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A NEW OVERLAY FOR PLAQUING ANIMAL VIRUSES (*)

by

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During a previous study of African horse sickness virus (AHSV) (1), the plaquing efficiency of this virus under agar overlay was compared with the plaquing efficiency under methylcellulose (2) and starch gel (3). The low efficiency of plating under agar that has been observed for several viruses (4) was also noticed for AHSV. However, a marked increase in plaque number was observed when methylcellulose or starch gel were substituted for agar. Because of the technical difficulties in the preparation of the two latter overlays, a substance that was simpler to handle was sought as a substitute for agar. This report describes the successful development of an overlay employing tragacanth gum and reports the results of a comparative study of plaque formation under tragacanth and agar with foot and mouth disease (FMDV), vesicular stomatitis virus (VSV), pseudorabies virus, AHSV, and measles virus.

Materials and Methods. Cell cultures. Primary chicken ebmryo cells (CC) and the stable monkey kidney cell line (MS) were grown in 2-oz bottles in Earle's solution containing 0.5% lactalbumin hydrolyzate, 5% yeast extract (Difco), 10% calf serum, and 100 units of penicillin and 50 μg of streptomycin per ml. The baby hamster kidney cell line, BHK21 (5), and the porcine kidney cell line, BD (6), were also grown in 2 oz bottles in Hanks' balanced salt solution containing the same amounts of lactalbumin hydrolyzate, yeast extract, calf serum, and antibiotics as described above. All media were adjusted to a pH of 7.2-7.4 with 7.5% $NaHCO_3$.

The BSC-1 cells, a line derived from African green monkey kidneys, were grown in 60-mm plastic petri dishes in Eagle's basal medium, supplemented with 10% fetal bovine serum, and 10% tryptose phosphate broth. This medium also contained 100 units of penicillin and 100 μg of streptomycin per ml.

The petri dishes were incubated at 37° in an atmosphere of 5% CO^2. All cells were grown for 3—4 days at 37° before being used for experimental studies.

Virus. The VSV (Indiana serotype) was supplied by the Research Institute, Pirbright, Surrey, England and was already adapted to chicken cells (7). The pseudorabies virus was recently isolated by Dr. Sohrab in the State

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Razi Institute, Iran, and had been through 15 passages in MS cells and 5 passages in chicken cells.

The FMDV, types SAT1,O and A, were kindly supplied by Dr. Amighi, of the Foot and Mouth Disease Unit, Razi Institute, Iran. These viruses had undergone 5 passages in BHK-21 cells; type A was also subsequently adapted to BD cells through 11 passages. The AHSV, type 4, strain VRY, is a vaccine strain adapted to MS cells (8). The virulent Edmonston strain of measles virus is the same as that used in previous studies in this laboratory (9) and has been passaged numerous times in BSC-1 cells. The mouse adapted measles (MAM) virus had been obtained from Dr. Imagawa (UCLA) and had undergone 30-40 passages in suckling mouse brains (10).

Plaque assay. The growth medium was removed from cell monolayers grown in bottles and0. 2 ml of Tris saline was added to the cells. The virus, at various dilutions in Tris saline, was then added in 0.1 ml amounts into each of 3-4 bottles or petri dish cultures and allowed to adsorb for 2 hr at room temperature with occasional swirling of the cultures. At that time, the appropriate overlay was added, the cultures incubated at 37° and plaques observed as described in Results."

Tragacantlh overlay. Tragacanth gum (available from Fisher Scientific Company) is obtained from the small thorny shrubs of the various species of Astragalus grown in the desert regions of the Middle East. The white leaf gum of tragacanth (called ketira) obtained in Iran is the best grade on the market (11). It is a water soluble demulcent drug with a high molecular weight, and is used to coat irritated or abraded tissue surfaces to protect the underlying cells from irritating contacts (12). The gum consists of 60-70% bassorin, and 30-40% soluble gum or tragacanthin. Bassorin, consisting of complex methoxylated acids, resembles pectin and swells in the presence of water to form a colloidal solution. Tragacanthin yields glucuronic acid and arabinose when hydrolyzed. The main advantage is that tragacanth is not toxic for cells and in various experiments, it was found that animal cells under tragacanth overlay appear healthier and survive longer than cells under agar.

The tragacanth solution was made with a fine powder of white tragacanth that had been washed twice previously with ethyl alcohol and air dried. The powder was suspended in double distilled water (1.6 g/100 ml) at 50° with vigorous shaking. The suspension was autoclaved at 120° for 30 min. and then cooled to 37°.

To this suspension was then added an equal volume of warm (37°) double strength growth medium free of serum and phenol red. The pH was adjusted to 7.4 with 7.5% $NaHCO_3$ just before use. Six ml of tragacanth overlay was added to each bottle, which were stoppered and placed at 37°. With measles virus, a double strength Eagle's medium containing 10% fetal bovine serum was used. In all experiments, 100 units of penicillin and 100 μg of streptomycin per ml were included in the overlay. The petri dishes were incubated in 5% Co_2 at 37°. After 2-4 days, the overlay was removed and the cell sheets stained with 2 ml of a solution of tetrazolium salt (13). The dye was removed

from the cells after an incubation period of 2 hr at 37°, and plaques were counted after an additional incubation of 4 hr at 37°.

Agar overlay. The agar overlay was made by mixing equal parts of melted 2% agar (Difco Nobel) with double distilled water and media similar to those described above. The pH was adjusted to 7.4 with a solution of 7.5% $NaHCO_3$ before overlaying the cells. Six ml of agar overlay was added to each bottle. The bottles were inverted after the agar solidified, and incubated at 37° for 2—4 days. The cell cultures were then stained with 3 ml of solution of 1:7500 neutral red for 4 hr at 37°. For staining measles plaques, 5 ml of a second overlay similar to the first but containing 1:20,000 of neutral red was added on the fourth day and plaques were enumerated after overnight incubation at 37° in a CO_2 incubator.

Results. Comparison of plaque numbers under agar and tragacanth overlays. The comparative plaque counts obtained with various viruses under the different overlays are summarized in Table I. Clear and well defined plaques developed under the tragacanth overlay with all viruses tested. In most of the experiments presented, the number of plaques that developed was slightly higher under tragacanth than under agar. Significant differences in plaque numbers were not observed. Difference in plaque sizes were also noticed. The FMDV type A under agar yielded large plaques of 9 to 11 mm and small plaques of less than 1 mm in diameter (Fig. 1). The same difference was observed for plaques of VSV under tragacanth and under agar the latter were larger than the former. Measles plaques were also somewhat smaller under tragacanth than under agar.

Dose-response relationship. It was repeatedly noted that the number of plaques developing under tragacanth following inoculation of the appropriate cell culture with the various viruses was directly proportional to the concentration of the virus. To ascertain that the phenomenon followed a linear rela-

TABLE I. Comparison of Plaque Counts under Tragacanth and Agar Overlays.[a]

Virus	Cells	Period of incubation (days)	(pfu/ml)	
			Tragacanth	Agar
VSV (Indiana serotype)	Chicken	3	1.2×10^9	0.9×10^9
Pseudorabies	Chicken	2	3.2×10^8	2.7×10^8
FMDV (type SAT 1)	BHK 21	2	3.2×10^8	2.7×10^8
FMDV (type O)	BHK 21	2	2.8×10^9	2.0×10^9
FMDV (type A)	BHK 21	2	8.6×10^9	3.2×10^9
FMDV (type A)	BD	3	2.2×10^6	1.8×10^6
ASHV (type 4, strain VRY)	MS	4	3.5×10^7	1.8×10^7
Measles (Edmonston)	BSC-1	4	4.4×10^6	4.6×10^6
Measles (mouse adapted)	BSC-1	4	1.8×10^4	1.8×10^4

[a] Abbrev.: pfu = plaque-forming units; VSV = vesicular stomatitis virus; FMDV = foot and mouth disease virus; and AHSV = African horse sickness virus.

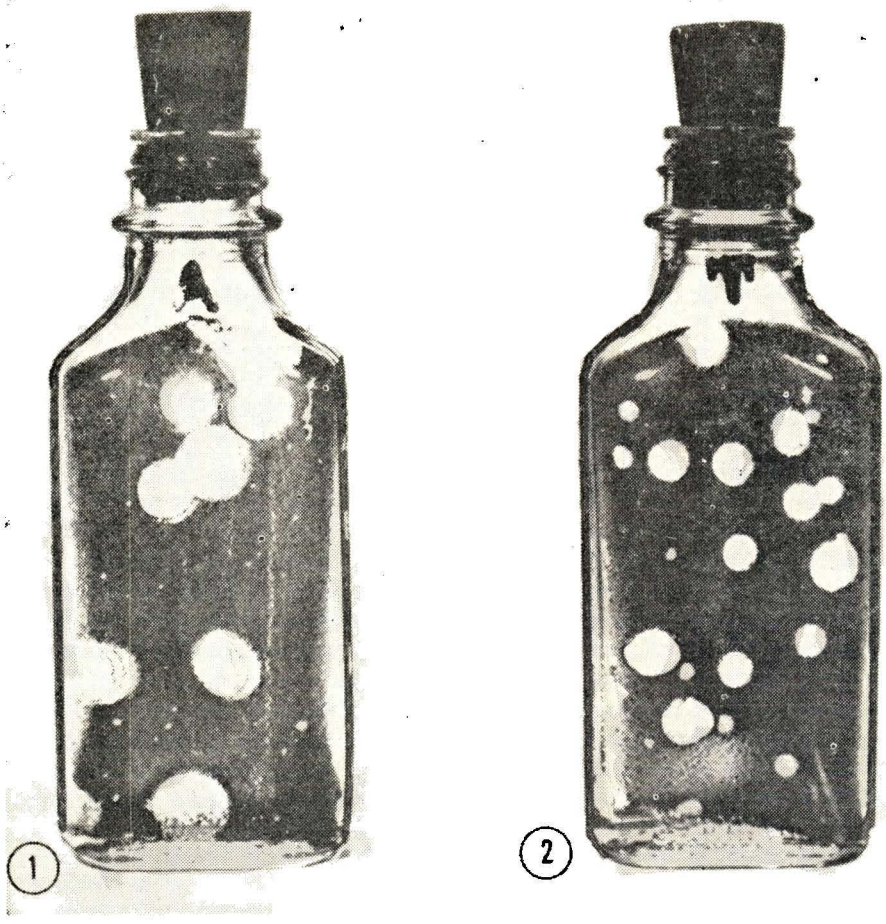

FIG. 1 (left) Plaques in BHK 21 cells produced by foot and mouth disease virus (Type A) under agar overlay 2 days after inoculation of the virus.

FIG. 2. (right) Plaques in BHK 21 cells produced by foot and mouth disease virus (Type A) under tragacanth overlay 2days after inoculation of the virus.

tionship, BSC-1 cell cultures were inoculated with various concentrations of measles virus. The virus was added in concentrations decreasing by a factor of 2-fold. The number of plaques developing was plotted against the virus concetration. As graphed in Fig. 3, it is evident that the development of measles virus plaques in BC-1 cells is directly proportional to the virus concentration used and that this relationship is a linear one.

Discussion. Our study with the viruses mentioned in this paper have shown that an overlay employing tragacanth instead of agar can be used with

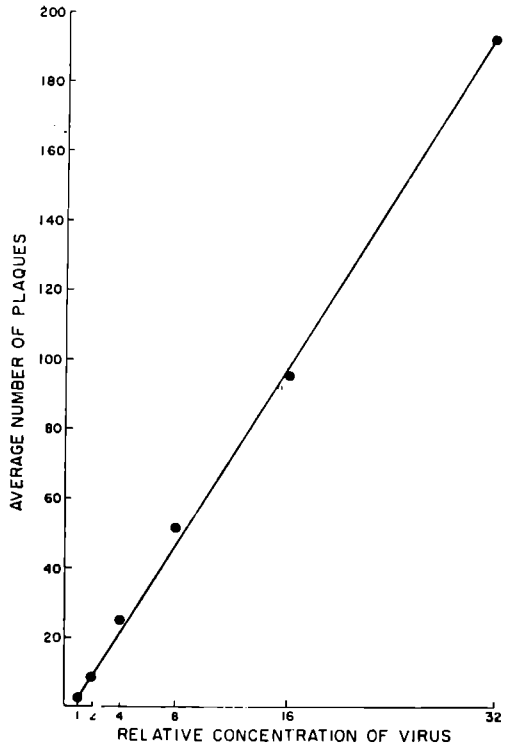

FIG. 3. Relationship of development of measles virus (Edmonston strain) plaques in BSC-1 cells to virus concentration under tragacanth overlay.

a variety of animal viruses. It is a matter of interest that all cell cultures used in this study looked much healthier under this new overlay than under agar; therefore, assays requiring long periods of incubation may be more feasible under tragacanth than under agar. We do not know whether the tragacanth is metabolized by the cells, but it is evident that the product is well tolerated by the cells used in this study.

Furthermore, tragacanth is easy to prepare and can be kept in the refrigerator for extended periods of time before use. Another technical advantage of tragacanth is that it can be easily removed for staining of the cultures. Preliminary experiments carried out in our laboratory have also revealed that cell cultures under tragacanth can be readily employed for the detection of virus antigens by the immunofluorescence technique. The well-known difficulty of removing either agar or methylcellulose from cultures does not appear to apply to the tragacanth overlay.

Summary. An overlay employing tragacanth gum as a substitute for agar in the plaque assay of animal viruses is described. The tragacanth overlay was successfully employed with vesicular stomatitis virus (a rhabdovirus), foot

and mouth disease virus (a picornavirus), pseudorabies virus (a herpes virus), African horse sickness virus, and measles virus (a paramyxovirus). Plaques under tragacanth were generally smaller but usually equalled or slightly exceeded counts obtained under the agar. The number of plaques developing with measles virus in BSC-1 cells was shown to be directly proportional to the virus concentration and the two variables were shown to have a linear relationship. Tragacanth is easy to prepare, the cells used tolerated long exposure to the material, and it was easier to remove than previously described overlays.

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