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Long Term Maintenance of Cell Cultures under Agar Overlay and Development of Herpes T Plaques (32835)

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Dulbecco's agar overlay plaque technique (1) has been widely and successfully employed in a variety of viral studies for more than a decade. None of these studies (2), however, provide enough information on long term maintenance of cell cultures under agar, period in which maximum plaque size is reached, nor as to virus recovery from the plaques after they have reached their maximum size.

During the course of studying the plaque characteristics of herpes viruses particularly that of herpes T virus (3) we were able to maintain cell cultures for extended periods of time under agar overlay. This allowed the herpes T plaques to develop to a maximum size, and to retain this characteristic for several days, with the recovery of viable virus from these plaques for as long as 53 days.

This report results from a study on the plaque characteristics of herpes viruses which is still being pursued. The purpose of this report is to describe the technique employed for the preparation and long term maintenance of various cell cultures under agar overlay, and the behavior of herpes T virus under the conditions studied.

Materials and Methods. A variety of primary cell cultures and cell lines was examined for their suitability for plaque morphology studies (Table I). The medium employed for growth of both primary cells and cell lines was Eagle's minimum essential medium supplemented with 10% fetal calf serum (MEM-10). Penicillin and streptomycin were added at a concentration of 250 units and 250 mg/ml, respectively. The agar overlay medium was Temin's modification of Eagle's medium (4,5). A commercial company prepared this medium for us as a 2× solution [Grand Island Biological Company, Buffalo, New York (GIBCO)]. The composition of Temin's

Eagle's medium is provided in Table II and the composition of the overlay medium is indicated in Table III.

Rabbit kidney primary cells (RKP) were prepared from single 1-week-old rabbits as follows: The kidneys were removed aseptically from etherized animals. After decapsulation cortical fragments were finely minced in Earle's balanced salt solution (EBSS). The tissue fragments were washed several times in EBSS to remove erythrocytes. After decanting the final rinse of EBSS, 30 ml of 0.2% trypsin (Difco 1:250) heated at 37°C was added to the tissues.

The tissues were then magnetically stirred at a uniform speed without foaming for 5 min. The supernatant fluid was decanted and 30 ml of warm trypsin were added and stirred for 15 to 20 min; then the trypsin solution was collected into a flask containing 100 ml of MEM-10. This procedure was repeated 3–4 times. The cell suspension was centrifuged for 5 min at 1000 rpm. The supernatant fluid was discarded and the cell sediment was suspended in a 1:150 dilution to set up 30-ml plastic flasks (Falcon) with 5 ml each. A 1:250 cell suspension was employed to set up 60 × 15-mm plastic dishes (Falcon) with 5 ml each. This higher dilution was employed for dishes as the growth was faster in the CO_2 incubator. A fully formed monolayer was obtained in 4–5 days. The cat kidney cell cultures were prepared similar to RKP.

Chick embryo fibroblasts were prepared from Rhode Island eggs obtained from a commercial supplier. Only 10-day-old embryos were employed for these studies and the preparation followed standard techniques with slight modifications, as follows: Ten ml of 0.2% trypsin was added for each embryo and trypsinization was continuous for 45 min at room temperature. At the end of this period the trypsin cell suspension was filtered through four layers of cheese cloth into a flask containing 2 ml of calf serum for each 100 ml of cell suspension. This was centrifuged for 5 min at 1000 rpm. Each ml of pellet was suspended in 150 ml of MEM-10. Each embryo yielded sufficient cells to seed six, 30-ml Falcon flasks. A fully formed monolayer was obtained in 48 hours at which time the cells

TABLE I. Plaque Development of Herpes T Virus in Various Cell Cultures and Cell Viability.

Cell cultures	Plaque size at 7 days (mm)	Days viable /size (mm)	Passage history of cell cultures
CF[d]	0.5–2	53/15	Primary cultures
CAM[a]	2 –3	17/5–6	Primary cultures
RKP	2 –3	24/10	Primary cultures
CKP[a]	3	21/5–6	Primary cultures
RKL	0.5–1.2	21/2.5	Cell line/57th[b]
HELA	0.5–1	24/2	M. Assoc/80th[c]
AG MK	0.5–1	13/2	Cell line/111th[b]
PC[a]	0.5–1	17/4	Cell line/25th[b]

[a] Cell monolayers inoculated with Newcastle disease virus have remained in good condition for over 20, 32, and 25 days, respectively without plaque development.

[b] Developed in our laboratory by one of us (L.V.M.).

[c] Microbiological Associates.

[d] CF = chick fibroblasts; CAM = chorioallantoic membrane; RKP = rabbit kidney primary; CKP = cat kidney primary; RKL = rabbit kidney cell line; HELA = HeLa; AG MK = African green monkey; PC = palm civit.

TABLE II. Formula[a] for Preparation of Temin's Eagle's Medium 2× Solution.

Solution no. 1		Solution no. 2	
NaCl	12.8 gm	$MgSO_4 \cdot 7H_2O$	0.4 gm
KCl	0.8 gm	$NaH_2PO_4H_2O$	0.25 gm
$CaCl_2 \cdot H_2O$	0.33 gm	Dextrose	9.0 gm
		$Fe(NO_3)_3$ Soln. (1.0 mg/ml)	1.0 ml
		$NaHCO_3$	8.4 gm
Dissolve in 400 ml of deionized water.		Dissolve in 200 ml of deionized water; add to salt solution no. 1.	

Add penicillin 2.5 × 10^5 units/ml and streptomycin 1.0 × 10^5 mg/ml.

Mix: Eagle's vitamins 100×, 40 ml; Eagle's amino acids 50×, 80 ml; add to above salt solution.

Add glutamine 0.87 gm to salt solution and dilute the above to 1 liter by adding 260 ml deionized water. The solution is 2× concentrated; sterilize by filtration.

[a] Personal communication of Dr. Gertrude Schloer, Department of Veterinary Science, University of Wisconsin, Madison, Wisconsin.

TABLE III. Composition of Agar-Overlay.

Overlay No. 1	
Bacto-agar	1 gm
Distilled water (demineralized)	45 ml
Temin's Eagle's medium 2×	50 ml
Calf serum	5 ml
1% Phenol red solution	0.5 ml
Overlay No. 2	
Bacto-agar	1 gm
Distilled water (demineralized)	43 ml
Temin's Eagle's medium 2×	50 ml
Calf serum	2 ml
Neutral red (1:300 solution) filtered	4 ml

were employed for plaque studies. Chorioallantoic membrane (CAM) cultures were prepared from the same embryonated eggs used for the preparation of fibroblasts and were prepared in a similar fashion. Each membrane yielded sufficient cells to seed 3 flasks or dishes. A fully formed monolayer was ready for use in 4–5 days.

Cell line monolayers were prepared as follows: Fully formed monolayers in flasks or dishes were washed with EBSS after aspiration of growth medium. The cell layer was rinsed with 3–4 ml of Tris trypsin (0.2%). After this 4 ml of trypsin was added and incubated at 37°C for 5–10 min. During this period the cells were examined microscopically. When more than 50% of the cells showed detachment the culture vessels were agitated for further dispersion of the cells. The trypsin-cell suspension was collected in a centrifuge tube containing MEM-10 equal to the amount of trypsin added.

The cell suspension was centrifuged for 5 min at 1000 rpm. The supernatant fluid was discarded and the pellet suspended in MEM-10; the volume depended on the splitting ratio of the cell line. Usually a ratio of 1 to 3 was employed, except in rabbit kidney line where a ratio of 1 to 5 was used.

Viruses. Herpes T virus squirrel monkey strain (3); herpes simplex; herpes suis; herpes canis; infectious bovine rhinotracheitis (IBR); sand rat nuclear inclusion agent (SRNIA) were obtained as previously described (6). An Adenovirus-like agent (Cebus monkey strain, unpublished) and another herpes virus squirrel monkey isolate no. 83 (7) isolated from squirrel monkey kidney cultures (unrelated to the above mentioned herpes viruses), and Newcastle disease virus, Delaware-Hickman strain, large, small, and red plaque-purified virus (8) were employed in this study.

Inoculation procedure. Fully grown monolayers were selected and after aspiration of growth medium, were washed once with EBSS and inoculated with an appropriate virus dilution. Virus adsorption was at 37°C for 1 hour. At the end of the adsorption period, unadsorbed virus was removed by washing cultures with 4–5 ml of EBSS and 5 ml of agar overlay no. 1 was added. After 20 min at room temperature the inoculated cultures were returned to the 37°C incubator in an inverted position. On the fifth day, 5 ml of the agar overlay no. 2 was added. The cultures were examined for plaque development 24 hours later. The development of the plaques was observed and recorded until complete cell lysis had occurred. From time to time plaques were picked with the aid of a capillary Pasteur pipette, both from the center and edge of a plaque for the detection of virus. Due to the fact that the 30-ml Falcon plastic flask has a narrow mouth, an opening was made by heating the plastic surface opposite the agar layer where the plaque was located. This was accomplished by melting the plastic surface with the aid of a heated glass tubing (the broad end of a Pasteur pipette); this produced a suitable perforation for removal by suction of a plug of agar from the plaque with another sterile Pasteur pipette. The plug of agar removed from the plaque area was suspended in 1 ml of MEM-10. Two-tenths ml of this suspension was inoculated in two RKP cultures grown on coverslips; the cultures were observed for a period of 2 weeks with one or two changes of medium as required. Coverslip preparations showing cytopathogenic effect were fixed and stained in hematoxylin eosin and examined for the presence of intranuclear inclusions. The specificity of the plaque virus was verified from time to time by serum neutralization tests using herpes T antiserum prepared in rabbits as described previously (6).

Results. Primary as well as continuous cell cultures were employed in this study (Table

I). These cell cultures were mainly observed for: (i) Maintenance of cell cultures under agar overlay, (ii) herpes T plaque development and virus recovery.

Of the three primary cultures tested the best plaque development was obtained in chick fibroblast. A maximum plaque size of 15 mm was observed 45 days after inoculation, and plaques remained at this size up to day 53. No further observation was possible due to cell lysis. In rabbit kidney primary cultures a plaque size of 10 mm was reached by day 12 postinoculation and plaque remained at this diameter up to 24 days. In cat kidney cell cultures a size of 5–6 mm was reached by day 13 and remained this way up to 21 days. The same cells inoculated with Newcastle disease virus, which did not develop plaques, remained viable for over 32 days. The maximum plaque size in chorioallantoic membrane was obtained on day 10 and remained with no further development until day 17.

The maximum plaque development in the continuous cell cultures was obtained between days 11 and 12. This size (Table I) was maintained until days 24, 21, 17, and 13 in HeLa, rabbit kidney cells line, palm civit cell line and African green monkey kidney cell line, respectively. The same palm civit cell line when inoculated with high dilutions of Newcastle disease virus remained viable for over 25 days with no plaque development, while cultures with plaques were destroyed within 14 days.

The duration of cell viability under agar overlay is indicated in Table I. Recovery of virus from the center as well as the edge of herpes T plaques developed in chick fibroblast has been indicated in Table IV. Figure 1 depicts various stages of intranuclear inclusions in a polykaryocyte developed in RKP infected with material obtained from a day 53 plaque. Figure 2 illustrates the development of herpes T plaques in chick fibroblast 17 days, 24 days, and 47 days after inoculation, ranging in size from 5 to 6 mm, 8 mm, and 15 mm, respectively.

Herpes simplex, herpes canis, IBR, SRNIA, Cebus monkey agent, and squirrel monkey isolate no. 83 failed to form plaques in chick fibroblasts. In RKP, herpes simplex, herpes suis, IBR and SRNIA formed

TABLE IV. Long-Term Maintenance of Chick Fibroblasts under Agar Overlay–Herpes T Plaque Development and Virus Recovery.

Days	Size (mm)	Virus recovery	
		Center	Edge
6	1.5	+	+
10	4	+	+
14	5	+	+
21	8	+	+
24	9	+	+
29	12	+	+
35	13	+	+
40	14	+	+
45	15	+	+
49	15	+	+
51	15	+	+
53	15	—	+

plaques. The herpes simplex plaques were large, about 5 mm when first seen on day 6 and developed up to 7–8 mm within 10–12 days. No further development was seen and cell cultures were destroyed within 14–20

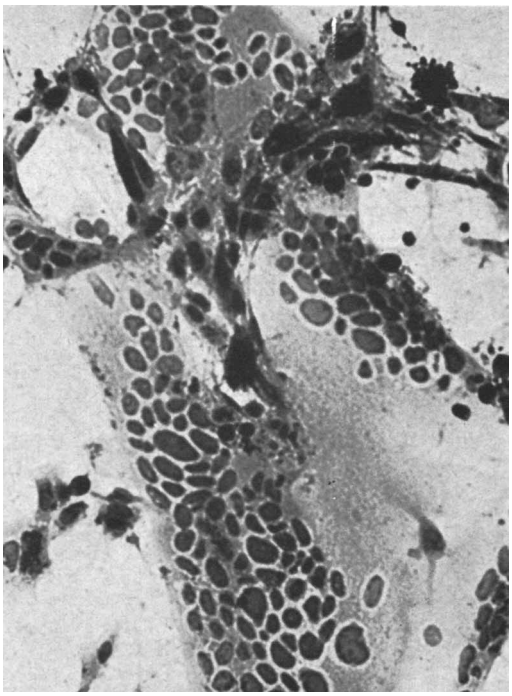

FIG. 1. Intranuclear inclusion in a polykaryocyte developed in RKP infected with material obtained from a day 53 plaque developed in chick fibroblast monolayer.

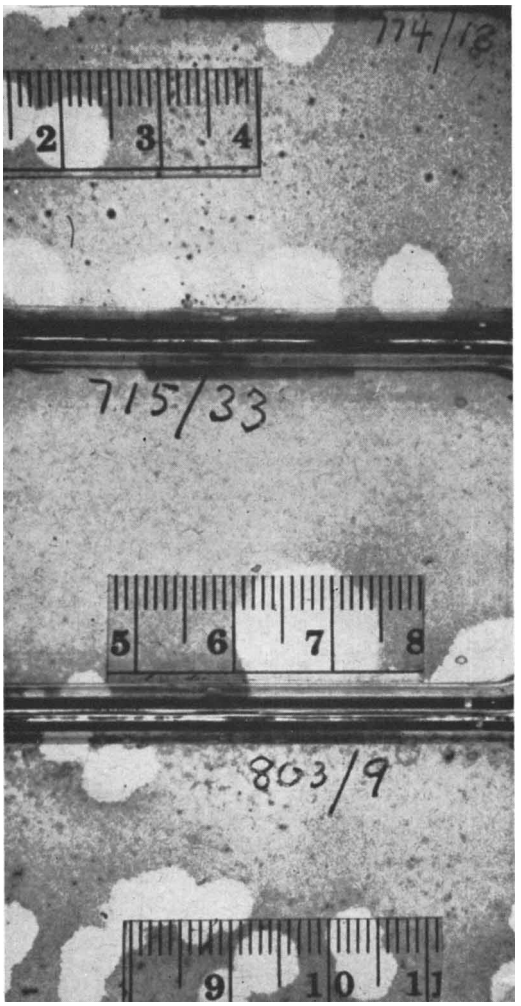

FIG. 2. Herpes T plaques developed in chick fibroblast monolayers (reading from left to right) 17 days, 47 days, and 24 days after inoculation with a 10^{-4} dilution. The size ranges from 5 to 6 mm, 8 mm, and 15 mm, respectively.

days. The IBR and herpes suis plaques were smaller and by day 14 they reached a size of 4 and 7 mm and destroyed the cell culture about the same time as herpes simplex virus. The SRNIA produced small plaques (1 mm) which could first be seen about day 7 or 8. At about 14 days they reached a size of 2–2.5 mm beyond which no increase in size was seen. The cells were destroyed within 3 weeks.

Newcastle disease virus (plaque-purified Delaware-Hickman strain) designated large, small and red, formed plaques in CF and CAM and in palm civit cell line (infection in rest of the cell cultures was not attempted). In CF the NDV large plaque reached a maximum size of 5 mm in 7–10 days and the cell cultures remained viable for 2–4 weeks depending on the number of plaques in a petri dish. Usually neighboring plaques tend to coalesce and destroy the cultures early. In CAM, NDV tends to destroy the cell cultures within 14–17 days, while in palm civit cell line the plaques were smaller than in CF and CAM and cell cultures were destroyed within 14 days depending on the number of plaques present. This palm civit cell line without viral plaques has been found to remain viable for periods over 25 days.

Discussion. Various animal viruses produce plaques under an agar overlay. The development of plaques is an aid to the characterization of a virus. Plaques may vary in their size, time of appearance, rate of development and their morphology. This variation may be due to the rate of adsorption of virus particle to cells, rate of virus multiplication, the number of virus particles formed per cell and to the thermal inactivation of newly released virus progeny. This latter condition may not apply to herpes T virus, as viable virus was recovered up to 53 days in chick embryo cells.

The survival of the herpes T virus for as long as 53 days at 37°C is remarkable and it might be an indication of its thermostability, about which enough information is lacking at present. Our preliminary results on thermostability of herpes T virus indicate its thermostable property. No loss in infectivity was observed after 24 hours at 37°C, while at room temperature (25°C) there was a loss of 90% infectivity after 7 days. In contrast herpes simplex virus loses more than 99% of infectivity in 24 hours at 37°C (9).

The ability of cells to survive under agar overlay for a long period of time would be an aid to the study of slowly developing viruses such as measles, mumps, pox virus, etc., or prove to be useful for detection of indigenous viral agents. Studies of this nature have been hampered due to the nonsurvival of cell cultures under agar overlay after the addition of neutral red stain. Most investigators have found that cultures began to degenerate within 24–48 hours after the addition of

neutral red. Filtered neutral red has been reported to be superior to the autoclaved solution (10). We found the same to be true and used filtered neutral red solution obtained from a commercial supplier (GIBCO). The use of autoclaved neutral red in our initial studies with the same media destroyed most of the cell cultures. Other investigators have found the presence of serum in the medium interfered with plaque formation and also brought about cell degeneration in 24–72 hours (11). In this study the use of calf serum in our medium did not affect plaque formation and permitted cells to remain viable under agar overlay for long periods.

The finding that herpes T virus plaques develop to a maximum size of 15 mm in CF in 45 days, and persist at this size up to 53 days, could be considered as a characteristic for the virus studied.

Kaplan (9) employing RKP cells found that herpes simplex virus plaques reached a maximum size and number after 4–6 days of incubation. He found that by the fourth day most of the plaques had reached a size of 1–2 mm in diameter and did not continue to increase in size. The strain of herpes simplex we used reached a size of 5 mm on the sixth day and increased to 7–8 mm by days 10–12 with no further increase in size. Herpes T virus plaque was smaller than herpes simplex on the sixth day but is increased to a size of 10 mm within 14 days and remained at this size up to 24 days in RKP cells. This continued growth of the herpes T virus plaque both in RKP and in CF distinguishes it from the rest of the herpes viruses examined in this study.

In our investigation of the plaque forming ability of some of the other members of the herpes group we found that herpes simplex, herpes canis, IBR, SRNIA and two recently isolated agents Cebus monkey strain and squirrel monkey isolate no. 83 failed to form plaques in CF and only herpes simplex, herpes suis, IBR, and SRNIA formed plaques in RKP cultures. Kaplan and Vatter (12) employing RKP cells found that herpes suis reached a maximum size (5–10 mm) in 3 days. Likewise with herpes B virus a maximum plaque size of 5 mm was obtained in 6–7 days in monkey kidney cultures (13). Therefore, maximum plaque size could also be considered as another feature for viral characterization, particularly when cell culture systems are available to maintain cells for a long period of time.

The continued development of herpes T virus plaques in CF (reaching a plaque size of 15 mm in 45 days and remaining at this plaque size until 53 days), while the other herpes viruses studied failed to form plaques in the same cultures perhaps may be explained by a lack of interferon activity. This action of interferon has been studied with vaccinia virus by Lindenmann and Gifford (14). They have suggested that a local accumulation of interferon within a cell might be a limiting factor for plaque growth.

The observations reported here might help in the designing of experiments for long term study of cell cultures, for purposes of viral plaque development, isolation of viral mutants and for viral genetic studies.

Summary. A method for the long term maintenance of various cell cultures under agar overlay with the use of Temin's Eagle's medium has been demonstrated. Primary cell cultures of chick fibroblast, cat kidney, rabbit kidney, and chorioallantoic membrane were maintained under agar overlay for 53, 32, 24, and 17 days, respectively. Cell lines of palm civit, HeLa, rabbit kidney, and African green monkey kidney were maintained for 25, 24, 21, and 13 days respectively under the same conditions. These long term cultures proved useful to study the plaque forming ability of several viruses (herpes T, herpes simplex, herpes canis, herpes suis, infectious bovine rhinotracheitis, sand rat nuclear inclusion agent, Cebus monkey strain (an adeno virus), another herpes virus (squirrel monkey isolate no. 83), and plaque-purified Newcastle disease virus (Delaware-Hickman strain). In chick fibroblast herpes T virus and Newcastle disease virus formed plaques. The herpes T virus plaques developed to a maximum plaque size of 15 mm in 45 days and remained at this plaque size up to 53 days with recovery of viable virus. The Newcastle disease virus-large plaque reached a maximum plaque size of 5 mm within 10 days. In rabbit kidney primary herpes T, herpes simplex, herpes suis, infectious bovine rhinotracheitis,

and sand rat nuclear inclusion agent formed plaques reaching maximum plaque sizes of 10 mm, 7–8 mm, 7 mm, 4 mm, and 2–2.5 mm, respectively within a period of 14 days. This cell culture remained viable up to 24 days with herpes T plaques. The rest of the viruses destroyed the rabbit kidney primary cells within 2–3 weeks.

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Glycosaminoglycans in Experimental Amyloidosis* (32836)

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Amyloidosis in animals is easily induced experimentally and serves as a useful model to study pathogenesis of amyloid disease as it occurs in man. The chemical nature of material recognized as amyloid and the mechanisms that are involved in amyloid formation are not well understood. It is still unknown whether amyloidosis is a single disease, appearing with varied clinical and anatomic expressions, or exists as several diseases. To explore such problems further chemical studies of amyloid material seem warranted.

The major components of amyloid consist of fibrillar protein (1), but complex carbohydrates are found in much smaller amounts (2–7). Carbohydrate materials occur, at least in part, as glycosaminoglycans [acid mucopolysaccharides (MPS)], and are suggested to contribute to the characteristic tinctorial quality of amyloid. The MPS are of particular interest, since they might play a basic role in formation of amyloid, and their characterization could aid in differentiation of specific forms of the disease.

As a further study of MPS in amyloidosis, these polysaccharides were determined in multiple organs from mice with amyloidosis induced experimentally. The results were compared to observations in human forms of the disease (6,7).

Experimental. Amyloidosis was produced in male C57 BL10J mice, 6–7 weeks old, obtained from Jackson Laboratory. The mice were divided into two groups, 50 animals each, consisting of mice with amyloidosis and controls. To induce the amylidosis the mice were given subcutaneous injection daily for a period of 120 days of 25–30 mg of casein, according to the method of Janigan (8). Those mice dying during the course of injections after 90 days were frozen and later pooled with those surviving the injection period. In order to obtain a maximum amount of tissue, no effort was made to study the sequence of amyloid production, or to quantitate the amount occurring in each mouse. At the end of the

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