# Virus-Like Plaque Formation in Human Cell Culture by *Pseudomonas aeruginosa** (33863)

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(Introduced by Wayburn S. Jeter)

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During a series of viral studies, we noted on two separate occasions that HeLa control cultures developed plaques which were indistinguishable from those present in cultures infected with virus (1). A cross contamination with virus was suspected initially but on further incubation the fluid became turbid, revealing the presence of bacteria.

Since bacteria had not been reported previously to produce plaques in tissue culture (2), we decided to investigate this unusual occurrence. This report describes this new bacterial plaque phenomenon as well as initial studies related to the possible mechanism involved.

*Materials and Methods. Cell cultures.* The propagation of HeLa $S_3$, other established cell lines, and primary cultures was described previously (3). Briefly, the cells were grown as monolayers on glass in milk-dilution bottles in 5% newborn calf serum (NBCS), Eagle's basal medium (EBM) with Earle's balanced salt solution (BSS) supplemented with 100 units of penicillin and 0.1 mg of streptomycin/ml. A 0.25% trypsin solution was used to release cells from the glass surface for subculture. Test tubes (16 × 150 mm) were seeded with 1 ml of the above medium containing $10^5$ cells and incubated horizontally in a stationary position at 37°. When the monolayer sheets were confluent (3–4 days) the medium was replaced and the tubes were infected with bacteria.

*Bacteria.* The *Pseudomonas aeruginosa* strain used was a pure culture contaminant of HeLa $S_3$ which was isolated on two separate occasions from a technician whose infant child developed summer diarrhea. Other bacteria employed were University of Michigan (UM) stock strains kindly supplied by G. S. Fearnehough and listed in Table I.

TABLE I. Plaque Forming Ability of Various Stock Strains of Bacteria on Monolayers of HeLa and RP HF 5.[a]

| Stock strain | Plaques |
|---|---|
| *P. aeruginosa*[b] UM 2-1[c] | Yes |
| *P. fluorescens* UM 2-2 | Yes |
| *A. fecalis* UM 7-1 | Yes |
| *E. coli* UM 8-9 | No |
| *S. typhosa* (Rawlings) UM 15-4 | No |
| *S. typhosa* (PC58) UM 15-6 | No |
| *P. vulgaris* UM 14-1 | No |
| *S. aureus* (P209) UM 23-2 | No |

[a] Assayed in 10% NBCS, EBM without antibiotics.

[b] Also positive in the presence of penicillin and streptomycin.

[c] University of Michigan numbers.

The organisms were grown for 6 hr at 37° in the medium described, except that penicillin and streptomycin were omitted. The bacterial suspensions were placed in 1-ml ampules and frozen in liquid nitrogen at −196° until needed.

*Plaque assay.* Prior to use an ampule of frozen bacteria was thawed rapidly in a 37° water bath and the contents were diluted with the same medium to contain the appropriate number of organisms per 0.1 ml. The 1 ml of medium in each tube containing a confluent monolayer of tissue cells was replaced and the cultures were infected in triplicate with 0.1 ml of bacterial suspension. These tubes were incubated horizontally in a stationary position at 37° and examined microscopically (50×) at hourly intervals for formation of plaques. Plaque counts were

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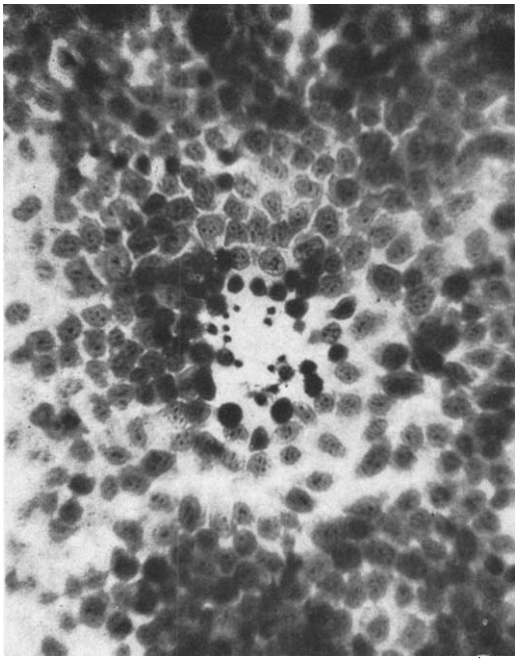

FIG. 1. Typical appearance of an early *P. aeruginosa* plaque in HeLa monolayer 12 hr after the inoculation of 100 organisms; 70×.

usually made 17–19 hr after infection, at which time the cultures were fixed with 10% formalin and subsequently stained with crystal violet in order to confirm the plaque counts.

*Bacterial assays.* Numbers of bacteria were estimated by the standard pour plate method using colony counts in Difco agar or by the most probable number procedure using tubes of nutrient broth.

*HeLa cell disruption.* Infected HeLa cells remaining on the glass were released with a 0.25% trypsin solution, centrifuged, and washed 3 times with BSS. The washed cell pellets were resuspended in medium and homogenized in Ten Broeck tissue grinders.

*Results and Discussion. Formation of* P. aeruginosa *plaques in HeLa.* The appearance of an early bacterial plaque in HeLa $S_3$ monolayers is illustrated in Fig. 1. The infected cells first round up, swell, and then slowly disintegrate as they pull away from the glass surface. This retraction leaves a homogenous space in the cell monolayer which is indistinguishable from a viral plaque. Figure 2 illustrates a more mature plaque in which the degenerating cells form a thickened edge at the periphery of the plaque.

*Proof plaques were formed by bacteria.* The question of whether the bacterial contamination was superimposed upon or concomitant with a viral infection of the cells was resolved by antibiotic and filtration experiments. The medium from tubes showing plaques was treated by adding neomycin at 0.1 mg/ml or by filtration through a no. 02 Selas filter. Both treatments yielded fluids which were incapable of producing plaques or turbidity when added to HeLa $S_3$ monolayers. These findings indicated that the bacteria were solely responsible for the formation of plaques.

*Identification of the plaque-forming bacteria.* The inoculation of medium from tubes showing plaques into nutrient broth or agar revealed the presence of a motile rod with gram-negative staining characteristics. Complete bacteriological and biochemical investigations indicated that the organism was an

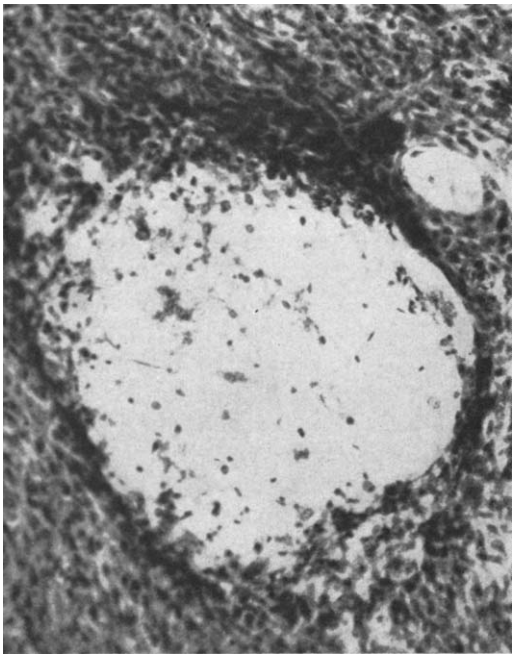

FIG. 2. Mature *P. aeruginosa* plaque in HeLa monolayer 19 hr after inoculation of 100 organisms; 70×.

achromogenic variant of *P. aeruginosa*.

*Bacterial types producing plaques in cell cultures.* Table I lists the results when various laboratory stock strains of bacteria were tested for their ability to produce plaques in the established cell lines HeLa and RP HF 5. Only the two Pseudomonas organisms and a strain *Alcaligenes fecalis* formed plaques. The other gram-negative rods as well as the gram-positive *Staphylococcus aureus* did not. However, all organisms multiplied in the culture fluid with the formation of turbidity and subsequent degeneration of the cell sheets. The UM stock strain of *P. aeruginosa* similar to the achromogenic variant isolated was able to produce plaques in the presence of penicillin and streptomycin indicating its resistance to these antibiotics.

*Cell types forming plaques.* Table II lists the variety of cell types which formed plaques when infected with *P. aeruginosa*. The four Reuben Petersen (RP) established cell lines were isolated in our laboratory (4). Primary culture of human foreskin formed plaques but similar cultures of human amnion did not. Studies are presently underway to determine whether the inability of primary amnion to form plaques is related to its poorer phagocytic activity. The deer mouse fibroblast cell line produced bacterial plaques indicating that the phenomenon was not restricted to human cells.

*Effect of bacterial number on the time plaques appear.* There was a linear relationship between the concentration of organisms inoculated and the time needed for plaques to form. With a concentration of $10^4$ organisms there was a lag of 8 hr before plaques were detectable while a lag of 14 hr occurred when only 10 organisms were employed. If the number of organisms added was increased to $10^5$ or greater, plaques did not develop and the cells appeared unaffected until turbidity became visible at 24 hr when generalized degeneration of the monolayers occurred. The reason for this plaque-inhibiting effect with large numbers of bacteria is not known. Viable organisms are required, however, since prior treatment with $10^5$ heat-inactivated bacteria failed to prevent formation by a small number of viable organisms. This finding rules out the possibility that plaques are caused by an endotoxin, but it does not eliminate the possible involvement of an exotoxin.

TABLE II. Cell Types Forming Plaques after Infection with *P. aeruginosa*.

| Type | Tissue origin |
|---|---|
| HeLa | Cervical carcinoma |
| RP CaCx 1 | Cervical carcinoma |
| RP CaCx 7 | Cervical carcinoma |
| RP HF 5 | Foreskin |
| RP HF 6 | Foreskin |
| Primary fibroblasts | Foreskin |
| F 120 E 90 | Deer mouse muscle |

*Effect of serum concentration on plaques.* The effect of using various concentrations of NBCS on the plaque number and detection time is presented in Table III. First, if serum was omitted from the medium, plaques were not formed. This finding has been consistently observed both with a liquid overlay (5) as well as with an agar overlay system (6). Second, plaques were detected as early as 8 hr with low concentrations of NBCS (1 and 2%) whereas with high concentrations (5–20%), plaques were not visible until sometime between 13 and 23 hr after infection. These findings indicate that while NBCS is actually essential for plaque formation it is at the same time inhibitory to the organism. This paradoxical behavior for NBCS may be

TABLE III. Effect of Serum Concentration on Plaque Number and Detection Time.[a]

| After infection (hr) | Serum concentration (%)[b] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 5 | 10 | 20 |
| 8 | 0 | 3[c] | 7 | 0 | 0 | 0 |
| 9 | 0 | 6 | 15 | 0 | 0 | 0 |
| 10 | 0 | 18 | 35 | 0 | 0 | 0 |
| 12 | 0 | 40 | 43 | 0 | 0 | 0 |
| 23 | 0 | — | — | 55 | 78 | 112 |

[a] Approximately 50 *P. aeruginosa* inoculated/HeLa tube culture.

[b] Newborn calf serum concentration used in the medium at the time of infection.

[c] Mean number of plaques from triplicate cultures.

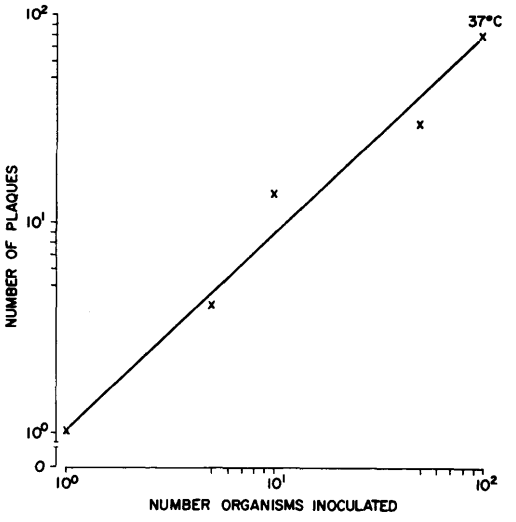


FIG. 3. Effect of *P. aeruginosa* input on the number of plaques formed in HeLa monolayer; Counted 19 hrs after infection.

explainable on the basis that serum may enhance HeLa cell phacocytosis of the organism while inhibiting extracellular multiplication (6).

*Effect of bacterial input on the number of plaques.* Figure 3 illustrates the linear relationship between the concentration of bacteria inoculated and the number of plaques formed in HeLa monolayers. This finding indicates that as with viral plaques (7), a bacterial plaque is the ultimate effect of a single bacterium. Confirmation of these results was obtained with a solid agar overlay procedure which will be reported later (6). One difficulty with the liquid overlay system used in this study is the formation of small satellite plaques which begin 20 hr after the inoculation of 100 organisms. For this reason plaque counts are only reliable between 17 and 19 hr after the addition of such an inoculum.

*Intracellular or extracellular multiplication of* P. aeruginosa. Addition of 0.52–0.75 mg/ml of neomycin at the time of bacterial inoculation was more effective in inhibiting bacterial plaque formation than addition of the antibiotic 1 hr before or after. The ineffectiveness of administering the antibiotic 1 hr prior to infection suggests some inactivation of the antibiotic. On the other hand the ineffectiveness of adding neomycin 1 hr after infection may simply indicate that the number of organisms increased during the hour before the antibiotic was added or it may indicate that some organisms entered the cells and were protected from the neomycin.

Growth curve studies were carried out to support the concept that the bacteria do enter and apparently multiply within the HeLa cells. A series of replicate HeLa monolayer cultures in tubes was inoculated with approximately 100 organisms. At various time intervals the number present in the medium plus those stripped from the cell surface by trypsinization was compared to the number present in glass-homogenized cells. Controls in which organisms were grown in HeLa cell-free medium also were employed. The findings presented in Fig. 4 indicate that the number of bacteria in the medium plus those released from the cell surface by trypsin exceeded the number in the homogenized cells for 11 hr. Then the reverse occurred and by

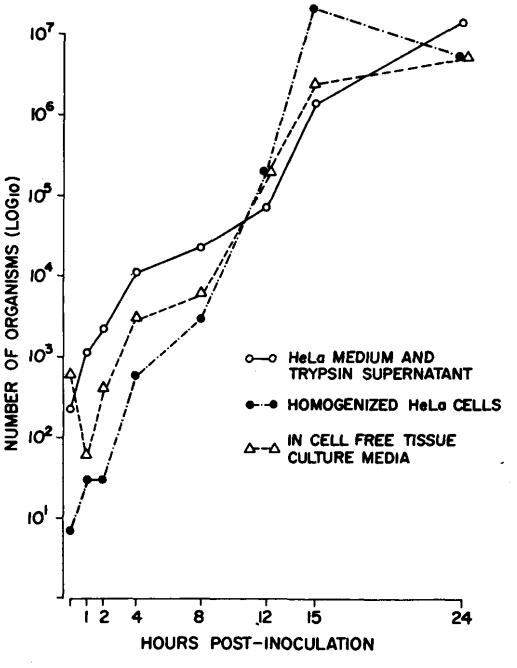


FIG. 4. Comparative growth curves of *P. aeruginosa* in medium plus trypsin supernatant fluid and in glass-homogenized HeLa cells: growth of the organism in HeLa cell-free medium served as a control.

15 hr there was at least 1 log greater number in the cells than in the combined fluid phase. By 24 hr, cell disintegration was sufficiently advanced to cause a release of organisms from the cells into the fluid yielding a 0.5 log increase in the fluid phase.

Bacteria grown in HeLa cultures did not show the customary lag period seen during the first hour of growth in HeLa-free medium. Also, the total yield of bacteria was considerably higher with HeLa than without.

The final piece of evidence in support of the idea that the bacteria are actually within the cells and not simply attached to the surface has been the consistent finding that four times as many viable organisms are found in HeLa cells that have been fractionated by glass-homogenization than in those left intact. Present evidence suggests a form of intracellular multiplication for *P. aeruginosa* which probably leads to orderly cell disintegration and in turn to plaque formation.

*Summary.* A chance contamination in the preparation of HeLa cultures in tubes with an organism identified as *P. aeruginosa* resulted in the formation of plaques within 24 hr which were indistinguishable from viral plaques. At 48 hr, the medium in such tubes became turbid, revealing the presence of bacteria. Filtration of the contaminated medium through a Selas filter or the addition of neomycin eliminated both plaques and turbidity. Stock strains of *P. aeruginosa, P. fluorescens,* and *Alcaligenes fecalis* formed similar plaques on HeLa and fibroblastic-like cell line cultured in antibiotic-free medium, whereas strains of *E. coli* and *S. aureus* did not. All human epitheloid and fibroblastic-like cell lines tested developed plaques with *P. aeruginosa.* A linear relationship was observed between bacterial concentration and plaque counts or the time required for plaques to appear. Bacterial plaques were not formed when newborn calf serum (NBCS) was omitted from the medium. At low concentrations of NBCS (1 and 2%), plaques were detected as early as 8 hr, whereas at high concentrations (5–20%), plaques were not detectable until sometime between 13 and 23 hr after infection. Addition of neomycin at the time of infection was more effective in inhibiting plaque formation than addition of the antibiotic 1 hr before or after infection. Growth curve studies indicated that the number of bacteria in the fluid phase (medium plus those released from the cell surface by trypsinization) exceeded the number in the cell phase (glass-homogenized HeLa) for 11 hr. Then the reverse occurred, for at 15 hr there was 1 log greater number in the cells than in the combined fluid. By 24 hr, cell disintegration due to plaques was sufficiently advanced to cause a release of organisms from the cells into the fluid.

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