Comparison of Agar and Agarose Preparations for Mengovirus Plaque Formation

ERNEST C. BORDEN, G. WILLIAM GARY, JR., AND FREDERICK A. MURPHY
National Communicable Disease Center, Atlanta, Georgia 30333

An agarose overlay yielded mengovirus plaques earlier and in greater size and number than overlays of chemically undefined agars with or without enhancers. Marked variability in plaque-forming efficacy of commercial agarose preparations was noted.

Agarose, a linear galactose polymer component of agar (2), is being used more frequently in place of agar in viral plaque assays. Agar is a relatively impure mixture of polysaccharides and contains at least one sulfated polysaccharide component which inhibits encephalomyocarditis virus (16), mengovirus (7), poliovirus (1), coxsackievirus (5), group A arboviruses (6), and herpesvirus (12) plaque formation. The plaque-inhibitory characteristic of agar can be partially overcome by the addition of polymers such as DEAE-dextran or protamine sulfate (10). However, the addition of these compounds may be undesirable since protamine has been reported to inhibit virus-cell interaction (7), and diethylaminoethyl (DEAE)-dextran has been shown to enhance interferon production (9). Agarose was used and evaluated in our laboratory for the plaquing of mengovirus in an interferon assay in which it was undesirable to add DEAE-dextran to the gel overlay.

Gel preparations were obtained from the following sources: Ionagar no. 2 (Oxoid), Consolidated Laboratories, Detroit, Mich.; special agar-Noble, Difco; Purified Agar, Difco; Seakem Agarose, lot 840806, Marine Colloids, distributed by Bausch & Lomb; Seravac Agarose, batch no. 150, distributed by Pallard-Schlesinger, Carle Place, N.Y. and manufactured by Seravac Laboratories (Pty.) Ltd., England; Agarose, L'Industrie Biologique, Francaise, S.A. Genneviilliers, France, A: lot F. 4871, B: lot F.F. 2743, C: lot F. 4872. Mengovirus strain was that used in the studies of Plagemann and Swim (11). It contained mutants with a plaque size variation reported in Table 1. Because plaque purification was followed by rapid back mutation, the wild strain was used for these studies.

Monolayers of L cells were prepared by seeding 100,000 cells in a volume of 2.5 ml into each well of trays containing six 35-mm wells (FB6-TC, Linbro, New Haven, Conn.). Cells were suspended in a growth medium consisting of 80% Eagle's minimum essential medium (MEM; Earle's base), 10% tryptose phosphate broth, and 10% newborn calf serum. The monolayer of cells was confluent at 48 hr, at which time 40 to 80 plaque-forming units (PFU) of mengovirus in 0.2 ml was added.

Virus was added to wells with a repeating dispenser and gas-tight syringe (model PB600-10 and 1010, Hamilton Company, Whittier, Calif.). After virus absorption for 1 hr at 37 C, 2.5 ml of the agar or agarose overlay was added to each well with a Cornwall pipette. The overlay consisted of 2% agar or agarose, dissolved in water with minimal boiling, and 2X medium, prepared to give a final concentration of 85% Eagle's MEM (Earle's base), 10% tryptose phosphate broth, and 5% newborn calf serum. Protamine sulfate (salmon, Sigma Chemical Co., St. Louis, Mo.), when used, was added to the 2X medium to achieve a final concentration of 200 μg/ml. After the overlay solidified, trays were incubated in a 3% CO2 atmosphere for 48 hr, after which 0.4 ml of neutral red solution (1:1,000 in water) was added to each well. Stain was allowed to diffuse into the overlay for 2 hr, at which time plaques were easily distinguishable.

The plaques formed by mengovirus beneath agarose were clear, circumscribed, and easily countable. With the exception of Purified Agar with 200 μg of added protamine per ml, plaques under agarose were superior to those under the other gel compounds in plaque size, mean plaque number, and time of initial plaque appearance (Table 1). Since these qualities were desirable for interferon assay, lots of agarose from various manufacturers were tested and compared to each other and to Purified Agar with added protamine. Significantly more plaques formed under agarose IBF-A than under the other gel preparations (Table 2). Another agarose from the same manufacturer, IBF-B, completely inhibited plaque formation for 48 hr; by 72 hr, tiny, barely count-
TABLE 1. Mengovirus plaque formation on different gelling preparations

| Gelling prep                  | Time of initial plaque appearance (hr) | Plaque size (mm) at 72 hr | Mean plaque number 48 hr | Mean plaque number 72 hr |
|------------------------------|----------------------------------------|---------------------------|--------------------------|--------------------------|
| Agarose IBF-A                | 12-14                                  | 1.0-4.0                   | 45                       | 50                       |
| Ionaagar No. 2               | 60                                     | 0.5-1.0                   | 0                        | 0                        |
| Difco Purified without protamine | 12-14                             | 1.0-4.0                   | 40                       | 58                       |
| Difco Noble without protamine | 60                                    | 0.5-1.0                   | 0                        | 32                       |
| Difco Noble with 200 µg of protamine per ml | 16                            | 0.5-3.0                   | 37                       | 47                       |

TABLE 2. Mean plaque numbers and standard deviations of different agar preparations

| Agar prep                  | Mean plaque no. | Standard deviation | Significance level |
|----------------------------|-----------------|--------------------|--------------------|
| Agarose IBF, A             | 71.0            | 7.5                |                    |
| Agarose IBF, B             | 0               |                    |                    |
| Agarose IBF, C             | 60.4            | 4.7                | 0.01               |
| Agarose Seravac            | 61.8            | 6.3                | 0.01               |
| Agarose Seakem             | 64.3            | 6.4                | 0.05               |
| Agar, Difco Purified with 200 µg of protamine per ml | 64.7 | 4.6 | 0.05 |

a Read at 48 hr.

b Significance levels for differences between the agarose A mean and other agarose means. Significance levels were determined by the procedure of Tukey for multiple comparisons (15).

able plaques had formed under this agar. The addition of protamine to this agarose resulted in plaque formation, suggesting that the variability might be due to unremoved sulfated polysaccharide inhibitor. However, there was no correlation between the manufacturer's specified sulfate content and plaque-forming efficacy of the agaroses. Similar differences in plaquing efficacy have been observed in lots of a purified agar from a single manufacturer (T. Stim, personal communication).

These comparisons were aided by the reproducible results obtained by using the repeating dispenser and gas-tight syringe for virus inoculation. With addition of virus by the repeating dispenser, a mean of 104 plaques with a standard deviation of 6.3 was obtained, whereas addition by manual pipetting gave a mean of 103 with a standard deviation of 13.0. The variance with the repeating dispenser was significantly less than with manual addition (5% level, F test). This device aided rapid, accurate dispensation of equal amounts of virus and could be applied to any assay requiring repetitive addition of constant amounts of virus.

Increase in mengovirus plaque size under agarose and the superiority of agarose in this respect to methylcellulose and Noble agar have previously been reported (4); in contrast to our findings, no difference in plaque numbers between the various gel preparations was found. Agarose has also been demonstrated to be equal or better than other less purified gel compounds for a wide range of viruses: Herpesvirus hominis (17), Newcastle disease virus (3), vaccinia (8), rhadoviruses, and arboviruses representative of all major antigenic groups (A. Chappell, personal communication). The inhibition by soluble sulfated polysaccharides of virus plaque formation has been demonstrated repeatedly (1, 5-7, 12, 14, 16), but the mechanism is still unclear (4). However, since agarose contains but small amounts of these inhibitors, it would seem to provide an ideal overlay gel.

LITERATURE CITED

1. Agol, V. I., and M. Y. Chumakov. 1963. Effect of polysaccharides on the multiplication of two variants of poliovirus. Acta Virol. 7:97-106.
2. Araki, C. 1958. Carbohydrate chemistry of substances of biologic interest, p. 15. 4th Int. Congr. Biochem., vol. I. Pergamon, Vienna.
3. Barahona, H. H., and R. P. Hanson. 1968. Plaque enhancement of Newcastle disease virus (lentogenic strains) by magnesium and diethylaminoethanol dextran. Avian Dis. 12:151-158.
4. 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.
5. Choppin, P. W., and H. J. Eggers. 1962. Heterogeneity of coxsackie B4 virus: two kinds of particles which differ in antibody sensitivity, growth rate, and plaque size. Virology 18:470-476.
6. Colón, J. I., J. B. Idione, O. M. Brand, and R. D. Costlow. 1965. Mode of action of an inhibitor from agar on growth and hemagglutination of group A arboviruses. J. Bacteriol. 90:172-179.
7. Colter, J. S., M. A. Davies, and J. Campbell. 1964. Studies of three variants of mengo encephalomyelitis virus II. Inhibition of interaction with L cells by an agar inhibitor and by protamine. Virology 24:578-585.
8. deMaeyer, E., and E. Schonne. 1964. Starch gel as an overlay for the plaque assay of animal viruses. Virology 24:13-18.
9. Dianzini, F., G. Riva, P. Cantagalli, and S. Gagnoni. 1969. Effect of DEAE dextran on interferon production and protective effect in mice treated with double-stranded polynucleotide complex polyinosinic-polycytidylic acid. J. Immunol. 102:24-27.
10. Liebhaber, H., and K. K. Takemoto. 1961. Virus polysaccharide interactions II. Alteration of plaque morphology of EMC virus with polycations. Virology 14:502-504.
11. Plagemann, P. G. W., and H. E. Swin. 1966. Replication of mengovirus. I. Effect on synthesis of macromolecules by host cell. J. Bacteriol. 91:2317-2326.
12. Rapp, F. 1963. Variants of herpes simplex virus: isolation,
characterization, and factors influencing plaque formation. J. Bacteriol. 86: 985-991.
13. Schulze, I. T. 1964. Reversible inhibition of type 2 dengue virus by agar polysaccharide. Virology 23: 79-90.
14. Schulze, I. T., and R. W. Schlesinger. 1963. Inhibition of infectious and hemagglutinating properties of type 2 dengue virus by aqueous agar extracts. Virology 19: 49-57.
15. Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics, p. 109-110. McGraw-Hill Book Co., New York.
16. Takemoto, K. K., and H. Liebhaber. 1961. Virus polysaccharide interactions. I. An agar polysaccharide determining plaque morphology of EMC virus. Virology 14: 456-462.
17. Wentworth, B. B., and L. French. 1969. Plaque assay of Herpesvirus hominis on human embryonic fibroblasts. Proc. Soc. Exp. Biol. Med. 131: 588-592.