multiply in tissue culture without visible CPE. Pollard and Starr(7) also were unable to detect histopathologic changes in chicken embryo explants that supported multiplication of DHV. Apparent lack of CPE may be a reflection of a low number or percentage of infected cells rather than an indication of viral multiplication without CPE. Observed titers could conceivably have been produced by release of a few hundred infective virus particles from as few as 10 cells per ml, a number virtually undetectable among the far larger number of cells in each tube.

Summary. Duck hepatitis virus was passed serially 7 times through monolayer chicken embryo liver cell cultures. Multiplication of virus was demonstrated by assay in embryonated chicken eggs and by gel diffusion precipitin test. Microscopic examination of stained tissue culture cells failed to reveal CPE.

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Received December 9, 1960. P.S.E.B.M., 1961, v106.

Cultivation *in vitro* of Adult Human Skin and Oral Mucosa on Gelatin Film.* (26466)

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Successful cultivation of human skin *in vitro* has been reported by numerous investigators(1-8), the majority of whom employed the plasma clot technic with small fragments of skin obtained from either living donors, or from cadavers. Plasma clot may present certain disadvantages for immunological, nutritional(9) and viral studies(10). Evans and Earle(9) have used perforated cellophane as a substitute for plasma clot for growth of some tissues. It would appear, however, that perforated cellophane is not a satisfactory substrate for primary cultivation of adult human skin(4). Attempts in this laboratory to cultivate single specimens of adult human skin from 9 donors with perforated cellophane were unsuccessful. Puck *et al.*(7) have used the method of trypsinization for successful growth of cells from fresh adult skin and other human tissues. Excision of 20 to 50 mg of skin(11), however, is not always feasible when repeated fresh specimens, particularly from the same area, are desired for cultivation. Ehrmann and Gey have reported growth of a number of cell strains and fresh explants of human tissue on transparent collagen gels(12) and Enders(13) has

* These studies were supported by grants from Nat. Inst. Health, Divisions of Allergy and Infect. Dis. and Dental Research, U. S. P. H. S.

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observed growth of cells from human embryonic tissue to occur on absorbable gelatin film which is available commercially.‡

This report is concerned with observations on cellular outgrowth from single small fragments of adult human skin on gelatin film as compared to plasma clot technic. Similar studies were carried out with adult human oral mucosa which to our knowledge has not been cultivated *in vitro*. There is some question as to whether the appearance of epithelioid cells from fragments of adult human skin represents cell migration or multiplication(2, 5). No attempts were made to clarify this question and accordingly the term, "growth," as applied to these cells, is used with this reservation in mind.

Materials and methods. One hundred and seventy-nine specimens of human skin§ were obtained from 72 donors most of whom were personnel or students at Tufts University ranging in age from 19-63 years. Fragments were usually taken from the lateral surface of the upper part of the right arm which was washed with sterile saline and the point of a 25 gauge needle was inserted very superficially into the skin. This was used to gently lift and hold the skin while a #11 Bard-Parker blade was placed just beneath the needle point and the thin layer of skin quickly excised. Stained histologic sections of representative specimens revealed some dermis as well as epidermis (Fig. 1). The specimens weighing from 0.15-0.40 mg and measuring from 1-2 mm were placed in about 4 ml of saline A(7) for one to 2 minutes before setting up for culture. Sixty-nine oral mucosa specimens were taken from the lower labial region of 32 different donors. The area was dried with sterile gauze, the tissue was lifted gently with small blunt forceps, and a fragment excised with a #11 Bard-Parker blade. Mucosal sections were not weighed but were similar in size to the skin fragments.

Plasma clot technic. A drop of chicken plasma (Difco dehydrated) was added to a #1 coverslip (22 mm^2) in a 60 mm Petri dish and a single fragment of skin or oral mucosa was placed in the plasma. No attempt was made to orient either surface of the tissue to the glass. One to 2 drops of 50% chick embryo extract in Hanks' salt solution (W/V), adjusted to pH 7.0 with NaOH, was then added to induce clotting which occurred in from 5 to 20 minutes. Three ml of medium (described below) were placed in the culture dish, which was then incubated at 36-37°C in an atmosphere of approximately 5% CO_2 in air.

Gelatin film technic. Pieces of gelatin film (about 10-20 mm^2) were cut from a sheet (100 × 125 mm and 0.075 mm in thickness) and sterilized at 140°C for 4 hours. These were washed 3 times with Hanks' salt solution containing 0.05% $NaHCO_3$ to neutralize the acidity of the gelatin film. The skin fragment was placed in a sterile 60 mm Petri dish with the keratin surface facing the glass. The dermal portion could be recognized as a domed white surface in contrast to the darker and flatter corneum. The square of prepared gelatin film was placed over the specimen in contact with the dermal surface of the fragment. If this were reversed, no growth or poor growth occurred almost without exception.

Media. The media consisted of Eagle's basal solution supplemented with 20% human serum (EHu_{20} medium), 10% human serum and 5% calf serum (EC_5 Hu_{10} medium), and 15% calf serum (EC_{15} medium). Eagle's solution was modified by addition of inositol(14) and replacement of Earle's salt solution with Hanks containing 0.05% $NaHCO_3$. Human serum consisted of a pool obtained from 10-12 young adult donors collected under sterile conditions and stored in small aliquots at approximately −25°C. Stored serum, after thawing, was used either immediately or held at 5°C to 10°C and used as needed over a period of about 2-3 weeks. Calf serum pools were obtained from the blood of 30-40 calves slaughtered at a local abattoir and sterilized by filtration through a Seitz filter which was first washed with 800 ml of distilled water and 200 ml of serum pool. It was stored and used in the

‡ Gelfilm, Upjohn Co., Kalamazoo, Mich.

§ A few of these skin specimens and several specimens of oral mucosa were cultured by Dr. William Gibson as part of another study to be reported later.

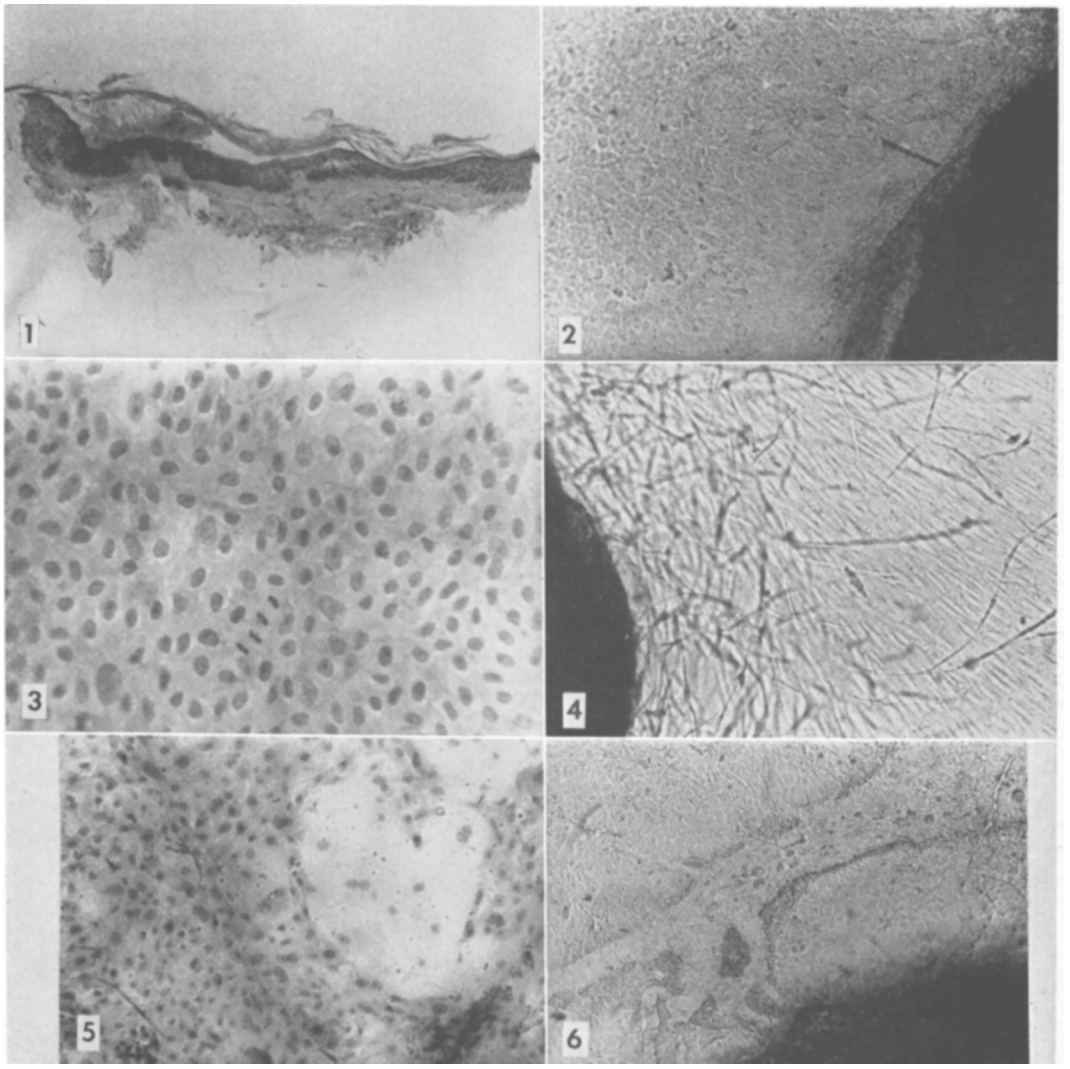

FIG. 1. Histologic section of skin fragment representative of specimens used for culture, showing epidermis and corium. Hematoxylin and Eosin. × 50.

FIG. 2. Skin fragment grown on gelatin film for 21 days showing epithelioid cell outgrowth. Unstained. × 50.

FIG. 3. Same as Fig. 2 after Hematoxylin and Eosin staining. × 215.

FIG. 4. Fibroblast-like cells growing from section of adult human skin cultured in plasma clot for 16 days. Note appearance of hyphae-like forms and typical compressed spindle forms in uniform sheath. Unstained. × 50.

FIG. 5. Outgrowth from adult human skin containing area of large pale cells with poorly stained cytoplasm bordered by characteristic epithelioid cells. Hematoxylin and Eosin. × 50.

FIG. 6. Epithelioid cells from adult oral mucosa. Eighteen-day-old culture. Hematoxylin and Eosin. × 50.

same manner as human serum. Soybean trypsin inhibitor(15,16) was added to all media to a final concentration of 0.05 mg per ml. Penicillin, streptomycin and mycostatin were added in final concentration of 100 units, 100 μg and 50 units per ml respectively. Initial pH of the media was 7.9 to 8.4. After 7 hours incubation under 5% CO_2 it dropped to 7.2-7.4 and remained more or less constant when tested on 3 successive days. Cultures were examined at 2 to 4 day intervals at which time fluid changes

were made. The majority of the cultures were held for 3-4 weeks in plasma clot and 4-6 weeks under gelatin film.

Staining of cultures. Cultures were washed for 15 minutes in 3 changes of saline A. Sections were then fixed in 10% formalin for 2 hours and stained with hematoxylin and eosin. Although the plasma clot took up the stain(s) and interfered with detail of underlying cells, nevertheless cells growing outside the clot were defined clearly. Staining of the gelatin film occurred which interfered similarly with examination of stained preparations(17). This difficulty was eliminated for the most part by removing the fragment and cell sheath with celloidin membrane(18). There was some staining in some areas of the celloidin membrane presumably due to adherence of a small amount of gelatin film material, but it did not interfere with observation of cellular detail.

Results. Similarities in growth characteristics of skin with plasma clot and on gelatin film. Primary outgrowth occurred in 76 of 77 specimens set up in plasma clot and cultured in EHu_{20} or EC_5 Hu_{10} medium and from 98 of 102 fragments cultured under gelatin film in the 3 media. Initial outgrowth by either technic in the 3 media usually consisted of a single zone of compact epithelial-like cells which appeared in 2-10 days in plasma clot and in 5-14 days in gelatin film cultures. Complete or almost complete zones of epithelioid cells were present on the average, in about 9 days in the plasma clot series and in about 13 days on gelatin film. The cells were small, compact, with relatively uniform nuclei (Fig. 2 and 3). Stained preparations revealed relatively few mitotic figures which is in accord with earlier observations by Hsu(5). In some cultures, filamentous-like processes occurred in the zone of polygonal cells and also cell forms which, morphologically, were neither epithelioid nor fibroblast-like. The latter included round forms which were seen within the 1st week in plasma clot and from the 2nd to 5th week in the gelatin film group. They did not seem to increase but disappeared eventually or were obscured by the epithelioid cells. Also, oval to pear shaped forms appeared during the 2nd or 3rd week, and, at about the same time, large, elongated and fusiform cells, often with protoplasmic processes. Degeneration occurred in the zone of epithelioid cells after initial outgrowth, usually between the 2nd and 3rd week in plasma clot and in the 3rd to 4th week on gelatin film.

Differences in growth characteristics of skin with plasma clot and on gelatin film. The most striking difference that was observed between plasma clot and gelatin film technics for cultivation of adult human skin was the development of fibroblast-like cells (Fig. 4) in the majority by the former, but not any by the latter method when EHu_{20} or EC_5Hu_{10} media were employed. However, 10 of 46 cultures which grew on gelatin film in EHu_{15} developed fibroblast-like cells, particularly in those cultures in which comparatively little epithelioid outgrowth occurred. One culture consisted of pure fibroblast-like cells which were first noted on the 14th day. These differences are summarized in Table I. There were also noted in about a fourth of the plasma clot cultures areas of larger polygonal cells in the zone of uniform epithelioid cells (Fig. 5) within 2 or 3 weeks and also in some preparations, usually within a week an amorphorous ring-like membrane

TABLE I. Relationship between Development of Epithelioid and Fibroblast-Like Cells from Adult Human Skin Cultured in Plasma Clot and under Gelatin Film.

Medium	Substrate	No. specimens cultured	No. with growth	No. with epithelioid growth	Time epithelioid-like cells appeared, days	No. with fibroblast-like growth	Time fibroblast-like cells appeared, days
EHu_{20} & EC_5 Hu_{10}	Plasma clot*	62	61	61	2-10	42	8-22
Idem	Gelatin film†	44	42	42	6-14	0	
EC_{15}	" "	48	46	45	5-14	10	10-25

* Plasma clot series cultivated for 3-4 wk. † Gelatin film series cultivated for 4-6 wk.

surrounding a portion, or all of the fragment. Large polygonal cells of this type were observed in only 2 of the 102 gelatin film cultures and the membrane structure was not seen in any of these preparations.

Outgrowth from fragments after subculture. Forty fragments of adult human skin were removed from the surrounding zones of cellular outgrowth and subcultured either in plasma clot or with gelatin film in the same medium employed for primary cultivation. Growth occurred from 23 of 26 fragments when subcultures were made from plasma clot to plasma clot, from plasma clot to gelatin film and from gelatin film to plasma clot. The outgrowth consisted only, or predominantly, of fibroblast-like cells from 20 of the subcultures, despite the fact that primary outgrowth was entirely or predominantly epithelioid at time of subculture (3-6 weeks). When subcultures were made with 14 fragments grown on gelatin film to the same substrate, outgrowth occurred from only 3 fragments (2 with fibroblast-like and 1 with epithelioid-like outgrowth).

Cultivation of adult oral mucosa. Twenty oral mucosa specimens from 14 donors were cultured in plasma clot with EHu_{20} or EC_5 Hu_{10} media and 49 from 21 donors on gelatin film with the 3 media. All of these cultures were held at least 3-4 weeks and a few as long as 10 weeks. Primary outgrowth occurred from 15 specimens in plasma clot and from 34 specimens cultured on gelatin film. The initial outgrowth, in all instances, was epithelioid and similar to that from human skin (Fig. 6). However, certain significant differences were noted. (1) Wider zones of epitheliod cells occurred in some cultures of oral mucosa than in any of the skin cultures, with no obvious degenerative changes even after 9 weeks. (2) Fibroblast-like cells developed from only 5 of 49 cultures which showed growth. These were seen in about 2 weeks in a plasma clot culture and from 5-8 weeks in 4 gelatin film cultures. (3) Attachment of fragments and subsequent epithelioid outgrowth occurred on glass equally as well as on gelatin film with glass-gelatin film interfaces.

Summary. Zones of epithelioid cells from single fragments of adult human skin and oral mucosa develop on gelatin film. Although the initial appearance of these cells was somewhat slower on this substrate, their subsequent migration or growth appeared to be comparable to that in plasma clot. Proliferation of fibroblast-like cells was observed in some skin cultures on gelatin film when the medium consisted of a modified Eagle's basal solution supplemented with 15% calf serum but not when supplemented with 20% human or 10% human and 5% calf serum. Cultural characteristics of adult human skin and oral mucosa were similar except that fibroblast-like cells were more rarely observed and extensive sheaths of epithelioid cells occurred on glass as well as on gelatin film from oral mucosa.

We wish to acknowledge the valuable suggestions and criticisms made by Dr. Bernard Appel, Dept. of Dermatology, Tufts Univ. School of Medicine.

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Received December 22, 1960. P.S.E.B.M., 1961, v106.

Cardiovascular Action of Adenosine and Other Nucleosides.* (26467)

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Several studies have been reported on the cardiovascular actions of adenosine and other purine and pyrimidine nucleosides(1-5), some of which exert a positive inotropic effect in isolated preparations(6-10). Similarly a negative chronotropic effect and a general vasodilator action have been found under a variety of experimental conditions(1,5,11). Due to the key biochemical role that some of these compounds may play in smooth and cardiac muscle energetics, it was desirable to test in the same preparation, *in vivo*, the inotropic, chronotropic and hypotensive activities of some of these compounds, to obtain quantitative data on specificity and potency.

Materials and methods. Mongrel dogs of either sex weighing from 7.8 to 14.2 kg were anesthetized with intraperitoneal injections of sodium pentobarbital. Blood pressures were measured through an Hg manometer. Myocardial contractility was measured with a Walton strain gauge arch(12) sutured on the left ventricular myocardium. Records of myocardial tension and ECG were obtained on a 2-channel Sanborn recorder. All injections were made intravenously into the jugular vein over a 10 minute period. Solutions were freshly prepared from the powdered compounds in 50 ml of normal saline. The list of compounds tested included: Adenosine, guanosine, inosine, cytidine, thymidine, uridine, adenine sulfate and guanine sulfate. All compounds were purchased from Nutritional Biochemicals, New York. Except as otherwise indicated, all animals were given 1 mg/kg of atropine at beginning of each experiment.

Results. The following compounds produced no detectable cardiovascular actions in the doses indicated: Adenine (2-10 mg/kg), guanine (2-10 mg/kg), guanosine (2-25 mg/kg), inosine (2-100 mg/kg), cytidine (2-5 mg/kg), thymidine (2-50 mg/kg), and uridine (2-50 mg/kg). These were tested in 2 to 4 animals each. In larger doses (25 mg/kg) cytidine produced moderate hypotension and negative chronotropic and inotropic effects, but such doses were considered too large to deserve further study.

Of all the compounds tested only adenosine exhibited cardiovascular actions of a sufficient magnitude and consistency to warrant further study.

Quantitative results on the inotropic, chronotropic and hypotensive effects of adenosine were obtained at several dose levels (0.1 to 2 mg/kg) from a total of 113 injections in 10 dogs (Fig. 1). Doses larger than 2 mg/kg, up to 25 mg/kg, produced no greater effects. In all instances recovery to control levels was attained within a few seconds (Fig. 2). Since in all of the above mentioned experiments the animals were fully atropinized, the observed actions of adenosine can not be classified as cholinergic in spite of the apparent similarity to the cardiovascular actions of acetylcholine. In these animals atropine was used to eliminate the reflex vagal effects on heart rate associated with hypotension.

Since there have been reports that atropine and vagotomy may abolish some of the cardiovascular actions of adenosine, experiments were designed to test this point using

* Supported by a grant from Massachusetts Heart Assn.

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