Agar Overlay Plaquing Technique for *Pseudomonas aeruginosa* in HeLa Monolayers

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The solid agar overlay procedure used for viral plaquing was adapted to the study of *Pseudomonas aeruginosa* plaques in HeLa monolayers. After adsorption of the organism to the HeLa monolayers, an overlay consisting of 10% newborn calf serum (NBCS), 90% Eagles basal medium (EBM), and 1.5% agar containing neutral red was added. Plaques developed after 24 hr of incubation at 37°C which were indistinguishable from viral lesions. Optimal conditions for plaques included: serum in the adsorption fluid as well as the overlay medium; 1-hr adsorption time; healthy HeLa monolayers; and the presence of EBM. The formation of plaques instead of colonies in agar was due to the inhibitory effect of NBCS or EBM. By omitting both of these components from the overlay, colonies appeared in place of plaques. Adult bovine serum acted in a similar bacterial colony-suppressing fashion, but fetal or agamma calf serum did not. Furthermore, NBCS or adult bovine serum when incorporated in the overlay medium instead of fetal or agamma calf serum displayed a plaque-suppressing effect by reducing the number and size of plaques formed. The findings lend further evidence to support the hypothesis that *P. aeruginosa* may produce plaques from a protected intracellular site within HeLa cells.

*Pseudomonas aeruginosa* produces plaques in HeLa monolayer cultures grown in a liquid medium (3) which are indistinguishable from viral plaques for approximately 30 hr. Thereafter, the medium becomes turbid, and eventually the entire HeLa sheet degenerates. The plaque phenomenon provides another approach to the study of factors responsible for the lethal effects of *Pseudomonas* infections (1).

The liquid system for bacterial plaque assays, however, has the same shortcomings as viral plaque tests with a fluid overlay. First, plaques have to be counted microscopically and, second, satellite plaques arise because the bacteria are not restricted in space. Consequently, we investigated an agar overlay plaquing technique similar to that used for viruses (2). This paper describes conditions required for the formation of bacterial plaques when solid agar overlay procedures are used, and reports the results of studies related to the mechanisms involved.

**MATERIALS AND METHODS**

**Cell culture.** The HeLa S3 stock was cultured in milk-dilution bottles according to methods previously reported (3). The medium used consisted of 10% newborn calf serum (NBCS) and 90% Eagle's basal medium (EBM) containing Earle's balanced salt solution (BSS). Antibiotics were not employed. A 0.25% trypsin solution was used to harvest cells for subculture.

**Bacteria.** The *P. aeruginosa* strain used was a pure culture contaminant of HeLa S3 which was isolated on two separate occasions from HeLa cultures handled by a technician whose infant child was sick with summer diarrhea (3). The organism was cultivated in the previously described medium for 5 hr at 37°C, placed in ampoules, and frozen in liquid nitrogen. When required, the contents of an ampoule were thawed rapidly in a water bath at 37°C and diluted with the same medium to the proper bacterial density. The number of bacteria in an inoculum was estimated by the standard pour plate method with the use of plate count agar.

**Standard agar overlay plaquing procedure.** Prescription bottles (1 oz; 28.4 ml) were seeded with 4 ml of medium containing 10⁶ HeLa cells per ml and incubated at 37°C. When the HeLa monolayers were confluent (3 to 4 days), they were washed once with BSS and inoculated with 41 to 100 bacteria in 0.1 ml of the same medium. The cultures were incubated at 37°C for 1 hr to permit bacterial adsorption to the HeLa cells. Then the adsorption fluid was removed by washing the monolayers with BSS prior to the addition of an agar overlay medium. This overlay consisted of equal parts of 3% agar (Difco) and double-strength EBM containing 10% NBCS and 3% neutral red solution (1:1,000). A 4-ml amount of the overlay medium at 45°C was added to the glass sur-
face opposite the infected HeLa sheet, and then the bottle was rotated slowly until the medium covered the monolayer of cells. After the agar solidified at room temperature, the bottles were inverted and incubated at 37 C for 24 to 36 hr when preliminary plaque counts were made.

The monolayers were then fixed by the addition of 5 ml of 10% Formalin. After standing overnight at room temperature, the agar overlays were discarded. The monolayers then were washed with tap water, stained with 2 ml of 1% crystal violet, washed with tap water, and permitted to dry. Confirmatory plaque counts were performed with a New Brunswick colony counter.

RESULTS

Formation of plaques or colonies. Since we were working with bacteria, mammalian cells, and agar, it was expected that both bacterial plaques and bacterial colonies would appear in the same culture. Surprisingly, however, only one or the other appeared, depending on the constituents of the overlay medium. The appearance of bacterial plaques and colonies is demonstrated in Fig. 1. The typical viruslike plaques (Fig. 1C) with clear central zones of dead cells did not stain with neutral red. In contrast, the bacterial colonies (Fig. 1B) embedded in the agar took up the neutral red dye. The plaques in the monolayer, after staining with crystal violet, appeared as clear unstained circular lesions (Fig. 1A).

Conditions for plaques or colonies. The major components of the overlay medium required for the production of P. aeruginosa plaques or colonies are presented in Table 1. The addition of NBCS and EBM to either type of agar induced the formation of plaques while suppressing colonies.

Table 2 lists the effect of omitting, individually and in combination, the various constituents of the overlay medium on the number of plaques or colonies produced. When either NBCS or EBM was omitted, neither plaques nor colonies developed, indicating that both are required for plaques; the presence of either one inhibited colonies. Similarly, when the amino acids, vitamins, and glutamine of EBM were eliminated as a group, no plaques or colonies appeared, indi-

![Fig. 1. HeLa monolayers infected with Pseudomonas aeruginosa and overlaid with solid agar medium. (A) Appearance of bacterial plaques after overnight fixation with Formalin and subsequent staining with crystal violet. (B) Neutral red uptake by bacterial colonies embedded in agar overlay 24 hr after infection of HeLa monolayers. (C) Typical viruslike appearance of P. aeruginosa plaques 24 hr after infection with clear central zones of dead cells not staining with neutral red.](image)
cating that Earle's BSS alone was insufficient. Conversely, if both NBCS and EBM or NBCS and Eagle's amino acids, vitamins, and glutamine were omitted, no plaques developed, but 9 and 15 colonies appeared, respectively. These results clearly indicate that NBCS and the amino acids, vitamins, and glutamine portion of EBM inhibited colony formation. Other results presented in Table 2 suggest that glutamine was the most important chemical in EBM for plaque production, whereas vitamins were less important.

Effect of bovine sera on bacterial colonies. Table 3 shows the effect of incorporating various bovine sera, treated and untreated, on bacterial colony formation. The serum was incorporated in plate count agar at the 10% level, but HeLa monolayers were omitted. Clearly, newborn and adult bovine sera were equally effective in suppressing colony development. Complement was not involved in the inhibition, because heat-inactivation of serum had no effect. Since adsorption of NBCS with living or dead *P. aeruginosa* failed to eliminate the colony suppressors, the inhibition was nonspecific in nature. When fetal or agamma calf sera was used, there was no inhibition of colonies.

| Serum type | Serum pretreatment | No. of colonies
|------------|--------------------|------------------|
| Newborn    | None               | 0                |
| Newborn    | Inactivated, 56 C, 0.5 hr | 0               |
| Newborn    | Adsorbed with *P. aeruginosa* | 0                |
| Adult      | None               | 0                |
| Fetal      | None               | 15               |
| Agamma     | None               | 14               |
| None       | None               | 21               |

* Serum incorporated at the 10% level.

Table 4 presents the effect of serum type in the adsorption fluid and in the overlay medium on the number and size of plaques formed. The type of serum used in the adsorption fluid did not affect the plaque number or size, but the type of serum used in the agar overlay made a significant difference in both the number and size of plaques formed. The use of fetal or agamma calf sera approximately doubled the number and size of plaques, but both plaques and colonies appeared in the same culture vessel.

**Table 3. Effect of various bovine sera on the formation of *P. aeruginosa* colonies in plate count agar without HeLa monolayers**

**Table 4. Effect of various bovine sera on plaque number and size**

| Serum type | Plaque |
|------------|--------|
| Adsorption fluid (10%) | Agar-EBM overlay (10%) | No. | Size |
| Newborn    | Newborn | 9  | 1-2 |
| Fetal      | Newborn | 8  | 1-2 |
| Agamma     | Newborn | 7  | 1-2 |
| Newborn    | Adult   | 6  | 1-2 |
| Newborn    | Fetal   | 22 | 4-5 |
| Fetal      | Fetal   | 15 | 4-5 |
| Agamma     | Agamma  | 18 | 2-3 |

* Mean in duplicate HeLa bottles 24 hr after the inoculation of 28 bacteria.

**Table 5. Effect of bacterial adsorption time on plaque number**

| Adsorption time | Plaque no. |
|-----------------|------------|
| min             |            |
| 0               | 0          |
| 15              | 0          |
| 30              | 2          |
| 45              | 5          |
| 60              | 7          |
| 120             | 5          |

* Mean in duplicate HeLa bottles 24 hr after inoculation of 77 bacteria.

**Table 6. Effect of serum concentration in the adsorption fluid on plaque number**

| Serum in adsorption fluid | Plaque no. |
|---------------------------|------------|
| %                         | 0          |
| 0                         | 1          |
| 2                         | 5          |
| 5                         | 4          |
| 10                        | 6          |

* NBCS in Eagle's basal medium with Earle's BSS.

b Mean in duplicate HeLa bottles 24 hr after inoculation of 77 bacteria.
sorption times on plaque number. Column one lists the time bacteria were allowed to adsorb to the HeLa monolayer at 37°C before the monolayer was washed with BSS and the overlay medium was added. Clearly, no plaques developed during the first 15 min. Plaques began to appear after 30 min of adsorption, but the maximal number of plaques occurred after 60 min.

**Effect of serum concentration in the adsorption fluid on plaque number.** Table 6 shows that when serum was omitted from the adsorption fluid the plaque number was reduced considerably, even though 10% NBCS was used in the overlay medium. The use of 2 to 10% NBCS in the adsorption fluid was equally effective in increasing the number of plaques subsequently formed.

**DISCUSSION**

The results of this study indicate that the solid agar overlay procedures used for viral plaquing can be adapted to the study of bacterial plaque formation in HeLa monolayers. In delineating conditions necessary for the formation of bacterial plaques in solid media, once again the requirement for NBCS was established as had been shown previously in the liquid overlay system (1, 3).

Surprisingly, however, the present study demonstrated a somewhat paradoxical behavior for NBCS; it was required for the formation of *P. aeruginosa* plaques in HeLa monolayers yet it inhibited colony formation both in Difco and plate count agar. This bacterial colony-suppressing effect was nonspecific, since adsorption of NBCS with dead or living *P. aeruginosa* cells did not eliminate it. Adult bovine serum acted in a similar bacterial colony-suppressing fashion, but fetal or gamma calf serum did not.

Furthermore, NBCS or adult bovine serum, when incorporated in the overlay medium instead of fetal or gamma calf serum, displayed a plaque-suppressing effect, as shown by a reduction in the number and size of plaques formed. These findings lend further evidence to support the previously presented hypothesis (3) that *P. aeruginosa* may form plaques from a protected intracellular multiplication site within the HeLa cells.

The finding in the present study that a bacterial adsorption time of 30 to 60 min was necessary for plaques to form also supports the above hypothesis. These data, coupled with the need for serum in the adsorption fluid, suggest that the organism is either phagocytized by the HeLa cells or becomes closely integrated with its cell surface before plaques can be formed. In support of the phagocytosis hypothesis is the well-established fact that serum facilitates phagocytosis of bacteria by various tissue cells (4).

At this stage of knowledge, then, one can postulate a dual role for NBCS in the *P. aeruginosa*-HeLa plaque-forming system. First, it is probably required in the adsorption fluid to facilitate phagocytosis of the organism by the HeLa cells, and, second, in the overlay medium it probably serves as a nutrient for the host cells to permit intracellular multiplication of the organism. Presumably the requirement for the vitamins and amino acids of EBM also may be for the latter purpose. Conceivably, both serum and EBM could be required by *P. aeruginosa* to produce an exotoxin which ultimately forms the plaques in HeLa monolayers (1).

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