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MOUSE EPIDERMAL CELL CULTURES.

I. ISOLATION AND CULTIVATION OF EPIDERMAL CELLS FROM ADULT MOUSE SKIN*

N. E. FUSENIG, M.D., AND P. K. M. WORST, M.D.†

ABSTRACT

A modified method was developed for rapid and continuous isolation of viable epidermal cells from adult mouse skin. Using a special type of trypsination apparatus (trypsinator) it was possible to obtain 3–6 $\times$ 10^6 epidermal cells per 30 cm^2 of depilated back skin. Dye exclusion tests indicated a viability of more than 80%. In suspension the isolated epidermal cells incorporated ^{3}H-thymidine into acid-insoluble material during a 4- to 6-hr period. After plating on plastic culture dishes the cells grew as primary cultures and could be maintained in a proliferative state for 2 months. After 7 days in culture the labeling index reached 17% and the mitotic count 0.6%, respectively. The epidermal nature of the cultures was characterized by morphologic features and by detection of a tissue-specific membrane antigen on cultivated cells with a heterologous antiepidermal antiserum. Attempts to increase the low plating efficiency and to maintain the initial proliferation rate were not successful.

The isolation and cultivation of epidermal cells from human or animal skin was accomplished previously mainly by two methods. Firstly, skin slices have been explanted in fluid media, as first reported by Medawar [1], using adult human and rabbit skin, or in plasma clots, as investigated chiefly by Pommerat and his associates [2, 3] and by Basset et al [4] working with adult human skin. Cultures established by these methods gave rise not only to an outgrowth of epithelial cell sheets but also to fibroblasts which soon overgrew the epithelial elements [5]. Secondly, the success of the trypsination method for dissociation of mammalian tissues into viable single cells led to the experients of Billingham et al [6] and Cruikshank et al [7], who were able to isolate viable epidermal cells by trypsin treatment of adult human, rabbit, and guinea-pig skin. This trypsin procedure, followed by mechanical separation of the loosened epidermis and its subsequent disintegration into single cells has been the method most widely used in the past. Other studies illustrating various modifications of this method have been reported by Giovanella and Heidelberger [8] and Briggaman et al [9].

Though several other successful attempts have been made to establish cultures of epidermal cells from adult human, monkey, rabbit, and guinea-pig skin using these isolation methods [9–14], single cell cultures from adult mouse skin have not been achieved so far. Mouse epidermal cultures could be established only if embryonic [15–17] or newborn [18,19] mouse skin was used. One of the main reasons for this failure has been the difficulty of isolating viable epidermal basal cells without fibroblast contamination. Moreover, the histologic composition of the adult mouse skin with its thin epithelium renders it very difficult to isolate large quantities of viable epidermal cells, a prerequisite for the successful cultivation of epithelial cells in vitro. Giovanella and Heidelberger [8] reported an isolation method for epidermal cells from adult mouse skin using three different enzymes. However, they did not report details of the behavior of these cells in culture. Laerum [20] achieved the isolation of epidermal basal and differentiating cells from adult hairless mouse skin with a modified trypsination method. By metabolic studies on single cells in suspension and by in vivo culture assays using diffusion chambers, the viability of the isolated cells could be demonstrated [21].

This study was undertaken to develop a simplified method for the isolation of epidermal cells from adult mouse skin by continual trypsin treatment. The method described below does not require additional mechanical separation procedures of the epidermis and leads to considerably larger quantities of viable epidermal cells. The cultivation of epidermal cells isolated by this procedure and preliminary observations on their nature and proliferation in vitro are reported.

MATERIALS AND METHODS

Animals

Female albino mice, 7–8 weeks old, of the inbred strain O_{20} (kindly supplied by Dr. L. M. Boot, The Netherlands Institute for Cancer Research, Amsterdam) and of the strain NMRI (purchased from the Institut für Versuchstierzucht, Hannover) were used.

Materials

Dulbecco's phosphate-buffered saline (PBS), trypsin (1:250), hyaluronidase (500 IU/mg), and elastase (lyophilized, 16 U/mg) were obtained from Serva, Heidelberg.

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* From the Institute for Biochemistry and † the Institute for Experimental Pathology, Deutsches Krebsforschungszentrum, 69 Heidelberg, Germany.

The enzymes were used as solutions in physiologic saline (0.8 gm NaCl, 0.04 gm KCl, 0.1 gm glucose per 100 ml bidistilled water). Tissue culture media concentrates (Eagle's Minimal Essential Medium, MEM [22]), calf sera, bovine and chick embryo extracts were obtained from Flow Laboratories Inc., Irvine, Scotland. Final outgrowth medium was prepared by further addition of amino acids and vitamins from stock concentrates to yield fourfold final concentrations, supplemented with nonessential amino acids (1%), glutamine (2 mM), and 17% fetal calf serum. The antibiotics penicillin (500 U/ml), streptomycin (100 μg/ml), and amphotericin B (2.5 μg/ml) were used both in PBS and culture media. All solutions and media were adjusted to pH 7.3.

Isolation Procedures

Mice were killed by cervical dislocation and thoroughly washed with soap, 70% ethanol, and PBS. The back skin (30 cm^2) was removed, pinned on a cork plate epidermis side down, and freed of subcutis by scraping with a scalpel blade. The skin was fixed dermis side down and the hairs were removed with depilatory wax (collophonium:wax/2:1). The melted wax (45–50°C) was quickly distributed with a brush on the fur and chilled with ice-cold PBS. The solid wax layer with the hairs sticking in it was peeled off, leaving behind the completely depilated skin. The skin was cut into 5-mm-broad strips, which were washed 3 times with PBS, and finally minced with scissors to slices of approximately 5 x 5 mm. These slices were suspended in 0.2% trypsin and poured into the trypsination vessel.

For the epidermal cell isolation we used a trypsination apparatus, developed by us, which facilitated continual enzymatic digestion of the epidermis in a closed system (Fig. 1). The temperature of the trypsinator itself and of the two storage vessels for trypsin and PBS, respectively, was maintained at 37°C using a water jacket. The trypsinator was 13 cm high, with an inner diameter of 4.5 cm, an outer diameter of 6 cm, and a volume of approximately 30 ml. The storage vessels were 17 cm high, with inner diameters of 7.5 cm, outer diameters of 9 cm, and volumes of approximately 75 ml. If skins of more than 6 animals are used in one experiment, vessels of larger sizes have to be used to prevent clogging of the nylon mesh. The storage vessels were connected with the trypsinator by silicon rubber tubings by way of a three-way tap. The skin slices were gently agitated in the trypsinator by a motor-driven glass rod at 80 rpm. A nylon filter (10–15 μm mesh width; Schweizer Seidengazefabrik, Zürich, Switzerland) at the bottom of the trypsinator permitted drainage of the trypsin suspension with the already isolated cells into centrifuge tubes and the influx of new prewarmed trypsin or PBS from the storage vessels into the trypsinator. The fresh solutions flowing through the nylon filter into the trypsinator simultaneously effected the detachment of the skin slices which tended to stick to the nylon net.

Trypsination was started with a 0.2% enzyme solution. As soon as turbidity in the trypsination vessel indicated the beginning of cell detachment, the suspension was drained as 30-ml fractions into precooled centrifuge tubes, containing 10 ml PBS with 10% calf serum (PBS/CS) for enzyme inactivation. The skin slices in the trypsinator were then whirled with PBS coming from the second storage vessel, in order to wash out all loosened cells, and again incubated with trypsin. This procedure was repeated at 5- to 10-min intervals. After 30–45 min the separation of the epidermis became macroscopically

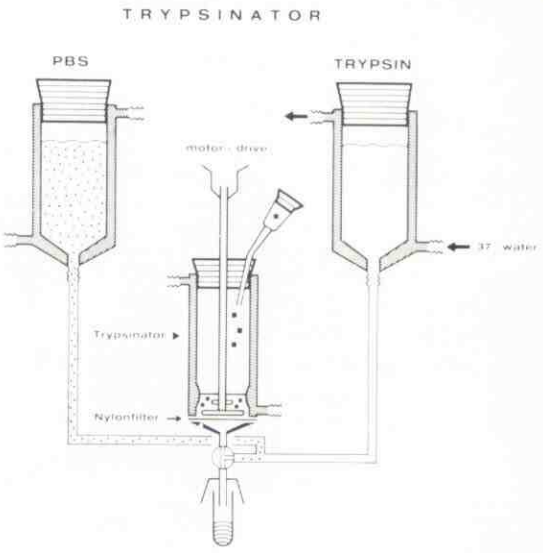

FIG. 1: Trypsination apparatus. For details see *Materials and Methods.*

visible by the detachment of the horny layers as intact sheets. The isolation was then continued using trypsin concentrations which were decreased stepwise by addition of PBS from the second storage vessel in order to minimize the cell damage exerted by the enzyme and to prevent the disintegration of the dermis. For the interval from 40–60 min the trypsin solution was diluted 1:1 (v:v) by PBS, yielding a concentration of 0.1% followed by dilutions of trypsin:PBS 1:2 and 1:3 (v:v), each for 20-min incubation time. The trypsination procedure was stopped after 90–120 min, leaving behind in the trypsinator the floating horny sheets and the dermal slices. The cell suspensions obtained during the first 20 min of the isolation procedure were discarded because they were often contaminated by fibroblasts probably loosened from the cut surface of the skin slices. The cell suspensions were centrifuged for 10 min at 500 × *g* at 4°C, pooled, and washed 3 times with PBS/CS, and finally resuspended in complete growth medium. These washing procedures combined with the antibiotic action were sufficient to eliminate bacterial or fungal contamination.

Cell Count and Viability Tests

Total cell count and viability count were made simultaneously using supravital staining procedures with trypan blue (0.2%) or nigrosin (0.2%) in PBS according to the method described by Briggaman et al [9].

As an additional viability test, thymidine-methyl-^3H (5 Ci/mM, Amersham/Buchler, Braunschweig) incorporation was studied in cell suspensions using the filter paper method as described by Süss and Volm [23]. Two-ml aliquots of the epidermal cell suspension in culture medium (5 × 10^5 cells/ml) were incubated at 37°C in a shaking water bath with 10 μCi/ml ^3H-thymidine. After 15, 30, and 60 min and 2, 4, 6, and 8 hr, 100-μl aliquots were spotted on filter papers, extracted with TCA (1 × 10%, 2 × 5%), dried with ether:ethanol (1:1) and assayed in a liquid scintillation counter. Supernatant medium of sedimented cell suspensions incubated at 37°C and cell suspensions incubated at 4°C were routinely run as controls. The measured radioactivity is expressed as cpm/number of cells.

Culture Techniques

For cell culture studies the cells were suspended at concentrations of 1–2 × 10^6 cells/ml in complete culture medium, plated in Falcon plastic Petri dishes (3.5 cm) and incubated at 37 °C in a humidified incubator with an atmosphere of 95% air and 5% CO_2. Medium change was first done 3 days after plating and then routinely carried out twice a week. Living cultures were observed and photographed in an inverted microscope with phase contrast optics (Wild, Heerbrugg, Switzerland). Skin slices were fixed in buffered 5% formalin and stained with hematoxylin and eosin. Cell smears were air dried and stained with Giemsa.

Autoradiography

Cultures from three different isolation experiments were exposed to 5 μCi ^{3}H-thymidine for 60 min on the third to ninth days of cultivation. The cultures were then washed 3 times with ice-cold PBS, fixed with ethanol:acetic acid (9:1), extracted for 10 min with 5% TCA, rinsed for 60 min under running tap water, and air dried. The rehydrated cultures were coated with Ilford K-5 nuclear emulsion, exposed for 8–10 days in light-proof boxes in 40% atmospheric humidity at 4 °C, developed in Kodak D-19 developer, fixed with Kodak Unifix (No. ½/1 size), and stained with Mayer's hematoxylin. Labeled cells and mitoses were counted in the same cultures.

Detection of a Tissue Antigen

Adult epidermal cells were tested for the presence of an epidermis-specific cell surface antigen. The technical procedure was essentially the same as recently described for embryonic mouse epidermal cells [24]. A rabbit antimouse epidermis serum, absorbed in vivo in a mouse of the appropriate strain was allowed to react against adult mouse epidermal cells growing in the small cups of a Terasaki micro-culture-plate (Falcon). The binding of antibodies to the surface of the cells was detected by a modification of the mixed hemadsorption test [25].

RESULTS

Histology

The epidermis from the depilated back skin of 7-week-old mice consists of 2–3 layers of nucleated cells with a definite basal and 1–2 differentiating cell layers. The depilation procedure did not cause visible morphologic damage to the epidermis (Fig. 2).

Cell Isolation

Preliminary attempts to isolate epidermal cells from adult mouse skin were performed according to the method reported by Giovanella and Heidelberger [8], using the enzymes hyaluronidase, elastase, and trypsin. In our hands the mechanical separation of the epidermis, after preincubation of the skin in hyaluronidase and elastase, was never complete. The total yield of epidermal cells which could be released with trypsin from the mechanically separated epidermis ranged from 5 × 10^5 to 2 × 10^6 per back skin. The viability of the cells isolated by this method did not exceed 50% as revealed by dye exclusion tests. The cells obtained by this method did not grow in culture. In addition, the mechanical separation of epidermal sheets from skin slices is time consuming and often complicated by microbiologic contamination.

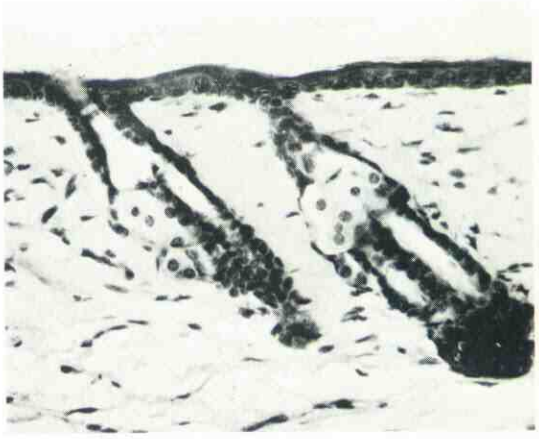

FIG. 2: Adult mouse skin, strain O_{20}, 7 weeks old, after epilation. (H & E; × 142)

Using the trypsination method described above, the yield of epidermal cells increased up to 3–6 × 10^6 per 30 cm^2 of back skin. Moreover, skin slices from the back skin of 4–6 animals could be handled in one experiment leading to total cell numbers of 20–40 × 10^6. Giemsa-stained cell smears contained mainly basal cells, uniform in size, with heavily stained nuclei and a high nuclear:cytoplasmic ratio (Fig. 3). Spinous cells were larger, showing a decreased nuclear:cytoplasmic ratio, and scattered chromatin. Attempts to separate these two cell types before plating by centrifugation on albumin or serum gradients [8] failed. Since the differentiated cells did not attach in culture [19, 26] and were washed away by medium changes, no further separation procedures were tried.

Eighty to 90% of the isolated, undifferentiated cells appeared viable by trypan blue or nigrosin. The data given in Figure 4 indicate that the isolated cells in suspension were also able to maintain thymidine incorporation for a limited period of time. The TCA-insoluble radioactivity increased up to 4 hr and reached a plateau after 8 hr. The incorporation rates showed good reproducibility using cells from different isolation tests (Mean value of incorporations after 120-min incubation in 15 assays: 738.5 ± 39.6 cpm/5 × 10^4 cells). The cells incubated at 4 °C did not incorporate radioactivity over the time period measured, nor did the incubation assays with supernatant media. Using the combined enzymatic–mechanical isolation methods, cell number and viability varied considerably from test to test. With the isolation method described in this report, in more than 30 experiments the cell yield per back skin was relatively constant (mean value: 5.5 ± 1.2 × 10^6) with reproducible trypan blue viability (mean value: 87% ± 4.5), plating efficiency, and outgrowth pattern.

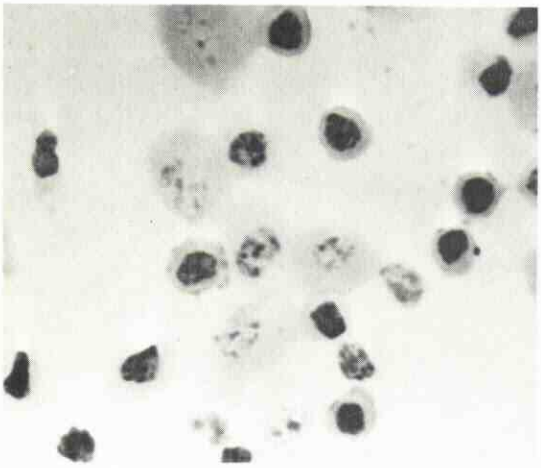

FIG. 3: Smear of isolated epidermal cells. (Giemsa; × 223)

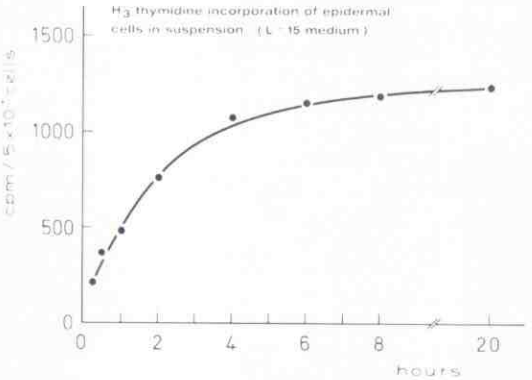

FIG. 4: ^{3}H-thymidine incorporation rate of epidermal cells in suspension. For details see text.

Cell Cultures

The plated epidermal cell suspension consisted predominantly of single cells, representing undifferentiated and differentiating cells (Fig. 5). During the first day in culture the basal cells formed progressively larger aggregates. The aggregation occurred before the cells attached to the culture surface and seemed to be a prerequisite for their attachment and further outgrowth. Similar observations were also made by Briggaman et al [9] in cultures of human epidermal cells. By the second and third day in culture the cell clumps attached to the plastic surface and the cells started to spread out forming cell islands of epithelial-like morphology (Fig. 6). The cells were round or polygonal and contained large nuclei with prominant nucleoli. Approximately 1–2% of the plated cells attached to the surface while the rest were washed away by medium changes. We have not been able to improve this low plating efficiency.

During subsequent cultivation the epidermal cell islands increased in size and became confluent (Fig. 7). Although the cells grew in close lateral apposition to each other, they were nevertheless clearly separated by a distinct intercellular space. Mitoses were first recognized after the cell islands had formed by spreading of the cell clumps, usually in 4- to 6-day-old cultures. During the first

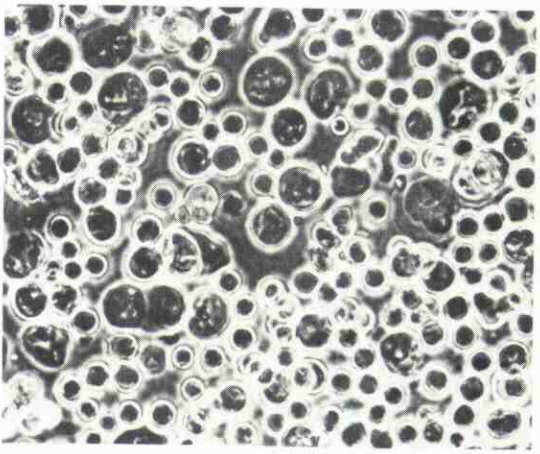

FIG. 5: Epidermal cell suspension 2 hr after plating. (Phase contrast; × 251)

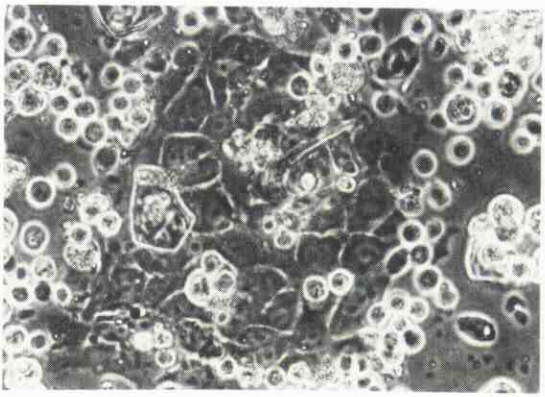

FIG. 6: Epidermal cell culture 4 days after plating; epidermal cell island. (Phase contrast; × 101)

FIG. 7: Epidermal cell culture 10 days after plating; monolayer of confluented epidermal cell islands. (Phase contrast; × 115)

10 days in culture the mitotic count did not exceed 0.6% (Table I). Since only meta-, ana-, and early telophases could clearly be distinguished in these studies, the actual mitotic index may be higher. Four weeks after plating mitotic figures were still present but, in a few cells, nuclear condensation or pyknosis had already occurred. These features were even more pronounced in 2-month-old cultures. With increasing cultivation time the cultures diminished as proliferation ceased.

Thymidine incorporation was measured starting 1 day after the cells had attached to the plastic dishes. The labeling index of the epidermal cells growing in cell islands of a few up to 50 cells increased during the first week in culture reaching a maximum of 17% after 7 days and then declined (Table I). This initial high labeling index could be due to a partial synchronization which has also been observed in embryonic epidermal cultures [17]. The proliferation slowed down in the following weeks and ceased after 2 months. Fibroblast colonies were rarely seen and only at later stages of the cultivation. They could be distinguished by their morphology and their faster proliferation rate. Although it has been reported that epidermals cells may alter their morphology after prolonged cultivation times [12], it seems that these colonies were fibroblasts starting from minor impurities of the cell preparation. Besides the fibroblasts, another cell type of different morphology was usually observed at the periphery of epithelial islands. Because of their long, branched processes these cells were classified as dendritic cells (Fig. 8). They could not be identified as melanocytes by the appearance of melanosomes or by the dopa reaction, as we used albino mice.

As morphologic criteria are uncertain if applied to cells growing in culture, further criteria were required to differentiate epidermal cells from fibroblasts. We attempted to characterize the epidermal nature of the cultivated cells by immunologic methods. A rabbit antiserum against mouse epidermis which after in vivo absorption had been demonstrated to react only against embryonic mouse epidermal cells [27] exhibited the same reactivity against cultured adult epidermal cells as compared to embryonic epidermal cells tested simultaneously. The serum did not react with cultivated fibroblasts from adult or embryonic mouse skin (Table II).

TABLE I

Labeling index (LI) and mitoses (M) in epidermal cell cultures from adult (NMRI, ♀) mouse skin

Cultivation time (days)	No. of experiments	% LI	% M
3	2	6.2–8.4	—
5	3	13.0–15.8	0.2–0.3
7	3	13.9–17.6	0.5–0.6
9	3	9.9–12.2	0.3–0.5

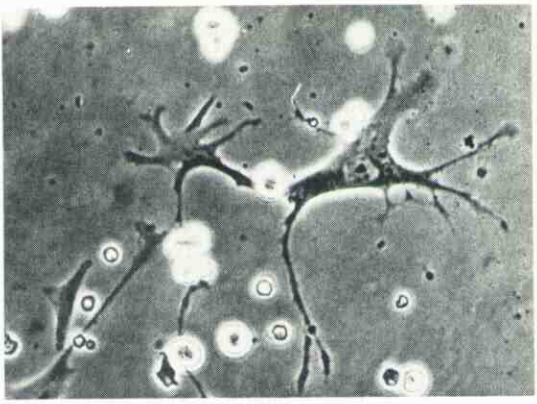

FIG. 8: Epidermal cell culture 3 days after plating; dendritic cells. (Phase contrast; × 262)

TABLE II

Reactivity of in vivo absorbed rabbit antimouse epidermis serum on cultivated epidermal cells or fibroblasts from adult (a) or newborn (n) mouse skin

Target cells	Mouse strain	Positive reactions/ no. of tests	End titers
(a) Epidermal cells	NMRI	8/(8)	<1/256
(a) Fibroblasts	NMRI	0/(5)	—
(n) Epidermal cells	C57BL/6	>30/(>30)	<1/256
(n) Fibroblasts	C57BL/6	0/(>30)	—

DISCUSSION

To our knowledge this is the first report on successful in vitro cultivation experiments with pure epidermal cell suspensions from adult mouse skin. The cultivated cells proliferated in vitro and were morphologically and immunologically identified as epidermal cells.

The procedure for the isolation of epidermal cells described in this report offers a modified one-step method for obtaining cell suspensions of rather pure viable epidermal composition in larger quantities. The closed system in which the isolation procedure takes place prevents microbiologic contamination of the cell suspension, a problem often encountered if larger amounts of cells have to be prepared in commonly used trypsination vessels or by mechanical separation of the epidermis. As revealed by dye exclusion tests and by the incorporation studies with ^{3}H-thymidine the isolated cells are viable and able to synthesize DNA in suspension. The stepwise lowering of the enzyme concentration during the isolation, correlated to the degree of epidermal detachment, together with the withdrawing of the freshly isolated cells at short time intervals, allowed prolonged trypsination without increased cell damage.

Only a small percentage of the seeded cells grew out in culture. This could be a consequence of inadequate culture conditions. Though the addition of various tissue culture media, embryo ex-

tracts, and sera did not significantly stimulate the plating efficiency, it cannot be ruled out that specific substrate or growth factors were lacking. The cells which grew out in culture had a slower multiplication rate compared to epidermal cells isolated from embryonic or newborn mice [15–19]. The labeling index rose to 17% in 7-day-old cultures but then declined again. Labeled cells and mitoses were predominantly located in larger cell islands during the first days of cultivation, indicating that epidermal proliferation in culture was favored by higher cell densities per surface unit. Attempts to increase the epidermal cell plating efficiency by stimulating the epidermal proliferation in vivo, either with the cocarcinogenic factor from croton oil 12-O-tetradecanoyl-phorbol-13-acetate (TPA) [30] or after partial synchrony with hydroxyurea failed.

Considerable difficulty is often encountered in establishing the identity of cultured epithelial cells by morphologic criteria, although some phenotypic traits of skin epithelial cells are maintained during growth in culture [18,29,31]. In contrast to fibroblasts, which generally grow as single cells, epidermal cells tend to grow in cell sheets. The polygonal shape of the individual cells and the characteristic cell contact by desmosome-like bridges are further indications of their epidermal nature [32]. In addition to morphologic criteria we were also able to prove the epidermal nature of our cells by demonstrating an epidermis-specific cell surface antigen present on adult mouse epidermal cells as well as on embryonic mouse epidermal cells but absent on embryonic or adult fibroblasts [27].

Contamination by fibroblasts is, nevertheless, one of the most serious problems in culturing epidermal cells. All known isolation procedures for obtaining epidermal cells, including the method used in this study, do not completely exclude fibroblast contamination. In our studies such contamination was minimal, due, we believe, to the minimal enzyme concentrations necessary for the separation of the epidermis.

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