

## *In vitro* Cultivation of Cells from the Chorioallantoic Membrane of Chick Embryos\*

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**Abstract.** By treatment of chorioallantoic membranes from embryonated eggs with collagenase and hyaluronidase before the conventional application of trypsin cells could be grown in culture which supported growth of a large variety of myxoviruses, herpesviruses, avian reoviruses and the infectious bronchitis virus of chickens. The cultures could be used for sensitive plaque assays and neutralization tests.

### Introduction

Besides the classical method to grow viruses in the allantoic cavity of embryonated eggs attempts have been made to have chorioallantoic cells available for studies which require host cells cultured *in vitro*. The deembryonated egg (Bernkopf, 1949) or cells outgrown from bits of chorioallantoic membranes in culture (Brandt, 1958) or the "allantois-on-shell" technique (Fazekas de St. Groth *et al.*, 1958) have been steps in this direction. The latter method has been of great value for special purposes in influenza virus research, but its applicability is too limited to be used for routine work in the laboratory.

In an attempt to plaque-purify and to quantitate some strains of influenza viruses and of avian reoviruses we succeeded to cultivate cells from the chorioallantoic membrane of chick embryos. The technique employed to prepare such cultures and some of their applications are described in this communication.

### Material and Methods

#### *Viruses*

The following strains were tested for their ability to grow, induce a cytopathic effect and form plaques in chorioallantoic cells of the chick embryo (CAM-cells).

1. Representative types of orthomyxoviruses: The human strains PR8 ( $H_0N_1$ ), FM1 ( $H_1N_1$ ) and the Iowa strain of swine influenza ( $H_{sw1}N_1$ ) were obtained from Dr. Davenport, Ann Arbor, Michigan; the human strain A2 Singapore 1/57 ( $H_2N_2$ ) and the Prague strain of equine influenza ( $H_{eq1}N_{eq1}$ ) from the World Influenza Centre, London, the Rostock strain of fowl plague virus (FPV,  $H_{av1}N_1$ ) and virus "N" ( $H_{av2}N_{eq1}$ ) from Dr. Schäfer, Tübingen, and the human strain X 31 ( $H_3N_2$ ) from Dr. Kilbourne, Mount Sinai Hospital, New York.

2. The Beaudette strain of NDV for paramyxoviruses (Rott, 1965).

3. *Herpesviruses*: Herpes simplex type I (HSV I, strain KOS, oral isolate), Herpes simplex type II (HSV II, strain 192, genital isolate), Pseudorabies (PsR, strain DEK). The history of these strains and their characterization have been described (Ludwig *et al.*, 1972).

Marek disease virus (MDV, strain J. M., from W. Okazakie, United States Department of Agriculture, East Lansing, Michigan).

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4. Avian viruses which supposedly belong to the reovirus group. Strain 2207 (Kösters *et al.*, 1972) which has been suspected to be the causative agent for infectious bursitis in chickens. Strain Wi was obtained from Dr. Kußmaul, Stuttgart. The history of this strain, which has been originally isolated by Winterfield as a potential cause of inclusion body hepatitis of chicken, had recently been reported (Woernle *et al.*, 1974).

5. *Coronavirus*: infectious bronchitis virus of chickens (Beaudette strain from Dr. Krauss, Gießen).

### *Media*

The commercially available medium 199 (Flow Laboratories) was used throughout.

### *Chemicals*

Collagenase (160 units/mg) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey, and hyaluronidase (ca. 500 I.E./mg) from Serva Feinbiochemica, Heidelberg.

*Buffer solutions* were made up according to standard procedures. Collagenase and hyaluronidase were dissolved in Hanks' solution without calcium and magnesium (GKN-buffer).

### *Antisera*

Antisera against the influenza virus strains A2 (Singapore) and X31 ( $H_3N_2$ ) were produced by injecting hemagglutinin from Tween/ether-dissociated virus incorporated in Freund's incomplete adjuvant subcutaneously into rabbits. Contaminating antibodies against cellular components were removed from the antisera by absorption with solid immunoadsorbents containing allantoic fluid as host antigens (Becht, 1971; Seto *et al.*, 1974). Antisera against the other influenza virus types were produced in adult chickens. The birds were inoculated 4 times intravenously with 1 ml of infectious allantoic fluid with intervals of 3 to 4 days. Blood was drawn 2 weeks after the last injection.

Antiserum against reovirus, strain 2207, was produced in chickens, 4 weeks old, by injecting concentrated virus subcutaneously, which had been purified as described (Kösters *et al.*, 1972). Three weeks later the birds were injected a second time and bled after 8 to 10 days. Anti-Wi serum was produced by injecting 2 ml of allantoic fluid containing  $2 \times 10^7$  plaque-forming-units (pfu)/ml according to the same schedule.

### *Preparation of Cultures and Plaque Titrations*

Eggs were purchased from 2 commercial farms and were incubated for 11 days for the routine preparation of chick embryo fibroblasts (Kraus *et al.*, 1963). After the embryo had been removed, the CAMs were harvested and minced carefully with a pair of scissors. The small pieces were suspended in prewarmed PBS without  $Ca^{++}$  and  $Mg^{++}$  and washed by letting them settle 5 to 7 times by gravity in PBS until the supernatant appeared clear. Virtually all erythrocytes are eliminated by this procedure. The tissue was then suspended in prewarmed GKN-buffer containing 0.01% collagenase and 0.001% hyaluronidase and stirred for 15 min at room temperature. The tissue fragments were allowed to sediment again, and after removal of the supernatant they were resuspended in 0.2% trypsin in versene-buffer (0.2% EDTA, pH 7.2) and stirred for 30 to 60 min at room temperature. After the residual crude material had settled, the supernatant cell suspension was centrifuged gently (about 400 g for 4 min), and the cell pellet was washed again in versene-buffer. After recentrifugation the cells were resuspended in medium 199 containing 10% fetal calf serum. The cells,  $3 \times 10^6$ , were seeded into 60 mm plastic Petri dishes (Greiner, Nürtingen) and incubated at 37° C in a water-saturated atmosphere with 6%  $CO_2$ . Before infection the monolayers were washed 3 times with PBS, inoculated with 1 ml of PBS containing the appropriate virus dilution and left at 37° C for 1 hr for adsorption. Four ml of maintenance medium was then added which contained 2% fetal calf serum plus 5% normal calf serum for the influenza viruses, 7% fetal calf serum for the avian reoviruses and 2% fetal calf serum for the herpesviruses and infectious bronchitis virus. For plaque assays the infected cell layers were covered with purified

agar (Difco) in a final concentration of 0.7% in maintenance medium at 42°C. For cultures infected with equine influenza virus the overlay was supplemented with 75 µg/ml of DEAE-dextran (Takemoto *et al.*, 1961). Plaques were visualized after 2 to 3 days by adding 0.05% trypan blue or 0.025% neutral red in PBS to the cultures. After 2 to 3 hrs at 37°C excess stain was removed and plaques were counted.

#### *Neutralization Tests*

About 100 pfu were mixed with equal volumes of the serially diluted antisera and left at room temperature for 30 min. Monolayers were inoculated and plaques were developed as described above. End-points were taken as the highest serum dilution which inhibited more than 80% of the plaques appearing in serum-free controls.

#### *Immunofluorescence*

The cells were grown on coverslips in plastic Petri dishes. The staining procedure with fluorescein-conjugated antibodies has been described (Becht, 1971).

#### *Hemagglutination Tests*

Media of CAM-cells or chick embryo fibroblasts infected with the various strains of myxoviruses were assayed for their hemagglutinating activity after an incubation period of 8 to 16 hrs p.i. according to Davenport *et al.*, 1960.

### **Results**

Treatment of the CAM fragments with collagenase-hyaluronidase (Hinz *et al.*, 1959; Pollard *et al.*, 1960) was a prerequisite for getting individual cells into suspension by trypsin. By conventional trypsinization alone no cells could be removed from untreated CAM. The use of a chelating agent during trypsinization and the subsequent washing prevented clumping of the cells. When buffers without EDTA were used, the cells had a high tendency to form large aggregates.

The cells usually formed monolayers within 2 days after seeding. The monolayer mainly consisted of epithelioid cells with relatively narrow cytoplasmic margins and round nuclei (Fig.1). Typical fibroblasts were usually present in a loose net-like arrangement. If the cells were seeded into the plates at a lower density (less than  $1 \times 10^6$  cells/ml), fibroblasts outgrew the epithelioid cells during the 2 to 3 days which were needed to form a monolayer.

All the virus strains tested replicated in CAM cells and induced cytopathic effects which consisted of rounding of cells and eventually detachment from the culture dish. Syncytia appeared in CAM-cultures, infected with PsR-virus, whereas infection by HSV I and II produced large clumps (Fig.2).

Table 1 shows that the yield of newly formed myxoviruses was higher in CAM-cells than in conventional chick fibroblasts. Hemagglutinating activity of virus released into the medium and plaque-counts on CAM-cells were considerably higher for almost all influenza virus strains. Of particular interest is the high yield of NDV, because it reached levels comparable to those in chicken kidney cells. Table 2 shows that the two strains of avian reoviruses and infectious bronchitis virus reached high titers in CAM-cells. Staining with specific fluorescent antibodies against the reoviruses showed that only epithelioid cells contained the virusspecific antigen, whereas fibroblasts remained unstained.

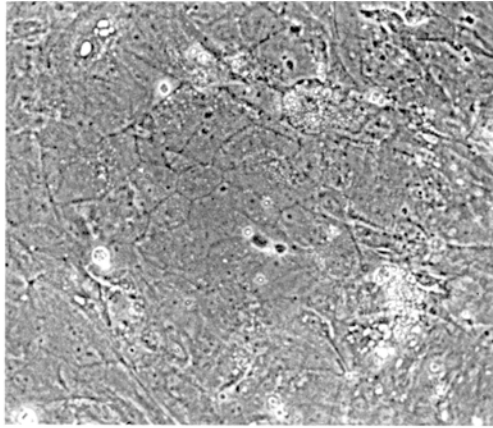


Fig. 1

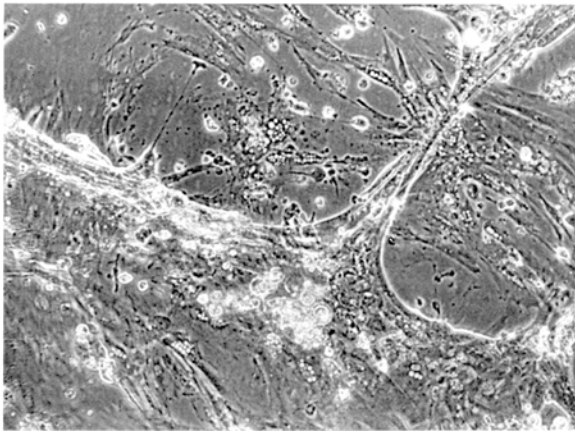


Fig. 2

Fig. 1. Monolayer of CAM-cells cultivated for 2 days at 37° C

Fig. 2. Typical cytopathic effect induced by the influenza virus strain FM 1

CAM cells could be used for plaque-assays of all virus strains tested including HSV I, HSV II and PsR. MDV was the only exception. Although the virus induced a cytopathic effect under liquid medium, no clearly discernible plaques appeared, even if the cells were kept up to 7 days before they were stained. After such a prolonged incubation period the monolayer showed signs of nonspecific disintegration such as vacuolization and formation of a web-like cell sheet. Incubation of the MDV-infected cells under liquid medium for 1 day before the agar overlay was added did not promote the appearance of plaques after 4 more days of incubation.

The number of plaques was directly proportional to the dilutions in all cases. After 2½ days of incubation the diameters of the plaques varied between 0.5 mm in the case of influenza virus A2 and 2.5 mm for FPV and NDV. The plaques had either sharp or irregular margins. The plaques of the equine influenza virus strain became visible after 4 to 5 days. Cultures infected with the 2 avian reoviruses produced large and small plaques (Fig. 3a—d). The genetic stability of these characteristics has not yet been tested.

Table 1. Replication of myxoviruses in CAM-cells and chick embryo fibroblasts cultivated *in vitro*

Virus strains	Virus grown in			
	CAM cells		chick embryo fibroblasts	
	pfu/ml of medium	hemagglutination titers of medium	pfu/ml of medium	hemagglutination titers of medium
A <sub>0</sub> (PR 8, H <sub>0</sub> N <sub>1</sub> )	2 × 10 <sup>7</sup>	2 <sup>-7</sup>	—	2 <sup>-7</sup>
A <sub>1</sub> (FM 1, H <sub>1</sub> N <sub>1</sub> )	1 × 10 <sup>7</sup>	2 <sup>-7</sup>	—	2 <sup>-4</sup>
A <sub>2</sub> (Sing., H <sub>2</sub> N <sub>2</sub> )	6 × 10 <sup>6</sup>	2 <sup>-7</sup>	—	2 <sup>-5</sup>
A <sub>3</sub> (X 31, H <sub>3</sub> N <sub>2</sub> )	8 × 10 <sup>6</sup>	2 <sup>-9</sup>	—	2 <sup>-7</sup>
A/Swine (H <sub>sw1</sub> N <sub>1</sub> )	2 × 10 <sup>7</sup>	2 <sup>-9</sup>	2 × 10 <sup>5</sup> <sup>a</sup>	2 <sup>-6</sup>
A/Equine 1 (Prague, H <sub>eq1</sub> N <sub>eq1</sub> )	2 × 10 <sup>5</sup>	2 <sup>-6</sup>	—	2 <sup>-3</sup>
N (H <sub>av2</sub> N <sub>eq1</sub> )	2 × 10 <sup>7</sup>	2 <sup>-11</sup>	—	2 <sup>-9</sup>
FPV (H <sub>av1</sub> N <sub>1</sub> )	3 × 10 <sup>8</sup>	2 <sup>-9</sup>	10 <sup>8</sup>	2 <sup>-8</sup>
NDV	2 × 10 <sup>10</sup>	2 <sup>-9</sup>	2 × 10 <sup>9</sup>	2 <sup>-4</sup>

<sup>a</sup> Fibroblasts prepared from 7-day-old chick embryos.

The plates were inoculated with 1 ml of infectious allantoic fluid. After an incubation period of 8 to 16 hrs the media were assayed for hemagglutinating activity and for infectivity by plaque tests on the same cell type used for growing the viruses, i.e. CAM cells or chick embryo fibroblasts. (—) No plaques visible.

Table 2. Plaques produced by avian reoviruses and by infectious bronchitis virus in different cell systems

Virus group	Virus	Number of plaques formed in:		
		CAM-cells	Chick embryo fibroblasts	Chick embryo kidney cells
		pfu/ml	pfu/ml	pfu/ml
Reovirus	Strain 2207	2 × 10 <sup>7</sup>	4 × 10 <sup>4</sup>	2 × 10 <sup>6</sup>
Reovirus	Strain Wi	2 × 10 <sup>7</sup>	2 × 10 <sup>3</sup>	2 × 10 <sup>5</sup>
Coronavirus	IBV	3 × 10 <sup>7</sup>	not done	2 × 10 <sup>5</sup>

Infectious allantoic fluid was serially diluted in PBS. 0.2 ml were inoculated onto monolayers of different cell types. Plaques were stained after an incubation period of 3 days with trypan blue. Chick embryo fibroblasts were prepared from embryos which were 7 days old. Cultivation of chick embryo kidney cells and plaque assays were done as described (Babiker and Rott, 1968).

Neutralization tests were carried out with a limited number of strains. Table 3 shows that CAM-cells represent a suitable indicator system for such neutralization tests.

### Discussion

A large variety of viruses have been propagated in the chorioallantoic membrane of the embryonated egg (Goodpasture *et al.*, 1931; Burnet *et al.*, 1934; Murphy *et al.*, 1952; Scott *et al.*, 1953; Bang, 1958; Brandt, 1958; Prince, 1958; Sweeny *et al.*, 1968). This commonly employed technique is less suitable, however,

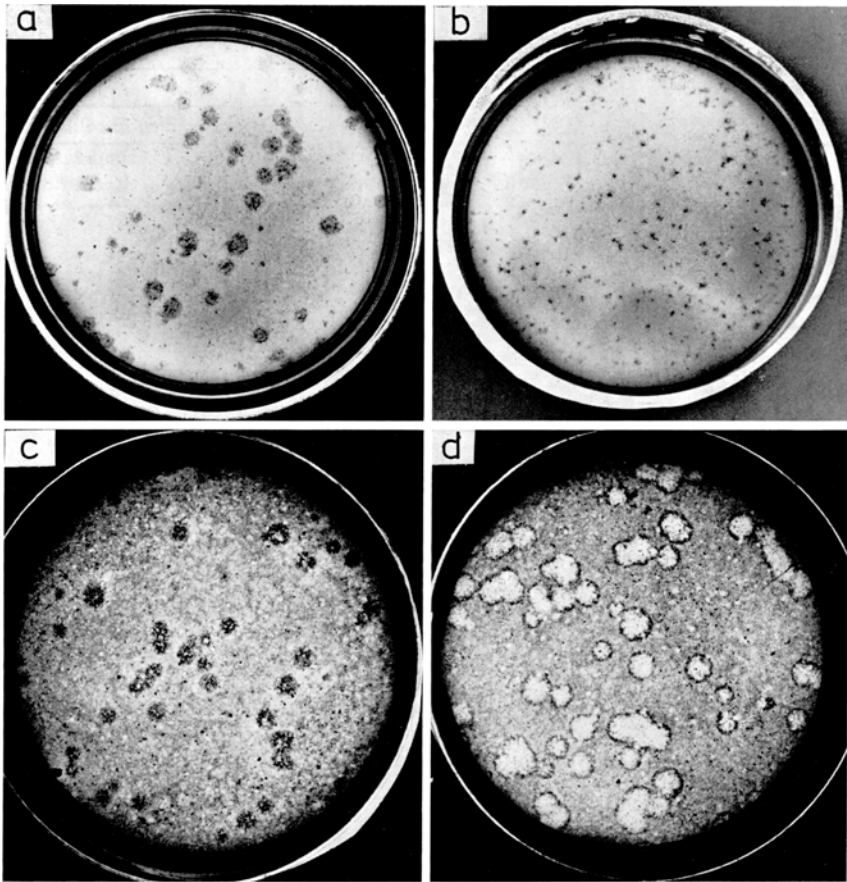


Fig. 3a—d. Plaque formation by different viruses in monolayers of CAM cells infected with (a) the avian reovirus strain Wi and (b) with the influenza virus X31. These cultures were stained with trypan blue after 2½ days. (c) Cells infected with the influenza virus strain PR8 and (d) with the avian influenza virus strain N. These cultures were stained with neutral red after 2½ days

Table 3. Neutralization of some viruses on CAM-cells

Virus group	Virus strain	Neutralization titer
Influenza	A <sub>0</sub> (PR 8, H <sub>0</sub> N <sub>1</sub> )	1:1024
Influenza	A <sub>1</sub> (FM1, H <sub>1</sub> N <sub>1</sub> )	1:1024
Influenza	A <sub>2</sub> (Sing., H <sub>2</sub> N <sub>2</sub> )	1:8192
Influenza	A <sub>3</sub> (X 31, H <sub>3</sub> N <sub>2</sub> )	1:512
Influenza	A/Swine (H <sub>sw1</sub> N <sub>1</sub> )	1:32764
Influenza	A/Equine 1 (Prague, H <sub>eq1</sub> N <sub>eq1</sub> )	1:1024
Influenza	N (H <sub>av2</sub> N <sub>eq1</sub> )	1:1024
Influenza	FPV (H <sub>av1</sub> N <sub>1</sub> )	1:800
Reovirus	Strain 2207	1:512
Reovirus	Strain Wi	1:1024

About 100 pfu were mixed with an equal volume of serially diluted antiserum, and 0,2 ml were inoculated per plate. Plaques were developed as described in the text. The last serum dilution which reduced the plaque number by 80% or more was taken as the end-point.

for a precise quantification of infectivity or biochemical and genetic studies. For such purposes it was desirable to grow these host cells *in vitro*. Removal of the mucin layer which is known to cover the whole CAM of chickens and especially the epithelioid cells (Conklin, 1968) was essential before the cells could be dispersed individually by the conventional trypsinization procedure.

Such cells form monolayers within a short time and support the growth of a variety of different viruses. Among the orthomyxoviruses representative strains of human and animal origin multiplied at high concentrations and produced plaques in these cells. The fact that all these strains replicate in the same cell system and form plaques could be used in this laboratory to produce recombinants among such strains. In many other instances these CAM-cells can replace chicken kidney cells, because NDV, avian reoviruses or infectious bronchitis virus replicate at least as efficiently in CAM-cells, which can be more readily supplied in large quantities.

The procedure outlined above is relatively simple to perform and not very costly. The starting materials are tissues which are usually discarded during the routine preparation of chick embryo fibroblasts.

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