Proliferation of Human Amnion Cells (FL Strain) in Submerged Culture.*† (23773)

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Ability of certain mammalian cells to proliferate in agitated fluid suspension (submerged culture) has been demonstrated utilizing a variety of equipment, nutritional media, and conditions(1-6). In our laboratories, strain HeLa (Gey) and L (Earle) have been cultured as discrete units in submerged culture in the spinner vessel, 25-500 ml(7); the New Brunswick fermentor, 1½-3 liters(8); and a 5 gallon stainless steel, water-jacketed, impeller agitated fermentor(9). The amnion cell as isolated from the human amniotic membrane has proved useful for isolation of certain viral agents(10). However, difficulties in obtaining a constant supply of satisfactory membranes are frequently encountered. Accordingly the establishment of a stable amnion cell line by Dr. Jorgen Fogh(11) presented the viral diagnostic laboratory with a procedure for procuring a uniform supply of this cell line. The present investigation presents data further simplifying the procedure demonstrating the ease of propagation of these cells in submerged culture thus making available at 2 or 3-day intervals fluid suspensions of these cells (1-1½ liters containing 1-1,500,000 cells/ml).

Methods. The strain of cells was supplied through the courtesy of Dr. Jorgen Fogh. The medium in our experiments consisted of 10% horse serum (heat inactivated at 56°C for 30 minutes) and 90% modified Eagle's medium.§ The equipment used consisted of the magnetic rotated Spinner system(7) and the standard impeller-agitated New Brunswick Fermentor (NBF)(8). Aeration of the culture was carried out by overlaying the fluid with compressed air which had previously been water-saturated in order to reduce rate of evaporation of the culture medium. The volume of fluid for suspension and growth of the cells was varied from 100 ml (Spinner) to as much as 3 liters (NBF). Agitation of the cultures was carried out at an impeller rate of 60-250 rpm depending on the volume. It has been observed that the smaller volumes do not demand the high rate of agitation that is required for the larger volumes. The cells in these experiments were initially cultured on the surface of Povitsky bottles in a medium consisting of 10% horse serum (heat inactivated) and 90% Eagle's medium. Appropriate antibiotics were incorporated. 0.25% trypsin was used to remove the cells from the glass surface, prior to establishing the submerged cultures. Trypsinization was allowed to take place during constant shaking and for a time (5 min. or less) sufficient to remove the cells from the glass surface. The cells were then washed twice with Eagle's medium and placed in the submerged culture medium. It has been demonstrated by Westfall *et al.*(12) that changes in concentrations of glutamine, isoleucine, leucine, methionine and arginine

§

L-amino acids	(g/l)
Arginine HCl	.021
Cystine	.012
Histidine HCl	.008
Isoleucine	.026
Leucine	.026
Lysine HCl	.026
Methionine	.008
Phenylalanine	.016
Threnine	.024
Tryptophane	.004
Tryosine	.018
Valine	.024
Glutamine	.300

Salts	(g/l)
NaCl	7.0
KCl	.4
$MgSO_4 \cdot 7H_2O$	.2
Na_2HPO_4	1.44

Vitamins	(mg/l)
Biotin	1
Choline	1
Folic acid	1
Nicotinamide	1
Pantothenic acid	1
Pyridoxal	1
Thiamin	1
Riboflavin	.1
Glucose	2.5 g/l
Phenol red	.01 "
Methocel (4000 CPS Dow Chem. Co. methylcellulose)	1 "
Serum	10%
Penicillin	100 units/ml
Streptomycin	50 μg/ml
Mycostatin	15 units/ml

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occurred following growth of HeLa cells in a medium containing known amounts of these substances. Accordingly, it seemed reasonable to consider these substrates as possible limiting factors in continual cell growth. In our laboratories, Thomas *et al.*(13) have demonstrated that periodic additions of one of these substrates, arginine, circumvents the necessity for frequent medium changes during the cultivation of L cells over an extended period of time. Therefore, on initiation of the Spinner cultures, during medium changes or upon addition of new medium, glutamine, leucine, isoleucine, arginine and methionine were added at concentrations of 2 to 10 times that normally present in Eagle's medium. Even at the higher concentrations no demonstrable toxicity was observed. Puck *et al.*(14) have demonstrated that 0.4 μg/ml of inositol increased the plating efficiency of various cell lines. It has also been reported that the concentration of inositol in blood plasma is in the range of 0.4-0.8 μg/ml(15). Accordingly, inositol was likewise added to the medium at a concentration of 0.4 μg/ml. Enumeration of the cell population was made by withdrawing 2-5 ml aliquots and making direct counts in a standard hemocytometer. Trypan blue staining of removed cells(7) was performed each day to determine the percentage of viable cells and thus the general state of the culture.

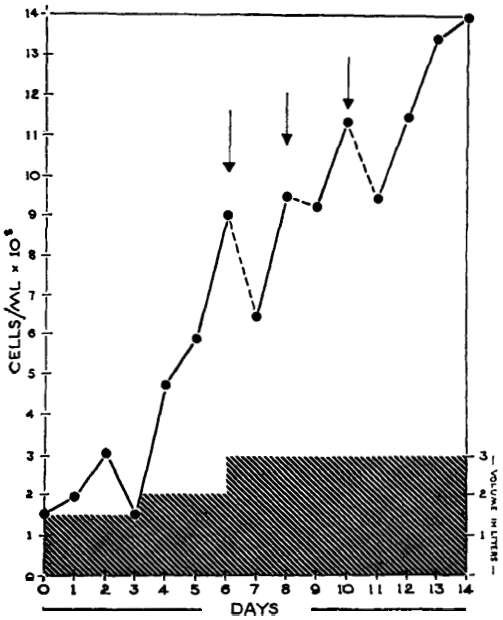

FIG. 1. Proliferation of F.L. strain in 3 1 New Brunswick fermentor. Arrows indicate time of cell harvest or medium change.

Results. The strain FL of human amnion cells readily proliferated in submerged culture, Fig. 1. Although the initial cell concentration was only 166,000 cells per ml, this population proved sufficient for initiating submerged growth. The cell multiplication rate approached a logarithmic growth curve with generation times of 25 to 36 hours (Fig. 1). Continuous cultivation in the submerged state for periods up to 1-2 months was readily achieved. At the points indicated in Fig. 1, cells were harvested from the stock tank (NBF) and placed in small Spinners, roller tubes or Povitsky bottles. (The cells showed no tendency to stick to the walls of the spinner-type culture vessel.) Greater than 98% of the total cell population remained viable as determined by the trypan blue staining procedure. The cells harvested at the indicated times grew well on the glass surfaces of the roller tubes or bottles, forming a typical monolayer sheet of cells in two days. The rapidity of the formation of the monolayer was directly related to the numbers of cells planted. Inoculation of glass monolayer or fluid suspension cultures with type 4 adenovirus demonstrated that these cells, as propagated, retained their susceptibility to this virus.

Summary. A method is described for propagation of human amnion cells (Strain FL) in Spinner-type culture vessels of volumes up to 3 liter capacity. Under these conditions, it is possible to maintain a stock culture which can supply cells for growth in roller tubes and Povitsky bottles without the necessity of a trypsinization procedure. The elimination of this dispersing procedure saves considerable time in handling of the cells. These cells after being propagated in submerged culture retain their susceptibility to type 4 adenovirus.‖

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Comparative Susceptibility of Cell Cultures to Vaccinia Virus: Application to the Standardization of Smallpox Vaccine. (23774)

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Although the vaccine employed for immunization of man against smallpox is prepared from bovine or avian tissues, the assay method generally used for determining vaccine potency has employed still another species, the rabbit(1). Because considerable natural resistance and variable sensitivity are encountered in the routine performance of vaccine potency assays in rabbit epithelium, in recent years titration of vaccinia in the embryonated egg has supplemented and in some cases supplanted the rabbit dermal techniques(2,3). Whether based upon pock counts or lethal dose determinations the chorioallantoic method appears simpler, cheaper and more reliable than the rabbit assay(4a,b,c). The growth of human, simian, and bovine cells in tissue culture and the marked cytopathic effects produced by vaccinia virus in all mammalian cells studied offers still another means of testing smallpox vaccine and the attractive possibility that this biologic could be simultaneously assayed in cell systems homologous to the source and to the primate recipient. In addition plaque techniques may permit the use of more quantitative procedures in the manufacture and standardization of this biologic.

The following report is concerned with the susceptibility of several tissue cultures to vaccinia virus derived from bovine embryonic cells, calf lymph or embryonated eggs. The high susceptibility of monkey kidney monolayers to vaccinia virus has been utilized for comparative titrations of this agent by roller tube and plaque methods.

Materials and methods. Vaccinia seed virus was obtained as the National Institutes of Health Calf Lymph Reference Standard, Lot 1b, a lyophilized preparation of dermal pulp. Serial passages of this strain were initiated in various cell lines by inoculating 2 or more cultures of each type. When all the cells in the culture showed cytopathic changes, the supernatant fluids from one type were pooled and 0.1 ml of the pool passed to fresh tubes of the same tissue culture. The remaining pooled fluids from each passage were stored at −20°C until used. Certain of the calf lymph and egg vaccines were generously supplied by Cutter Laboratories, Lederle Laboratories, Merck, Sharp and Dohme, National Drug Co., Parke, Davis and Co., Wyeth Laboratories, Inc., and the New York City Board of Health. *Titrations* in rabbits (weighing 3-4 kg) were performed in two