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Biological Synthesis of a Growth Factor for Mammalian Cells in Tissue Culture.* (25636)

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The search for simplified or chemically defined media has long occupied the efforts of those interested in growth of mammalian cells *in vitro*. Much of the recent reported work in this area has been done with the L strain of mouse skin fibroblasts isolated by Earle(1). Merchant and Rahn(2), Kuchler and Merchant (unpublished), and Waymouth(3) have shown the effects of peptone components on growth of L strain fibroblasts, and Gerarde and Jones(4) reported that bacto-peptone substituted for dialyzed embryo extract promoted collagen formation in chick embryo lung cultures. The chief difficulty encountered in preparation of defined media is that of replacing the serum which is usually present to the extent of 10-40% of volume. Lieberman and Ove(5) reported that a protein factor isolated from bovine serum stimulated growth *in vitro* of human appendix cells for short periods of time in presence of peptone-albumin, but in absence of whole serum. Recent experiments by Kutsky(6) have shown that a nucleoprotein isolated from embryo extract stimulated growth of chick embryo heart fibroblasts in cell cultures. Human cell culture lines have not as yet been adapted to growth in serum-free media, either chemically defined or containing peptone as a serum replacement. Preliminary experiments in our laboratory have shown that a stable cell line of mouse lung fibroblasts adapted to a serum-free medium by Pumper(7) release into the medium proteinaceous substance(s) capable of replacing the serum requirement of a human liver cell line isolated by Chang(8). Human liver cells were incapable of growing on the peptone-containing medium unless serum or protein from mouse lung cell growth fluid was added. Following this initial finding experiments were designed to separate the material responsible for stimulation of human liver cells from the dialyzable components of the growth fluid, and to study the physiological activity of the unpurified factor.

Methods. A. *Cell lines.* 1. Mouse lung cells adapted to a serum-free medium(7): Mouse lung cells were grown in milk dilution bottles on a medium consisting of 99% medium 199, 1 per cent Difco Bacto-peptone, 100 mg % dextrose, and 50 units each of penicillin and streptomycin per ml. The peptone was prepared in a 10% solution, and

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sterilized by autoclaving for exactly 10 minutes at 10 lb pressure. The pH was then adjusted to 7.2 with sodium bicarbonate. The cells were passed every 6-10 days by scraping them free from the glass with a bent tip pipette. The cell suspension was centrifuged and resuspended in complete medium to contain 3.0 × 10^5 cells per ml (hemocytometer count). These are now beyond their 104th serum-free passage. 2. Human liver cells: This cell line was isolated from normal human embryonic tissue by Chang(8). The cells were routinely grown on 90 per cent medium 199, 10 per cent horse serum, and penicillin-streptomycin (50 units each per ml), in T-60 Earle flasks. A normal inoculum consisted of 2.5 × 10^5 cells per ml, and the cells were passed every 6-7 days.

Results. Dialysis and lyophilization of growth fluid. As a preliminary step in separating the material responsible for the stimulation of the liver cells from salts and other dialyzable substances, the pooled growth fluid from serum-free mouse lung cells was dialyzed against running distilled water at 4°C for 72 hours, lyophilized, and stored at 4°C. An equal volume of uninoculated complete medium was treated in an identical fashion. The dry weight yield from uninoculated complete media was 1.8 mg/ml, representing the non-dialyzable portion of peptone + medium 199. The growth fluid, which was collected and pooled after maximum cell growth (6-10 days), gave a non-dialyzable fraction yield of 2.44 mg per ml. Both of these results represent mean values for 7 runs carried out on an original volume of 200 ml of fluid.

TABLE I. Effect of Heat on Stimulatory Activity of Material from Growth Fluid for Human Liver Cells.*

Added to medium 199	Treatment	Response (outgrowth) of liver cells after 24 hr
None	None	—
10% horse serum	"	+++
10% undialyzed fluid	56°C 0-45 min.	+++
Idem	" 60	—
10% dialyzed fluid	" 0-45	+++
Idem	" 60	—
Lyophilized material†	" 0-30	+++
Idem	" 45	++
"	" 60	—
"	100°C 5	—

* Washed human liver cells resuspended in medium 199 and used in a conc. of 1.0 × 10^5/ml.

† .5 mg/ml.

+++, max outgrowth compared to serum-containing cells. ++, poor outgrowth. —, absence of growth.

TABLE II. Growth Promoting Activity of Lyophilized Material from Serum-Free Mouse Lung Cell Growth Fluid for Human Liver Cells.*

Sample	Conc. lyophilized material, mg/ml	Response (outgrowth) of liver cells after 24 hr	Cell count after 48 hr, cells/ml × 10^5
Medium 199 only	.0	—	1.1
10% horse serum	.0	+++	4.0
Lyophilized material	.006	—	1.0
	.0125	—	1.8
	.25	++	2.0
	.5	+++	2.8
	1.0	+++	3.2
	2.0	+++	3.0
	2.5	+++	3.1

* Initial liver cell count = 1.0 × 10^5/ml.

+++, max outgrowth compared to serum-containing cells. ++, poor outgrowth. —, absence of growth.

Effect of heat on material from growth fluid. The effect of heat on the growth promoting activity of the material from growth fluid was determined, and the results are shown in Table I. It was shown that the substance responsible for stimulation of human liver cells in the absence of serum protein was stable to heat at 56°C for 45 minutes but rapidly lost the ability to support growth after this period. The material was coagulated at 100°C, and no stimulatory activity was detectable in the coagulum or supernatant fluid from this material. It was also shown that the stimulatory material from growth fluid was non-dialyzable and stable to lyophilization.

Stimulatory activity of lyophilized material from serum-free cell growth fluid. The stimulatory activity of the lyophilized materials is shown in Table II, which represents a typical experiment. The dried material was dissolved in balanced salt solution and varying concentrations were tested for physiological activity for human liver cells. Prior to stimulation experiments the liver cells were

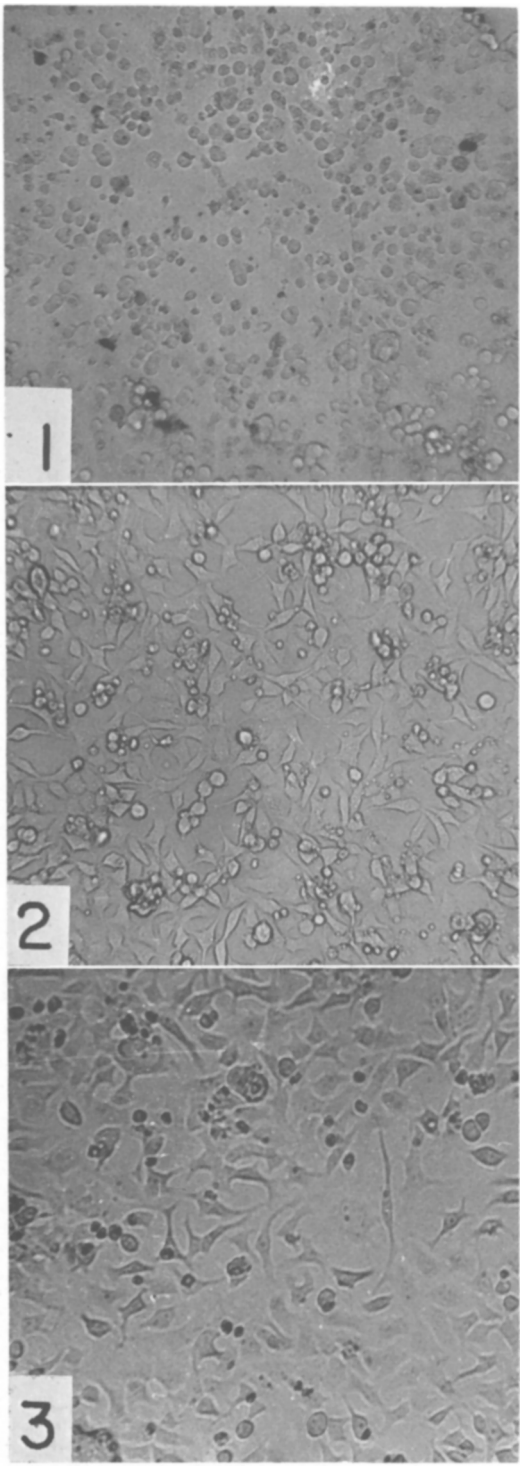

FIG. 1. 1. Culture of human liver cells grown on medium 199 only. × 100. 2. Normal culture of human liver cells grown on medium 199 + 10% horse serum. × 100. 3. Culture of human liver cells grown on medium 199 + lyophilized material from serum-free mouse lung cell growth fluid (conc. 0.5 mg/ml). × 100.

washed 3 times in balanced salt solution, once in medium 199, and counted with a hemocytometer. The stimulation was carried out in T-30 flasks, each of which contained medium 199, the sample material at varying concentrations, and 1 × 10^5 cells per ml. Observations for growth were made after 24 and 48 hours, and the cells trypsinized and counted after the 48 hour period. The results (Table II) show that the active substance present in the lyophilized material can replace serum protein in human liver cell cultures at least for short periods of time. It is further indicated that there is a graded response to the stimulatory factor. A series of photographs showing liver cell response to growth factor is shown in Fig. 1.

Discussion. This phenomenon was first observed when human liver and mouse lung cells were experimentally cultured on serum-free medium in the same flask. The human cells were able to grow very well as long as the serum-free mouse lung culture was present, but could not be propagated in its absence. Further attempts to grow human liver cells on a medium consisting of bactopeptone and constituents of medium 199 were unsuccessful. However, as shown in these studies mouse lung cells adapted to a serum-free medium (7) appear to synthesize a protein-containing substance that can be used for the growth of human liver cells in the absence of serum or its derivatives. Response of the liver cells to this factor appears to be quite similar to that obtained when 10% serum is added to the medium. There is a graded response proportional to concentration of material added.

Experiments are underway to purify and to determine the chemical nature and mechanism of action of the material produced in growth fluid of the serum-free mouse lung cell cultures.

Summary. A non-dialyzable substance was recovered in crude form from growth fluid of mouse lung fibroblasts adapted to a serum-free medium. This substance was capable of replacing the serum protein normally required

for human liver cell growth. The substance in the material from mouse lung cell growth fluid is heat labile. The crude material was heat coagulable with loss of growth promoting activity for human liver cells. A graded dose response of growth of liver cells was obtained.

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Centrifugation of Rickettsiae and Viruses Onto Cells and Its Effect on Infection. (25637)

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Chick embryo entodermal cells have been extensively used in investigations of viruses of the psittacosis group and rickettsiae(1-4). During attempts to improve and simplify the basic procedure, it was found that centrifugation of the infectious agents directly onto the cells greatly enhanced infection. Preliminary results also indicated that the centrifugation technic might be profitably applied to other host cells. These studies were prompted by the report by Macdougald *et al.*(5) that chick embryo explants can withstand exceedingly high centrifugal forces and by the studies by Gey *et al.*(6) who effectively sedimented eastern equine encephalomyelitis virus onto HeLa cells.

Materials and methods. Microorganisms. The following strains were used: *Rickettsia prowazekii* (Madrid E), *Coxiella burnetii* (California), psittacosis virus (6 BC), and trachoma virus (TW 29, isolated in the laboratory of Dr. J. T. Grayston, Naval Med. Research Unit No. 2, Taiwan). Pools of these microorganisms were prepared from infected yolk sacs and diluted in Bovarnick's isotonic sucrose solution(7). Purified suspensions of typhus rickettsiae and trachoma virus were prepared by minor modifications of commonly used procedures(8,9) which included the following steps: 1. Treatment with trypsin ("Tryptar" Armour), 0.25% final dilution, for 1 hour at room temperature; 2. Centrifugation at 2,500 g for 45 minutes at 2°C in an angle centrifuge (Spinco); 3. Suspension of pellet in sucrose solution containing 0.6% bovine plasma albumin. The suspension was cleared with an amount of celite (Johns Manville) corresponding to 10% of weight of the yolk sacs and by centrifugation in an horizontal centrifuge at 500 g for 10 minutes at room temperature. The slightly opalescent supernatant was saved and used directly or subjected to step 4, centrifugation at 11,000 g for 20 minutes and suspension of pellet in tissue culture medium. Q fever rickettsiae were partially purified by a somewhat simpler procedure, while psittacosis virus was used as a crude suspension. All 4 agents were titrated in eggs, viruses by the routine serial 10-fold dilution method, rickettsiae by the single dilution method as described previously (9,10), or as explained in a later section on *C. burnetii*. *Entodermal cell cultures.* Explants were obtained from the avascular membranes of the 4-day-embryo as described previously (1-3). When the experiments did not require microscopic observation, explants were placed in standard round-bottomed 16 × 125 mm screw-cap tubes. The nutrient, 0.6 ml per tube, consisted of 20% rooster serum diluted in Hanks'(11) balanced salt solution. Tubes were incubated at 35°C in an upright position