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Plaque Formation by *Rickettsia conori* in WI-38, DBS-FRhL-2, L-929, HeLa, and Chicken Embryo Cells

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Mammalian cells particularly suitable for the study of specialized aspects of rickettsial biology were tested for their ability to support plaque formation by *Rickettsia conori*. The detection of plaques was substantially influenced by the combination of growth medium and cell type used. Large plaques (2.0 to 3.0 mm in diameter) occurred by 8 days postinfection in WI-38 and DBS-FRhL-2 cells supported by medium 199. Smaller plaques (0.5 to 1.0 mm in diameter) were seen in L-929 and HeLa cells at 8 to 11 days postinfection and were more discernible in cells supported with Eagle minimal essential medium. Chicken embryo cells maintained in Dulbecco's modified Eagle medium exhibited large spherical plaques with a diameter of approximately 1.5 mm by 8 days postinfection.

Plaque formation by *Rickettsia conori* or other spotted fever group rickettsiae has been reported in chicken embryo (CE) cells (4, 9), Vero cells (9), and irradiated L-929 cells (10), but the major emphasis of studies with pathogenic rickettsiae has been on their plaquing ability in CE cells. These cells have been utilized in the identification of factors influencing plaque formation (13), including the effect of suspending medium (12), and in the plaque isolation of rickettsiae directly from a vector (11).

The purpose of this investigation was to study the ability of a representative spotted fever group organism, *R. conori*, to form plaques in cells that are particularly suitable for either vaccine development or the study of the molecular biology of rickettsia-host cell interaction. The WI-38 and DBS-FRhL-2 cells are candidate substrates for virus vaccine production (3, 8) and were logical choices for inclusion in this work. Extensive biochemical studies with HeLa and L-929 cells have revealed substantial information concerning their molecular processes (2, 7) and makes them a valuable substrate for molecular studies of rickettsial interaction with mammalian cells.

MATERIALS AND METHODS

Rickettsial seed. The initial seed material of *R. conori* was generously supplied by Charles L. Wisseman, Jr. (University of Maryland School of Medicine, Baltimore) at the 43rd yolk sac passage. Additional seeds were prepared by homogenizing infected yolk sacs in a blender with sucrose phosphate glutamate solution (1) to make a 20% (wt/vol) yolk sac suspension, which was dispensed in glass vials, shell-frozen in dry ice-95% ethanol, and stored at −70 C.

Tissue culture procedure. All cells were routinely propagated at 37 C in a humidified atmosphere of 5% CO_2 in air, using Eagle minimal essential medium (MEM) supplemented with glutamine, nonessential amino acids, and 10% fetal calf serum, all purchased from Grand Island Biological Co., Grand Island, N.Y. (GIBCO). WI-38 cells were purchased from GIBCO and used between passage 22 and 30. DBS-FRhL-2 cells were generously supplied by John Petricciani (National Institutes of Health, Bethesda, Md.) and used between passages 25 and 30. HeLa and L-929 cells were purchased from GIBCO at an unknown passage level. CE cells were prepared by standard techniques (6) from embryonated 11-day-old SPAFAS eggs (Spafas, Inc., Norwich, Conn.).

Plaque assay procedure. Cells were grown to confluency in 60-mm culture dishes (Falcon Plastics, Oxnard, Calif.), growth medium was removed, and 0.1 ml of rickettsial inoculum, diluted in brain heart infusion (Difco, Detroit, Mich.), was applied to the surface of the monolayer. Rickettsiae were adsorbed for 60 min at room temperature with frequent rocking and then overlaid with 5 ml of growth medium containing 10% fetal calf serum and 0.5% agarose (Marine Colloids, Inc., Rockland, Me.). The various growth media used were MEM, Dulbecco's modified Eagle medium (DMEM), and medium 199 (M199), all purchased from GIBCO. Plates were incubated at 32 C in 5% CO_2 in air, usually for 7 days, and then overlaid with growth medium containing 10% fetal calf serum, 0.5% agarose, and neutral red at a final dilution of 1:10,000. Plaques were counted and photographed at 8 and 11 days postinfection.

RESULTS

Plaque formation in WI-38 cells. Large stellate plaque morphology was observed in

M199, and smaller irregular plaques were seen in DMEM. MEM was consistently the poorest medium, with plaques either not evident or barely discernible due to poor contrast after staining with neutral red. Plaques formed in M199 measured approximately 2 by 3 mm (Fig. 1), and microscopic observation indicated that they conformed to the elongated, swirled growth pattern of the fibroblastic cells. All cultures were stained with neutral red 7 days postinfection and photographed the following day, but could not be further maintained for observation of increase in plaque size because of deterioration of the cell monolayer.

Plaque formation in DBS-FRhL-2 cells. Large plaques of both round and stellate morphology were observed in M199 and DMEM. The size was slightly smaller than seen in WI-38 cells, with plaques measuring approximately 1 by 2 mm (Fig. 2). No plaques were evident in MEM. All cultures were stained with neutral red 7 days postinfection and photographed the following day, but as with WI-38 cells, rapid deterioration of the monolayer was observed.

Plaque formation in HeLa cells. At 8 days postinfection, tiny spherical plaques, less than 0.5 mm in diameter, were evident in MEM and DMEM, although fewer numbers were observed in the latter medium. Continued incubation until 11 days postinfection caused an increase in plaque size to approximately 0.75 mm (Fig. 3). Plaque formation at rickettsial dilutions of both 10^{-4} and 10^{-5} was shown to aid in plaque identification. At this time tiny spherical plaques, less than 0.5 mm in diameter, became evident in M199.

Plaque formation in L-929 cells. Initial attempts to plaque *R. conori* in these cells by using the standard staining protocol were unsatisfactory. Plaque formation was not visible until 11 days postinfection, when infected cells maintained in MEM evidenced tiny plaques, less than 0.5 mm in diameter, which could not be reliably quantitated. Subsequent studies indicated that the time of application of the neutral red staining overlay was critical. If the staining overlay was applied 3 days postinfection rather than the normal 7 days, plaques were easily visualized by 8 days and increased in size through 11 days postinfection. This phenomenon was apparently not related to the nutritional status of the cells, because L-929 monolayers refed at 3 days postinfection and then stained at 7 days postinfection did not evidence plaque formation. Figure 4 indicates the results obtained with MEM when the neutral red staining overlay was applied 3 days postinfection and incubation was continued until 11 days postinfection. Small spherical plaques, approximately 0.5 mm in diameter, were initially observed at 8 days postinfection, and by 11 days postinfection had increased in size to approximately 1 mm. At this time tiny plaques, less than 0.5 mm in diameter, were observed in DMEM, but none were apparent in M199.

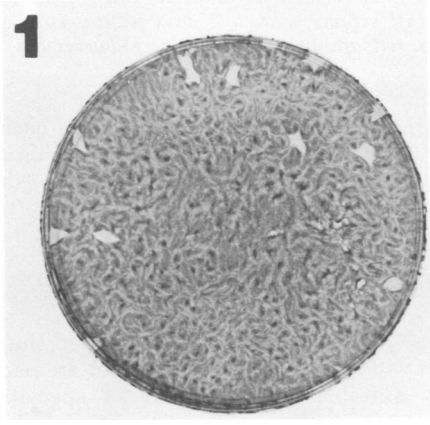

FIG. 1. *Plaque formation in WI-38 cells by standard inoculum of R. conori diluted 10^{-5} in brain heart infusion. Cells were maintained in M199 with 10% fetal calf serum, stained 7 days postinfection with neutral red, and photographed the following day.*

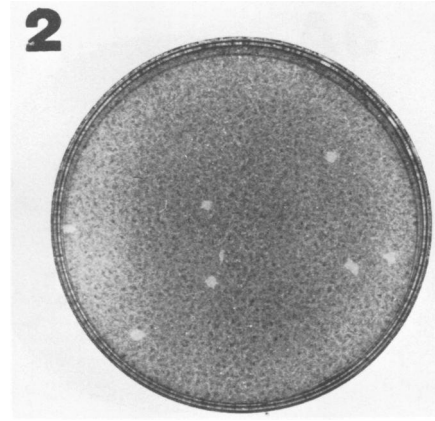

FIG. 2. *Plaque formation in DBS-FRhL-2 cells by standard inoculum of R. conori diluted 10^{-5} in brain heart infusion. Cells were treated as in Fig. 1.*

Plaque formation in CE cells. Plaque formation in these cells has been carefully investigated by others (4, 9, 11 to 13) and was included in this study to provide a basis for comparison of plaque size and to initially test the feasibility of plaquing in unsealed culture dishes. Sharply defined, large, spherical plaques measuring approximately 1.5 mm in diameter were evident in DMEM by 8 days postinfection (Fig. 5). Plaques formed in M199 were irregular and smaller, with a diameter of

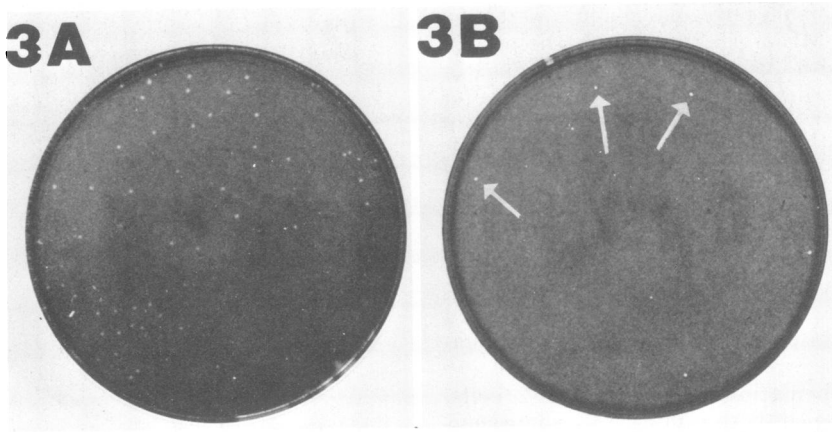

FIG. 3. *Plaque formation in HeLa cells by standard inoculum of R. conori diluted in brain heart infusion. Cells were maintained in MEM with 10% fetal calf serum, stained 7 days postinfection with neutral red, and photographed 11 days postinfection, (A) Inoculum diluted 10^{-4}; (B) inoculum diluted 10^{-5}.*

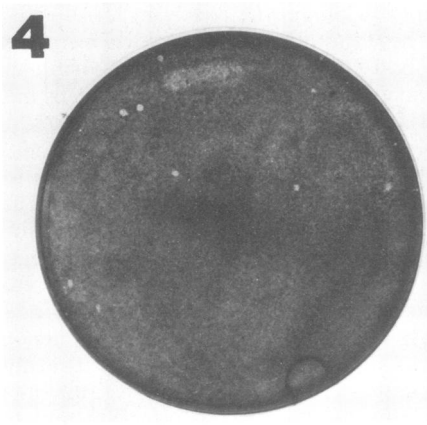

FIG. 4. *Plaque formation in L-929 cells by standard inoculum of R. conori diluted 10^{-5} in brain heart infusion. Cells were maintained in MEM with 10% fetal calf serum, stained 3 days postinfection with neutral red, refed at 7 days postinfection, and photographed 11 days postinfection.*

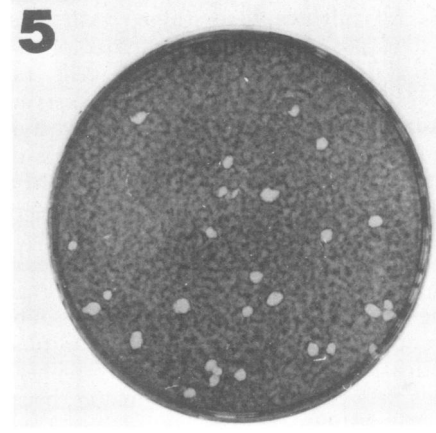

FIG. 5. *Plaque formation in CE cells by standard inoculum of R. conori diluted 10^{-5} in brain heart infusion. Cells were maintained in DMEM with 10% fetal calf serum, stained 7 days postinfection with neutral red, and photographed the following day.*

approximately 0.75 mm. MEM gave unsatisfactory results, with very hazy plaques poorly discernible due to lack of contrast after staining.

Quantitative comparison of plaque formation. Quantitation of plaques formed in each cell type cannot be rigorously analyzed by statistical techniques because of differences in growth medium and period of incubation, but the arithmetic mean number of plaques per milliliter of standard rickettsial inoculum does show the following interesting pattern: WI-38, 15×10^6 plaque-forming units (PFU)/ml; DBS-FRhL-2, 12×10^6 PFU/ml; HeLa, 17×10^6 PFU/ml; L-929, 13×10^6 PFU/ml; CE, 37×10^6 PFU/ml. It is apparent that the first four cell types may be grouped together on the basis of their ability to support plaque formation and that CE cells stand apart as the most sensitive indicator of plaque formation for rickettsiae propagated by serial passage in embryonated eggs.

DISCUSSION

This study has clearly demonstrated that *R. conori* will form plaques in cells that are particularly suitable for the study of specialized aspects of rickettsial biology. Successful plaquing in WI-38 and DBS-FRhL-2 cells is an important observation for future studies in vaccine development. Wike and Burgdorfer (11) have demonstrated the feasibility of isolating

rickettsiae directly from tick hemolymph and rickettsemic blood of guinea pigs by plaque formation in CE cells. It is reasonable to expect that plaque formation by rickettsiae from the blood of infected humans can similarly be accomplished, thus providing a means for isolation and growth of rickettsiae in cells considered to be free of adventitious agents. The ability to plaque rickettsiae in such cells will also be important in studies concerned with the development and plaque purification of attenuated living vaccine agents, where the presence of adventitious agents would present a severe problem. The plaquing and growth of rickettsiae and L-929 and HeLa cells provides an opportunity to study the biochemistry of these agents and perhaps the molecular basis for their obligate parasitism in a host whose own biochemical functions are currently under intensive study at the molecular level. Our plaquing system in culture dishes, rather than the sealed flasks used by other investigators, affords substantial advantage in ease of plaque recovery. This factor is particularly important with the large number of plaques that must be transferred or recovered during genetic studies with rickettsiae.

There was little difference between WI-38 and DBS-FRhL-2 cells in their ability to support plaque formation by *R. conori*. Large plaques were easily and routinely obtained by 8 days postinfection, before deterioration of the monolayers.

Plaque formation was achieved with both continuous cell lines tested, but each had its technical difficulties. HeLa cells evidenced plaques of sufficient size for reliable enumeration or recovery only after 11 days of incubation. Plaques were also observed in L-929 cells at 11 days postinfection but required a special staining technique. The basis for enhanced plaque formation in L-929 cells after early application of neutral red is unknown, but a similar observation has been made with St. Louis encephalitis virus plaque formation in duck embryo cells (5). We plan to explore this observation further to determine whether it has general applicability to rickettsial plaque formation in other cell lines.

CE cells show greater sensitivity for rickettsial plaque formation than the other cell types tested. It is possible that this is the result of multiple passages of rickettsiae in embryonated eggs and the selection of a population of organisms particularly suited to growth in cells derived from chicken embryos. Similar passage of rickettsiae in another type of cultured cell could lead to enhanced plaque formation in that cell type, and studies are currently in progress to test this possibility.

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LITERATURE CITED

1. Bovarnick, M. R., J. C. Miller, and J. C. Snyder. 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of rickettsiae. J. Bacteriol. **59**:509-522.
2. Goldstein, E. S., and S. Penman. 1973. Regulation of protein synthesis in mammalian cells. V. Further studies on the effect of Actinomycin D on translational control in HeLa cells. J. Mol. Biol. **80**:243-254.
3. Hayflick, L., and P. S. Moorhead. 1961. The serial cultivation of human diploid cell strains. Exp. Cell Res. **25**:585-621.
4. Kordova, N. 1966. Plaque assay of rickettsiae. Acta Virol. **10**:278.
5. Marcovici, M., and J. E. Prier. 1968. Enhancement of St. Louis arbovirus plaque formation by neutral red. J. Virol. **2**:178-181.
6. Mascoli, C. C., and R. G. Burrell. 1965. Experimental virology. Burgess Publishing Co., Minneapolis.
7. Perry, R. P., and D. E. Kelley. 1973. Messenger RNA turnover in mouse L cells. J. Mol. Biol. **79**:681-696.
8. Petricciani, J. C., H. E. Hopps, and D. E. Lorenz. 1971. Subhuman primate diploid cells: possible substrates for production of virus vaccines. Science **174**:1025-1027.
9. Weinberg, E. H., J. R. Stakebake, and P. J. Gerone. 1969. Plaque assay for *Rickettsia rickettsii*. J. Bacteriol. **98**:398-402.
10. Weiss, E., L. W. Newman, R. Grays, and A. E. Green. 1972. Metabolism of *Rickettsia typhi* and *Rickettsia akari* in irradiated L cells. Infect. Immunity **6**:50-57.
11. Wike, D. A., and W. Burgdorfer. 1972. Plaque formation in tissue cultures by *Rickettsia rickettsi* isolated directly from whole blood and tick hemolymph. Infect. Immunity **6**:736-738.
12. Wike, D. A., R. A. Ormsbee, G. Tallent, and M. G. Peacock. 1972. Effects of various suspending media on plaque formation by rickettsiae in tissue culture. Infect. Immunity **6**:550-556.
13. Wike, D. A., G. Tallent, M. G. Peacock, and R. A. Ormsbee. 1972. Studies of the rickettsial plaque assay technique. Infect. Immunity **5**:715-722.