Modified Procedure for the Microscopic and Macroscopic Study of Viral Plaques

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Received for publication 24 August 1966

A virus plaque method that consistently gives good visual contrast for macroscopic observation and also permits microscopic study is described. Cells are grown in plastic flasks and the gelled overlay medium can be of any desired agar concentration or volume. A fixing solution is used prior to removal of the agar overlay, and dye is added to the fixing solution or staining can follow fixation and agar removal. The bottom of the flask with the fixed monolayer is separated from the rest of the container and handled as a slide. A new mounting medium is described.

The development of good contrast for the morphological study and counting of virus plaques in cell monolayers is frequently difficult. Vital stains, like the most commonly used neutral red, yield variable results and have other well-known disadvantages (1). In this laboratory, vesicular stomatitis virus, herpes simplex virus, and polio vaccine viruses, types I and II, have been grown in the following plaque tissue culture systems: primary human amnion, a human amnion cell line, monkey kidney, and HEp2. Neutral red, in final concentrations of 1:5,000 to 1:100,000, was found to stain both the amnion cell line and primary amnion tissue cultures poorly and to be toxic to the latter cell type. The same stain gave good results with rhesus kidney cell sheets. All systems stained well with crystal violet. The following is a modification and further development of the procedure of Holland and McLaren (3) which, in addition to other advantages, allows both microscopic and macroscopic study of viral plaques.

EXPERIMENTAL

Tissue culture monolayers are grown in 30-ml screw-capped plastic tissue culture flasks (Falcon Plastics, Baltimore, Md.). Eagle's basal medium with bicarbonate is usually used for both growth and maintenance, but this can be varied with different cell types. Calf serum, deprived of γ-globulin, is used as a medium supplement. It is our impression that in the presence of this serum cells attach better, not only to glass but also to plastic surfaces. Cultures are used as soon as possible after a continuous cell sheet is formed. Inoculation and adsorption are varied depending upon the virus under study. Any desired final concentration of agar (Bacto or Noble Agar, Difco Laboratories, Detroit, Mich.) in the overlay medium may be used, including concentrations that give fairly firm gels (0.9 to 1.2% or even higher). Cultures are incubated and observed daily for plaque formation, either with the naked eye, by use of oblique or direct light against a dark or clear background, or under the inverted (soft gel-containing flasks) or regular (firm gel-containing flasks) microscope. When plaques are optimally formed, the gelled medium is flooded with a few milliliters of fixative (10 ml of a saturated solution of methyl violet in 95% ethyl alcohol and 90 ml of neutral buffered Formalin) which is allowed to stay for one to several hours, depending on the agar content and amount of the overlay. Other combinations of fixative and dye may be used.

Firm gel overlays are then separated from the cell sheet by vigorously hitting the opposite top side of the flask against the palm of the hand and holding the flask by the neck with the other hand. The cell sheet is firmly fixed to the bottom by the fixing solution and is not affected by this procedure. After adding tap water, the flask is shaken, and the agar is broken in small pieces that are washed out with additional tap water (hot water may be used). If a different stain is desired, the monolayer may be destained with 95% ethyl alcohol (plain or with 1% concentrated HCl). As an alternative, plain 10% buffered Formalin or another fixing solution may be used initially, and the resulting colorless sheet can be stained at a later time by other procedures. For more involved staining techniques and microscopic observation,
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the bottom part of the flask with the monolayer is transformed into a slide by using pliers to shatter the neck, top, and sides of the flask. The resulting section is as long as a regular microscopic slide, fits into staining racks, and may be filed as a permanent record.

Any staining and mounting procedures that do not attack the plastic are adequate. Hematoxylin and eosin staining is routinely used. After the final eosin rinse in 95% or absolute alcohol, the slide is allowed to dry and a cover glass is mounted. A novel mounting medium developed in this laboratory was found to be the most satisfactory. This is made by dissolving 70 g of polyvinylpyrrolidone in 100 ml of absolute ethyl alcohol. No adjustment of the pH is necessary, and the mounting is permanent.

DISCUSSION

Several advantages are derived from the use of Formalin fixation and the possibility of utilizing gels firmer than the soft 0.6% agar of Holland and McLaren (3). The amount of overlay medium (2) and its agar content are not indifferent factors for various cell-virus systems (1). The limiting concentrations of agar which prevent diffusion of viruses are approximately 1% for 65-mu particles and 0.7% for 100-mu particles (4). The present method permits variation of these factors as needed. In general, a firm gel is desirable. It permits incubation in the frequently preferred inverted position. Soft gels, even if not inverted, have a tendency to slip, which makes handling of cultures cumbersome. With the use of Formalin, cells with advanced degrees of cytopathic effect remain firmly attached to the plastic; therefore, there is little difficulty in distinguishing cell gaps from virus plaques. The fixing agent also inactivates virus, adding a safety factor. The conversion of plaque bottles to microscopic slides enables observation with high-power dry and immersion objectives, which facilitates the correlation of microscopic and macroscopic features of plaques. This is useful for virus "spot tests," cloning, and other techniques.

ACKNOWLEDGMENT

This investigation was supported by the John A. Hartford Foundation, Inc.

LITERATURE CITED