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Albert Joseph Banes

The Induced Differentiation of Mouse Ectoderm to Epidermis by Growth on Freeze-Thawed Dermis

By

Albert Joseph Banes

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Approved

Committee Chairman

Examining Committee

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Abstract

Previous investigators have grown or maintained adult or embryonic skin in tissue culture and on the chorio-allantoic membrane (CAM) of chickens. These studies have revealed two important factors involved in culturing skin: (1) the presence of an inducer which originates in the dermis and affects the phenotype of epithelial cells; and (2) the influence of the physical surface of the substrate on which epidermis is cultured.

The current investigation presents evidence that ectoderm from the limb buds of 10 to 12 day-old mouse embryos can form an epidermis when combined with dermis killed by alternate freezing and thawing, and cultured on the chorio-allantoic membrane of chick embryos.

The possible effects that the inducer, physical substrate, and experimental treatment have on the graft material are discussed.

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Introduction

Mesenchyme has been shown to play an essential role in the differentiation of epithelial tissue (3, 4, 9, 20, 21, 25, 26, 27, 31, 42, 43, 44, 45). Mesenchymal cells apparently pass a physiological cue to epithelial cells to effect some pre-determined phenotypic expression (3, 20, 21, 30, 31). The necessity for the direct apposition of these two tissues to achieve differentiation of the epithelium indicates a possible two factor relationship: 1) the passage of an inducer from mesenchyme to epithelium; and 2) the influence of the physical surface between mesenchyme and epithelium.

The chemical nature of the hypothetical inducer is not clear, however numerous studies substantiate the logic of pursuing research to determine what key molecules cause differentiation (3, 21, 27, 31).

Rawles (27) demonstrated that mid-dorsal epidermis of a 5 to 8-1/2 day-old Silver Campine chick embryo, which normally produces feathers, formed scales when placed over 12 day-old, embryonic, tarsometatarsal dermis from the same source in chorio-allantoic membrane (CAM)

grafts. Tarsometatarsal dermis taken from an embryo younger than 12 days did not induce scale formation in overlying, mid-dorsal epidermis. When epidermis from 8 to 8-1/2 day-old embryos was combined with tarsometatarsal dermis from 13 day-old embryos, feathers rather than scales developed in the epidermis. But when the same age epidermis (8 to 8-1/2 day) was combined with beak dermis, a normal, featherless, beak formed. A difference was found in regional intensity of inductive capacities, both in and between dermal tissues, and in the ontogenetic timing of dermal inductive capacity. Rawles concluded that the ectoderm acquired specific properties in reaction to inductive stimuli arising in the underlying mesoderm, that the reaction was mutual, and dependent on properties of both epidermis and dermis.

McLoughlin (21) found that epidermis isolated from five day-old chick embryos and grown on gizzard mesenchyme, proventriculus mesenchyme, or heart myoblasts resembled the epithelium that normally clothes the particular mesenchyme. He noted that when a cyst of epidermis collapsed in gizzard mesenchyme or heart myoblasts, and the epithelium piled up so that it was removed from

direct contact with the mesenchyme, it keratinized. McLoughlin interpreted the keratinization as a breakdown in mesenchymal influence which must act continuously to maintain the modified differentiation of the epidermis. He speculated that the characteristic effects of mesenchyme on epidermis were mediated by fibroblasts; more specifically, by the intercellular material between fibroblasts and epithelium, because the content of the material varied from tissue to tissue.

Slavkin et al. (31) showed that ribonucleic acid (RNA) was a component of the intercellular matrix interposed between epithelial and mesenchymal cells during embryonic rabbit odontogenesis. The isolated matrix, shown to originate from the two tissues by autoradiographic methods, enhanced differentiation of several different cell types. A subsequent report (30) indicated that the matrix was acellular, and consisted of membrane bound, electron-dense bodies that contained methylated, RNAase sensitive RNA.

Briggaman and Wheeler (3) found that adult, human epidermis could be maintained in tissue culture in recombinants with dermis that had been heated for one hour at 56 C, but degenerated in recombinants with dermis that

had been heated for four hours at 56 C. The heat stability of the dermal inducer (3) correlates with the properties of RNA (31), but a protein, or protein-nucleic acid complex, or some other molecule cannot be ruled out.

The nature of the physical substrate and its relationship to the epithelium is important in the inductive effect. Briggaman and Wheeler (3, 4), Dodson (9), and Karasek and Charlton (18) have grown or maintained epidermis on collagen gels, while Dodson, in addition, has used frozen-thawed dermis, heat-killed dermis, agar, alginate gels, fibrin clots, gelfilm, and Millipore filters (9). Of the latter substrates, collagen gel, frozen-thawed dermis, and Millipore filters supported maintenance of chick epidermis in tissue culture. Briggaman and Wheeler (3) in a more recent publication, have maintained human epidermis in tissue culture with preparations of tendon, tendon sheath, fascia, and powdered dermis. They also reported the failure to maintain epidermis on collagen gels when a medium of chemically defined components was used rather than a chick embryo extract - plasma clot fluid. Karasek and Charlton (18) however, have demonstrated the capacity of collagen gels to support the growth of postembryonic skin epithelial

cells using a defined medium.

Whole skin and epidermis alone have been grown in tissue culture and on the CAM. The purpose of the present investigation was to see if frozen-thawed dermis could cause differentiation into epidermis of ectoderm removed from the limb buds of 10 to 12 day-old mouse embryos. The problem was approached by culturing grafts of ectoderm combined with frozen-thawed dermis on the CAM of chick embryos and in tissue culture.

MATERIALS AND METHODS

All fluids were sterilized by filtration through a 0.22 u Millipore filter apparatus. Sterile, distilled water and sterile techniques were employed throughout the investigation.

1. Preparation of Biological and Physiological Fluids

a. Basic Culture Fluid

Minimum essential medium (MEM) (Flow Laboratories, Rockville, Md., cat. no. 1F-020) with Earle's salts, without sodium bicarbonate was supplemented with fetal calf serum (Flow cat. no. 4-055M and Gibco, Grand Island, N. Y., cat. no. 614-11) and used as culture fluid. Antibiotics were added to reduce microbial contamination (all values in units/ml or ug/ml of culture fluid: 200 units sodium penicillin G (Squibb, New York, N. Y. cat. no. 6737), 100 ug streptomycin sulfate (Lilly, Indianapolis, Ind., cat. no. 588), and 10 ug amphotericin B (Squibb, cat. no. 4373). Fetal calf serum, heat-inactivated at 56 C

for one hour, was used as 5, 10, and 20% of the culture fluid. Whole embryo juice prepared after the method of Rutter (43) was used in culture fluid in volumes from zero to 4%. Sodium bicarbonate was added to a final concentration of 0.75 mg/ml of culture fluid. Because a pH lower than 6.8 or greater than 7.8 has been shown to be contact inhibiting for cells in culture (6), the pH of the culture fluid in the present investigation was 7.6 in the freshly made fluid and after 24 hours in the CO_2 incubator dropped to 7.0 at which time the fluid was changed.

The various volumes of serum and embryo juice used had no apparent effect on the differentiation of ectoderm. Components of the basic culture fluid used throughout the course of the investigation appear in table 1. The culture fluid was filtered and stored frozen at -25 C until use.

b. Preparation of Wistar Medium

Components of Wistar medium appear in table 2. Essential amino acids 100X, cat. no. 13-602, vitamin mixture 100X, cat. no. 13-601F, and non-essential amino acids, cat. no. 13-114, were purchased from Microbiologi-

cal Associates (Bethesda, Md.). The medium was mixed on a magnetic stirrer and filtered under positive pressure to avoid denaturing the components.

c. Preparation of Physiological Saline

Earle's Basic Salt Solution (Flow cat. no. 3-821) was used at 1X concentration.

d. Preparation of Trypsin

A 10% suspension of crude Difco 1:250 Trypsin (Detroit, Mich., cat. no. 0152-15) was prepared at 4 C in buffer consisting of the components listed in table 3 (40). The suspension was stirred on a magnetic stirring unit for ten minutes at 4 C, then centrifuged at 10,000 RPM for one hour at 4 C, yielding a clear, brownish-yellow supernatant. The resulting 50X trypsin concentrate was filtered, brought to pH 7.6 with 0.1 M NaOH, pipetted into tubes in 2 ml lots, and stored at -25 C until use (40). Addition of 0.2 ml of the 50X trypsin concentrate to 5.0 ml of the salts used to make up the buffer, with the omission of glucose, is an

0.4% trypsin solution. The latter solution was used in all trypsinizations.

2. Preparation of Mouse Tissue

a. Mating

Runs were made using eight females per trial, rotating among three sets of males. In each run, a sexually mature, female mouse (PET/Wmr strain) was placed in a clear, plastic cage with two sexually mature male mice of the same strain at time zero. Twenty-four hours later the female was removed to another cage. Eleven to twelve days from time zero, the females were examined for bulging sides which suggested pregnancy.

b. Isolation of Ectoderm

The working area of a laminar air flow hood was scrubbed with 70% ethanol as was the stage of the dissecting microscope. All instruments were soaked in 70% ethanol, heated to a red glow in an alcohol flame to reduce

contamination, then quenched in sterile, distilled water before use. All operations were performed under the magnification of the microscope.

A pregnant female was injected intraperitoneally with 0.1 ml Diabutal (Diamond Laboratories Inc. cat. no. 8721). After five minutes, the narcotized mouse was immobilized on a dissection board and the abdomen scrubbed with 70% ethanol. With scissors, the ventral abdominal wall of the mouse was opened from the pelvis to the diaphragm. Three 60 mm x 15 mm Falcon Plastics petri dishes (Los Angeles, Calif. cat. no. 1007) were prepared, each containing 10 ml of sterile Earle's saline. The intact uterine horns with embryos were dissected from the mouse, washed in two changes of saline, and placed in the third change. The uteri were transferred from saline to culture fluid.

Starting at the distal end of the uterine horns, a cut was made through the uterine wall surrounding the embryo. Frequently the embryo emerged from its amnion with the initial cut in the uterus. More often, continued dissection was needed to release the embryo from its membranes. The embryo was placed in a 10 mm x 35 mm petri dish (Falcon Plastics cat. no. 3001) with 3.0 ml of culture

fluid. With two jeweler's forceps, any remaining extra-embryonic membranes were pulled away. Freed of all membranes, the embryo was placed in another 10 mm x 30 mm dish of culture fluid. A translucent, blue glass disc was placed under the petri dish to increase the definition of the embryo limb buds, which at the 10 to 12 day stage resemble small paddles about 0.5 mm wide.

The embryo was immobilized at the head region with one forcep while the limb buds were snipped off with after-cataract scissors. Each embryo body was removed from the dish and discarded. When all limb buds were free in the medium, they were removed to a 10 mm x 35 mm petri dish with a Pasteur pipette and thoroughly washed in saline to remove any adhering exudate. The saline was pipetted off and 2.0 ml of 0.4% trypsin was added to the limb buds. The buds in trypsin were placed in the refrigerator for two hours at 4 C.

At the end of two hours, the petri dish of limb buds was taken from the refrigerator and placed on an ice-water bath with a blue glass disc between the dish and the ice, and examined to see if the ectoderm had separated from the mesoderm. If the ectoderm had separated from the

mesoderm, the trypsin was pipetted off and 3.0 ml of Earle's saline at 4 C was added to wash the ectoderm. The saline was pipetted off and 3.0 ml of culture fluid at 4 C with 20% fetal calf serum was added to inactivate the trypsin (4, 9).

If the limb buds had a swollen appearance, that is, the ectoderm had pulled away from the mesoderm without the limb bud falling apart from the trypsin action, the ectoderm was teased away from the mesoderm as an intact cap, using as instruments, two, hooked micro-needles inserted in drawn glass handles. One needle was placed at the severed end to immobilize the bud, while the other needle was used to tease back the ectoderm until it fell cleanly away. The ectoderm was washed twice in culture fluid to dilute out mesodermal cells left floating in the fluid carried over from the dissection dish.

Histological examination of the ectoderm with the light microscope revealed a bilayered ectodermal structure; an upper peridermal layer and a lower ectodermal layer. No mesodermal cells were observed adhering to the preparation (figure 1).

c. Preparation of Dermis

The back and sides of 5 to 6 day, 2 to 3 week and five week-old mice were covered with Nair (a depilatory by Carter-Wallace, Inc.). After five minutes the hair was washed away with tap water and the skin was scrubbed with 70% ethanol. After decapitation, a square of skin was removed from the dorsum. The skin was placed in saline and scraped with a scalpel to remove large fat deposits from the hypodermis. The skin was washed again in saline and placed spread-out, hypodermis-side-up in a 60 mm x 15 mm petri dish. Culture fluid was added up to the level of the hypodermis. The petri dish containing the skin was placed in a metal pan with tap water filled to a level just below the top cover of the petri dish. The pan and petri dish with skin were placed level in a freezer at -60 C for 15 minutes. After the water was frozen, the tray was taken from the freezer, leaving the petri dish of skin in the anchoring ice. With a no. 22 scalpel blade, the hypodermis was scraped away from the dermis. The same procedure was used to remove the epidermis. If necessary, the procedure was repeated until all the hypodermis

and epidermis were removed.

After the scraping procedure, the dermis was washed in saline and inspected for any attached epidermis. If epidermis was present, these portions were excised and discarded.

The prepared dermis was cut into 3 mm x 3 mm sections, placed in 60 mm x 15 mm petri dish, and frozen and thawed at 30 minute intervals four times at -60 C alternately to lyse any viable cells in the dermal preparation (4, 9).

Freeze-thawed dermis did not show fibroblastic outgrowth in tissue culture or viable cells after histological preparation for light microscopy.

3. Preparation of Grafts

a. Preparation of Frozen-Thawed Dermis-Ectoderm Grafts

The dermis was removed from the freezer in preportioned lots. The dish of ectoderm and the dish of dermis were placed side-by-side on blue glass discs. The ectoderm was removed from the dish with a sterile, fine

capillary pipette and placed on the dermis. The orientation of the ectoderm was difficult to determine but when removed from limb buds using the above methods, the ectoderm turned inside-out. All ectoderm was assumed to be in this conformation and was flipped 180 degrees when positioned on dermis, slit from an edge to the center, and spread over the dermal surface. Three to four ectodermal caps spread in this fashion were placed on each piece of dermis.

b. Preparation of Control Grafts.

Grafts of frozen-thawed dermis were cultured alone in tissue culture and on the CAM. Whole limb buds, and separately, baby hamster kidney cells (BHK_{21} cells from the American Type Culture Collection) were grown on frozen-thawed dermis in tissue culture to test for any possible toxicity of the dermal preparation.

Ectoderm was grown at 37 C in whole pieces and after trituration through a 27 gauge needle ten times in tissue culture in a closed flask; in petri dishes in a 5% CO_2-21%O_2 atmosphere; and in petri dishes in a 5%CO_2-40%O_2 atmo-

sphere.

Grafts of frozen-thawed dermis-epidermis, live dermis-live epidermis, live dermis-ectoderm, heat-killed dermis-ectoderm, live dermis alone, live epidermis alone, and live, intact four day neonatal, and 18 to 21 day-old embryonic skin were cultured on the CAM for ten days (down on the CAM; see preparation of CAM grafts).

Live epidermis and live dermis were prepared by treating whole skin with 0.4% trypsin for two hours at 4.0 C, followed by mechanical separation of the tissues (modified from 4, 9, 33).

Heat-killed dermis was prepared by immersing freshly excised whole skin in sterile, boiling water for 30 seconds, pealing off the epidermis, and using the dermis for graft material (4).

4. Incubation Procedure

a. Preparation of CAM Grafts

White Leghorn chick eggs that were incubated with periodic turning for seven days in a Jamesway incubator,

were candled, and the shell was marked over a highly vascularized area. The area was swabbed with 70% ethanol and an isolateral, triangular opening, 10 mm on a side, was cut in the shell with an egg saw over the vascular area. The shell piece was removed, the shell membrane flushed with saline and removed with jeweler's forceps. The graft was transferred with forceps to an area on the CAM directly over a blood vessel. The opening was covered with Scotch tape and the edges sealed with paraffin (modified from 12 and 27).

Grafts were cultured in two ways: in the first method, the dermis was placed on the CAM with the ectoderm spread on top of the dermis (up on the CAM); in the second method, the graft was inverted so the ectoderm was in direct contact with the CAM (down on the CAM).

Eggs were incubated at 60% relative humidity at 38 C for 10 or 20 days to allow ample time for the ectoderm to differentiate. Control grafts were also incubated for ten days. Grafts that were cultured for 20 days were transferred after an initial incubation of ten days, to the CAM of a second seven day-old embryo CAM and cultured an additional ten days. At the time of transfer, the graft was

passed to the second CAM with membranes adhering from the first culture. These membranes were not removed because the trauma would disrupt the graft.

b. Preparation of Tissue Culture Grafts

Frozen-thawed dermis-ectoderm grafts were handled in three ways: (1) they were incubated for three or ten days at 37 C in a closed container in culture fluid; (2) they were incubated 24 hours in a 5% CO_2-21% O_2 incubator at 37 C with just enough growth medium to submerge the ectoderm until it attached to the dermis, then they were gently flooded with 3.0 ml of culture fluid and incubated 1, 2, 3, 5, 7, 10, and 20 days with daily culture fluid changes, and rocked on a platform rocker to improve diffusion of nutrients into the grafts; (3) they were handled as in part two above but were placed in a 5% CO_2-40% O_2 incubator and incubated 3, 10, or 20 days. The latter environment was used because Reaven and Cox (28) reported that 40% oxygen tension is optimal for growth of skin in tissue culture.

Of 18 grafts incubated in 5% CO_2-21% O_2 at 37 C in Wistar medium, nine were collected after 32 hours, and

nine were collected after 72 hours in culture.

A problem that occurred in the tissue culture system was getting the ectoderm to adhere to the dermis when the graft was covered with culture fluid. During the first 24 hours in the incubator, only enough culture fluid was added to the grafts to keep them moist. During this early period, the ectoderm contacted and adhered to the dermis; however, many grafts separated when fluid was added after 24 hours.

Dermis poorly adhered to the petri dish at the onset of experimentation and several methods were tried to overcome the problem. A drop of nutrient agar at 56 C from a 27 gauge needle was placed between the dermis and the plastic with the expectation that the agar would act as an adhesive to hold the dermis in place. This method failings, plates of nutrient agar were poured and dermis was placed in a depression in the agar. With a warmed glass needle the surrounding agar was liquified, resulting in the containment of the dermis. Although dermis did not float off the agar, this method was abandoned because diffusion of nutrient to the grafts was limited. Finally, the dermis, directly out of culture fluid containing 20% fetal calf serum,

was placed in a petri dish and frozen until use. The last technique was successful, possibly because the albumin in the serum acted as an adhesive to hold the dermis in place.

The possibility existed that fat globules in the dermis and hypodermis prevented the ectoderm from properly adhering to the dermis. An idea for future experimentation is to submerge the scraped dermis in hexane at 4 C overnight to remove lipids, and evaporate excess hexane under negative pressure, then use this preparation for grafting.

5. Recovery of Grafts and Histotechnique

a. Recovery of CAM Grafts

At the end of the culture period, the egg was removed from the incubator, the tape was removed, and the graft located. The shell around the false airspace was cut away with scissors. Disruption of the circulatory system was avoided because excess bleeding hindered graft retrieval. The CAM was securely gripped with forceps, 3 mm from the graft, while a circular cut was made around the graft. If the graft was to be recultured, sterile procedures were

used to excise the graft. The recovered graft was placed in Bouin's fixative for 48 hours.

b. Recovery of Tissue Culture Grafts

At the end of the culture period, the grafts were removed from the petri dish with forceps and placed in Bouin's fixative for 48 hours.

c. Histotechnique

After fixation, the grafts from CAM or tissue culture were dehydrated in ethanol and benzene (one hour in 70% ethanol, two hours in 95% ethanol with one change at one hour, one hour in 100% ethanol with one change at one-half hour, then one hour in benzene). The grafts were infiltrated overnight in Paraplast at 62 C, embedded, and serially sectioned at 8 u. The sections were placed on slides, floated with an albumin solution, warmed to spread the sections, dehydrated at 45 C overnight, stained with Delafield's hematoxylin and eosin Y, coverslipped, and examined with the light microscope.

RESULTS

Grafts of ectoderm on frozen-thawed dermis showed differentiation of the ectoderm to epidermis in the CAM system, and showed stratification of the ectoderm in one case in the tissue culture system.

The criteria used in these studies to delineate a mature epidermis were: (1) a stratified maturation of epidermal cells derived from the germinal population, (2) elaboration of a stratum corneum, the end product of epidermal maturation.

1. CAM Grafts

Of 111 CAM grafts attempted, six were positive for differentiated epidermis. Of the grafts that showed positive epidermal differentiation, five were grafted down on the CAM and one was grafted up on the CAM (table 3). In these grafts, ectoderm from the limb buds of 10 to 12 day-old mouse embryos showed differentiation into epidermis when combined with frozen-thawed dermis and grafted to the CAM of chick embryos for 10 or 20 days (figure 2 a, b, c).

2. Tissue Culture Grafts

Of 381 frozen-thawed dermis-ectoderm grafts prepared for culture, only one showed differentiation of ectoderm (table 4 and figure 3). In no case was a stratum corneum seen. The bilayered ectoderm grew to a thickness of four to six cells in several cultures and to 12 cells thickness in one culture, but did not resemble the several histologically recognizable layers of epidermis. When ectoderm grew only four to six cells in thickness, no orderly stratification was noted.

Often the ectoderm folded back on itself in tissue culture because the ectoderm lifted off the dermis when culture fluid was added after the first 24 hours. The lapping gave the impression of increased thickness, but on close examination, the overlapping layers could easily be seen (figure 4).

Of 18 grafts grown in Wistar medium, the single case that grew to 12 cells thickness did not show overlapping of layers but did show stratification. The cells closest to the frozen-thawed dermis were squamus, while those in the upper layers showed increased flattening. No highly corni-

fied layer was present, but a single strand of what appeared to be cornified cells was seen at the uppermost layer of the differentiated tissue.

3. Control Grafts

None of 14 grafts of frozen-thawed dermis alone on the CAM (down on the CAM) showed any signs of epidermis present (figure 5). No apparent structure of chick origin was induced by the dermis. There was no outgrowth of fibroblasts from frozen-thawed dermis in tissue culture.

None of 14 heat-killed dermis-ectoderm grafts (down on the CAM) showed any signs of epidermal differentiation.

Six grafts of whole skin from 18 to 21 day-old mouse embryos and three grafts from a four day-old neonatal mouse showed a thickened stratum corneum, a decrease in columnarity of the basal cells, and a loss of orderly stratification (figure 6). The basal cells and keratinocytes were prematurely squamus.

Five dermis-epidermis recombinants prepared from 18 to 21 day-old embryonic skin by trypsinizing dermis from epidermis also showed squamus change in the epi-

dermis (figure 7a), while one showed normal histology (figure 7b).

In six frozen-thawed dermis-18 to 21 day-old embryonic epidermis grafts, the epidermis also appeared squamus.

None of six grafts of 18 to 21 day-old embryonic epidermis placed directly on the CAM, with basal cells apposing the CAM, could be located aften ten days incubation and were presumed to have degenerated or integrated with the CAM beyond recognition.

In five grafts of dead dermis with limb buds in tissue culture, one graft showed an entire bud firmly attached and growing on the dermis (figure 8). A focus of blood cell formation occurred in the center of the bud. At the margins of the limb bud-dermis interface, the ectodermal layer had attached and had spread approximately 20 u from the bud periphery and had even invaded into the dermal hair follicles (figure 9). Attachment and growth of the limb bud to frozen-thawed dermis in tissue culture indicates that the dermis offered a suitable substrate for cell adhesion and growth.

In another experiment, BHK_{21} cells were grown on the prepared dermis in tissue culture. The BHK_{21} cells piled

up over each other to form an amorphous nodule of cells attached to the hairs in the dermis (figure 10). The cells did not form a confluent layer as they would on glass, but did migrate into the hair follicles.

When whole mouse limb bud ectoderm was cultured alone under any of the culture conditions listed under incubation procedures, the ectoderm deteriorated within 24 to 72 hours as judged by observation of the detached cells. If the ectoderm adhered to the culture dish bottom, epithelial cells would spread out at the periphery, but focal degeneration would occur in the cell sheet within five days. If the ectoderm was passed through a 27 gauge needle ten times and suspended in growth medium in any of the above mentioned incubation systems, the cells adhered to the culture dish bottom, but very little cell division occurred, as opposed to growth of isolated epidermal cells in tissue culture (17, 19). The cells spread out somewhat over the plastic bottom but rather than being fibroblastic, were epithelioid, resembling HeLa cells. At 5 to 8 days in culture, the cells lost contact with the substrate and deteriorated in the fluid. Although the cell population could not be maintained in excess of 5 to 8 days even with

daily culture fluid changes, BHK_{21} cells could be grown indefinitely under the same conditions.

DISCUSSION

1. General

These experiments indicate that ectoderm from the limb buds of 10 to 12 day-old embryonic mice (PET/Wmr) combined with frozen-thawed dermis and cultured on the CAM of White Leghorn chick embryos for 10 or 20 days can form an epidermis.

An easily demonstrable criterion by which epidermis was identified in the grafts in these experiments was the presence of a well developed stratum corneum with a stratification of cells below. Cornification of cells ended abruptly on either side of the frozen-thawed dermis, and because not all grafts showed the stratification-cornification, and none of the controls showed this histology, the stratification-cornification gave evidence that the mouse ectoderm had been induced to form an epidermis under the conditions of the experiment. In support of the latter finding, Briggaman and Wheeler (4) reported that in grafts constructed so that the epidermal component overlapped the dermis, leaving isolated areas of epidermis in direct

contact with the CAM, the epidermis contacting the CAM degenerated whereas the epidermis contacting the dermis was maintained. Both Briggaman and Wheeler (4) and Dodson (9) showed that epidermis cultured alone on the CAM degenerated. In the present study, the presence of a stratum corneum only over the area of the frozen-thawed dermis indicated that the induction of ectoderm to cornify was caused by the dermis.

The histology of the cells in the CAM grafts located between the stratem corneum and dermis was different from the histology of uncultured mouse epidermis. The basal layer of the graft epidermis ranged from cuboidal to squamus while succeeding epidermal cells derived from the basal layer were flattened. Uncultured epidermis showed a cuboidal to columnar basal layer of germinal cells, discrete Malpighian and granular cell layers, and a stratum corneum. The progression of cells from the basal layer to the stratum corneum was atypical in the frozen-thawed dermis-ectoderm graft after 10 or 20 days cultivation on the CAM. Dodson (9) reported a rapid keratinization in grafts of metatarsal epidermis of 12 day-old embryonic chickens when recombined with dermis

killed by freezing or on collagen gel. The rapid keratinization of ectoderm grown on freeze-killed dermis may be the cause of the atypical histology.

In the present study it was noted that in control grafts of whole skin cultured on the CAM for ten days, the histology of the epidermis was abnormal. The columnarity of the basal layer was reduced or absent, flattening of the Malpighian and granular layers occurred, and thickening of the stratum corneum with parakeratosis was evident. The control grafts of epidermis-dermis recombinants that had been separated by trypsinization, recombined, and cultured on the CAM also showed squamus change in the epidermis in several cases and normal histology in one case.

Briggaman and Wheeler (4) cultured on the CAM for five days human, adult, whole skin and recombinants that had been separated with trypsin, but demonstrated no change in the histology of the epidermis. The characteristics of an organized, viable epidermis, which included a columnar basal layer, were preserved. The major differences between this author's procedure and that of the latter study were: (1) the use in the present study of embryonic mouse ectoderm rather than adult human skin, and

(2) the length of culturing time on the CAM. The embryonic tissues are more readily affected by experimental treatment and are more sensitive to the effects of the chemical and physical environment. Longer culture periods of Briggaman and Wheeler's tissue on the CAM might have resulted in squamus changes similar to that found for ectoderm in the present study.

Dodson (9) reported an alteration in the normal morphology of epidermis in tissue cultures of 12 day-old embryonic chicken epidermis on unsuitable substrates such as plasma-embryo extract clots, heat-killed dermis, or agar. He found that the columnar orientation of the basal cells was not always maintained. The basal cells often rapidly flattened and became bipolar when epidermis was isolated on an unsuitable surface. Both changes were prevented when the epidermis was placed on a suitable substrate, such as frozen-thawed dermis, frozen-thawed dermis treated with trypsin, or collagen gel. From these observations he concluded that the primary effects of attachment not only keep the basal cells in a columnar orientation, but maintain polarization of the epithelium. In addition, epidermis grown on collagen gels for more

than two days showed flattened basal cells, indicating that columnarity was not essential at all stages of epidermal development (9).

2. Factors Influencing Differentiation of Ectoderm

It is evident that many factors influence the direction of differentiation of ectoderm apposed to frozen-thawed dermis in culture. The presence of an inducer molecule, some physiological cue activating certain genes in the epithelial cells to form epidermis, may be the most important factor (5); but the physical substrate also influences ectoderm to modify its phenotype. The exposure of ectoderm to the unnatural chemical and physical environment in the experimental procedure obviously had an effect on the phenotype.

a. An Inducer Molecule

Briggaman and Wheeler (3) demonstrated a loss of inductive capacity for the maintenance of epidermis on dermis incubated at 56 C for four hours whereas dermis

incubated for one hour at 56 C retained the inductive capacity. Earlier they (4) had submerged dermis for 30 seconds in water at 100 C and found that it could not support maintenance or growth of epidermis cultured on the CAM. The present study confirmed the latter experiment.

What is the chemical nature of the inducer? Most proteins are inactivated when heated to 56 C for one hour. The latter treatment is a standard method for heat-inactivating components of serum (complement) which might otherwise adversely affect tissues in culture. Yet at least one protein, RNAase, can withstand 56 C for one hour and longer without losing activity when returned to physiological temperatures (37 C). Nucleic acids can also withstand this treatment (31). The inducer may be a protein, but in support of the nucleic acid, Slavkin et al. (31) and others (1), have demonstrated the presence of RNA in the extracellular matrix between epithelial and mesenchymal cells during embryonic rabbit odontogenesis, and in the periphery of lymphocytes. In another publication, Slavkin et al. (30) showed that membrane-bound bodies in the matrix may contain RNA. Preparations of the matrix have morphogenetic effects on epithelial cells. Messenger

RNA, which has been conclusively shown to direct protein systhesis (8, 23, 24), may be the inducer present in dermis. Grafts should be made of RNAase-treated dermis combined with epidermis to see how well this preparation supports epidermal maintenance and growth.

McLoughlin (20, 21) and Rawles (21) also indicated that the inducer may be present in the intercellular matrix, and that the inducer can vary in intensity in various ontogenetic stages within the same tissue, and can vary in relative intensity between tissues at their respective peaks of induction.

Studies designed to detect the diffusibility of an inducer where Millipore filter was interposed between epithelium and mesenchyme have indicated the presence of a diffusible inducer (9).

b. Physical Substrate

Several authors have demonstrated that epidermis cannot be maintained on all substrates tested (3, 4, 9, 10, 18, 20, 21, 27, 43). The highest percentage of maintenance of viable epidermis in culture was obtained when

some form of dermis, collagen gel, or other collagen derivative was used (3).

Dodson (9) found limited maintenance of epidermal characteristics when epidermis was cultured on Millipore filter in tissue culture. In 17 of 45 explants the lower cells of the epidermis were flattened and arranged in layers though the upper cells were unoriented. Basal cells next to the filter were almost always flattened. If this effect is non-specific, then epidermis may have the inherent capacity to maintain a somewhat orderly, stratified composition without the presence of an inducer. The effect of an inducer may be to refine the appearance of specific epithelia for particular functions.

Although epidermis has been maintained on smooth surfaced substrates (9, 18), and on materials other than dermis (3, 9, 18), the special grooves of the dermal surface may somehow cause the epidermis to modify the phenotypic expression of the epithelium. That fibroblasts respond to grooved surfaces was shown by Rovensky et al. (29). Mouse and chick embryo fibroblasts fell into the grooves when seeded over a polyvinychloride, polymethylmethacrylate, polyethylene, or nickle plate with groove

dimensions ranging from 5 to 40 u deep and 5 to 200 u wide. Twenty-four to 48 hours after seeding, the cell density in the grooves was several times lower than the density in the intervals between the grooves. Rovensky attributed the movement of cells out of the grooves to differences in the interactions of cells with various parts of the substrate. The decreased attachment of cells to the grooves may be due to some peculiarities of the surface microstructure. Cell-surface and cell-cell interactions are implicated in communication and morphogenesis (2, 36, 37) and most likely play a role in skin. Precisely what that role is, and how the surface of dermis communicates with epidermis is obscure.

The surface of the scraped dermis used in the present investigation was flat when observed with the light microscope. The grooved topography of unscraped dermis was removed. The surface of collagen gels is smooth yet epidermis can still adhere to it. Briggaman and Wheeler (3, 4) showed that epidermis could be maintained as well on inverted dermis which presents a non-grooved hypodermal side for grafting, tendon preparations composed almost totally of collagen bundles, and powered dermis

which has the consistancy of a gel, as on dermis. When each of these preparations was observed with the electron microscope, the surfaces were indistinguishable from each other (3). Even though the macroscopic architecture was disturbed, the microarchitecture was the same: all preparations had a typical molecular periodicity of collagen.

In the present study, the author had difficulty maintaining contact of ectoderm to frozen-thawed dermis. Ectoderm did not adhere to dermis in tissue culture, whereas BHK_{21} cells trypsinized and seeded over the dermis, aggregated, adhered very well, and even migrated into the follicles.

The differences described between adhering and non-adhering surfaces could be properties of the cells, the dermis, or both; or involve an interaction between the ectoderm and substrate resulting from treatment during preparation.

c. Effect of Experimental Treatment on Ectoderm

Precisely how the chemical and physical treatments received by tissues during the experimental procedure

influence their phenotypic expressions in not known with certainty, but several thorough investigations have dealt with this problem (11, 14, 22, 25, 39).

c_1. Chemical Environment

The ectoderm (and dermis) used in the present study was immersed only in culture fluid, Earle's saline, trypsin, or CAM fluid. Although the concentrations of certain ions such as sodium, potassium, and calcium have been shown to vary throughout development in the chick, rat, and rabbit (14), fluid in this study remained constant in composition and within physiological limits for mouse tissue (22).

Skin from many different animals has been cultured on the CAM. The effect the CAM has on skin seems to be minimal according to the investigations of Briggaman and Wheeler (4) and Goodpasture (12), although the latter author indicated that heterologous skin grafts to the chick CAM rapidly degenerate beginning on the 18th or 19th day of incubation. Eggs used in the present study were incubated up to the 17th day to minimize the possibility of

degeneration of the grafts.

Trypsinization has been shown to produce growth static or degenerative effects on tissue cultured in vitro (25, 39). Tissue used in skin studies in which the duration of trypsin on cells was from 15 minutes to 1.5 hours at 37 C was probably damaged at the plasma membranes (25) and may have been induced to undergo some phenotypic change. Figure 11 shows epidermis of an 18 to 21 day-old embryonic mouse at 2 hours in 0.4% trypsin at 4 C. The basal layer was greatly disrupted. Individual basal cells were swollen and some were broken. The combination of trypsin and mechanical treatment caused degenerative changes in the epidermis. Trypsin and other proteases have been shown to adhere to the plasma membrane where continued enzyme activity interferes with the formation of glycoprotein cell coat materials, attachment, spreading, and growth of cells on glass (25). Thorough washing of cells with saline and serum reduces the harmful effects of residual trypsin to a negligible level (25). The ectoderm used in the present study was washed once in Earle's saline, then washed in culture fluid containing 20% fetal calf serum. It is possible that because these washings occurred at 4 C,

and because the ectoderm was bilayered and continuous, thus slowing down the removal by diffusion of residual trypsin, all of the enzyme may not have eluted or reacted with the trypsin inactivating components of serum. Subsequent trypsin degradation of the ectoderm could have occurred to some extent although the ectoderm was cultured successfully in a closed flask in culture fluid for 5 to 8 days, on frozen-thawed dermis on the chick CAM, and in one case, on frozen-thawed dermis in tissue culture. One should be aware however, that a dilution of 1:1,000,000 trypsin is capable of digesting 10 mg of casein over a period of 72 to 96 hours at 37 C, and that cells grown in serum-free medium containing 0.0001% trypsin fail to manifest growth (39).

Alternative agents may be used in lieu of trypsin, such as Versene or collagenase, but the complete removal of these agents after use is still a problem.

The role of serum in supporting growth of tissues in culture has been clarified in recent publications (16, 34, 39). Wallis et al. (39) have shown that anti-protease factors may be responsible for growth enhancing properties of serum because trypsin treated cells grow more rapidly

in medium with serum than in medium without serum, whereas,non-protease treated cells grown as well in either medium.

Heat inactivation, which was carried out in the present investigation, may reduce the ability of serum to inactivate trypsin, permitting residual tryptic degradation of plasma membranes (39).

c_2. Physical Trauma

Physical trauma to the ectoderm on excision of the limb bud and removal of the ectoderm probably enhanced cell division because wound healing is accompanied by cell proliferation (38).

Apart from the wounding of the tissue, complicated by the possibility that cytotoxins could be released (39), air-drying of the tissue culture and CAM grafts could have caused cornification of tissues, mimicking a stratum corneum. Air-drying in tissue culture was a possibility only during the first 24 hours when the grafts were kept moist with culture fluid, but were not fully submerged. Air-drying in the CAM system was more likely because

the false airspace directly exposed the grafts to a potentially drying atmosphere. In grafts that were cultured up on the CAM, the ectoderm could have dried out, but in grafts that were cultured down on the CAM, the ectoderm continually contacted the moist CAM directly over a blood vessel, alleviating any potential dessication hazard. The results in table 2 show that five of 83 grafts cultured down on the CAM were positive for an epidermis, while one of 28 cultured up on the CAM was positive indicating that ectoderm survived more often under moist conditions.

The osmolarity of the culture systems was important because rabbit embryos have been shown to require 285 milliosmoles in the bathing fluid and degenerate at 308 milliosmoles, a value for adult rabbit serum (22). The optimum osmolarity for developing mouse embryos is 276 milliosmoles. The fluid of the CAM and of bovine serum have a value of approximately 256 milliosmoles, a value within the limits for the growth of mouse tissue (22).

CONCLUSION

The influence of the physical substrate on the expression of a particular phenotype of the ectoderm is important because ectoderm exhibits a range of capacities to adhere to different substrates. Experimental treatment of the ectoderm during the course of the handling procedures could have directed the tissue toward a particular expression of the phenotype, that is, to flatten, or to degenerate; however the author tried to maintain the tissue under conditions that simulated a physiologically acceptable environment.

Under these conditions, ectoderm from the limb buds of 10 to 12 day-old mouse embryos formed an epidermis when placed on frozen-thawed dermis and cultured on the CAM of chick embryos. It is concluded in this study that dermis, whether living or dead, provides factors essential for the maintenance of epidermis and the differentiation of ectoderm to epidermis. Available evidence suggests that dermis does contain an inducer which this author speculates may be messenger RNA.

Many authors have previously maintained or grown adult, whole skin, or multilayered, embryonic skin in tissue culture and on the CAM. In this investigation a bi-layered ectoderm was grown to an epidermis on the CAM, and in one case, in tissue culture to a stratified tissue. This author cannot say with certainty that the ectoderm had not received genetic instruction to form an epidermis at the time of removal from the limb bud; however, the 10 to 12 day period is the earliest stage at which it is technically feasible to obtain material in the quantity required for investigation. Future refinements of the experimental method will possibly give further insights into the degree of inherent differentiation of the ectoderm.

Summary

From the results obtained in the present investigation, the following was demonstrated: (1) ectoderm from the limb buds of 10 to 12 day-old PET/Wmr mouse embryos combined with freeze-thawed dermis and with control substrates was cultured on the CAM of chick embryos and in tissue culture; (2) ectoderm formed an epidermis when cultured on the CAM of chick embryos for ten days as judged by the formation of cornified cell layers directly over the freeze-thawed dermis, but did not form cornified layers in tissue culture; (3) control grafts of ectoderm combined with freeze-thawed dermis showed no formation of cornified cell layers over the dermis; (4) the dermis apparently contained an inducer which directed the phenotype of ectoderm; (5) the substrate that ectoderm was combined with and the chemical and physical treatment that ectoderm received, altered the phenotypic expression of ectoderm.

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Table 1

Basic Culture Fluid Components Per 100 ml Fluid

MEM	89.5 ml
Fetal Calf Serum	10.0 ml
Antibiotics	0.5 ml
Sodium Bicarbonate Solution (7.5% in $H_2 0$)	0.5 ml

Table 2

Components of Wistar Medium Per Five Liters Medium	
MEM with Earle's salts without sodium bicarbonate	49.00 g
Sodium bicarbonate	3.75 g
Essential amino acids 50X	20.00 ml
Non-essential amino acids	10.00 ml
Vitamins 100X	10.00 ml
Glucose (50% in water)	35.00 ml
Ferric nitrate (0.1 g/100 ml water)	0.50 ml
Sterile distilled water	4880.00 ml

Table 3

Buffer Components of 50X Trypsin Solution after Wallis (22)

All Components in g/l at 4.0 C

NaCl	8.00
KCl	0.40
Na_2HPO_4	0.06
KH_2PO_4	0.06
Glucose	50.00

Table 4

Number of Attempts and Successes of Grafts Cultured on the Chorio-Allantoic Membrane (CAM) of White Leghorn Chick Embryos.

Type of Graft	a/b
[1]Frozen-thawed dermis - [2]ectoderm ([3]up)	28/1
*Frozen-thawed dermis-ectoderm ([4]down)	83/5
[5]Heat-killed dermis-ectoderm (down)	14/0
Frozen-thawed dermis alone	14/0
Whole mouse skin, four day neonatal	3/3
Whole mouse skin, 18 to 21 day embryonic	6/6
Dermis-epidermis recombinant after trypsin	6/6
Freeze-thawed dermis-live epidermis	6/6
Live epidermis alone	6/6

a represents the number of grafts cultured on the CAM.

b represents the number of positive cases of differentiation of ectoderm to epidermis or maintenance of epidermis when stratified epidermis was the starting material.

1 Frozen-thawed dermis is dermis that was alternately frozen at -60 C and thawed at 37 C four times to lyse all viable cells.

2 Ectoderm is the bilayered tissue removed from the limb buds of 10 to 12 day old mouse embryos by the action of 0.4% trypsin for two hours to 4.0 C.

3 "Up" indicates that the dermis was situated between the ectoderm or epidermis and the CAM.

4 "Down" indicates that the ectoderm or epidermis was situated between the dermis and the CAM.

5 Heat-killed dermis is dermis that was submerged in sterile, boiling water for 30 seconds.

Note: All grafts were grown on the CAM for ten days or where marked with an asterisk, for ten and 20 days.

Table 5

Number of Attempts and Successes of Grafts Grown in Tissue Culture.

	a/b
Grafts incubated in 5% CO_2-21% O_2 at 37 C	
Frozen-thawed dermis-ectoderm, total number	231/1
Frozen-thawed dermis-ectoderm in [1]Wistar medium:	
Recovered at 32 hours:	9/0
Recovered at 72 hours:	9/1
Frozen-thawed dermis -[2]limb bud	5/1
Frozen-thawed dermis-[3]BHK_{21} cells	5/5
Grafts Incubated in 5% CO_2-40% O_2 at 37 C	a/b
Frozen-thawed dermis-ectoderm	132/0

Note: See table 4 for legend to terms in table 5.

1 Wistar medium is a culture medium fortified with vitamins and amino acids.

2 A limb bud is the paddle-like appendage protruding from the lateral side of the embryo.

3 BHK_{21} cells are a special strain of hamster fibroblasts isolated in pure culture.

Figure 1

Ectoderm removed from the limb bud of a 10 to 12 day-old embryonic mouse limb bud is a bipartite structure consisting of a simple outer periderm and a lower cuboidal ectodermal layer. 1000X.

ecto - ectoderm

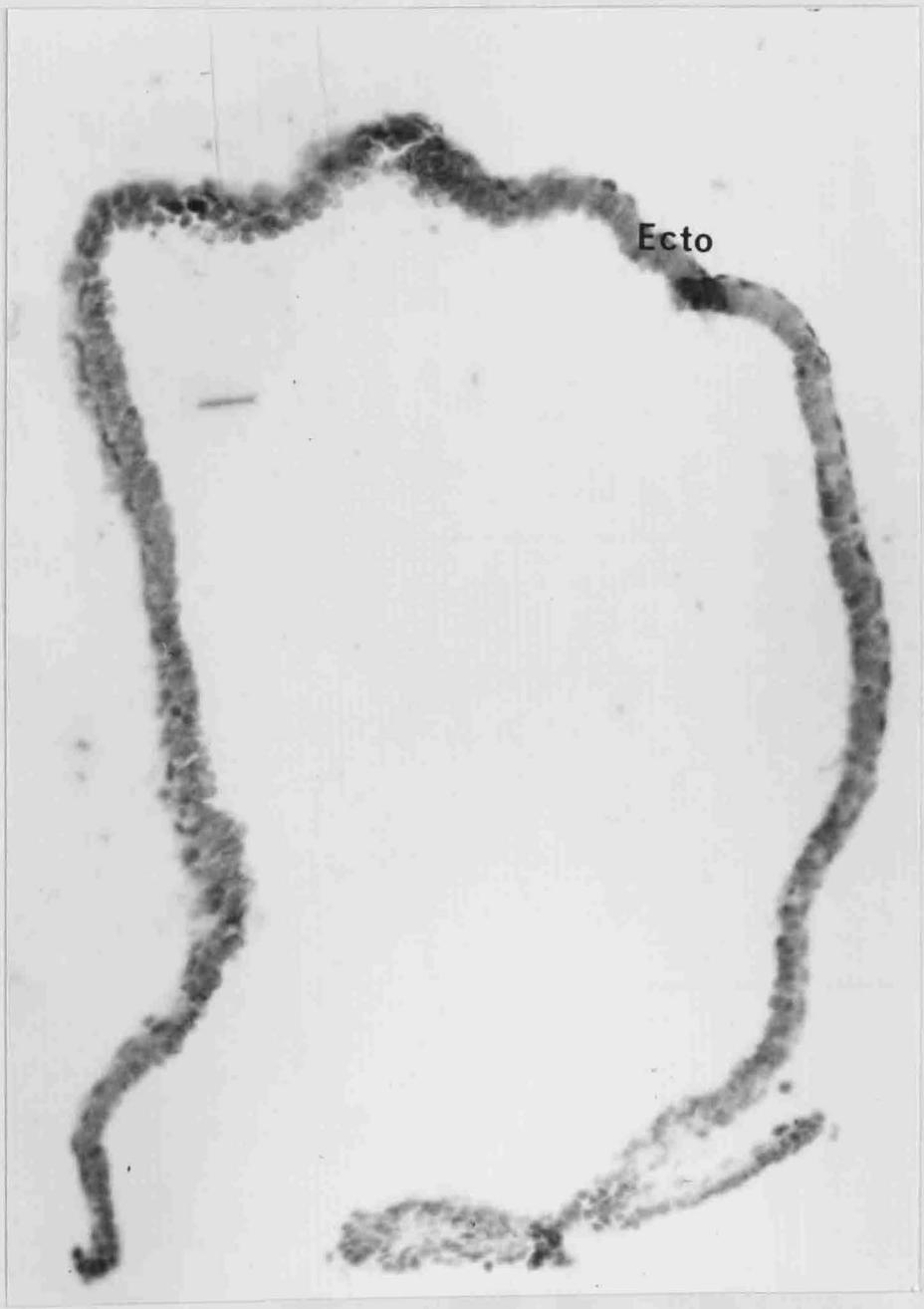

Ecto

Figure 2

Grafts of ectoderm from the limb buds of 10-12 day-old embryonic mice and frozen-thawed dermis grown on the chick embryo CAM showed differentiation of ectoderm to epidermis. In figure 2a the graft was cultured "up" on the CAM. CAM covered the graft preventing dessication of the ectoderm. A well defined stratum corneum is present but the intervening layers between the stratum corneum and dermis are atypical. 450X.

CAM - Chorio-allantoic membrane

Sc - Stratum corneum

E - epidermis

D - dermis

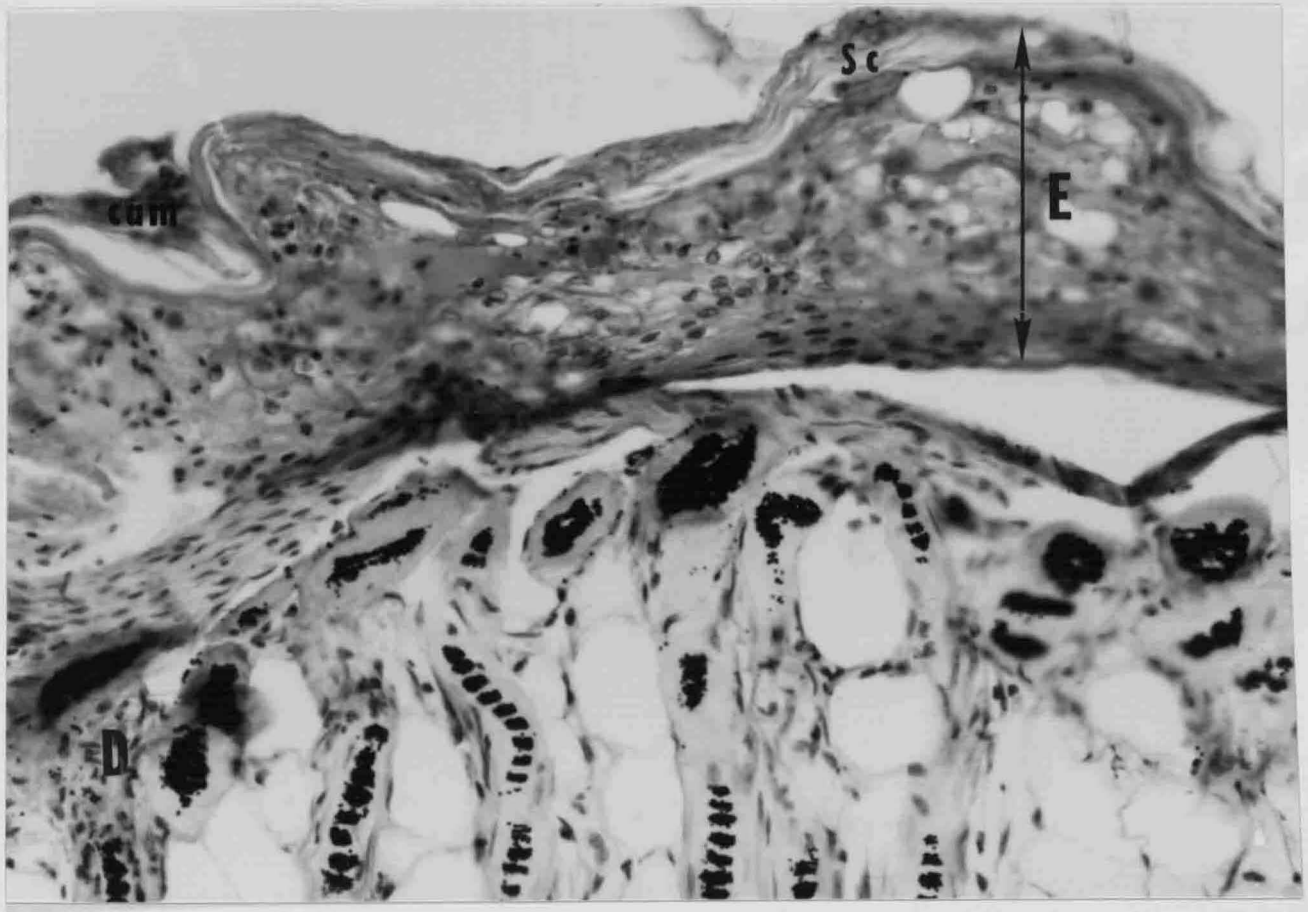

Sc
E
cum
D

Figure 2b

In figure 2b the epidermis has a well defined stratum corneum but the cell layers between the stratum corneum and dermis are squamus. 450X.

CAM - Chorio-allantoic membrane

d - dermis

e - epidermis

cam
e
p

Figure 2c

Figure 2c is an enlargement of figure 2b illustrating the stratum corneum arising from the squamus cell layers apposed to the dermis. 450X.

CAM - chorio-allantoic membrane

d - dermis

e - epidermis

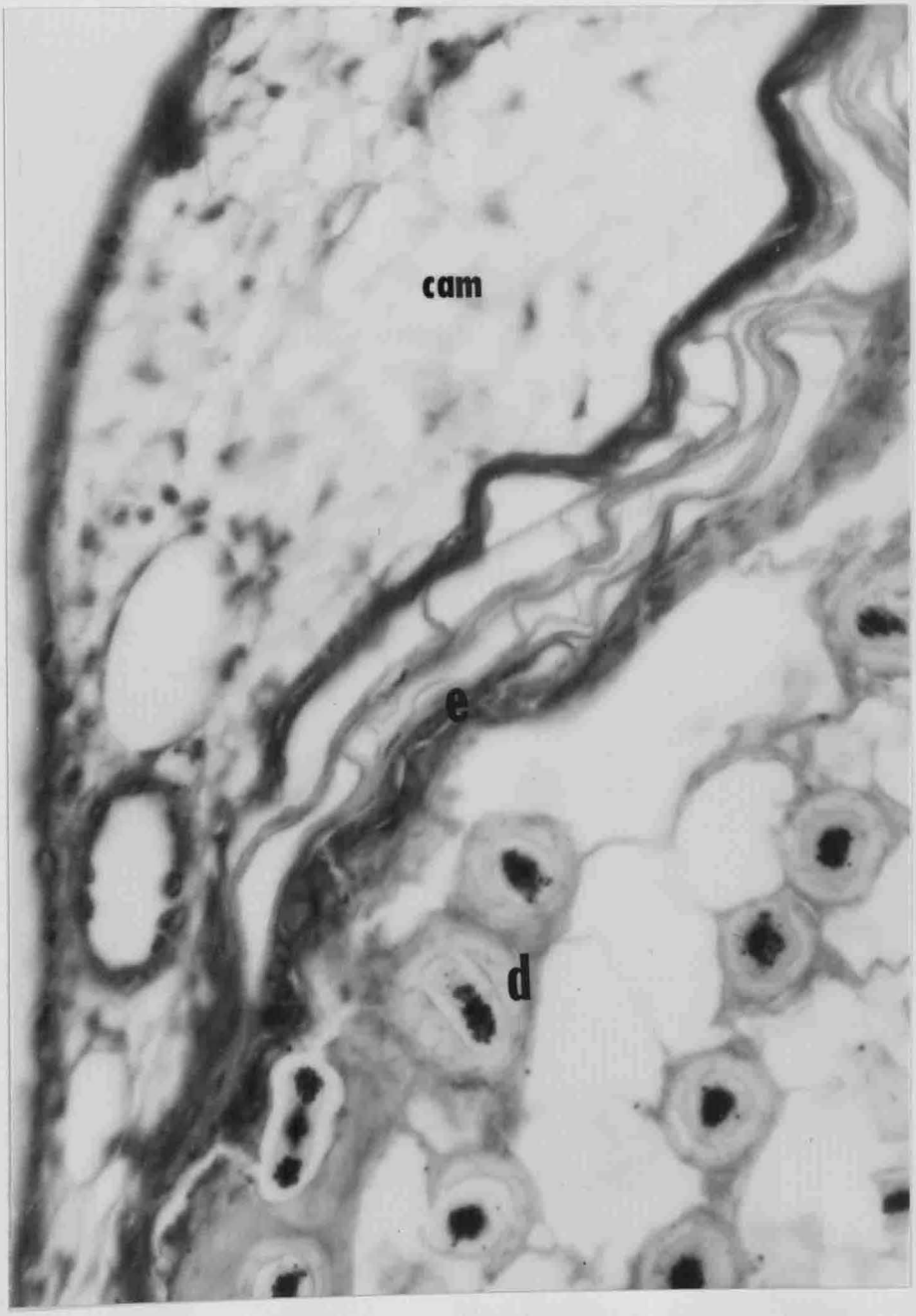

cam
e
d

Figure 3

Ectoderm removed from the limb buds of 10-12 day-old mouse embryos was grown on frozen-thawed dermis in tissue culture in Wistar medium. 1000X.

ecto - ectoderm differentiated to several layers

D - dermis


Ecto
D

Figure 4

When ectoderm cultured on frozen-thawed dermis in tissue culture folded back on itself, the cells degenerated and the nuclei stained heavily. Overlapping of ectoderm is indicated by the loop of dark nuclei and loss of cellular form in the tissue. 1000X.

ecto - ectoderm

d - dermis

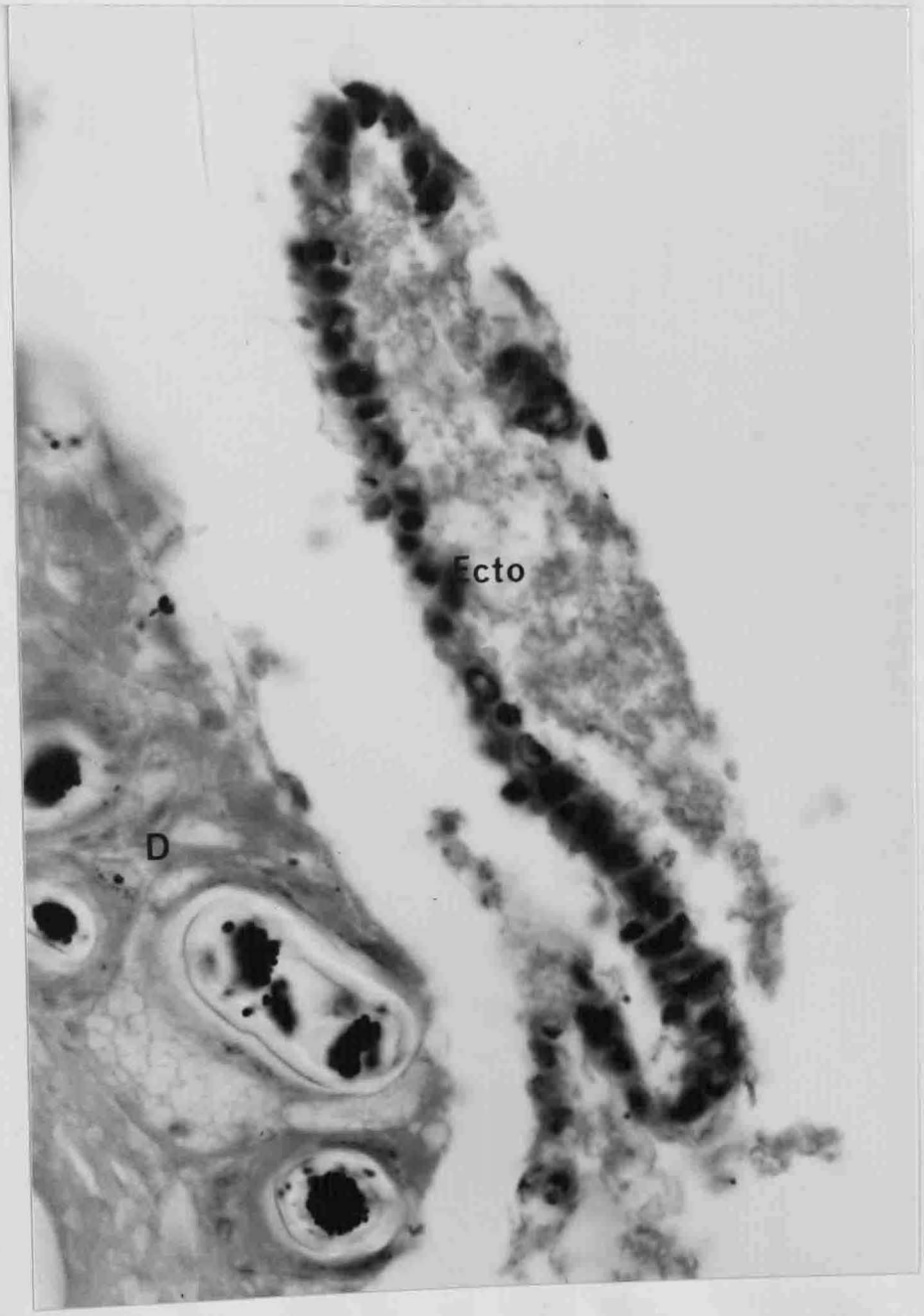

Ecto
D

Figure 5

Frozen-thawed dermis cultured alone on the CAM of a chick embryo for ten days. The CAM completely engulfed the dermis but no stratum corneum was induced to form by dermis in the control graft. 100 X.

CAM - chorio-allantoic membrane

CD - control dermis

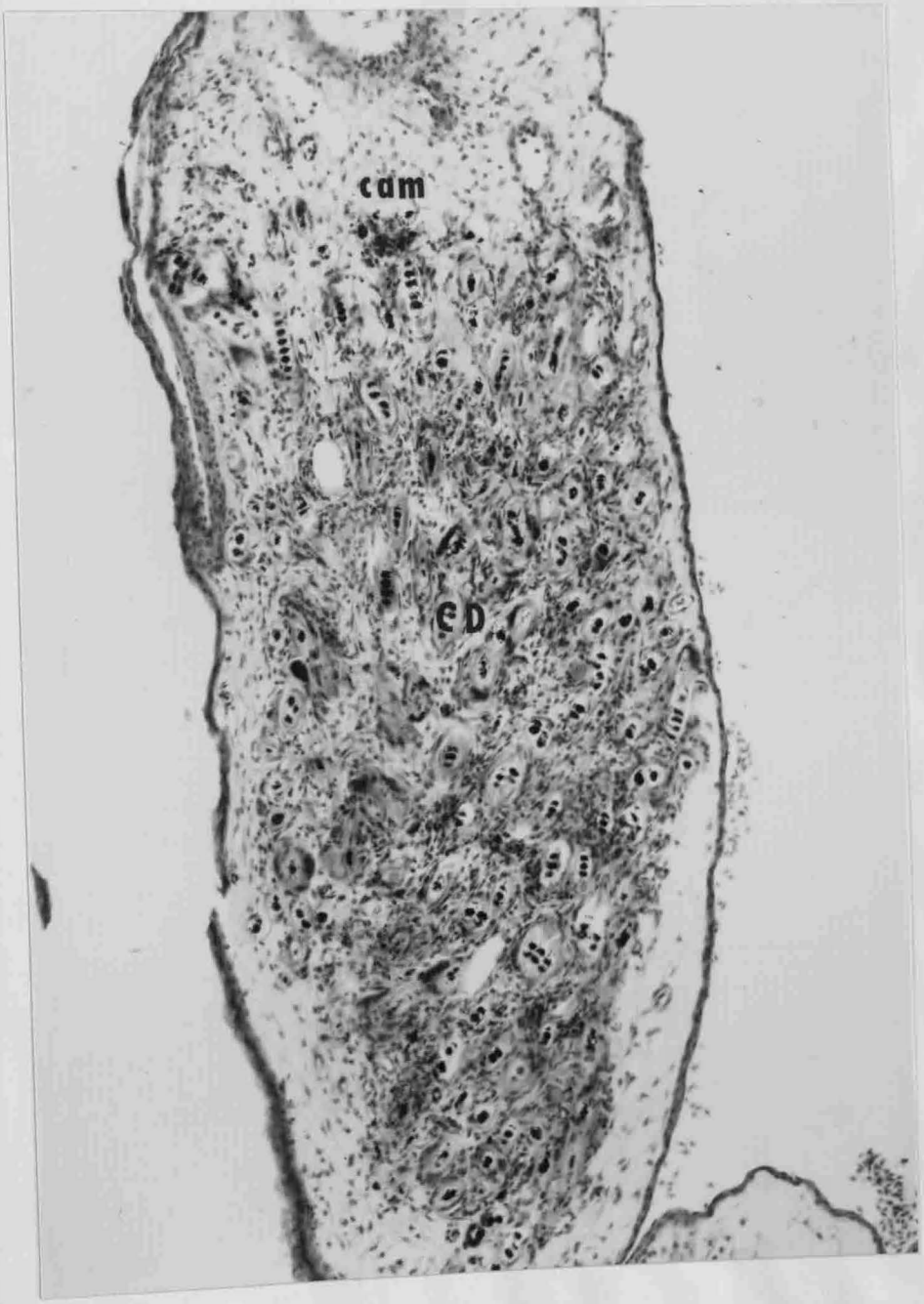

cam
ED

Figure 6

Eighteen - 21 day-old embryonic mouse skin was cultured on the CAM of a chick embryo for ten days. The dermis coalesced with the CAM, the epidermis was maintained although the cells tended to become squamus, and the stratum corneum greatly increased in thickness. 100X.

CAM - chorio-allantoic membrane

D - dermis

E - epidermis

Sc - Stratum corneum


sc
E
D
cam

Figure 7a

A graft of live mouse epidermis and live dermis after trypsinization, separation, recombination, and cultivation for ten days on the CAM of a chick embryo showed epidermal degeneration. 450X.

CAM - chorio-allantoic membrane

D - dermis

Sc - stratum corneum

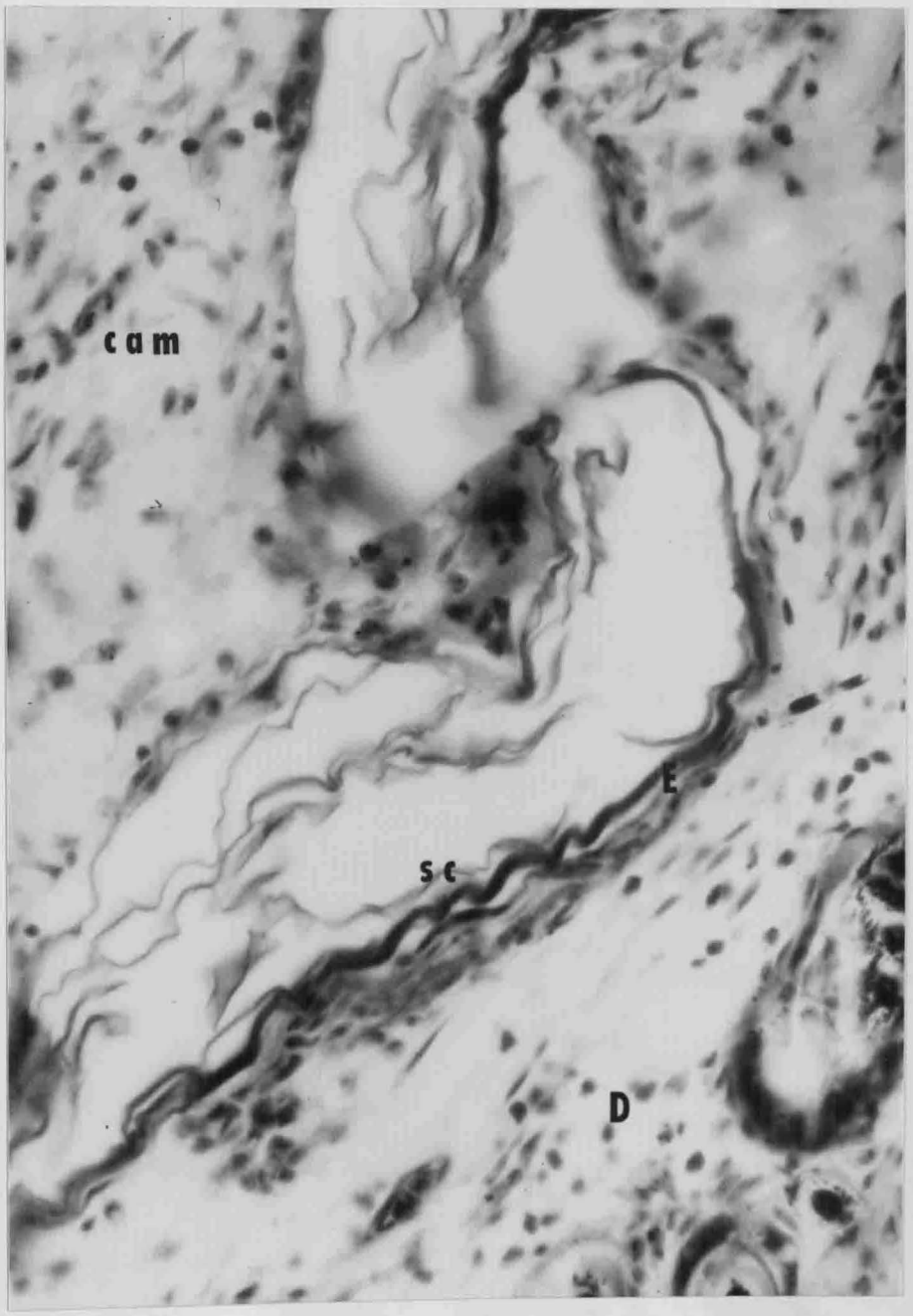

cam
E
sc
D

Figure 7b

A graft of live mouse epidermis and live dermis treated as in figure 7a but showing normal development of epidermis. The CAM covered the graft. 450X.

CAM - chorio-allantoic membrane

D - dermis

G - germinal layer

Sc - stratum corneum

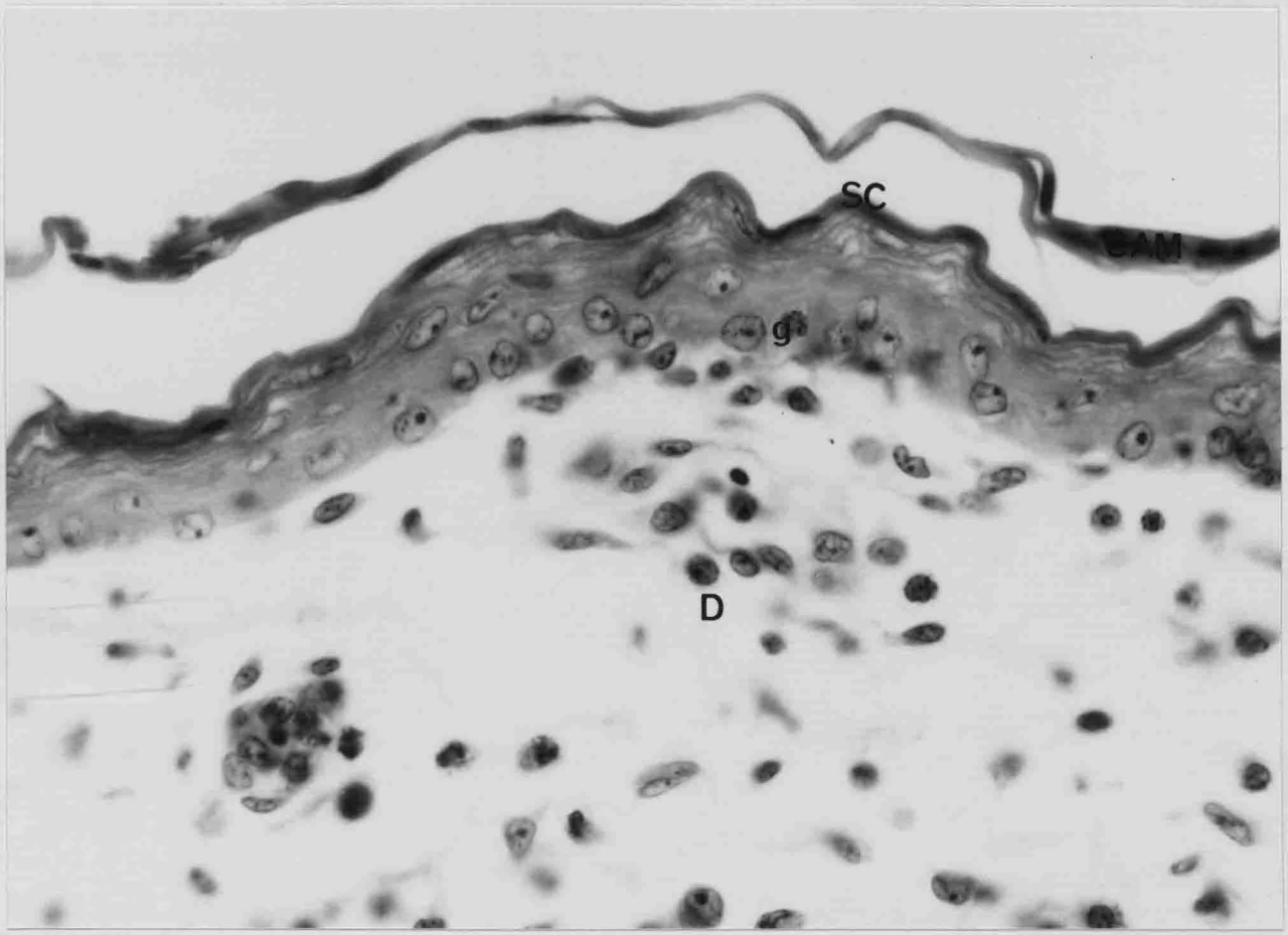

SC
g
D

Figure 8

A limb bud from a 10-12 day-old embryonic mouse was grown on frozen-thawed dermis in tissue culture. The multi-celled mass was maintained and fibroblasts migrated into the dead hair follicles. 450X.

D - dermis

LB - Limb bud

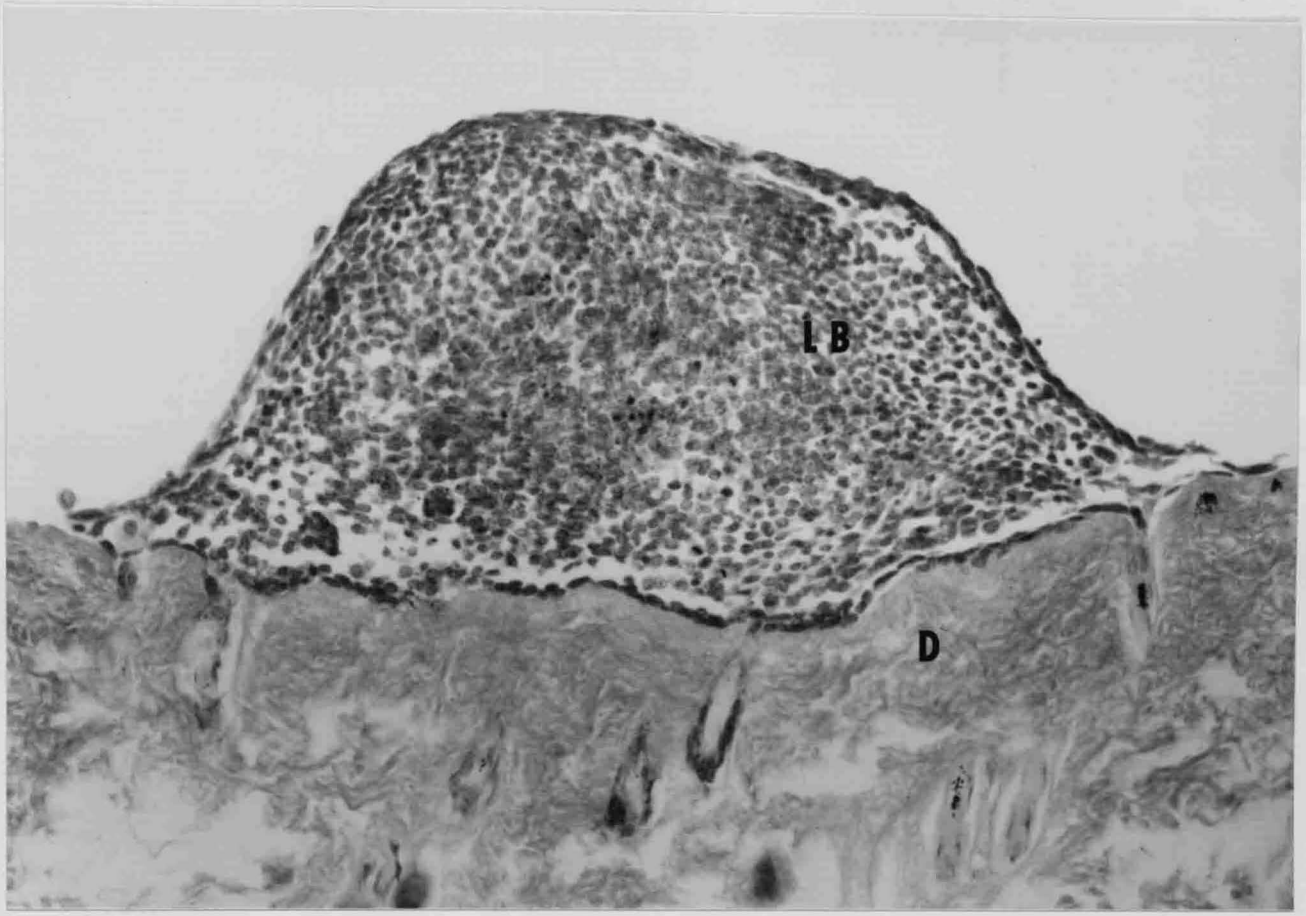

LB
D

Figure 9

Cells from the limb bud shown in figure 8 migrated into the hair follicles of the frozen-thawed dermis. 1000X.

D - dermis

F - follicle

LB - limb bud

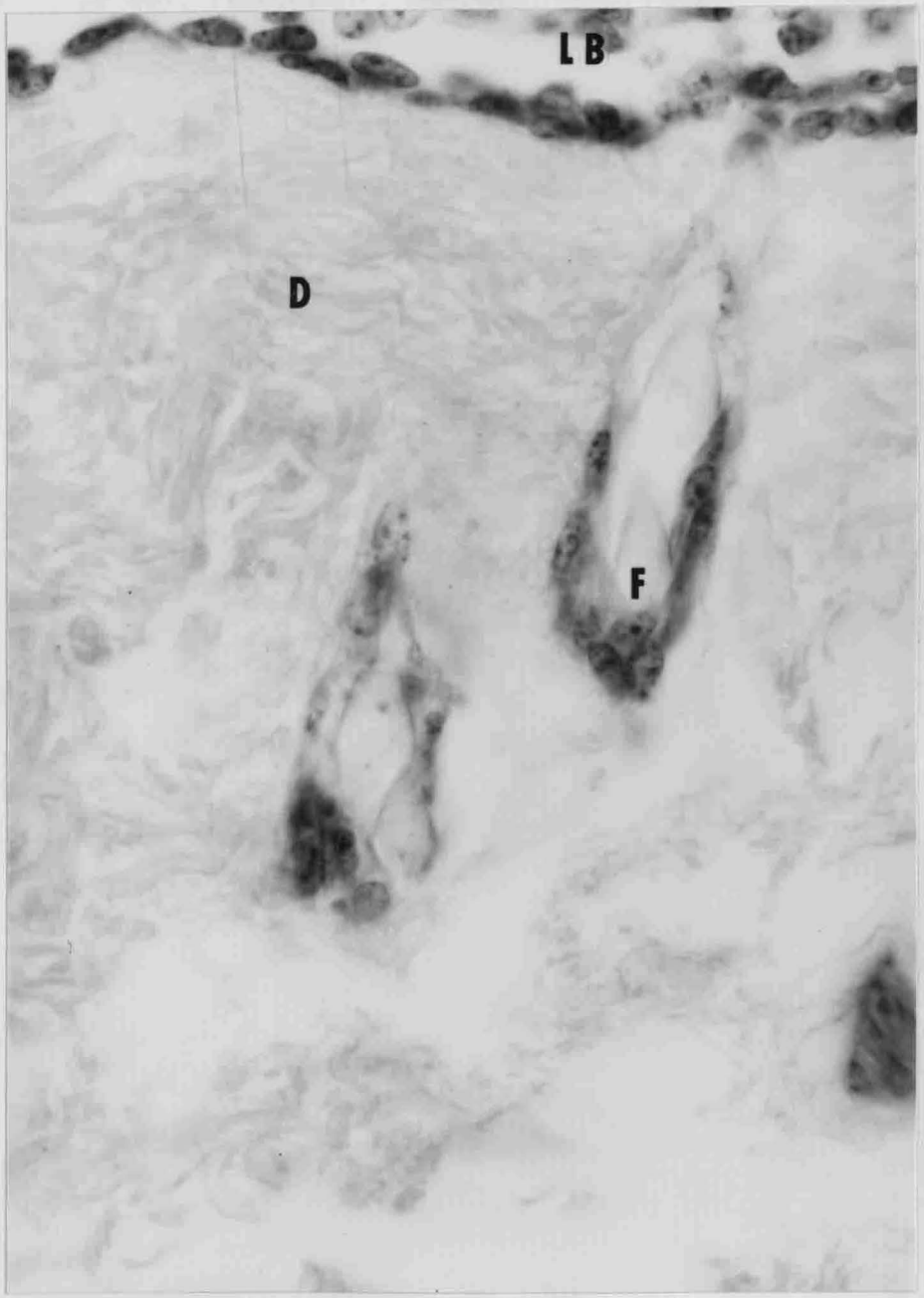

L B
D
F

Figure 10

A tissue culture preparation of BHK_{21} cells cultured on frozen-thawed dermis. 450X.

BHK - baby hamster kidney cells

D - dermis

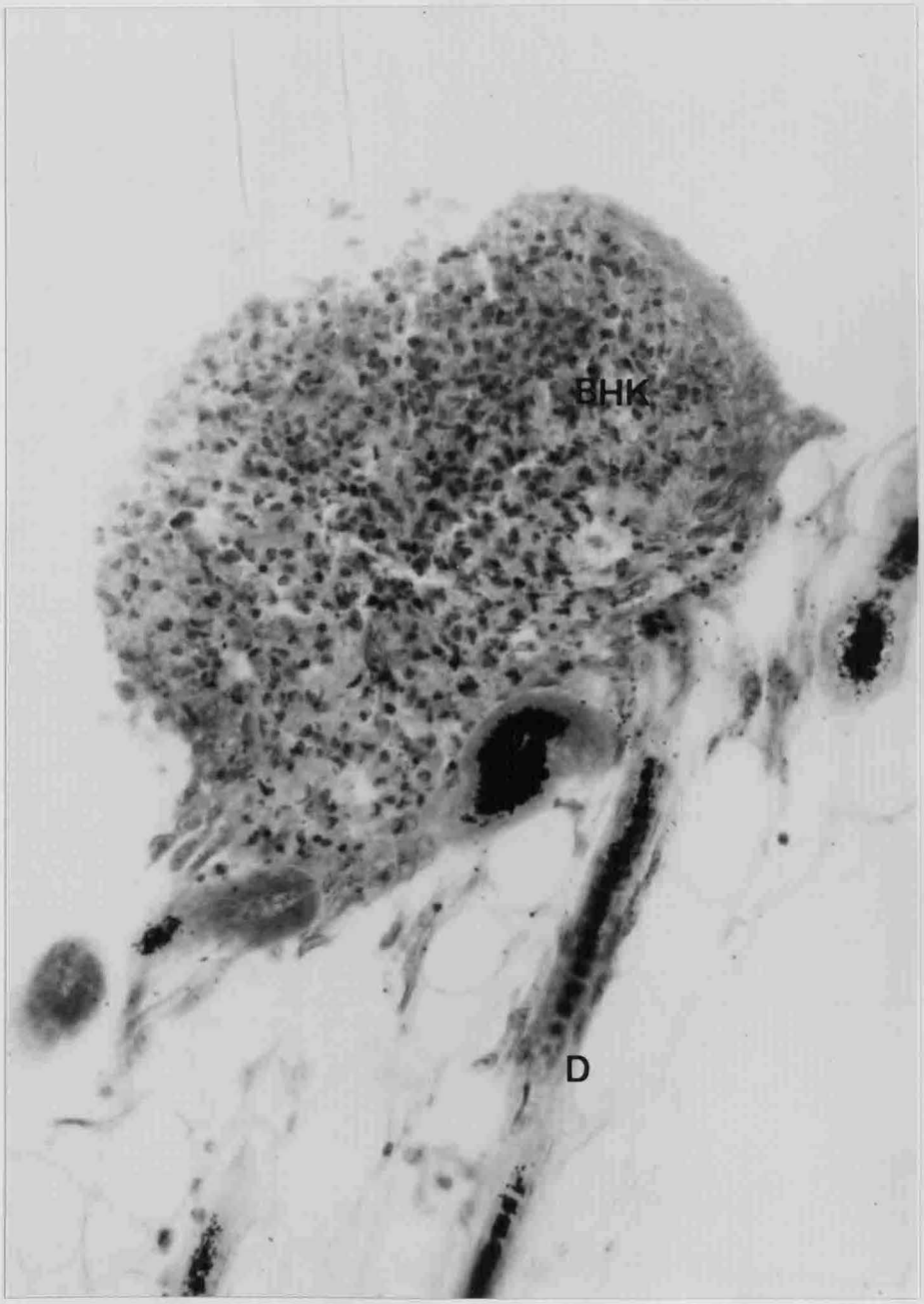
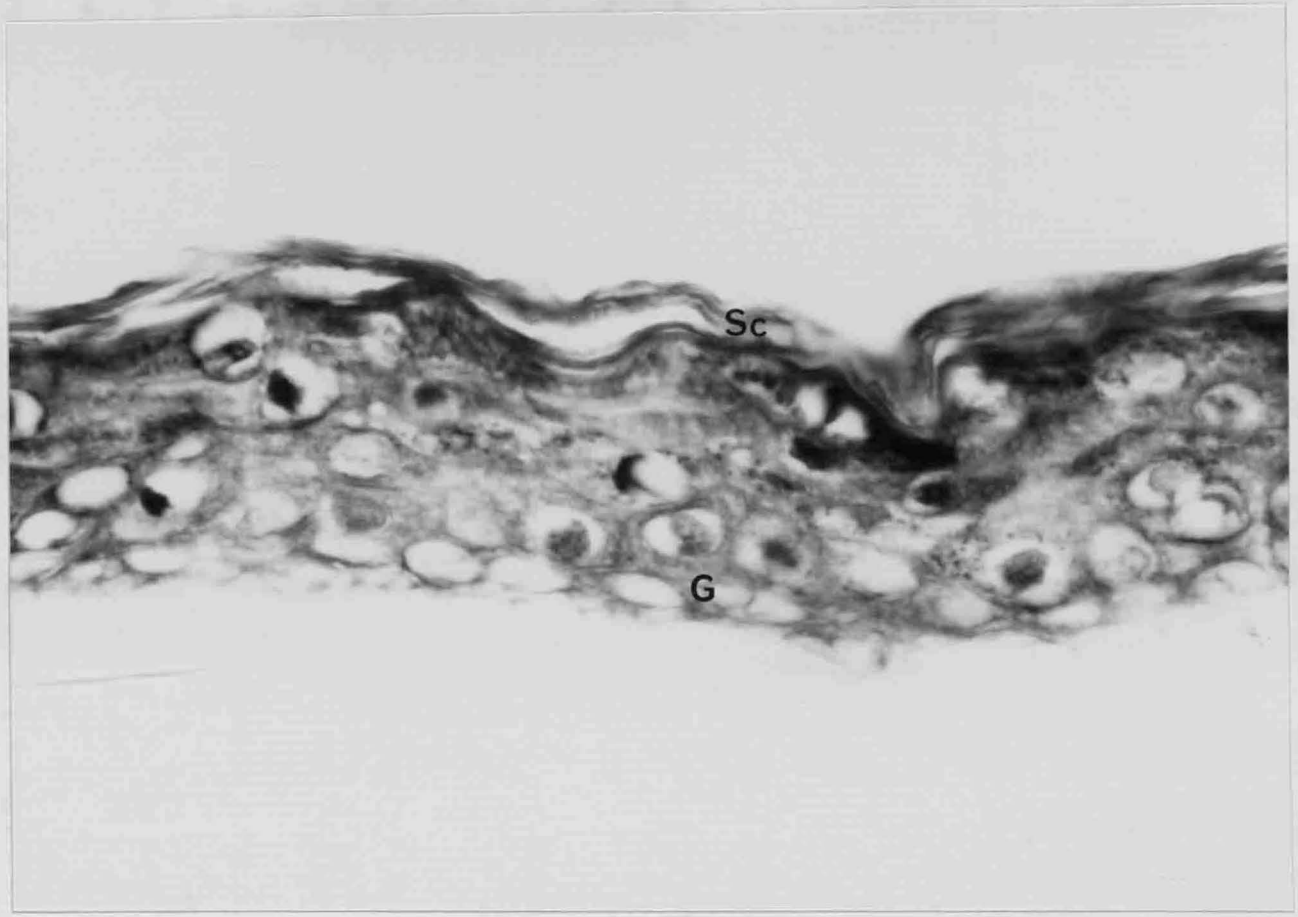

BHK
D

Figure 11

Epidermis from a 4 day-old neonatal mouse mechanically separated from underlying dermis after incubation at 4 C in 0.4% trypsin. Note the swollen appearance of germinal layer cells and those cells above the germinal layer. 1000X.

g - germinal layer

Sc - stratum corneum


Sc
G

VITA

Albert Joseph Banes was born in McKeesport, Pennsylvania on August 26, 1947. He received his elementary education in the Allegheny County Public Schools and secondary education at Shadyside Academy, a private school for boys in Fox Chapel, Pennsylvania. He graduated from Shadyside in 1965. He matriculated at Lehigh University in September 1965 and graduated with a B.A. in biology in June 1969.

In September 1969 he entered graduate school at the University of Richmond to pursue studies leading to the Master of Science degree in biology.

Upon receiving the master's degree, he will enter graduate school at the Medical College of Virginia in the microbiology department and will specialize in animal virology.

He is married to the former Ann Churchill Edelen.