubation at 34°C. Plaques are approximately 1 mm in diameter and indistinct (15). Using conventional overlay media, we were unable to reproducibly detect *R. prowazekii* plaques on Vero76 cells, consistent with the findings of other investigators (3). Although this species replicates in Vero76 cells, undefined aspects of this host cell system appear to block or retard the repeated rounds of entry, multiplication, and lysis necessary for plaque development. It was noted during early studies of rickettsial cultivation that low levels of metabolic activity in the host cell favored rickettsial growth (5, 34). The NaF-emetine modification of overlay conditions may metabolically favor productive infection of the initial host cell and survival of uninfected cells during additional rounds of infection.

The dextran sulfate method may enhance *R. prowazekii* plaque formation on Vero76 cells by increasing the rate of reinfection after the initial burst. In contrast to observations in

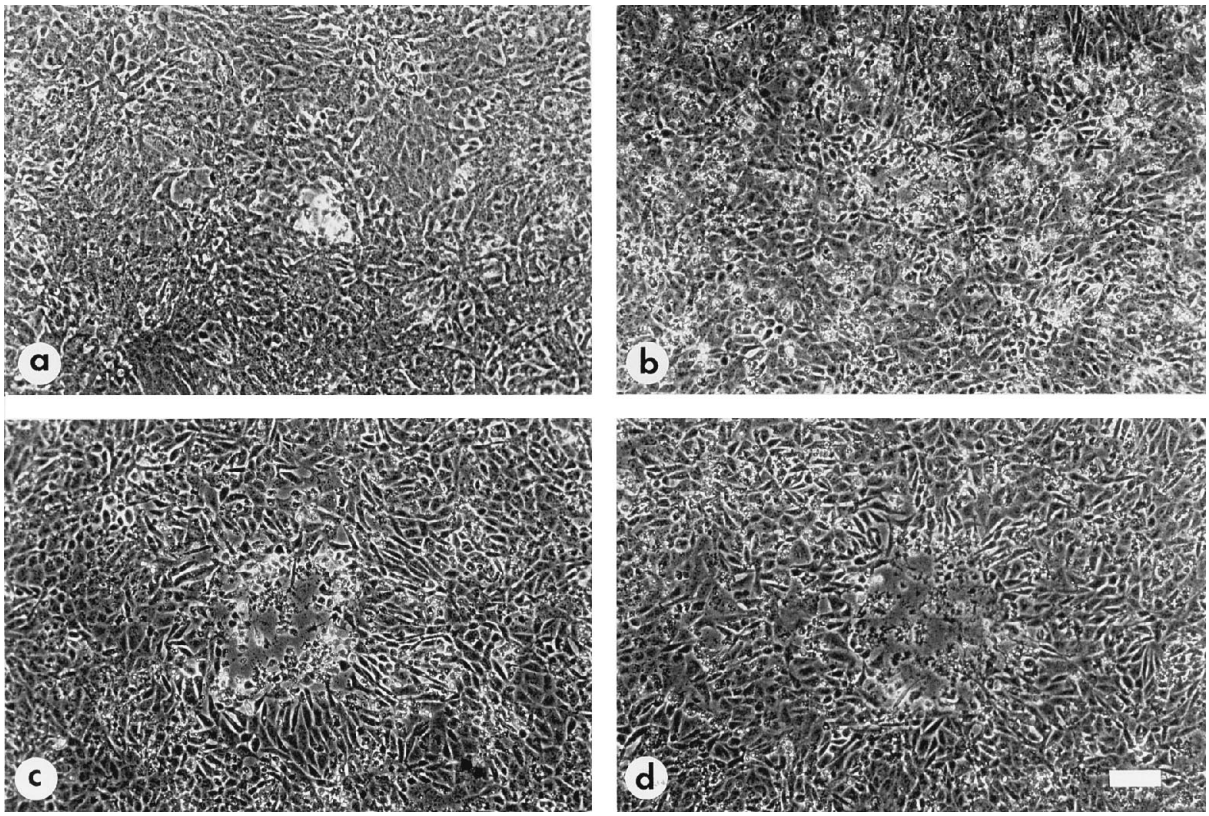

FIG. 2. Development of *R. prowazekii* E plaques on Vero76 cells. Cell monolayers were treated as described in the legend to Fig. 1, except that a secondary overlay was applied at 2 days p.i. (a) 6 days p.i.; (b) 7 days p.i.; (c) 8 days p.i.; (d) 9 days p.i. Bar, 0.1 mm.

virus plaque formation studies (24, 25) and bacterial adhesion studies (2, 12–14, 17), dextran sulfate apparently enhanced rather than inhibited *R. prowazekii* infection. The mechanism of this enhancement and its significance to host cell adhesion and invasion by rickettsiae require further study.

We found that the titer of viable *R. prowazekii* cells determined by the NaF-emetine overlay method is higher than that determined by a parallel focus-forming assay. The data in Table 2 indicate that *R. prowazekii* inocula show higher percentages of rickettsiae that are able to generate plaques in 10 days compared with the percentages generating a distinct infected cell in 3 to 4 days. Wisseman et al. (33) noted that *R. prowazekii* cells harvested from an infected CEF monolayer during the logarithmic phase of growth were capable of reinitiating the logarithmic phase of growth with little or no delay upon inoculation on new host cells. When harvested during the stationary or late phase, rickettsiae showed a lag period before initiating a new round of logarithmic-phase growth. This lag period was estimated to be 7.5 h (33). The status of the rickettsiae at the time of harvest, when they were reinoculated onto a new monolayer, may influence the number of progeny at 4 days p.i. A larger percentage of the population, however, may be capable of reaching the logarithmic phase of growth and thereby of producing a plaque during the 8- to 10-day period of the plaque assay. Part of this enhancement of viable particle detection may be due to the initial pH 6.8 overlay, since this

TABLE 2. Vero76 cell limits of infectivity with *R. prowazekii* E

Inoculum R for *R. prowazekii*[a]		PFU[b]	R/PFU	FFU[c]	R/FFU
T23	2.8×10^9 (3)[d]	2.0×10^7 (2)	140	1.1×10^7 (4)	255
T79	6.0×10^8 (2)	4.5×10^7 (14)	13	9.2×10^6 (1)	65
T80	8.9×10^8 (2)	3.04×10^7 (10)	29	7.8×10^6 (3)	114

[a] Direct rickettsial count by acridine orange method. R, titer per milliliter of seed material.
[b] PFU by emetine-NaF overlay method.
[c] FFU, focus-forming units by immunofluorescence method.
[d] Values in parentheses are the number of observations.

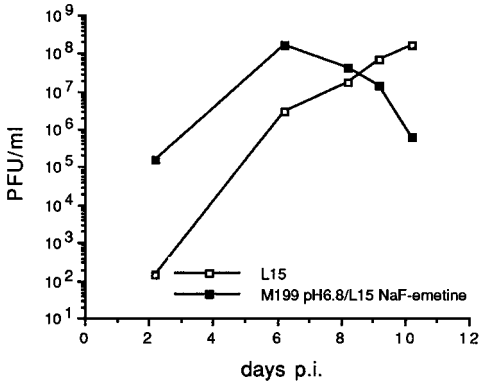

FIG. 3. Recovery of *R. prowazekii* E PFU from infected Vero76 monolayers. Inoculated cells were fed, harvested, and assayed for PFU as described in Materials and Methods. L15, standard L15 medium at 0 and 2 days p.i. M199 pH 6.8/L15 NaF-emetine, medium 199 adjusted to pH 6.8 with HEPES buffer at 0 days p.i. and L15 medium containing 2 μg of emetine per ml and 40 μg of NaF per ml at 2 days p.i.

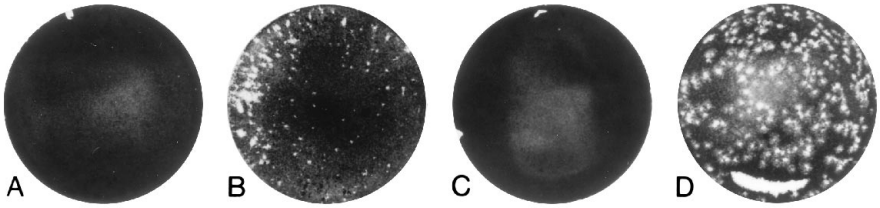

FIG. 4. Comparison of *R. prowazekii* E plaque morphology on Vero76 cells. Inoculated cells were overlaid, incubated, and stained with crystal violet at 8 days p.i. as described in Materials and Methods. (A) Medium 199 (pH 6.8) primary overlay and L15 medium secondary overlay without NaF or emetine at 2 days p.i. (B) Medium 199 (pH 6.8) primary overlay and L15 medium secondary overlay containing NaF and emetine at 2 days p.i. (C) L15 medium overlay without dextran sulfate. (D) L15 medium overlay with 50 ng of dextran sulfate per ml.

treatment in open cultures appears to enhance initial uptake or intracellular survival (Fig. 3).

Rickettsiae are isolated from arthropod and vertebrate tissues by inoculation of mice, guinea pigs, or a variety of cultured cells. It may be possible to isolate a much wider range of rickettsiae that do not produce infection in animals or standard cell culture systems by using treatments such as those described here to modify the rickettsia-host cell interaction.

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