Growth of Mouse L Cells in Shaken Culture and Mengovirus Plaque Formation on L Cells Suspended in Agar

MILTON W. SLEIN AND GERALD F. LOGAN, JR.

Biological Sciences Laboratories, Department of the Army, Fort Detrick, Frederick, Maryland 21701

Received for publication 29 July 1970

Procedures are described that require a minimum of equipment and maintenance for growing mouse L cells suspended in liquid medium and for plaquing mengovirus on L cells suspended in agar. Viability of L cells during storage for 1 to 2 hr at relatively high concentrations was better in media at 30 C than at 0 C, as measured by viable counts after growth for 24 hr at 35 C. The number and size of plaques increased with increasing concentration of NaHCO3 in the agar layers, but the relative difference in plaque size was maintained. Large- and small-plaque-size variants had similar virulence as determined by the rates of viability loss of L cells in liquid suspension cultures.

While initiating studies of the infection of mouse L cells with mengovirus, we developed simplified procedures that required a minimum of equipment and maintenance for growing cells suspended in liquid medium and for plaquing the virus on cells suspended in agar. Some observations made during this work are reported.

MATERIALS AND METHODS

L-cell culture. The mouse L-cell line was derived through several laboratories at Fort Detrick from Earle's 929 strain of fibroblasts (9). The chemically defined medium, containing no serum or antibiotics, was originally formulated by Nagle (7, 8). It is commercially available as a dry preparation from Grand Island Biological Co. (USA-1 with dextrose). Modifications were based on suggestions by Higuchi before his improved medium was completely developed (5). All solutions were prepared with triply distilled water. The growth medium (1 X) contained the following solutions: 120 ml of 2.5 X USA-1 with dextrose, 147 ml of water, 12 ml of supplements, 15 ml of 2% methyl cellulose (15 centipoises), 3 ml of 1% NaH2PO4·H2O, and 3 ml of 5% (about 0.6 ml) NaHCO3 containing 0.001% phenol red. The supplements comprised: 245 mg of choline chloride, 575 mg of L-proline, 525 mg of L-serine, 750 mg of L-asparagine, and 10 g of dextrose dissolved with water to give a final volume of about 194.5 ml. To this were added 5 ml of 0.005 M FeNH4(SO4)2 and 0.5 ml of 0.005 M zinc acetate. The USA-1 and supplement solutions were sterilized by filtration through membrane filters (Millipore Corp., 0.22-μm pores). The other solutions were autoclaved at 121 C for 15 min, the bicarbonate in a bottle with a tightly closed lid filled nearly to the top. Before the bicarbonate solution was added, CO2 was gently bubbled into it until the phenol red color changed to pale pink. The CO2 was generated from dry ice and was passed through a sterile filter packed with non-absorbent cotton.

Cell cultures were centrifuged for 3 min at about 200 X g, the supernatant fluid was aspirated, and the cells were suspended in growth medium to give about 1.6 X 10^6 viable cells per ml. Viable cells were determined in a hemocytometer by dye exclusion immediately after diluting the cells with growth medium and 0.5% aqueous trypan blue to give 0.125 to 0.167% dye. A 1-ml amount of the inoculum was added to 50 ml of growth medium in a 250-ml Pyrex, flat-bottomed centrifuge bottle, which was shaken at 35 C in a New Brunswick G25 gyrotory incubator shaker at 140 cycles per min. Flat-bottomed bottles were used because they do not require support, are more stable in the shaker, and allow the cells to be packed more conveniently in an angle centrifuge head than do round-bottomed bottles. The bottles were closed with no. 5.5 rubber stoppers fitted with glass filter tubes, which served as vents (10 cm in length with 0.6-cm stems, and 2-cm tops filled with nonabsorbent cotton). When desired, the vents were closed with no. 3 rubber stoppers to prevent gas exchange. Growth was about the same with 25 ml of medium per bottle as with 50 ml.

Although the medium contained no antibiotics, contamination problems were avoided by making all additions and transfers aseptically in partially closed hoods with no airflow. Ultraviolet sterilizing lamps were kept on when the hoods were not being used.

Plaque formation. In general, the principles of Cooper were used for agar-cell suspensions (3). Medium was prepared at twice the concentration of growth medium, but with the omission of methyl cellulose and with the bicarbonate increased eightfold. This gave a concentration of about 0.024 M NaHCO3.
in the final agar layers compared with 0.006 m in growth medium. A mixture of 2% Difco Special Agar-Noble and 0.015% diethylaminoethyl (DEAE)-dextran (Sigma Chemical Co.) was dissolved at 100 C and allowed to incubate overnight at 45 C. In a few tests, 0.08 to 0.1% proteamine sulfate (Nutritional Biochemicals Corp.) was substituted for the DEAE-dextran. The flocculent precipitate was centrifuged for 5 min at about 10,000 x g in an SS-1 Sorvall centrifuge, the head of which had been heated to 50 C in an oven. Centrifugation was simpler and more effective than filtration for removing the precipitate. The clear agar was decanted and autoclaved for 15 min at 121 C. If not removed, the precipitate stained with trypan blue and made it more difficult to count tiny plaques 1 mm or less in diameter. Equal volumes of the 2X medium and 2% agar, both warmed to 45 C, were mixed to form the underlayer solution. A 4-ml amount of the solution was transferred to each T30 flask (Falcon Plastics) by using pipettes and flasks that had been warmed in an oven at 50 C. The caps were closed tightly, and the agar was spread and allowed to harden at room temperature.

L cells were suspended to give about 6 x 10^6 viable cells per ml of cold 2X medium that had been prepared without methyl cellulose or bicarbonate. Mengovirus was kindly supplied by R. J. Kuchler, Rutgers, The State University, New Brunswick, N.J. The virus was added to the cells in an ice bath, and adsorption proceeded for periods as long as 1 hr with occasional mixing. Cold 2X medium, without methyl cellulose but with augmented NaHCO_3, was added to the cells to give about 10^6 viable cells per ml and about 0.048 m bicarbonate. One-milliliter samples were transferred to tubes in an ice bath. Each sample was warmed for 2 min in a water bath at 30 C, and 1 ml of 2% agar-0.015% DEAE-dextran, warmed to 45 C in a water bath, was added. After mixing, 1.5 ml of the suspension was pipetted into a flask containing the solid underlayer agar that had been warmed in the oven at 50 C. The lid was closed tightly, and the cell suspension was spread and allowed to harden at room temperature for about 15 min. The flask was then inverted and incubated at 37 C.

About 40 hr after infection, the flasks were cooled at 5 C for about 30 min, and 1 ml of cold 0.05% trypan blue (Allied Chemical Corp.) in 0.9% NaCl was added to each. The stain was spread over the agar layers and left at room temperature for 30 min to 1 hr. Each flask was then inverted, and the stain was spread to wet the plastic evenly. This allowed uniform illumination for viewing the plaques with a dissecting microscope with light reflected from below. Counting the stained plaques at 6.6 or 13X magnification was facilitated by a ruled plastic grid cut to fit the shape of the flask. Chilling the agar layers before staining prevented flaking of the agar cell suspension. Once the plaques were stained, they were relatively stable for several days at room temperature. But if the agar-cell suspensions were chilled too long before staining (e.g., overnight), cells outside the plaques gradually died so that the plaques lost their sharpness and became indiscernible.

Agarose, which was used in a few tests, was a product of L'Industrie Biologique Francaise, distributed by Fisher Scientific Co.

**Virus replication.** Mengovirus was replicated in fresh liquid medium containing about 2 x 10^6 viable L cells per ml. When the viable count approached zero, samples of the culture were transferred to screw-capped tubes and were stored at -20 and -60 C. Titters of approximately 10^6 plaque-forming units (PFU) per ml were obtained and were stable for months even at -20 C. L-cell cultures grown for 1 week without changing the medium before infection were not suitable for mengovirus replication.

**Mengovirus plaque size variants.** Two sizes were arbitrarily selected for cloning: small (1 mm, or less, in diameter) and large (4 to 6 mm in diameter). It is difficult to see tiny plaques without staining, but staining with a solution of dye is not feasible when cloning plaque size variants because of cross-contamination with free virions. An unsuccessful attempt was made to stain plaques by incorporating trypan blue into the nutrient agar layers at the time of infection. With 0.005% stain in the final agar layers, no plaques formed, although the L cells remained viable as judged by dye exclusion. With 0.0025% trypan blue, only a few very tiny plaques formed. With 0.001% dye, the number of plaques was about the same as in controls without dye, but all of the plaques were tiny instead of having the size distribution found in the controls.

After plaques had formed, the inverted flasks were observed at 6.6X magnification with light reflected from below at an angle giving the best visibility of small plaques. The positions of plaques to be cloned were marked on the surface of each flask. Sterile syringe needles were used to transfer bits of agar from selected plaques to small tubes containing 0.5 ml of 0.9% NaCl. The samples were vigorously mixed with a Lab-Line Super-Mixer for about 0.5 min. A 0.1-ml amount of the suspension was added to 1 ml of fresh 1X medium containing about 2 x 10^6 viable L cells in a screw-capped tube, which was incubated in a shaker at 35 C. Most of the cells were killed by 24 to 30 hr, and the samples were stored frozen until titrated for virus. The agar cell suspensions remaining in the flasks from which plaque samples had been removed were stained with trypan blue to determine the effectiveness of plaque selection.

**RESULTS**

**L-cell growth and maintenance.** The procedure described above resulted in good cell growth without frequent medium changes and without having to incubate the cultures in a controlled atmosphere with CO_2. In liquid suspension cultures, the initial concentration was 2 x 10^9 to 3 x 10^9 viable cells per ml. To prevent an increase in pH by the excessive loss of CO_2, the filter vents were usually kept stoppered for 3 to 4 days until the cultures contained about 10^6 viable cells per ml. However, it was found that growth rates were not markedly different whether the vents were left open from the time of inoculation or were kept closed for 4 to 7 days (Fig. 1). The medium was
not changed during the 8 days of incubation. When started with $3 \times 10^5$ viable cells per ml, maximal counts of about $5 \times 10^6$ viable cells per ml were reached in about 1 week and remained at that level for as long as 7 more days without changing the medium.

For maintaining cells suspended in agar during plaque formation studies, the effects of different agars were tested. Cells remained viable for at least 3 days in flasks kept tightly closed with about 0.024 M NaHCO$_3$ in the agar layers. The initial pH was about 7.5, as indicated by the pink color of phenol red in the agar, and dropped somewhat by the 3rd day (orange). Starting with 0.006 M bicarbonate, the final pH was slightly lower (yellow-orange), but L-cell survival was still good. In the absence of bicarbonate, most of the cells died within 40 hr.

**Effect of low temperature on L cells.** To minimize metabolic effects during virus adsorption in $2\times$ medium without bicarbonate at high cell concentrations for as long as 1 hr, the adsorption was carried out in an ice bath. During the relatively short procedure for subculturing L cells in $1\times$ growth medium, the cells were not chilled. A comparison was made of cell survival in the two media during storage at 30 and 0 C, with occasional shaking (Table 1). Cells were suspended at about $1.8 \times 10^5$ viable cells per ml in $2\times$ medium without bicarbonate or methyl cellulose. Samples were immediately diluted 10-fold with $1\times$ medium to give $1.8 \times 10^5$ viable cells per ml of $1.1\times$ medium for storage at the two temperatures. Samples of the undiluted cells in $2\times$ medium were also stored at each temperature. At intervals, duplicate 1-ml portions of the cells in $1.1\times$ medium were transferred to screw-capped tubes (20 by 150 mm). Duplicate 0.1-ml portions of cells in $2\times$ medium were added to 0.9 ml of $1\times$ medium in other tubes. All of these 1-ml samples, containing about $1.8 \times 10^6$ viable cells per ml of $1.1\times$ medium, were then incubated in a shaker at 35 C for about 24 hr, at which time viable counts were made. Little or no loss of viability was found immediately after storage in either medium for 2 hr at 30 or 0 C, or after growth at 35 C for 24 hr after storage for 105 min at 30 C in either medium. A slight increase in nonviable cells occurred during growth for 24 hr after storage in $2\times$ medium at 0 C for 105 min, but a more significant increase was found after the subsequent growth of cells stored in $1.1\times$ medium at 0 C for 75 and 105 min. In other tests, as many as 30% nonviable cells were obtained during growth for 24 hr after storage in $1\times$ medium at 0 C for 1 hr.

**Effect of agar variations on plaque formation.** Although 1% Noble agar and 0.5% agarose produced clear gels without centrifugation and did not impair the maintenance of L cells, each prevented plaque formation. Inclusion of DEAE-dextran (0.0075% in the final layers) allowed plaque formation, but in the case of agarose all plaques were tiny, whereas with Noble agar a normal distribution of sizes was obtained with a mixture of plaque size variants.

A final concentration of 0.04 to 0.05% protamine sulfate instead of 0.0075% DEAE-dextran
in 1% Noble agar also allowed plaque formation. But the agar gel was very fragile, and syneresis apparently occurred with oozing of fluid from the agar layers while inverted during incubation at 37 C. The small-plaque variant gave large plaques under these conditions.

Other tests with plaque size variants. Small and large plaques are shown in Fig. 2. The two variants were incubated under identical conditions before staining with trypan blue. Figure 3 demonstrates that virulence was the same for both variants as determined by the rates of viability loss of L cells in liquid suspension cultures.

The presence of 0.024 m NaHCO₃ in the 2X medium during adsorption of mengovirus at 0 C markedly inhibited plaque formation. However, bicarbonate was necessary for cell maintenance in the final suspensions in agar and greatly improved plaque formation (Table 2). After virus adsorption in the absence of bicarbonate, samples were diluted with 2X medium containing different amounts of NaHCO₃ to give the final concentrations indicated. The underlayer agars had been prepared with corresponding final concentrations of bicarbonate. No plaques formed in the absence of NaHCO₃ because the L cells did not survive. The number of plaques increased as the bicarbonate was raised from 0.006 to 0.024 m. Both small and large plaques enlarged with increasing bicarbonate, but their relative size differences were maintained.

The effect of time of adsorption of mengovirus was tested at 0 C in the absence of bicarbonate. The final bicarbonate concentration in the agar layers was 0.024 m. Both plaque size variants gave increasing numbers of plaques, which leveled off at about 1 hr for the small variant but still appeared to be increasing for the large variant. Time of adsorption had no significant effect on the final plaque sizes at 41 hr after infection.

For most PFU titrations, mengovirus was added to cells within 10 min after dilution with 0.9% cold NaCl. However, in one test, diluted virus was kept at 0 C for 2 to 7 hr before it was added to L cells. The number of plaques obtained was much lower than expected. Since the number of plaques increased with time of adsorption in 2X medium without bicarbonate or methyl cellulose, this solution was compared with 0.9% NaCl as a virus diluent. After storage in these solutions at 0 C, a small volume of mengovirus was added

![Fig. 2. Large- and small-plaque-size variants of mengovirus. The plaques were formed in 4 hr at 37 C with L cells suspended in 1% Noble agar containing 0.0075% DEAE-dextran and 0.024 m NaHCO₃.](http://aem.asm.org/)
to L cells in 2x medium, and adsorption was allowed to proceed for 1 hr at 0°C. Virus diluted in NaCl lost 50% of its infectivity in 1 hr and 90% in 4.5 hr at 0°C, whereas virus diluted in 2x medium lost only 50% of its infectivity in 4.5 hr at 0°C. Only the large-plaque-size variant was tested under these conditions. It is likely that protein present in the concentrated suspensions of L cells helped to protect the virus during the adsorption interval.

**DISCUSSION**

A method has been described for growing L cells with a minimum of equipment and maintenance. Cells were suspended in a completely synthetic liquid medium and were shaken in centrifuge bottles in which they could be harvested directly. To avoid any possible physiological side effects, antibiotics were omitted from the medium. With appropriate aseptic techniques, contamination has been minimal even without the use of closed or laminar flow hoods. Although good growth was obtained in 1 week without changing the medium, a fivefold increase in cell yield would be expected with daily changes of medium (5).

L cells required some bicarbonate for survival under our conditions of maintenance when suspended in agar, but viability, as defined by trypan blue exclusion, did not seem to be markedly different with final concentrations of 0.006 to 0.024 M NaHCO3. The effect of bicarbonate may have been due to pH, since L cells also survived in agar at about pH 7.2 in the presence of other buffers such as tris(hydroxymethyl)aminomethane or hydroxyethyl piperazine ethane sulfonic acid. Although the presence of bicarbonate during adsorption of mengovirus inhibited plaque formation, both the number and size of plaques increased with increasing concentration of NaHCO3 in the final agar layers. With a poliovirus variant, Vogt et al. also obtained more plaques at higher concentrations of bicarbonate in agar placed over infected monolayers (10).

It was surprising that no plaques were obtained in our tests with mouse L cells suspended in agarose or Noble agar, because others have reported plaque formation with mengovirus on L-cell monolayers overlaid with these agars (1, 2). Although the addition of DEAE-dextran to the agars allowed plaque formation, plaque size variation was expressed only with the Noble agar, whereas all plaques obtained with agarose were very small. It appears that such variations in response may depend on the source and batch of agar as well as on the specific procedures used in plaque formation. However, the addition of DEAE-dextran to agar was effective in releasing the inhibition of plaque formation in agreement with the findings of others (4, 6).

Protamine has also been used to overcome plaque inhibition by agar overlays. Campbell and Colter reported that the addition of protamine to Noble agar resulted in the formation of large plaques with all plaque size variants of mengovirus (2). We also found that small-plaque variants formed large plaques when 0.04 to 0.05% protamine sulfate was present in the 1% Noble agar-cell suspension and underlayers. But we do not recommend the use of protamine in our system because the warm-agar underlayer was very fragile and was easily torn when the infected cell suspension in agar was added as an overlay. Furthermore, syneresis seemed to occur so that the resulting fluid had to be removed from the inverted flasks underlayers before use and, again, before the stain was added for plaque counts. The relatively soft agar may partially explain the larger plaques obtained with our agar-cell suspensions. Although the excess fluid was removed before use and the flasks were kept inverted during incubation, it is possible that some spreading of free virions on the agar surface was responsible for the large plaques obtained with the small-plaque-size variant. The spread of virions was also indicated by the fact that many more dead cells were present in the "background" areas than with layers prepared with DEAE-dextran, so that the plaques were less sharply defined.

Wallis and Melnick reported that agar viscosity affects plaque size and numbers and found evidence that echovirus particles diffuse more rapidly through agar with an increased positive charge due to polycations such as protamine and DEAE-dextran (11).

In several tests, no significant difference was found in the rates of killing of L cells by the smaller-large-plaque-size variants of mengovirus. This does not agree with the report of Amako and Dales (1), but the results may be at variance because of differences in the strains of virus, L cells, media, procedure, or other factors.

**LITERATURE CITED**

1. Amako, K., and S. Dales. 1967. Cytology of mengovirus infection. I. Relationship between cellular disintegration and virulence. Virology 32:184-200.

2. Campbell, J. B., and J. S. Colter. 1965. Studies of three variants of mengo encephalomyelitis virus. III. Effect of overlay and polyanions on plaque size. Virology 25:608-619.

3. Cooper, P. D. 1967. The plaque assay of animal viruses, p. 243-311. In K. Maramorosch and H. Koprowski (ed.), Methods in virology, vol. 3. Academic Press Inc., New York.

4. Craighead, J. E. 1965. Some properties of the encephalomyocarditis, Columbia SK and mengo viruses. Proc. Soc. Exp. Biol. Med. 119:408-412.

5. Higuchi, K. 1970. An improved chemically defined culture medium for strain L mouse cells based on growth responses to graded levels of nutrients including iron and zinc ions. J. Cell. Physiol. 75:65-72.
6. Liebhaber, H., and K. K. Takemoto. 1961. Alteration of plaque morphology of EMC virus with polycations. Virol-
ogy 14:502–503.
7. Nagle, S. C., Jr. 1968. Heat-stable chemically defined medium for growth of animal cells in suspension. Appl. Microbiol. 16:53–55.
8. Nagle, S. C., Jr. 1969. Improved growth of mammalian and insect cells in media containing increased levels of choline. Appl. Microbiol. 17:318–319.
9. Sanford, K. K., W. R. Earle, and G. D. Likely. 1948. Growth in vitro of single isolated tissue cells. J. Nat. Cancer Inst. 9:229–246.
10. Vogt, M., R. Dulbecco, and H. A. Wenner. 1957. Mutants of poliomyelitis viruses with reduced efficiency of plating in acid medium and reduced neuropathogenicity. Virology 4:141–155.
11. Wallis, C., and J. L. Melnick. 1968. Mechanism of enhancement of virus plaques by cationic polymers. J. Virol. 2:267–274.