GROWTH AND DIFFERENTIATION OF PRIMARY RAT KERATINOCYTES ON SYNTHETIC MEMBRANES

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SUMMARY

The attachment, proliferation, and differentiation of primary cultures of keratinocytes isolated from murine epidermis were monitored after purified cell suspensions were seeded and incubated in vitro on various synthetic membranes. Concomitant studies of the effects of attachment factors added to synthetic membranes before use as substrata for keratinocytes were also done. The study demonstrated that a synthetic membrane composed of nylon was superior to other membranes and to plastic control culture vessels in supporting the growth of murine keratinocytes. Although laminin enhanced initial attachment and proliferation of cells on nylon membranes, the untreated substratum was more effective for extended incubation. Stratification and differentiation of these keratinocytes on the nylon substratum was enhanced by raising confluent cultures (7 d) to the air-medium interface so that they were in contact with medium only from the bottom. Cultures raised for 14 d produced many morphologic markers of the epidermis and closely resembled the architecture of this tissue in situ.

Key words: keratinocytes; differentiation; stratification; synthetic membranes; attachment factors.

INTRODUCTION

A keratinocyte cell culture which can undergo terminal differentiation in a manner that closely resembles the ordered differentiation seen in situ would be of considerable value in epidermal research. It would offer the investigator a model for studying epidermal differentiation and homeostasis in vitro and for investigating the effects of environmental agents on those factors that are important in normal epidermal function. Most cell culture models used in biomedical investigations utilize the growth and metabolism of cells attached to an artificial substratum submerged in nutrient medium. On the other hand, keratinocytes isolated from mammalian epidermis and raised to the air-medium interface while in culture show physiological and differentiative activities that more closely resemble epidermal behavior in situ than when they are incubated totally submerged. Reviews that include most of the methods of cultivating keratinocytes at the air-medium interface have been reported (4,15,22,23). Most of the published procedures involved the inclusion of mesenchymal elements. Some investigators believe that dermal equivalents are necessary for proper keratinocyte differentiation (9,13).

We have developed a stratifying and differentiating culture of keratinocytes for evaluating the toxicity of chemical and physical agents as determined by changes in morphology, macromolecular metabolism, and cell surface properties. The goal was to obtain a culture that contained most of the biochemical, morphologic, and ultrastructural markers identified with the epidermis in situ. Ideally, such a culture should have a simple, easily prepared material as its substratum and thus could be used by laboratories involved in toxicity testing to obtain assays with significant reproducibility. Here, the results of growth and differentiation of rat keratinocytes on synthetic membranes constructed from materials containing cellulose, nylon, and other substances are reported.

MATERIALS AND METHODS

Establishing primary cultures of rat keratinocytes. Animals used for the isolation of keratinocytes from the epidermis were obtained from the randomly inbred colony of CFN albino rats maintained in this laboratory. The procedure for preparing these cells for primary cultivation was based on modifications in the method of combined stretching and trypsinization described in previous reports (28,30). One-day-old animals were killed by cervical dislocation and the total body cleansed with 70% ethanol. Skins from the backs of the animals were then dissected and stretched on the surface of a sterile petri dish so that the stratum corneum side of the tissue adhered to the bottom of the dish (three to four skins/100-mm dish). The petri dishes containing the stretched skins were then chilled to 4° C, after which approximately 20 ml of 1% trypsin, 1:250 (Difco Laboratories, Detroit, MI), prechilled to 4° C, was added to completely cover the adhering skins. After 2.5 to 3 h at 4° C, the enzyme solution was removed and the skins were

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TABLE 1

SYNTHETIC MEMBRANES SELECTED FOR STUDY OF
ATTACHMENT AND PROLIFERATION OF
RAT KERATINOCYTES IN VITRO

Membrane	Pore Size, μM	Material	Source
Puropor-200[a]	0.02	nylon	Gelman Sciences, Ann Arbor, MI
Puropor-450[a]	0.45	nylon	Gelman Sciences, Ann Arbor, MI
TCM-200	0.20	cellulose triacetate	Gelman Sciences, Ann Arbor, MI
TCM-450	0.45	cellulose triacetate	Gelman Sciences, Ann Arbor, MI
HA-TF	0.45	cellulose nitrate cellulose acetate	Millipore, Bedford, MA
RA-TF	1.20	cellulose nitrate cellulose acetate	Millipore, Bedford, MA
HT-200W[a]	0.20	polysulfone	Gelman Sciences, Ann Arbor, MI
ACRO[a]	0.00	polyethylene	Gelman Sciences, Ann Arbor, MI
MEM[b]	1.00	silicon-polycarbonate	General Electric, Schenectady, NY
Nylon-66	0.45	nylon	Rainin, Woburn, MA
Nuclepore	0.40	polycarbonate	Nuclepore, Pleasanton, CA

[a] These membranes were supplied through the courtesy of Gelman
Sciences, Inc., and are not available commercially.

[b] This membrane was still in the experimental stage and obtained
from General Electric by request.

washed with Earle's balanced salt solution (EBSS)
(GIBCO, Grand Island, NY) which had been prechilled to
4° C. The dermis was then carefully lifted from the
epidermis which remained on the surface of the petri
dish. The dermal tissue was discarded and the epidermal
cells on the underside of the epidermis were suspended
in complete growth medium [90% Eagle's minimum
essential medium (MEM) (KC Biologicals, Kansas City,
MO); 10% fetal bovine serum (Flow Laboratories,
McLean, VA); 10 $\mu g/m$ insulin and 10 $\mu g/ml$ hydrocorti-
zone (both from Sigma Chemical Company, St. Louis,
MO); 100 $\mu g/ml$ penicillin and 100 $\mu g/ml$ streptomycin
(both from Pfizer Laboratories, New York, NY); and 0.05
$\mu g/ml$ Fungizone (GIBCO)] by stroking the surface with a
sable-hair brush. Most debris and fibroblasts were
removed by centrifuging the cell suspension through
Ficoll gradients as described in a previous report (30).
The keratinocytes were then seeded onto selected
substrata at a concentration of 0.2% (vol/vol) which
contained approximately 5 × 10⁵ cells/ml as determined
by electronic counting (Coulter counter). Inocula consist-
ed of 0.5 ml of suspended keratinocytes per well in
culture vessels containing 24 wells (Costar, Cambridge,
MA). These primary cultures were incubated at 35° C in a
95% air:5% CO_2 atmosphere with 95% humidity.

*Membrane filters used as substrata for keratinocyte
cultures.* The various commercially available and experi-
mental synthetic membrane filters selected for this
investigation are listed in Table 1. The source of each

membrane and the material from which it was construct-
ed are also given in the table. Membranes TCM-200,
TCM-450, HA-TF, and RA-TF were selected for study
because they are specifically treated to be "tissue culture
compatible" (detergent-free). Membranes Acroshield L
(ACRO) and MEM were included because they are
transparent and allow cell growth to be continuously
monitored via phase contrast microscopy. The silicon-
polycarbonate filter (MEM) was prepared in our
laboratory by a method described in another report (29).
Filters were sterilized by (a) autoclaving, (b) immersion in
70% ethanol, (c) UV irradiation, or (d) gamma irradiation.
All filters were 13 mm in diameter and were placed in the
wells of the dishes before seeding with keratinocytes as
described above. In each experiment, wells without
membranes served as controls for keratinocyte attach-
ment and proliferation.

*Pretreatment of membranes with biological attachment
factors.* Two attachment factors were studied for their
ability to enhance the attachment and proliferation of rat
keratinocytes on selected membranes. Human fibronec-
tin (HFN) (Collaborative Research, Waltham, MA) was
applied to substrata at 5 $\mu g/cm^2$, allowed to remain for 2
h, and the excess fluid then removed. Laminin (LMN)
(Bethesda Research Laboratories, Inc., Gaithersburg, MD)
was applied at 1 mg/cm^2 and allowed to evaporate to
dryness. Untreated membranes served as controls for
attachment and proliferation of murine keratinocytes on
treated membranes.

*Establishing stratified, differentiated rat keratinocytes in
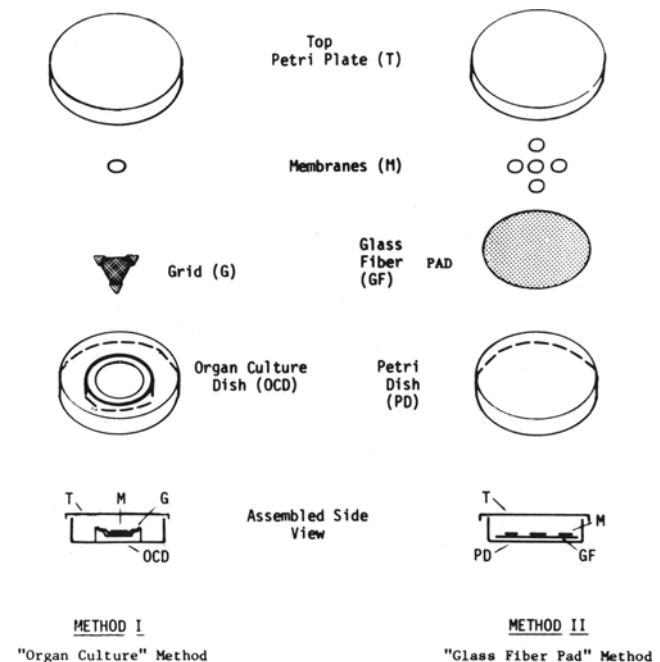vitro.* Isolated keratinocytes were seeded on selected

FIG. 1. Two methods for lifting keratinocyte monolayers
grown on membranes to the air-medium interface for continued
cultivation. Method I used the standard organ culture procedure
for providing nutrients and in Method II cultures were placed on
the surface of filter pads saturated with nutrient medium.

membranes and incubated as submerged cultures until monolayers resulted or up to 14 d. In method I, the membranes containing the monolayers were transferred to stainless steel screens in standard commercial organ culture dishes (Falcon Plastics, Cockeysville, MD) or, in method II, placed on the surface of glass filter pads (Gelman Sciences, Ann Arbor, MI) saturated with growth medium. Both methods are illustrated in Fig. 1. Cultivation was then continued for varying periods with the cells at the air-medium interface. Method I was used in early experiments but method II was used exclusively in later experiments because of simplicity and convenience. No difference in culture development was detected using the two methods.

Microscopy and quantitation procedures. Initial attachment and proliferation of rat keratinocytes on selected membranes and plastic culture vessels (Corning Glass Works, Corning, NY) were compared. Most of the membranes studied were opaque and thus cellular growth could not be followed via phase contrast microscopy. The extent of attachment and growth of cells on these membranes were determined after fixing the cells and substratum in 10% phosphate buffered formalin or Cornoy's solution (90% butanol, 10% acetic acid). The preparation was then stained with hematoxylin, cleared with xylene, and mounted on microscopic slides with permount and cover slips. Quantification of initial attachment/or proliferation, or both, was accomplished by counting stained nuclei per unit area of the substratum. This was performed manually using an ocular micrometer and electronically with the Bioquant Image Analysis system on the Apple IIe microcomputer (R. and M. Biometrics, Inc., Nashville, TN) with a video overlay and digitizing tablet. To obtain cross sections for microscopic observation after selected incubation periods under particular cultural conditions, the membranes were fixed in 10% buffered formalin, embedded in paraffin, sectioned with a rotary microtome, placed on microscopic slides, and stained with hematoxylin and eosin.

Keratinocyte cultures grown on the various membranes were also examined at the ultrastructural level. Cultures were fixed in Karnovsky's fixative containing 0.1 *M* sodium cacodylate buffer at room temperature for 1 h followed by incubation for approximately 12 to 24 h at 4 to 6° C. The fixative was then removed, the specimens washed with buffer and poststained with a 2% osmium tetroxide solution for 2 h, washed with distilled water, and stained enblock for 30 min with 2% aqueous uranyl acetate. The specimens were then dehydrated with a graded series of ethyl alcohol and finally with propylene oxide. The specimens were rotated for 2 to 4 h in a 1:1 mixture of propylene oxide and epoxy resin, transferred to a 100% resin mixture overnight, and finally embedded in resin. The embedded specimens were polymerized at 60 to 65° C for 48 h in flat embedding molds. Sections were cut at 700 to 1000 Å, mounted on 200 mesh, poststained with 2% aqueous uranyl acetate and with lead citrate, observed with an AEI Corinth 275 transmission electron microscope (TEM) at 60 kV, and photographed to obtain permanent records.

RESULTS

Comparative attachment and growth of rat keratinocytes on untreated synthetic membranes in submerged cultivation. Growth of primary rat keratinocytes on various membranes was compared by determining the number of cells present after cultivation for 1 and 10 d. Keratinocytes grown on plastic culture vessels were used as controls. Resulting attachment and proliferation were also recorded via photomicroscopy of the stained cultures. Figure 2 *A* and *B* depict the number of keratinocytes per square millimeter on various substrata after cultivation for 1 and 10 d, respectively. Several representative areas (20 to 30) on each substratum were included in the quantification, and each histogram represents the data from four or five experiments. Also included in Fig. 2 *A*
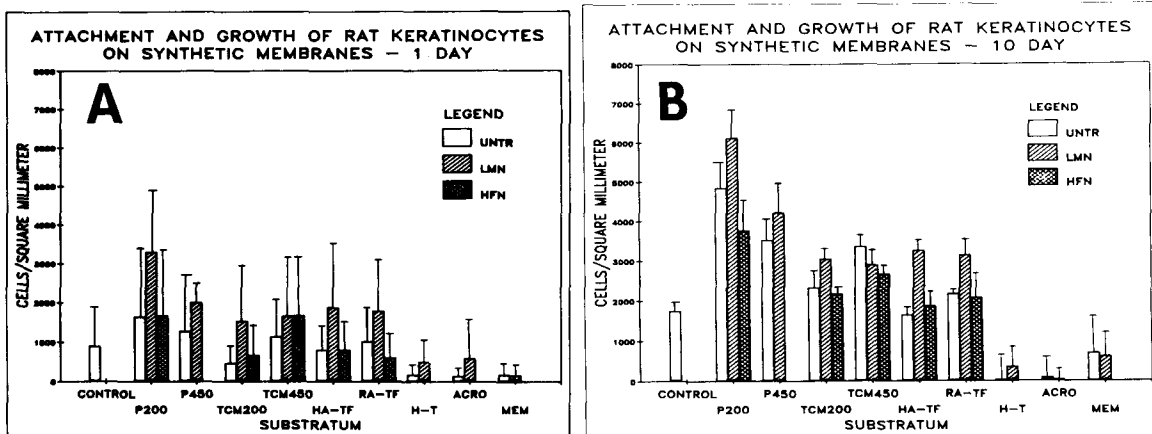

FIG. 2. Attachment and growth of rat keratinocytes on synthetic membranes. *A*, growth for 1 d; *B*, growth for 10 d. Substrata were untreated (*UNTR*), precoated with LMN, or precoated with HFN. Data are presented as mean + SD of four or five determination.

and *B* are data on growth after pretreatment of substrata with HFN or LMN. Nylon-66 and Nuclepore were not included because several attempts to obtain satisfactory attachment on these membranes were unsuccessful. The data in Fig. 2 *A* show that initial attachment and growth of rat keratinocytes on some of the selected membranes were superior to that on plastic culture vessels used as controls. Observation of cultures after 1 d of incubation showed that aggregates of keratinocytes had attached and spread out as single cell layers, forming growth centers (30). The large variation in cell number per unit area reflects the different sizes and distribution of growth centers in early cultures. After 10 d of incubation, monolayers had formed with varying degrees of compactness depending on the substratum (Fig. 2 *B*). Membranes specially prepared for tissue culture procedures, i.e. TCM-200, TCM-450, HA-TF, and RA-TF, supported attachment and proliferation equal to or better than the plastic culture vessels. The increased attachment and proliferation of keratinocytes on Puropor-200 over controls was statistically significant ($P < 0.001$). Also, more proliferation resulted on this membrane than on the others examined. It was therefore selected for further study in producing stratified, differentiated keratinocyte cultures in vitro. None of the transparent membranes

(HT-200W, Acroshield and MEM) supported attachment and proliferation as well as controls seeded on plastic culture vessels as demonstrated in Fig. 2 *A* and *B*.

Effects of attachment factors on keratinocytes cultured on synthetic membranes: HFN treatment. The membranes precoated with HFN did not support the attachment and proliferation of keratinocytes better than untreated membranes ($P > 0.05$) as illustrated in Fig. 2 *A* and *B*. In some cases (on membranes P200, TCM200, and TCM-450) there may have been less proliferation on HFN-coated than on uncoated membranes as observed after 10 d of incubation (Fig. 2 *B*).

Laminin treatment. Coating membranes with LMN enhanced the initial attachment compared to untreated controls after 1 d of incubation. Spreading of seeded keratinocytes across the substratum within the first day was also facilitated (Fig. 2 *A*). This was particularly evident when untreated and LMN-pretreated Puropor nylon membranes seeded with primary keratinocytes were compared after incubation for 1 d. However, only slight, although significant, differences in the total number of cells on untreated or pretreated nylon membranes were observed after incubation for 10 d ($P < 0.01$) (Fig. 2 *B*). Therefore, although precoating membranes with LMN seemed to enhance initial attachment,
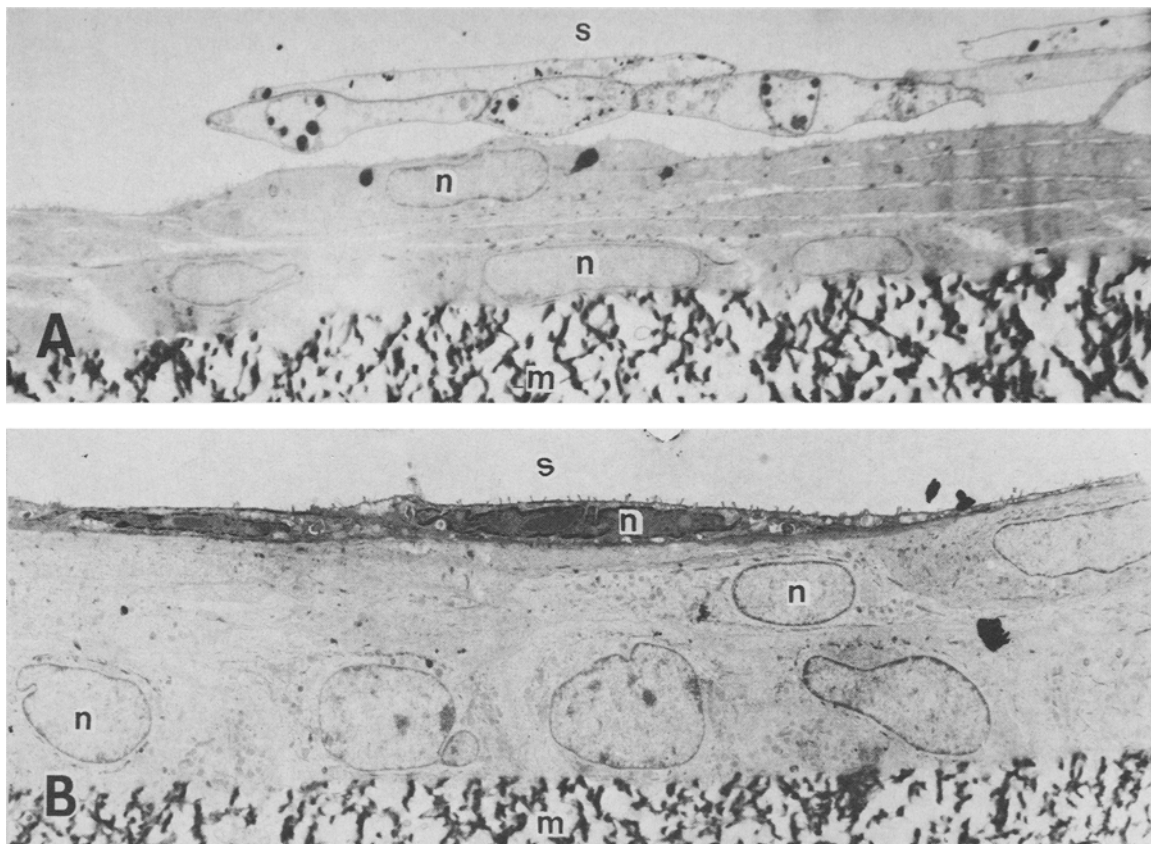

FIG. 3. Transmission electron micrographs of murine keratinocyte cultures on nylon membranes. *A*, culture remained submerged for the entire incubation period (21 d); *B*, culture incubated submerged 7 d then at the air-medium interface 4 d. The nylon membrane (*m*), the cell layers as indicated by their nuclei (*n*) and the surface of the cultures (*s*) are shown. ×2800.

proliferation did not much exceed that on untreated membranes. However, LMN-coated membranes HA-TF and RA-TF did support keratinocyte growth substantially better than uncoated membranes ($P < 0.001$). In addition,
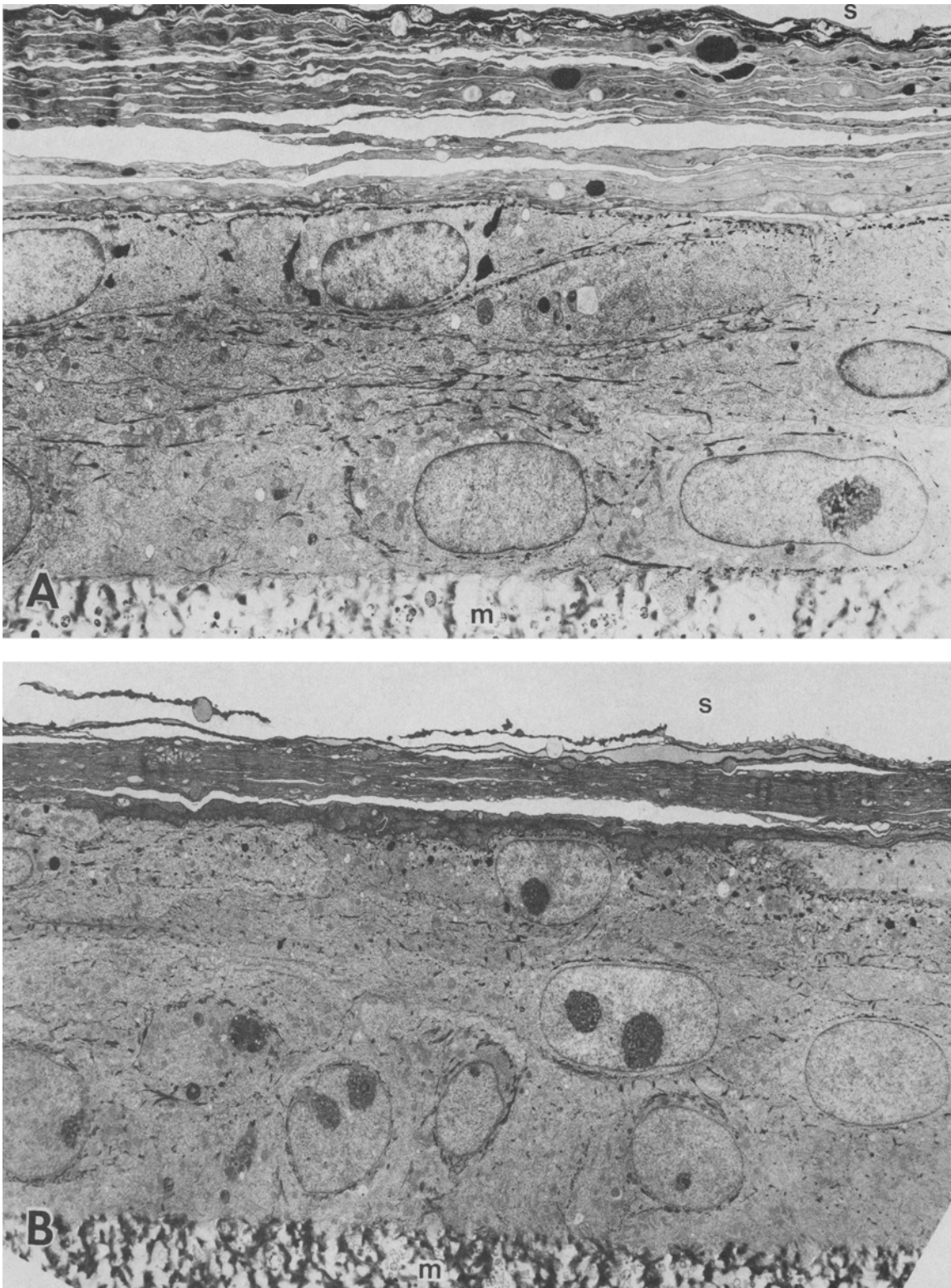

FIG. 4. Transmission electron micrographs of murine keratinocyte cultures on nylon membranes. *A*, culture incubated submerged 7 d then at the air-medium interface 8 d; *B*, culture incubated submerged 7 d then at the air-medium interface 14 d. The surface (*s*) of the cultures and the nylon membrane (*m*) below are shown. ×2800.

detachment of keratinocytes as sheets from the membranes and plastic culture vessels precoated with LMN

was observed in most cultures after incubation for approximately 14 d (data not shown). Because of these
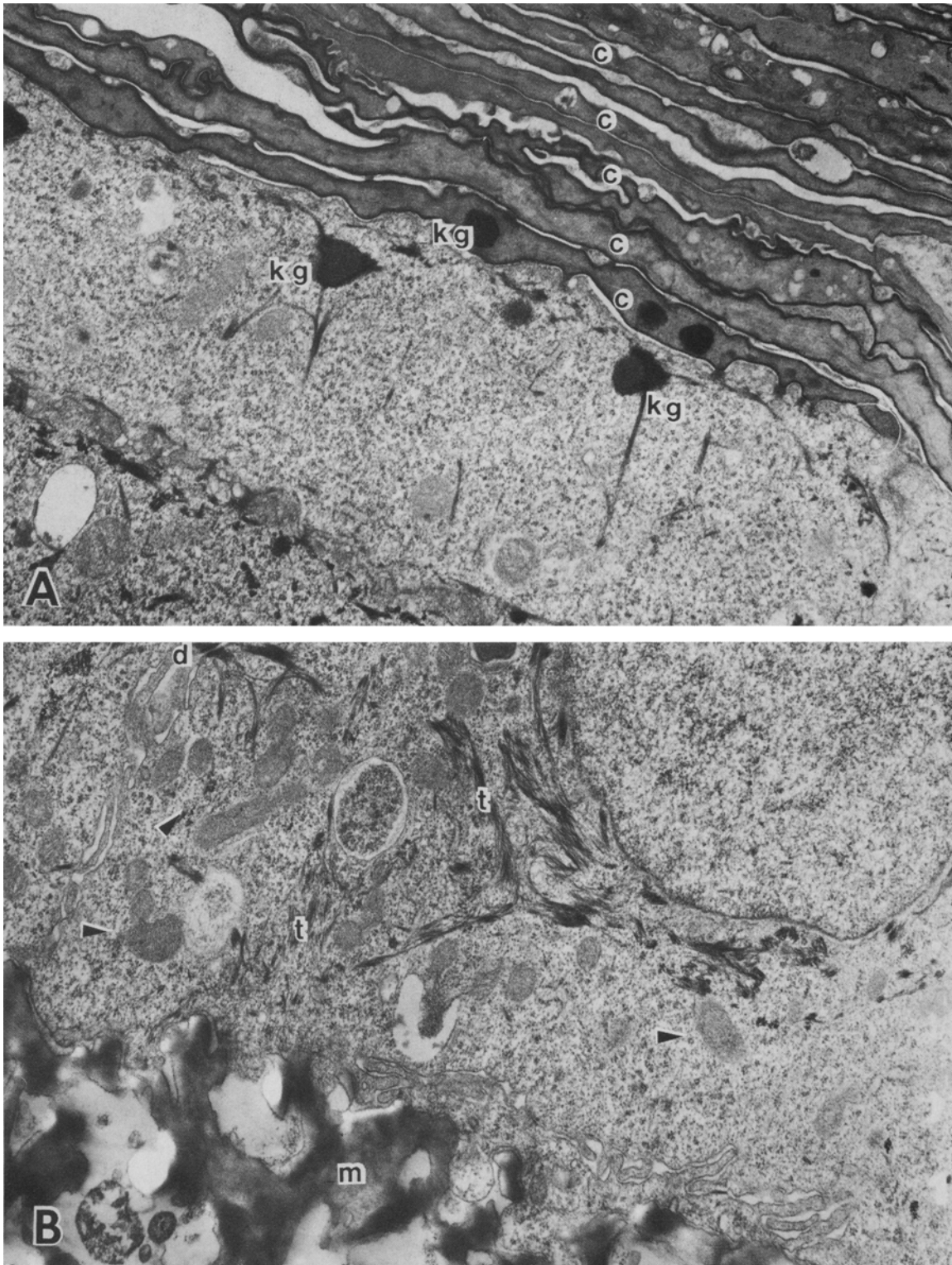

FIG. 5. Transmission electron micrographs of murine keratinocyte cultures on nylon membranes. Cultures were incubated submerged 7 d then at the air-medium interface 14 d. *A*, upper cell layers showing granular layer with keratohyalinlike granules (*kg*) and cornified cells (*c*) above; *B*, basal cell layer on the nylon membrane (*m*) with tonofilaments (*t*), desmosomes (*d*), and numerous mitochondria (*arrows*). ×14 800.

observations, neither HFN nor LMN were used in further studies of the cultivation of keratinocytes on synthetic membranes.

Differentiation and stratification of keratinocytes on nylon membranes. The two-stage cultivation procedure described for producing stratified squamous epithelium in vitro resulted in differentiated multilayers of keratinocytes on the nylon synthetic membrane. Various stages of differentiation were also observed. Multilayering and differentiation increased with the age of the lifted culture up to approximately 12 to 14 d, after which time the number of layers remaining and the extent of differentiation did not change. Figures 3-5 are representative electron micrographs of these observations. Figure 3 *A* illustrates a keratinocyte culture submerged for 21 d. Some multilayering resulted and squamous cells accumulated at the surface; however, the outermost layers tended to detach and float into the medium. Figures 3 *B*, 4 *A*, and *B* are examples of cultures submerged for 7 d and lifted for 4, 8, and 14 d, respectively. Slight variations existed between areas of the same culture, and the photographs presented represent typical examples. The culture lifted for 4 d seems to have produced approximately three nucleated and one to two enucleated cell layers (Fig. 3 *B*). Note that the flat nuclei are still present in the uppermost layers. Enucleated cell profiles were also present at this time. Numerous microvilli can be observed on the upper surface.

Considerable differentiation took place between Days 4 and 8. The culture lifted for 8 d produced 4 to 5 nucleated layers and 15 to 20 enucleated layers (Fig. 4 *A*). Normal markers of differentiation are evident, i.e. keratohyalin-like granules, desmosomes, tonofilaments, etc. The culture that was lifted for 14 d resulted in 5 to 6 nucleated and 15 to 20 enucleated cell layers (Fig. 4 *B*). Thus, most multilayering and differentiation had developed 7 to 8 d after incubation at the air-medium interface and not much difference was observed after 14 d, although the outer layers may have become more compact. Substantially more stratification and differentiation was seen in the culture submerged for 7 d and lifted for 14 d (Fig. 4 *B*) when compared to a culture submerged for the entire cultivation period (21 d) (Fig. 3 *B*).

Various markers of epidermal differentiation were clearly demonstrated in electron micrographs at higher magnifications. A culture lifted for 14 d showed abundant desmosomes, tonofilaments, and mitochondria in the basal layer (Fig. 5 *B*). The keratinocyte-filter interface showed the basal cells protruding a few micrometers into the porous filter, thus forming a tight connection to the substratum. Electron dense granules, morphologically similar to in situ keratohyalin granules, and cells resembling those in the in situ stratum corneum were also evident in some areas of the lifted cultures. Figure 5 *A* is a photomicrograph of a culture lifted for 14 d and displaying those two morphological characteristics found in the intact epidermis. Thus far, no evidence of basement membrane components have been observed in these stratified, differentiated primary cultures growing on nylon or other membranes.

DISCUSSION

Earlier cultivation methods employing various substrata to enhance the attachment and proliferation of epidermal keratinocytes were based on the assumption that these isolated cells required factors produced by dermal fibroblasts. Improved cultivation of human keratinocytes was observed when they were seeded onto collagen gels (9,13) on feeder layers of irradiated fibroblasts obtained from an established cell line (25) or on culture vessels in medium previously conditioned by metabolizing fibroblasts (10). However, a number of investigators have shown that comparable results with human and animal keratinocytes can be obtained with the inclusion of growth factors in selected basal media (7,16,20,21,31). Hydrocortisone was shown to increase the life span of human (8) and rat (30) keratinocytes and make serial passage possible. Also, higher concentrations of insulin (50 μg/ml) increased cell densities before contact inhibition (30). Bovine pituitary extract (26), epidermal growth factor (8), and cholera toxin (19) have also been used to increase human keratinocyte proliferation in vitro. Attachment factors have been shown to promote the cultivation of various epithelial cells. Human fibronectin enhanced the attachment of keratinocytes isolated from human foreskins (7). Laminin was shown to enhance the adhesion of the PAM 212 established cell line (27) and epithelial cells isolated from regenerating liver (1). Epidermal cells from adult guinea-pig skin attached preferentially to type IV collagen when compared to other attachment factors (18). Lowering the calcium level of the growth medium below that found in most commercial media (11) seemed to enhance proliferation and delay differentiation of mouse keratinocytes.

Most of these innovations offered some improvement in the attachment and proliferation of keratinocytes in vitro with some retention of metabolic and structural characteristics. More characteristic differentiation resulted when keratinocyte cultures were incubated for varying periods at the air-medium interface rather than completely submerged in culture medium. This was accomplished by seeding keratinocytes on collagen gels (6,13,14,17) and on dissected dermal elements from pig (24) or human (5) skin. Early attempts to grow mammalian cells on synthetic membranes included using millipore (3) and nuclepore (2) filters. More recently human keratinocytes were grown on membranes coated with collagen or collagen and endothelial cells (23). The latter method used corneal endothelial cells which were seeded on collagen-coated membranes and allowed to grow until a basement membrane equivalent was produced. The latter substance was reported to contain both LMN and type IV collagen—two of the constituents of the basement membrane to which keratinocytes attach in the intact epidermis.

The present investigations have demonstrated that rat keratinocytes attach, proliferate, and differentiate on membranes that are untreated except for sterilization. Growth on Puropor nylon membranes was superior to that on other membranes although those composed of cellulose triacetate also supported keratinocyte cultivation

equal to or better than commercial plastic culture vessels. Membranes coated with HFN gave inconsistent results which indicated that the procedure was less effective. No increase in attachment or proliferation of murine keratinocytes after treatment of plastics or synthetic membranes with type IV collagen obtained commercially was demonstrable. The success reported by Murray et al. (18) using guinea-pig epidermal cells may have been a result of a substantive difference in the collagen they prepared compared with the commercial preparation used in the present study. Another possibility is that guinea-pig keratinocytes have attachment requirements that differ from those of the rat.

Laminin, another substance found in the basement membrane of mammalian skin, initially supported attachment and growth of rat keratinocytes better than the other attachment factors investigated. Again, the inability of this substance to promote continued cultivation may have been a result of the nature of the commercial preparation with which we were working.

Attachment and proliferation of keratinocytes on selected substrata in vitro had to be established before use of these cultures for toxicological studies of agents that adversely affect the epidermis. However, differentiation of cultured keratinocytes may be as important as proliferation. The homeostatic relationship between keratinocytes in various stages of differentiation may have significant effects on the ability of an agent to cause tissue damage. Thus, establishing a microenvironment that fosters differentiation as well as controlled proliferation is a desired goal in our investigations to develop a culture for use in toxicological studies. The epidermis is characterized by the development of a stratified tissue with the outer surface composed of flat, enucleated cells that eventually desquamate. More specific ultrastructural differentiation markers are evident on TEM examination. Those markers were listed in a review by Holbrook (12) and include tonofilaments, desmosomes, hemidesmosomes, membrane-coating granules (intracellular and extracellular), keratohyalin granules, gap junctions, thickenings of the membrane, and the flat squamous cells that make up most of the stratum corneum. Many of these markers do not develop in submerged cultures. Altering the microenvironment by incubating cultures of keratinocytes on Puropor nylon membranes at the air-medium interface has resulted in differentiation more characteristic of the intact epidermis. Although the resulting stratified cultures are not morphologically identical to the in situ epidermis in every respect, we believe that the similarity is sufficient to begin studies on the selective effects chemical and physical agents have on the different populations of stratified epithelium, i.e. the basal, spinous, granular, and cornified cells, individually.

The main goal in this study was to develop a differentiated keratinocyte culture, closely resembling the intact epidermis, with the potential for use in studying the effects of environmental agents on the function of this tissue at the cellular or molecular level. For this system to be accepted widely for screening potentially toxic agents it must be simple in construction

and reproducible. These criteria are necessary if data obtained from different laboratories are to be used collectively in evaluating the effects of selected xenobiotic agents. The successful cultivation and differentiation of rat keratinocytes on nylon membranes fulfills these criteria.

Puropor nylon is a synthetic material that can be manufactured with minimal variation in its chemical nature, whereas the preparation of collagen from rat tissue, for example, can vary significantly from lot to lot and is thus difficult to standardize. Moreover, the nylon membrane can be sterilized by UV irradiation, by autoclaving, or with ethanol. No further treatment is necessary. A comparatively simple medium seems to be sufficient, although this phase of culture development has not been studied exhaustively. The only ingredient whose reproducibilty cannot be controlled is animal sera. There are investigations into the growth of keratinocytes in serum-free medium, and substances contained in serum are being identified to the degree that a chemically defined medium may be available in the future.

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