Cultivation of Human Epithelial Cells in Tissue Culture.* (22271)

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Several investigators have studied serial propagation of normal cells in tissue culture, particularly in the last 3 years, partly because of need for a substitute for fresh monkey kidney cells in mass production of poliomyelitis vaccine. Many other uses can be envisaged. Chang(1,2) accomplished this and 2 cell lines established by him from presumably normal human tissues were received in this laboratory early in 1955. This communication describes our experience in serial subcultivation of those 2 cell lines to determine their adaptability to various laboratory technics or use in diagnostic and investigative studies. Data are presented on media requirements, growth in various culture vessels, preservation at reduced temperatures, lyophilization, adaptation to growth in nonhuman sera, and treatment of contaminated cultures. The use of these cells for propagation of various viruses will be presented later.

Materials. Cell lines. Human conjunctiva had been originally removed from the bulbar conjunctiva of a child at strabismus operation, Feb. 17, 1954. The tissue was then considered nonmalignant. When received in this laboratory Feb. 3, 1955 it was in the 37th serial tissue culture passage. The human kidney had been removed from a hydrocephalic child at the time of subarachnoid ureteral anastomosis on 5-12-54. The kidney was then considered normal on gross examination. When received in this laboratory on 2-3-54, the cell line was in the 25th serial tissue culture passage. *Media.* Human, horse, calf, monkey, rabbit, and chicken sera were used in these experiments. Solution 199 (3) and Hank's balanced salt solution were prepared in the usual way. Eagle's medium (EM)(4) was prepared in 1x concentration and neutralized with 4.4% sodium bicarbonate prior to use. Soybean trypsin inhibitor was prepared in a 1% stock solution in Hank's balanced salt solution. Trypsin (Difco 1:250) was prepared in a 0.125% solution in phosphate buffer solution (pH 7.6). The above solutions were sterilized by sintered glass filtration. Chick embryo extract was prepared fresh weekly from 9 day viable embryos. The standard medium for cultivation of cells was Chang's basic medium (CM), *i.e.*, 20% human serum, 5% chick embryo extract, 75% balanced salt solution, and 0.001% crystalline soybean trypsin inhibitor. Antibiotics were added to a final concentration of 100 units per ml of penicillin, 100 μg of streptomycin, and 25 μg of nystatin.

Results. The 2 cell strains received were cultivated in 3 basic media to which were added varying percentages of human or animal sera. No difficulty was encountered in growing these cells. To evaluate quantitatively ability of these media and sera to support proliferation of the 2 cell strains, the cell count method described by investigators (2,5-8) was modified to fit our needs, *viz.*, preparation of standard cell suspension for inoculation by trypsinizing the cells growing in a milk dilution bottle culture. Total viable cells were enumerated by inclusion of 1:50000 neutral red in the trypsin, dispersing cells by gentle agitation and counting in a hemocytometer. Trypsin was removed after centrifugation at 1000 rpm for 10 minutes in International No. 1 centrifuge. The sedimented cells were resuspended in balanced salt solution and homogenously dispersed by a magnetic stirrer. Replicate roller tubes were inoculated with equal number of cells contained in 0.25 ml of suspension using automatic syringe and 4 tube cultures were fed with each medium. Replicate cultures were incubated at 37°C and refed with the same medium after 4 days. After 7 days incubation cells from replicate tubes were removed by trypsin containing 1:50000 neutral red,

* Aided by grant from National Foundation for Infantile Paralysis.

TABLE I. Effect of Animal Sera on Proliferation of Human Conjunctiva Cells Cultivated in Chang's Medium plus 20% Serum.

Serum	Avg No. cells per tube × 10^3	Proliferation index (7 days)
Horse 1	0*	0
2	0†	0
3	49	7.4
4	41	6.2
Calf 1	47	7.1
Rabbit 1	6.7	1.0
2	17.2	2.6
Sheep 1	14	2.1
Chick	21	3.1
Control (human #20)	77	11.6

* Inoculum/tube = 6.6 × 10^3 cells of Passage C_{55}.
† 0 indicates that all original cells inoculated into tubes were degenerated or lysed.

pooled, dispersed, and enumerated. Two separate cell counts were done for each pool of 4 cultures. A standard human serum was included in each test for comparison. Human sera from 38 separate donors were employed. Because of Chang's early experience (1) that 1 out of 5 human sera were toxic for conjunctiva cells and 2 out of 5 supported multiplication slowly, great pains were taken by us to collect these various sera carefully and in large quantity so that if a good serum was obtained enough would be on hand to carry the cells for a number of passages.

Only 2 of the 38 sera proved to be toxic for the tissue culture cells; both were toxic to both conjunctiva and kidney cells. Toxicity consisted of rounding and clumping of cells. All other sera were non-toxic and maintained both cell strains in normal morphologic state of clear epithelial-like sheets.

Sera from different donors varied greatly in ability to support cellular multiplication. The proliferation index (number of cells at end of 7 days ÷ number of cells inoculated) varied from 5.4 to 9 for conjunctiva and from 2.3 to 18.2 for kidney cells when different human sera were employed in Chang's medium. Sera from various animals varied in ability to support multiplication of conjunctiva cells when substituted for human sera in CM (Table I). Horse and calf sera seemed superior to rabbit, sheep, and chick sera tested but were not as good as human serum control. Two of the 4 horse sera tested were toxic.

Multiplication of conjunctiva and kidney cells when cultivated in CM, EM, and 199 media with added human, horse, or calf serum in 10 and 20% concentrations is shown in Tables II and III. These data indicate that both cell types multiplied faster in media with 20% serum than with 10% serum. Conjunctiva cells multiplied fastest in solution 199, CM and EM in that order. EM with human serum supported good growth but with horse or calf serum it was clearly inferior. Kidney cells responded in a similar manner. Beginning June 1955 efforts were made to adapt both cell lines to media containing horse and calf serum and no human serum. The adaptation was easily accomplished and conjunctiva have been carried since 6-24-55 for 18 serial passages from C_{50} to C_{68} in both 20% horse or calf serum in EM and 199 solution. Kidney cells have been serially subcultivated

TABLE II. Effect of Serum Concentration on Proliferation of Human Conjunctiva Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3 10% serum	20% serum	Proliferation index (7 days) 10%	20%
Chang's + human serum (21)	42*	54	7.0	9.
Eagle's human (21)	31	55	5.1	9.
" horse	10	26	1.6	4.3
" calf	12	11	2.0	1.8
199 horse	16	66	2.6	11.
199 calf	27	66	4.5	11.

* Inoculum/tube = 6 × 10^3 cells of Passage C_{56}.

TABLE III. Effect of Serum Concentration on Proliferation of Human Kidney Cells Cultivated in Chang's, Eagle's, or 199 Medium with Added Human, Horse or Calf Serum.

Media	Avg No. cells per tube × 10^3 10% serum	20% serum	Proliferation index (7 days) 10%	20%
Chang's + human serum (23)	13*	60	2.6	12
Eagle's human (23)	7	16	1.4	3.2
" horse	1.4	25	.28	5.0
" calf	1.1	4.4	.22	.88
199 horse	20	42	4.0	8.4
199 calf	24	64	4.8	12.8

* Inoculum/tube = 5 × 10^3 cells of Passage K_{48}.

TABLE IV. Total Cell Yield in Various Culture Vessels Inoculated with Either Human Conjunctiva or Kidney Cells.

Vessel (vol, ml)	Vol of media (ml)	Range of cell population after 7 days cultivation (human or horse serum) (× 1000)
Roller tube (18)	1	26 to 182
Milk dilution bottle (230)	10	250 to 3150
Blake bottle (1000)	50	1600 to 5800
Povitsky bottle (5000)	250-500	10000 to 30733

in these 4 media since 7-15-55 for 14 serial passages from K_{41} to K_{55}.

The 2 cell lines have been cultivated in microscopic glass chambers and hanging drop preparation for direct microscopy; roller tubes, milk dilution bottles, Blake bottles, and Povitsky bottles. In each type of culture vessel the range of cell population after approximately 7 days cultivation is shown in Table IV. Both types of tissue may be maintained in good morphologic condition for 2-3 weeks at 37°C by refeeding cultures twice weekly. For maintenance of conjunctiva cell cultures at a reduced metabolic rate they have been stored at 32°C, at 5°C, and at −70°C. Kidney cell cultures have been maintained at 32°C and 5°C. At 32° C cultures were stored with the usual volume of nutrient media and refed once weekly. At this temperature cultures maintained their usual morphologic state for 4-5 weeks. At 5°C they were stored with only a thin layer of nutrient media for 2 months. Conjunctiva roller tube cultures were quick frozen in acetone-CO_2 bath after removal of supernatant fluid, stored 2 days and revived upon feeding and incubation at 37°C. Eleven such conjunctiva tubes were lyophilized and stored at room temperature for one day. When they were refed and incubated at 37°C for 5 weeks with medium change once a week, 3 of the 11 cultures revived and formed sheets of new cells.

Centrifugally sedimented conjunctiva cells without added fluid were alternately quick frozen and thawed 10 times. When tested for viability, 1 of 4 cultures grew a sheet of cells in 7 days. When kidney cells were suspended in EM plus 20% horse serum in concentration of 1000000 cells/ml and then frozen and thawed 10 times, no viable cells were noted upon subsequent cultivation.

Non-viable cell extracts of both conjunctiva and kidney cells were prepared by this latter method. These were each injected into monkey kidney tissue cultures, 10 embryonated eggs, 4 rabbits, 4 guinea pigs and 10 mice to note presence of herpes virus simiae, lymphocytic choriomeningitis virus, tubercle bacilli, bacteria or any orphan virus which would manifest itself in these tests. No agents were found in either cell line by any of these tests.

Contamination of a batch of monkey tissue cultures is disappointing but economically not too serious because they can be discarded and duplicated within a week by setting up new tubes with fresh monkey kidney. However, bacterial or mold contamination is a severe threat to an established strain of cells which may have taken months to establish in serial tissue culture. To overcome this, we have applied the principles used in clinical medicine. The tissue culture fluid is streaked on agar plates and antibiotic discs added. The antibiotic of choice is added to tissue culture medium for several passages. Cultures contaminated with molds or yeasts have been successfully treated by adding 50 μg of nystatin for several passages.

To note the virus yield from poliomyelitis stool specimens inoculated into tissue cultures of conjunctiva cells as compared to tissue cultures of monkey kidney cells, 2 stool specimens from poliomyelitis patients were inoculated into 3 roller tube cultures each of conjunctiva and monkey kidney. Cytopathogenicity occurred after 1 day in monkey kidney cultures and after 2 days in conjunctiva cultures. After 5 days, supernates from each cell type were pooled and typed with specific antisera and titrated in monkey kidney cells to determine the virus titer in the fluids. The poliovirus isolated was Type I in stool A and the virus titer in the 5-day supernate from monkey kidney cells was $10^{6.33}$ per ml as compared to $10^{6.41}$ per ml from conjunc-

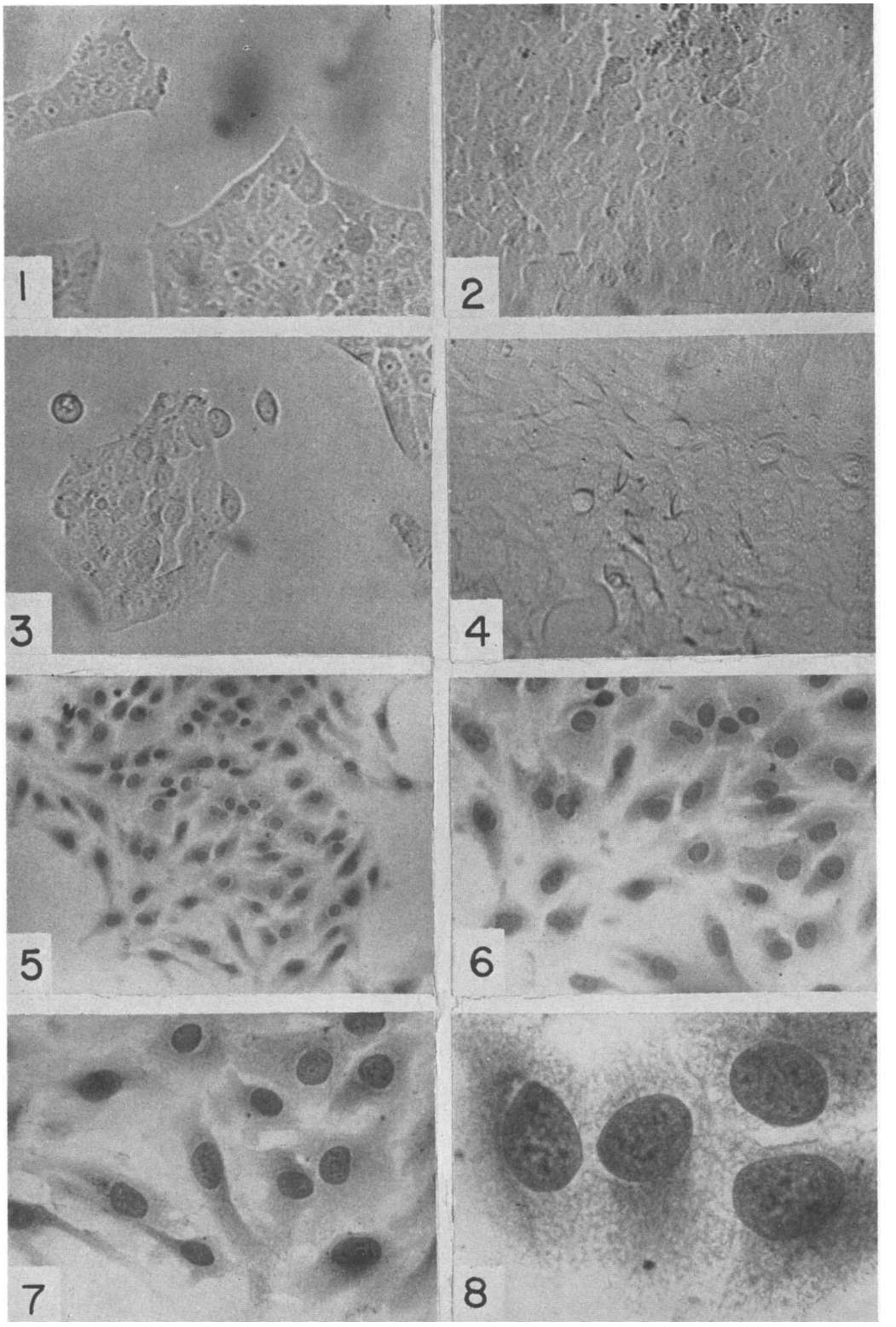

Plate 1

FIG. 1. Conjunctiva cells, 2 days after subcultivation showing islands of flat monolayer epithelial cells. Unstained. × 220.

FIG. 2. Conjunctiva cells, 10 days after subcultivation showing thick sheet of cells with some clumping. Unstained. × 190.

FIG. 3. Kidney cells, 2 days after subcultivation showing islands of cells. Unstained. × 220.

FIG. 4. Kidney cells, 7 days after subcultivation showing thick sheet of cells. Individual cell borders are indistinct because of light diffraction by underlying cells. Unstained. × 190.

FIG. 5-8. Conjunctiva cells, 2 days after subcultivation, showing cellular and nuclear detail. Magnification × 100, 250, 400, and 1000, respectively. Osmic acid fixation, toluidine blue stain.

tiva cells. The virus isolated was Type II in stool B and the virus titer in the 5th day supernate from monkey kidney cells was $10^{6.71}$ per ml as compared to $10^{6.76}$ per ml from conjunctiva cells.

Histology. The morphologic appearance of these 2 cell lines is shown in Plate 1. Both produce large epithelial-like cells with a nuclear cytoplasmic ratio of about 1-6. The cells fit together in flat monolayer sheets in young cultures but form thick sheets, sworls, and thick clumps in older cultures. There is remarkable uniformity of cells although occasional abnormal mitotic figures are observed and rarely a binucleate cell. Polyploidy has been observed but its significance cannot be interpreted in the present state of knowledge of how cells behave in tissue culture. It is of interest that the microscopic appearance of these cells has not changed since the original publication by Chang(1).

Discussion. It is believed that these cell lines established by Chang represent the first human cells derived from normal tissues and successfully cultivated serially *in vitro* over a prolonged period of time. As such, they offer a research tool of great potential usefulness in study of virus diseases and other problems.

These cells can be grown in a variety of containers for microscopic studies or for mass production or chemical analysis. They thrive in media free from human serum and in relatively simple synthetic media. The toxicity of many human sera for these cells, observed by Chang during earlier subcultures, has not been observed by us, nor has it been observed by Chang in recent months. Presumably this is a reflection of a change in the cells, the nature of which is still quite obscure. The cells can be stored for prolonged periods at low temperature and some will survive freezing and lyophilization. Tests for presence of a resident virus in these cultures have been negative, and sublines have been maintained for many generations without any human serum added. This was done to prevent contaminating cultures with a hepatitis virus or other human pathogenic agent(9).

In very limited studies one of the cell lines appeared to be as sensitive as monkey kidney cells for the isolation of poliomyelitis viruses. If this proves to be true, they might be very useful for laboratory diagnosis. This is now being tested. Because of the ease of handling these cells and because they are of human origin, they should be useful in the search for a laboratory method to grow viruses which as yet have not been cultivated in a practical manner such as measles and German measles. Other diseases such as infectious mononucleosis, infectious polyneuritis, mesenteric adenitis and hepatitis may be explored with such cell lines.

Since the conjunctiva and kidney cells grow so well in tissue culture, one might ask whether they have also acquired the power of independent growth *in vivo*. Studies are under way using the technics of Greene(10) of implantation in the brain and anterior chamber of guinea pigs, and the technics of Toolan(11-14) and Moore(15) of inoculation of irradiated rats.

Summary. 1. Experience is described in the serial subcultivation of 2 human cell lines derived from conjunctiva and kidney and established in serial passage *in vitro* by Chang. 2. Thirty-eight human sera were compared in their ability to support multiplication of the 2 cell lines. Two of the sera were toxic to both cell lines. The remaining 36 sera were non-toxic but varied in their ability to support cellular multiplication. Horse and calf

sera were superior to rabbit, sheep, and chicken sera but inferior to the human serum control. 3. Comparison was made of the multiplication of the 2 cell lines when cultivated in Chang's, Eagle's, and 199 media with added human, horse, or calf serum in 10 and 20% concentration. 4. Conjunctiva cells have been serially subcultivated for 18 passages in media containing horse or calf serum but no human serum. Kidney cells have been carried for 14 passages in such media. 5. Conjunctiva and kidney cells survived for varying periods when stored at 32°C or 5°C. Some sedimented conjunctiva cells survived 10 freezing-thawing cycles and formed a sheet of cells when incubated at 37°C. 6. Three of 11 conjunctiva cultures survived lyophilization and revived when reincubated at 37°C for 5 weeks. No contaminating bacterium or virus could be detected in these cell lines by the injection of nonviable cell extracts into tissue cultures of monkey kidney, embryonated eggs, rabbits, guinea pigs, and mice. 7. A method is described for the treatment of contaminated tissue cultures. 8. The conjunctiva cells gave results comparable to monkey kidney cells when used for isolation of poliomyelitis virus from 2 stool specimens.

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Received January 20, 1956. P.S.E.B.M., 1956, v91.

Serial Biochemistry of Serum and Cerebrospinal Fluid in Amaurotic Family Idiocy (Tay-Sachs Disease).* (22272)

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Studies in Amaurotic Family Idiocy (AFI) have thus far been principally concerned with histologic examination and biochemical analysis of brain tissue. These latter investigations resulted in isolation of a new cerebroside-like substance called a "ganglioside" and differing from other cerebrosides by the presence of neuraminic acid(1-3). In contrast to other lipoidoses, it is generally assumed that there are no characteristic serum changes in AFI.

The present report is concerned with serial studies of the neuraminic acid, protein and lipid fractions in serum, and cerebrospinal fluid (CSF) proteins in a group of 7 children with infantile AFI, the diagnoses in most instances confirmed at autopsy. Similar biochemical studies of 2 children with Schilder's disease and gargoylism, serving as controls, were also carried out. Most children in this survey were admitted to this institution shortly after clinical inception of the disease and remained hospitalized until after death. The increased longevity of some patients with AFI in this study(4), afforded greater opportunity for carrying out such serial biochemical

* Supported by grant from U. S. Public Health Service dealing with Protein Studies in Chronic Diseases.