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Plaque Assay for Herpes Simplex Virus in L-929 (Earle) Mouse Fibroblasts.* (32508)

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Primary cell cultures of chick embryo and rabbit kidney (RK) cells have been used commonly for plaque assays of herpes simplex virus(1-3). The preparation of primary cell cultures for assay purposes requires sacrifice of animals and chick embryos and is expensive, time consuming, and cumbersome. Several continuous-passage cell lines have been utilized for plaque assays of herpes simplex virus with varying degrees of success (4-8) and cytologic changes produced by the virus in Earle's mouse fibroblasts have been described(9). Likewise, an agar suspension method for plaquing the virus in the L-M cell line has been outlined(10) which is employed successfully for plaque assays of small viruses, but is not recommended for use with medium and large-size viruses(11).

The present communication describes a plaque assay method for herpes simplex virus, (HF) strain, in L-929 (Earle) mouse fibroblasts (L-cells) under methyl cellulose (MC) overlay, which is simple to perform, inexpensive, reproducible, and the results are obtainable within 36-48 hours. The methods described here can be utilized for classroom experiments as well as for research purposes which require exact biological assays of herpes simplex virus.

Materials and methods. The HF strain of herpes simplex virus, originally obtained from the National Communicable Disease Center, Atlanta, Ga., was used throughout these experiments. Before starting the experiments, the virus was passaged twice through HeLa cells and seed from the second passage was used for plaque assays. The L-cells were obtained from the Microbiological Associates, Bethesda, Md. and were grown in a growth medium (GM) containing lactalbumin hydrolysate, 0.5%, yeast extract, 0.1%, glucose, 0.5%, newborn calf serum, 8%, and antibiotics (penicillin, 100 U/ml; streptomycin, 100 μg/ml) in Hanks' balanced salt solution. Monolayers were prepared in 30 ml plastic flasks (Falcon Co., Los Angeles, Calif.) using 4 ml of a cell suspension in GM containing 600-700 thousand cells per ml. Cell monolayers were formed within 48-72 hours of incubation at 36°C.

The MC overlay was prepared, with some modification, according to a procedure described elsewhere(12,13). MC, 4000 centipoises (Dow Chemical Co., Midland, Mich.), was washed once with absolute ethyl alcohol and air dried. One and one-half grams of the material was then suspended in 50 ml of triple distilled water and maintained in a water bath at 60°C for 30 minutes with occasional vigorous shaking. The suspension was then autoclaved at 121°C for 20 minutes and cooled to 45°C. An equal volume of double strength GM, chilled to 4°C, was added to it and the mixture was stored at 4°C, at which temperature the MC was completely dissolved within 24 hours. Thereafter, the overlay was stored at either 4°C or —20°C until used. For plaque assays, the GM from cell monolayers was decanted and 0.3 ml of the appropriate dilution of virus was added to each flask. Adsorption was carried

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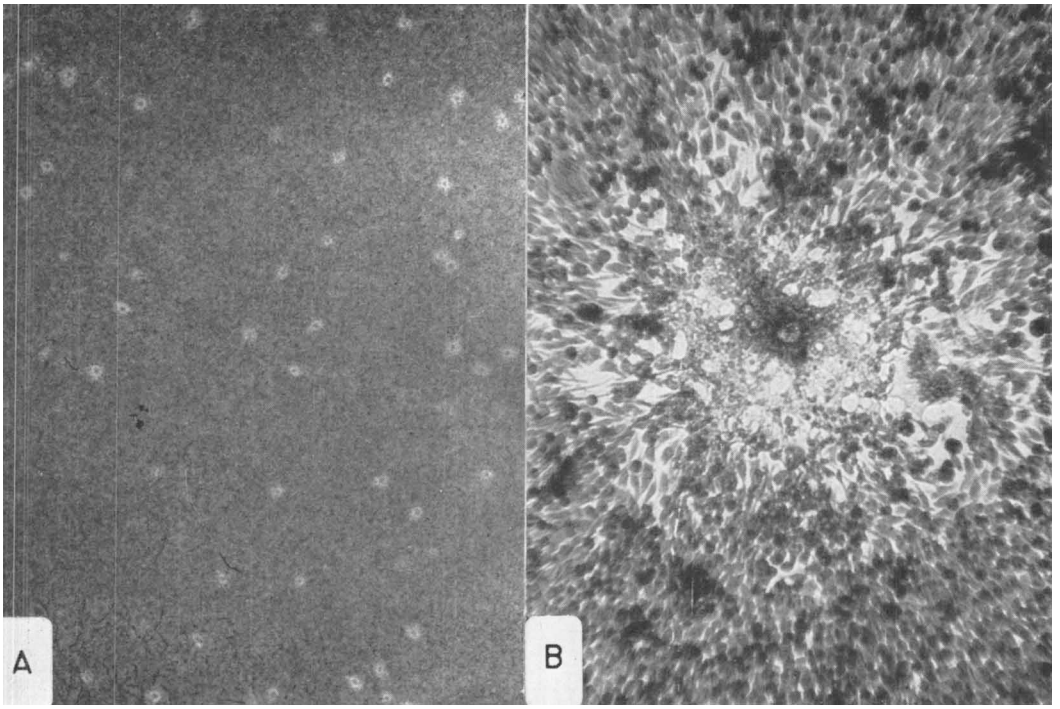

FIG. 1. (A) Plaques of herpes simplex virus in L-929 (Earle) mouse fibroblasts 48 hr after inoculation of virus (×3). (B) Photomicrograph of a plague 36 hr after inoculation of virus. Note dense necrotic center and cytoplasmic bridges (×150). Methylene blue stain.

out for 2 hours at 36°C with occasional tilting of the flasks. At the end of this period, the fluid from the monolayers was poured off and 2 ml of cold MC overlay were added to each flask with a chilled pipette. The MC was then spread evenly over the monolayers and the flasks were placed in an incubator at 36°C for 36-48 hours. They were then removed from the incubator, the MC was diluted by the addition of a few ml of cold 0.85% NaCl and poured off. The monolayers were fixed with 97% methyl alcohol and stained with 0.5% methylene blue in distilled water for 2 minutes. Plaques were counted macroscopically or by the use of a hand lens.

Agar overlays for plaque assays were prepared according to standard procedures(1). Both 1% and 0.5% agar overlays were used for these assays. Rabbit kidney (RK) monolayers were prepared by trypsinization of kidneys from a 3-week old rabbit and were grown in a medium containing lactalbumin hydrolysate, 1%, and newborn calf serum, 8%, in Hanks' balanced salt solution.

Results. Within 21 hours after inoculation of virus, herpes simplex plaques in L-cells under MC appeared as tiny specks about 3/4 mm in diameter and they became larger and more distinct within 36-48 hours. With further incubation, the plaques occasionally became more diffuse. When they were fixed with methyl alcohol and stained with methylene blue, the plaques attained a densely stained center and a halo periphery against a blue background of stained L-cells. Microscopically, each plaque presented a central aggregation of nuclei (multinucleated giant cell) separated from the rest of the cells by an acellular area or by the remnants of cytoplasmic bridges. The plaques were distinct, uniform in size, easily visible with the naked eye or by the use of a hand lens (Fig. 1), and could be counted easily. Furthermore, their number was reproducible (Table I), and they could be differentiated from plaque-like artifacts in the cell monolayer by their dense-staining centers.

The use of agar as overlay, instead of MC, resulted in a 3-fold reduction in the number of plaques. Counts were slightly higher under

TABLE I. Plaque Assays of Herpes Simplex Virus in L-929 (Earle) Cells Under Methyl Cellulose and Agar Overlays.

Type of overlay	Avg No. of plaques per 0.3 ml of virus*	Total No. of plaques per 1 ml of virus
Methyl cellulose	142	4.73×10^5
Agar, 0.5%	50	1.67×10^5
Agar, 1.0%	38	1.27×10^5

* Virus dilution, 10^{-3}. Avg of 4 counts.

0.5% agar than under 1% agar (Table I). When RK cells were used instead of L-cells in plaque assays the counts were approximately 10-fold higher (Table II) and the plaques were appreciably larger in size. However, they were less sharply differentiated and much more difficult to count.

Discussion. The cytological changes in cell cultures which result from infection with herpes simplex virus have been reviewed elsewhere(14). The HF strain of herpes simplex virus used in the present experiments has the remarkable ability of producing localized degenerative foci in L-cells resulting in the formation of multinucleated giant cells. At this stage, the plaques are already visible. Within 36-48 hours, about 50-100 or more nuclei aggregate in the center of the cell. The centrally located nuclei eventually become necrotic and attain a granular appearance. The most prominent feature of the plaque is its dense center which stains heavily with methylene blue and gives the plaque a distinct appearance which differentiates it from nonspecific plaque-like artifacts on the cellular monolayers. Of all the other plaque technics studied, including agar overlays(1), starch gel overlays(15), singly dispersed cell suspension(6), and the agar cell suspension(16), the MC technic was found to be the most satisfactory. With agar overlays the plaques were smaller in size and considerably reduced in number. The other three technics were also found to be unsatisfactory in these experiments.

TABLE II. Plaque Assays of Herpes Simplex Virus in L-929 (Earle) and Primary RK Cells Under Methyl Cellulose Overlay.

Virus dilution	No. of plaques* per 0.3 ml of virus: L-929 cells	RK cells
10^{-3}	54†	TNC‡
10^{-4}	5	67 §
10^{-5}	0	5

* Avg of 4 counts.
† 1.73×10^5 per ml.
‡ Too numerous to count.
§ 1.95×10^6 per ml.

The advantages of plaque assays using L-cells under MC overlays are: (a) ease in preparation of monolayers, (b) use of GM both for cellular proliferation and in overlays, (c) ease in handling of MC overlays, which can be stored at —20°C for long periods of time, (d) distinctness of plaques, which show densely stained centers when they are stained with methylene blue, (e) reproducibility of results, and (f) the short period of time required for the plaques to become apparent and countable. This technic was used, with considerable success, in a medical school class of 117 students.

Replacing the L-cells by HeLa cells in these assays resulted in the appearance, within 72-96 hours, of microscopic plaques only. When compared with the RK cells, the L-cells have the disadvantage of producing fewer and smaller plaques. However, the uniform susceptibility of the L-cells to herpes simplex virus and the distinctness of the plaques which result from infection with the virus make the L-cells a suitable tool for use in biological assays of herpes simplex virus.

Summary. A plaque assay method for the HF strain of herpes simplex virus using L-cells under methyl cellulose has been described. Its ease in preparation, the reproducibility of results, the distinctness of the plaques, and the short period of time required for plaques to appear make it a suitable technic for use in classroom experiments and in research.

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Use of Alveolar Macrophages for Cultivation of Canine Distemper Virus.* (32509)

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Lack of methods to demonstrate and assay virulent canine distemper virus (CDV) *in vitro* has hampered studies of the disease that it causes. Adaptation of CDV in eggs and in tissue culture required serial transfers(1,2, 3,4). Once adapted, CDV strains have been propagated readily in a variety of primary cell culture systems and in continuous cell lines with typical cellular changes(5), but an assay method for virulent virus remained elusive. Cultivation of CDV from infected dogs has succeeded in ferret kidney cell cultures, but demonstration of virus required staining procedures(6). This report describes a method for demonstration and assay of virulent CDV that makes use of dog lung macrophages.

Materials and methods. Tissue culture. Puppies 2 to 6 weeks of age were used for dog lung cell (DL) culture. A puppy was given deep anesthesia and then exsanguinated by severing brachial arteries. Lungs were removed under sterile conditions, minced with scissors and washed in phosphate buffered saline (PBS) at pH 7.2. Lung pieces were stirred in Medium 199 (M 199) plus 0.5% lactalbumin hydrolysate for one-half to 1 hour at room temperature. The suspension was filtered through cheesecloth and filtrate was centrifuged 2 minutes at 1000 rpm. Sedimented cells were resuspended 1:150 by volume in M 199 plus 0.5% lactabumin and either 20% newborn lamb serum or 20% rabbit serum that had been heated at 56°C for 30 minutes. Bovine serum, dog serum, horse serum, pig serum and guinea pig serum did not always yield good virus growth and none were used for these studies. An antibiotic mixture was added that consisted of 500 units of penicillin, 100 μg of streptomycin, 100 units of mycostatin and 2 μg of fungizone per ml. Final pH was approximately 7.1. The suspension contained approximately 8×10^5 cells per ml. Into each Leighton tube with coverslip, 1 ml of cell suspension was placed and incubated in a stationary rack at 36°C. A lung yielded approximately 100 to 150 tubes. These cultures consisted of epitheloid, fibroblastic-type cells and macrophages. Epitheloid and fibroblastic cells began formation of monolayers after 5 days and could be maintained for several weeks. Macrophages degenerated after cultivation for 2 weeks.

After Myrvik(7) reported that almost pure cultures of macrophages were obtained from washings of bronchial and alveolar surfaces of intact lungs of rabbits, his method was applied to dog lungs in order to study this cellular element present in the minced lung preparations. Lungs were removed from euthanized dogs; the trachea or 1 bronchus at the bifurcation was then tied with a hemostat and PBS was injected with syringe and needle until the lungs were distended. After slight

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