Plaque Assay of Rickettsiae in a Mammalian Cell Line

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Clear-cut and repeatable plaque assays were obtained for three rickettsiae of the spotted fever group (Rickettsia rickettsii, R. conorii, and R. montana) in Vero cells in a manner similar to that for arboviruses. In addition, three typhus group agents (R. typhi, R. canadensis, R. prowazeki) induced plaques in these cells. In preliminary tests Coxiella burnetii (Nine Mile strain) failed to produce plaques. Comparable results were obtained in plastic flasks and plastic culture trays incubated in ambient air with or without addition of N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer. Larger and more well defined R. rickettsii plaques were produced when cultures were overlaid with Leibovitz (L15) medium than with either medium 199 or Eagle medium. Phosphate-buffered saline containing bovine plasma albumin (fraction V), in contrast to brain heart infusion broth, was a diluent for preparing inocula consistently permitted development of larger and more numerous plaques with three agents: R. rickettsii, R. conorii, and R. montana. When R. rickettsii and R. typhi were assayed in parallel in primary chicken embryo cultures and Vero cells, comparable results were obtained, but with R. canadensis results in Vero cells were superior. In contrast, R. prowazeki produced inconsistent results in Vero cells.

Methods for plaque assays of rickettsiae in primary monolayer cultures of chicken embryo cells were described by Kordova (2), McDade et al. (3), Weinberg et al. (4), and Wike et al. (6). Weinberg also reported plaque production by Rickettsia rickettsii in monolayer cultures of a monkey-kidney cell line (Vero cells). To our knowledge, no further investigations of rickettsial plaquing in established cell lines have been reported.

We describe here improved methods for plaquing six pathogenic rickettsiae in Vero cell monolayers, and report effects of various diluents, buffers, incubation temperatures, and overlay media on plaque size and titer.

MATERIALS AND METHODS

Rickettsial seeds. Sources of the organisms tested were infected yolk sacs of embryonated egg passage. Two had undergone prior passages in guinea pigs.

Agents tested were R. rickettsii "R" strain (53EP), R. conorii Simko strain (13EP), R. montana M/S-6B strain (20EP), R. typhi Wilmington strain (112EP/15GP/1EP), R. prowazeki ZRS strain (15GP/2EP) and (4EP), R. prowazeki Breini strain (15GP/2EP), R. canadensis (6EP), and Coxiella burnetii Nine Mile strain Phase I (90GP/2EP).

Cells. The Vero cell line derived from African green monkey kidney by Yasamura and Kawakata (7) was obtained in its 122nd passage from the American Type Culture Collection, Rockville, Md. It was used in its 135th to 145th passage.

Culture vessels. Plaque assay culture vessels were 30-ml plastic flasks (Falcon Plastics, Los Angeles, Calif.), or plastic trays (model FB16-24TC) containing 24 wells with adhesive plastic-sheet tray covers from Linbro Chemical Co., New Haven, Conn.

Media and additives. All cell culture media were obtained in powdered form from Grand Island Biological Co., Grand Island, N.Y., and prepared according to manufacturer’s instructions. Both growth and overlay media were used at pH 7.4.

Stock cell cultures were grown in medium 199 containing Earle salts to which had been added 5% fetal bovine serum and 10% tryptose phosphate broth (Difco, Detroit, Mich.) plus 100 units of sodium penicillin G and 100 µg of streptomycin sulfate per ml. The serum, obtained from Microbiological Associates, Inc., Bethesda, Md., was inactivated for 1 h at 56 C.

Primary overlay media were medium 199, Leibovitz medium (L15), and Eagle minimum essential medium (MEM) containing 5% fetal bovine serum, 10% tryptose phosphate broth, and 1% agarose (Seakem, Marine Colloids, Inc., Rockland, Me.). Secondary overlay media differed from these only by the addition of 1 ml of 1% neutral red dye per 100 ml of overlay medium (1:10,000 final concentration neutral red). Effects of the addition of N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid buffer (HEPES; Calbiochem, San Diego, Calif.) to overlay media was tested.
in Vero cell cultures in flasks and trays. Parallel titrations of *R. rickettsii* were performed in each type vessel, with and without 10 mM HEPES buffer, in each of the overlay media. Cultures were incubated in ambient air at 32 C for 7 days before receiving the second overlay.

Dilutions of rickettsial seeds were made in either 0.15 M phosphate-buffered saline (PBS) containing 0.75% bovine plasma albumin (BPA) fraction V (Armour Pharmaceutical Co., Kankakee, Ill.) or brain heart infusion broth (BHI, Difco) as prepared by Wike et al. (5). Both were used at pH 7.0 and at 0 C.

**Cell cultivation.** Methods used for maintenance of Vero cell stock cultures and preparation of cultures for plaque assays were as described by Earley et al. (1), but modified for use of 30-ml plastic flasks or 24-well plastic trays. Flasks were plated with 5 ml of a cell suspension containing 5 x 10⁷ cells per ml; wells of trays received 1 ml of this suspension. Monolayers were used for plaque assay after 48 h of incubation at 37 C.

**Plaques procedures.** Inoculations of plaque assay cultures were performed with a precision pipetting system (Medical Laboratory Automation, Inc., Mount Vernon, N.Y.). Flasks from which medium had been decanted were inoculated with 0.1 ml, and wells of trays received 0.65 ml of inoculum. Inocula were allowed to adsorb for 1 h at room temperature on a mechanical rocking platform. Then, 5 ml of the appropriate overlay medium equilibrated to 44 C was applied to monolayers in flasks and 1 ml was applied to those in tray wells. After incubation at a temperature and time found appropriate for the particular rickettsia, 2 ml per flask or 0.5 ml per tray well of secondary overlay was applied in subdued room light. Vessels were covered with aluminum foil and reincubated. Plaques, often visible after 4 h, were usually read after overnight incubation. Incubation temperatures after primary overlay were 27, 32, 35, or 37 C. Secondary overlays were usually applied after 5 or 6 days of incubation, but various intervals up to 20 days were sometimes used depending on the development of satisfactory plaques.

Comparative titrations of four rickettsiae were done in Vero cell monolayers and in primary cultures of chicken embryo (PCE) as described by Wike et al. (6).

**RESULTS**

**Effects of incubation time and temperature.** In all cases, the sizes of plaques in Vero cell cultures reflected time and temperature of incubation (Fig. 1). Spotted fever group organisms caused plaques 1.0 to 2.0 mm in diameter within the 32 to 35 C range, providing that the secondary overlay was applied at a satisfactory time. Distinct plaques were produced by *R. rickettsii* when stained with secondary overlay after 5 days of incubation at 37 C (1.0 mm), by *R. montana* after 6 days at 35 C (1.5 mm), and by *R. conorii* after 6 days at 35 C (2 mm). *R. prowazekii* ZRS strain (4EP) repeatedly produced well-defined plaques of 1.0 mm diameter after 6 to 8 days at 37 C. However, another seed of ZRS strain (15GP/2EP) as well as of the Breinl strain (15GP/2EP) only occasionally formed plaques; these were less than 1 mm in diameter, poorly defined, and visible only after 20 days of incubation at 32 C. *R. canadensis* plaques, which varied from 1 to 2 mm in diameter, were not well defined until 12 days after incubation at 37 C (6 days after secondary overlay had been applied). *R. typhi* formed plaques of 1.0-mm diameter only after 20 days of incubation at 27 C. *C. burnetii* (Nine Mile strain), tested at 32, 35, and 37 C for 14 days and at 27 C for 20 days, did not form plaques.

**Effects of overlay media and modifications.** In parallel assays of *R. rickettsii* performed in flasks and trays incubated at 32 C for 7 days in ambient atmosphere, only moderately well-defined plaques, about 1.0 mm or less in diameter, were produced in cultures that received medium 199 or MEM overlay medium (Fig. 2). Cultures that received L15 overlay medium produced well-defined plaques 2.0 mm in diameter. HEPES buffer had no effect on plaque development.

**Effects of diluents on plaque size and titer.** Parallel plaque assays were performed in Vero cell cultures to compare PBS + BPA with BHI as a vehicle for dilution of seeds of *R. rickettsii*, *R. montana*, and *R. conorii*. Various incubation times and temperatures were used. Plaques of *R. rickettsii* obtained through use of PBS + BPA were larger (1.5 to 2.0 mm diameter) than those resulting from use of BHI (1.0 to 1.5 mm diameter) (Table 1). Titers of *R. rickettsii* seeds in which PBS + BPA was used were equal to or higher than (up to 0.53 dex) those obtained with BHI. Results with *R. montana* were similar. *R. conorii* plaques were 2.0 mm in diameter regardless of diluent used; the titer was only 0.20 dex higher when diluted in PBS + BPA. Although plaque values did not differ greatly, titers were consistently higher and plaques larger when PBS + BPA was used as diluent.

**Rickettsial titers compared in Vero and PCE cultures.** Parallel plaque assays were performed in Vero and PCE cultures with identical inocula of *R. rickettsii*, *R. typhi*, *R. canadensis*, and *R. prowazekii* (15GP/2EP). All tests were performed with two or four replicate culture vessels for each dilution tested. Results of four such tests with *R. rickettsii*, performed at different times, yielded nearly identical plaque values in both systems. Plaque values with *R. typhi* also were nearly equal in both systems (Table 2).

Comparative tests with the other two agents
FIG. 1. Plaque production in Vero cells by certain rickettsiae: (1) *Rickettsia typhi* stained after 20 days of incubation at 27°C; (2) *R. montana* after 8 days at 37°C; (3) *R. conori* after 8 days at 37°C; (4) *R. canadensis* after 12 days at 35°C.

FIG. 2. *Rickettsia rickettsii* plaques in Vero cells. Effects of vessels (above, flasks; below, same in trays); different overlay media; and addition of 10 mM HEPES buffer. (1) Medium 199; (1a) same, with HEPES buffer; (2) Leibovitz medium; (2a) same, with HEPES; (3) Eagle minimum essential medium; (3a) same, with HEPES. All incubated for 7 days at 32°C.
revealed a difference in susceptibility between Vero and PCE cells. *R. canada* produced plaques in Vero cell cultures but produced either indistinct and noncountable plaques or no plaques at all in PCE cultures. In contrast, *R. prowazekii* (15GP/2EP) produced clear, countable plaques in PCE cultures but not in Vero cells.

**DISCUSSION**

We have shown plaque production with six rickettsial agents of the spotted fever and typhus groups in an established mammalian cell line (Vero) widely used, especially in arbovirus studies. Previously, *R. rickettsii* had been plaqued in this line (Weinberg et al. [4]), but details of the methods used were not given. Except for omission of antibiotics from overlay medium, our Vero cell methods are standard virologic plaquing techniques. Agents of the spotted fever group produced reproducible plaque assay titers and were comparable to those in PCE cultures. Certain typhus group

| Table 1. Plaque production in Vero cells by certain rickettsiae: effects of two diluents on plaque number and size |
|---|---|---|---|
| **Seed** | **BHI**<sup>a</sup> | **PBS + BPA**<sup>a</sup> |
| | **PFU** | **Size**<sup>b</sup> | **PFU** | **Size**<sup>b</sup> |
| *R. rickettsii* (test 1) | 7.7 | 1.5 | 8.0 | 2.0 |
| *R. rickettsii* (test 2) | 8.4 | 1.0 | 8.9 | 2.0 |
| *R. rickettsii* (test 3) | 6.9 | 1.0 | 6.9 | 1.5 |
| *R. conori* (test 1) | 5.3 | 2.0 | 5.5 | 2.0 |
| *R. montana* (test 1) | 8.2 | <1.0 | 8.7 | 1.0 |

<sup>a</sup>Brain heart infusion broth (Difco).

<sup>b</sup>Phosphate-buffered saline (0.15 M) with 0.75% bovine plasma albumin (Fraction V, Armour Co.).

<sup>c</sup>Log<sub>10</sub> plaque-forming units per milliliter.

<sup>d</sup>Average plaque diameter in millimeters.

| Table 2. Plaque production in Vero cells by certain rickettsiae: comparison of titers between Vero cells and primary chick embryo (PCE) cell cultures receiving identical yolk sac inocula |
|---|---|---|---|
| **Seed** | **Vero**<sup>a</sup> (PFU) | **PCE**<sup>a</sup> (PFU) |
| *R. rickettsii* (test 1) | 7.9 | 7.9 |
| *R. rickettsii* (test 2) | 7.9 | 7.7 |
| *R. rickettsii* (test 3) | 7.3 | 7.8 |
| *R. rickettsii* (test 4) | 8.4 | 8.2 |
| *R. typhi* (test 1) | 8.5 | 8.4 |

<sup>a</sup>Expressed as log<sub>10</sub> values of numbers of plaques per gram of yolk sac tissue. Each assay was performed with two to four cell culture vessels for each dilution tested.

agents, however, yielded superior assays in Vero cells when compared to PCE, whereas results with certain *R. prowazekii* seeds were inconsistent. *R. rickettsii* and *R. typhi* have previously been shown to give plaque-forming unit values in PCE cultures equal to or higher than corresponding mean lethal dose or mean infective dose values in embryonated eggs (4, 6). *C. burnetii* failed to produce plaques in Vero cells under the conditions tested, whereas Wike et al. (6) induced this agent to plaque in PCE culture by modifications of overlay medium and extended incubation. BHI, as used by Weinberg et al. (4) and later determined by Wike et al. (5) to be the best of numerous diluents tested for preparing rickettsial inocula for PCE culture plaque assay, was compared to a standard viral diluent. We found 0.15 M PBS + BPA superior to BHI for assays in Vero cells. As used by Wike et al., however, PBS did not contain BPA.

Use of established cell lines, such as Vero cells, avoids some problems of preparation and contamination inherent in the use of primary tissue cultures. Our findings indicate that Vero cells are at least equal to PCE cells for plaquing certain rickettsial agents. Therefore, Vero cells offer an alternative to PCE cells for rickettsial plaque assays. In addition, Vero cells may provide a practical and economical means to test for more than one infectious agent in a single sample. Both *R. rickettsii*, the causal agent of Rocky Mountain spotted fever, and the virus of Colorado tick fever are transmitted to man by the same species of tick, *Dermacentor andersoni*. Moreover, distinguishing between these causal agents in cases of febrile illness associated with tick bite requires individually distinct laboratory procedures. In our experience, the Vero cell plaquing technique is a highly efficient method for isolation and identification of Colorado tick fever virus in samples of human or rodent bloods and in ticks (unpublished observations). It remains to be determined if this technique will prove as sensitive in detecting rickettsiae from clinical or field specimens as from laboratory-produced seeds. If so, Vero cells offer a practical and economical means to test for either agent in routine or large-scale operations.

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