The Plaque-Antiserum Method: an Assay of Virus Infectivity and an Experimental Model of Virus Infection

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Areas of cytopathic effect can be circumscribed in cell monolayers by adding antiserum to the liquid nutrient medium after adsorption of virus. This procedure represents a simple and reliable tool for the titration of virus infectivity and provides an experimental model for studying some aspects of virus infection.

At the end of the replication cycle in infected cells, the progeny particles of most animal viruses are freely released into the liquid medium and spread over the cell monolayer to yield a generalized cytopathic effect (CPE). Areas of CPE, each corresponding theoretically to a single virus infective unit, can be, however, circumscribed by covering the cell monolayer with a solid gel nutrient medium, according to the principle of the usual plaque assay technique (6).

On the other hand, some large deoxyribonucleic acid viruses diffuse from the focus of infection by a cell-to-cell transmission (2, 9). Hence, areas of CPE become detectable macroscopically even in the absence of a solid overlay medium and before the spreading of infective progeny virus produces secondary lesions (4, 11, 12).

In the present paper, a simple method is described for focal assay of viruses spreading from cells after replication, through which the use of agar, double-strength media, and vital dyes can be avoided. This plaque-antiserum method is based on the addition of antiserum to the liquid medium overlaying the cell monolayer, after the adsorption of virus.

MATERIALS AND METHODS

Cell cultures. Primary cultures of African green monkey kidney (AGMK) and continuous-line KB cells were grown as monolayers in Earle minimum essential medium (MEM) containing 10% fetal calf serum, 100 U of penicillin per ml and 100 μg of streptomycin per ml. Cells were cultured at 37 C in various types of containers with a fairly flat base and were maintained in Earle MEM with 3% fetal calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml.

Viruses. The experimental conditions for the plaque-antiserum method were determined for polioviruses (Brunhilde, MEF-1, and Saukett wild strains and L. Sc 2ab, P 712 Ch 2ab, and Leon 12 a,b Sabin vaccine strains of type 1, 2, and 3, respectively). Some preliminary tests were also made with the A2/England/42/72 influenza virus. All strains were obtained from the collection of the Institute of Hygiene, University of Genoa, and were stored at – 80 C in a KW freezer (Siena, Italy).

Source of antibody. Whole sera from rabbits immunized against polioviruses were commercially available (Italidagnostic, Ciampino, Italy). Anti-influenza serum was prepared in the ferret by collecting the blood 14 days after a single intranasal inoculation of A2/England/42/72 virus. Human immune sera were also used in some tests. The neutralizing activity of sera was expressed in neutralizing units (NU). One NU was considered the reciprocal of titer of neutralizing antibodies per ml of serum, challenged with 100 mean infective doses of virus in AGMK cultures (polioviruses) or in the allantois of embryonated eggs (influenza virus).

Plaque-agar assay. Confluent cell monolayers (2 to 8 cm²) were inoculated with 0.1 to 0.2 ml of poliovirus suspension after removal of maintenance medium. During the adsorption period, the inoculum was respread on the cell cultures every 15 min by rocking the containers. The inoculum was aspirated after 90 min of adsorption at 37 C, and the cultures were washed once with Earle MEM and covered with 0.5 to 3.5 ml of a solid overlay medium. The medium consisted of 1.8% purified agar (Difco) mixed with an equal volume of a double-strength medium (20 ml % of 10× concentrated Earle MEM, 80 ml % of deionized water, 1 g % of lactalbumin hydrolysate, 0.03 g % of glutamine, 0.45 g % of glucose, 200 U of penicillin per ml, and 200 μg of streptomycin per ml). The resulting mixture was supplemented with a NaHCO₃ solution, the final concentration of which was 0.5%.

After 40 to 60 h at 37 C, the agar overlay was removed, the cell monolayer was fixed with 99.9% methanol for 2 min, and the contrasting background of uninfected cells was enhanced by means of usual histological stains (e.g., fuscin, methylene blue, or hematoxylin and eosin). Details of this procedure have been previously described (4, 5).
Plaque-antiserum assay. Preparation of cell monolayers and adsorption of virus (poliovirus in AGMK or KB cultures, influenza virus in AGMK cultures) were performed exactly as for the agar method. After adsorption, rather than overlaying the infected monolayers with a solid nutrient medium, the cultures were overlaid with 0.3 to 3.5 ml of a liquid medium, supplemented with a suitable amount (see Results) of specific antiserum. The liquid medium was fresh maintenance medium or, in some cases, the same maintenance medium which had been removed from cultures before addition of the virus inoculum. Cultures were then held stationary at 37 C and, at the end of a suitable incubation time (see Results), the liquid medium was discarded and the cell monolayer was fixed and stained as for the agar assay. The plaques yielded by influenza virus were identified also by means of hemadsorption with guinea pig erythrocytes.

RESULTS

Standardization of antiserum concentration. The addition of antiserum to the liquid medium overlaying the cell monolayer, immediately after adsorption of virus, resulted in the development of local lesions which were detectable macroscopically within a short incubation time.

The amount of antiserum affected only the size of the lesions, the plaque diameter being inversely related to the antibody concentration in the culture fluid (Fig. 1). When an excess of antiserum was added, no lesions could be detected, whereas a low concentration of antiserum resulted in the dissemination of CPE.

However, the dosage of antiserum was not critical and optimal conditions could be observed over a rather wide range of antibody concentrations. Standard calibration curves showed that antibody levels from 0.5 to 4 NU per ml of medium elicited the optimal development of poliovirus plaques after incubation for 50 h in KB and AGMK cells. The optimal range of neutralizing antibody for the development of plaques yielded by A2/England/42/72 influenza virus, after incubation for 72 h in AGMK cells, was 0.8 to 9.6 NU per ml of medium. No significant difference could be observed among the different sources of antibody used in the experiments.

Reading of the assay. Preliminary chess board assays, performed by varying the antiserum concentration in the culture fluid and the length of the incubation time, showed that the optimal time for reading the test, which was chosen for all the following experiments, was 50 h for polioviruses and 72 h for influenza virus (Fig. 2).

However, this time is not critical and may eventually be slightly shortened or extended by

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**Fig. 1.** Incorporation of antiserum into the liquid nutrient medium, after adsorption of virus (Brunhilde type 1 poliovirus), led to a plurifocal involvement of the cell monolayer (KB cells grown in plastic petri dishes of 32-mm diameter, containing 3.5 ml of nutrient liquid medium). The number of foci depended on the initial infecting dose and their size on the concentration of antibody over the monolayer (16, 4, and 1 NU per culture, respectively, corresponding to 4.57, 1.14, and 0.28 NU per ml of medium). The background of uninfected cells was stained with fucsin 50 h after infection.

**Fig. 2.** The plaques yielded by the nonadapted A2/England/42/72 influenza virus on AGMK cultures were sometimes less regularly shaped and less contrasted against the background of uninfected cells in comparison with plaques yielded by polioviruses. Nevertheless, an accurate enumeration of lesions could be made by microscope scanning of the monolayer at a low power magnification. The lesion shown in the micrograph was circumscribed by adding, 2 h after infection, 6.5 NU of antibody to 3 ml of liquid nutrient medium (2.17 NU/ml of medium) of an AGMK monolayer cultured on the cover slip of a Leighton tube (culture area, 5.5 cm²). Cultures were fixed 72 h after infection by means of methanol and stained with hematoxylin and eosin.
increasing or decreasing, respectively, the antiserum concentration.

It is noteworthy that the development of lesions could be readily stopped at any time, by adding an excess of antibody to the medium during the course of infection, prior to CPE dissemination (Fig. 3).

**Relationship between concentration of virus inoculated and plaques observed.** The number of plaques yielded by all of the virus strains tested was independent of the antiserum concentration in the culture fluid, within the optimal range of concentration (Fig. 1), thus proving the reliability and reproducibility of the assay.

The number of plaques was only correlated with the virus concentration in the inoculum. Such a finding provides evidence that all the plaques formed under optimal concentrations of antiserum do represent primary lesions. An example showing the linearity of plaque count with dose is presented in Fig. 4.

**Size of plaques.** As previously stated, the size of plaques was inversely related to the antibody concentration in the culture fluid. With the same incubation time, the cytolytic areas localized by means of antiserum were smaller than those circumscribed by means of agar gel.

These findings indicate that a number of progeny virus particles are neutralized by the antibody molecules overlaying the focus of infection, whereas the remaining particles, the number of which is inversely related to the amount of antibody molecules, escape neutralization and can adsorb to adjacent cells. Accordingly, the possibility that progeny particles leave the focus of infection to yield secondary lesions appears to be unlikely, since they are easily neutralized by antibody during spreading. This view is supported by the finding that development of secondary plaques could be observed only under largely improper conditions, e.g., when cultures containing very low amounts of antiserum were rotated or were repeatedly moved during incubation.

**Shape of plaques.** The plaques localized by means of antiserum are regularly round-shaped when the culture is layered on a horizontal and flat surface. However, the enlargement of plaques is affected by gravity when the surface of the culture is uneven (Fig. 5). Therefore, linear lesions can be obtained by holding the cultures at an inclined angle during incubation.

**Accuracy and sensitivity of the assay.** The very high number of nonoverlapping lesions which are detectable in small cultures lends considerable accuracy to the plaque-antiserum assay technique. In fact, the distribution of infective particles in cultures is Poissonian, and accordingly the accuracy increases with the number of plaques counted (n), being the coefficient of variation equal to √n/n.

Moreover, localization of lesions by means of antiserum improves the sensitivity of the plaque assay. In fact, the number of plaques yielded by all strains of polioviruses was signifi-
tion between progeny virus particles and antibody molecules in the fluid overlaying the necrotic cell.

The affinity of antibody toward virus particles may also affect the size of plaques. This was suggested by preliminary tests, in which the principle of the plaque-antiserum method was successfully applied to the intratypic serodifferentiation of polioviruses (S. De Flora, unpublished data).

Although a number of factors may interfere in the whole organism with immunological mechanisms, the patterns of lesion development in tissue cultures, as related to virus-antibody interaction, can be regarded as a presumptive model of histopathological changes involved during the infection in vivo. Thus, according to results obtained with influenza and polioviruses, a high concentration of antibody should prevent virus implanting in the target tissue and even stop the course of infection when virus particles have already adsorbed to cells. Conversely, the total lack of antibody molecules may result in a diffuse spreading of the histopathological damage, and thereafter the recovery from infection in vivo relies upon other host defense mechanisms.

A weak antibody barrier should determine a plurifocal involvement of the tissue, the number of foci depending on the amount of parent virus particles which have escaped neutralization and have therefore started the infective cycle. The size of the resulting lesions is inversely related to the concentration of antibody in the overlaying fluid. During the enlargement of lesions, spreading of virus particles is affected by me-

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**TABLE 1. Number of plaques yielded by the antiserum method as compared with the agar method**

| Expt no. | No. of plaques |
|----------|----------------|
|          | Agar method | Antiserum method |
| 1        | 135          | 198              |
| 2        | 156          | 210              |
| 3        | 149          | 189              |
| 4        | 127          | 203              |
| 5        | 158          | 219              |
| Mean     | 145          | 204              |
| ±2σ      | ±24          | ±28.5            |

*The number of plaques yielded by Brunhilde type 1 poliovirus was significantly higher (p = 0.05) when lesions were localized by means of antiserum instead of agar gel. The coefficient of variation of multiple assays was below the value statistically expected from the total number of plaques counted.*
FIG. 6. Tentative scheme of mechanisms responsible for the development of plaques, on the basis of a quantitative interaction between antibody molecules (-) and progeny virus particles (*) released from a cell in the fluid overlaying the focus of infection. (A) In the absence of antibody molecules, all progeny particles can freely adsorb to neighboring cells and spread to yield a generalized CPE. (B) An excess of antibody molecules results in the complete neutralization of progeny particles. Therefore, progress of infection is stopped, and no lesion is detectable macroscopically. (C) When progeny particles are in excess, some of them escape neutralization and adsorb to adjacent cells. The development of lesions and, accordingly, their size are inversely related to the amount of antibody molecules (see Fig. 1). No secondary lesion is formed, due to neutralization of virus particles as they leave the focus of infection. If cultures are layered on a flat and horizontal surface, the resulting plaques are regularly round-shaped. (D) When the surface of the culture is inclined, the progeny particles accumulate downward, and the enlargement of lesions follows the direction of gravity (see Fig. 5).

Mechanical factors, such as movements and gravity.

The bulk of previous investigations indicated that local antibodies may not always be involved in recovery from an established infection of a body tissue (1). It was in fact calculated that the time required for the neutralization of viruses in extracellular fluids is longer than the time required for most of the virus to penetrate adjacent cells (8, 10). The results reported in this paper show that, even when the infection has started its course, the progression of histopathological changes can be blocked at any time, provided that a full degree of local immunity is restored.

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