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The Growth and Differentiation of Cultured Newborn Rat Keratinocytes

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Keratinocytes were cultured from adult and newborn rat epidermis using the 3T3 feeder cell technique. By modifying culture conditions a long-lived line of newborn rat keratinocytes was developed which showed a plating efficiency of 40% and a doubling time of 16 h. The cells produced stratified colonies with tonofilaments, desmosomes, cell envelopes, and keratohyaline granules. When the cells were grown on a collagen gel they formed a thick stratum corneum and many keratohyaline granules.

The fibrous proteins synthesized by the newborn rat cultured keratinocytes were different than those of newborn rat epidermis but similar to those of adult rat cultured keratinocytes. A histidine-rich basic protein was identified by immunologic techniques but it appeared to be more heterogeneous than that of newborn rat epidermis. A cell envelope precursor protein was identified by dansyl cadaverine incorporation studies and was identical to a major envelope precursor of newborn rat epidermis.

The growth characteristics, colony morphology, and biochemical markers did not change for up to 40 passages and there was no evidence of malignant transformation. Because of their ease of growth and long-term survival these cells are useful for studying a variety of problems related to keratinization.

There have been significant advances in the techniques for culturing normal epidermal cells using tissue from a number of animals [1–8]. Although the colonies of cultured cells are not identical in appearance to normal epidermis, they form a number of products of differentiation such as desmosomes, tonofilaments, and cornified envelopes. Furthermore it has been shown that at least some cultured cells are able to assume the appearance of normal epidermis when transplanted onto an animal [8]. Long-term cultivation of keratinocytes with the establishment of permanent lines has been reported only with adult rat lingual epithelium [9,10] and newborn mouse epidermis [11].

This report describes the culture of epidermal cells for more than 40 passages using newborn rat skin and a modification of the 3T3 feeder technique. The cells retain the morphologic characteristics of cultured keratinocytes and continue to synthesize a number of structural proteins characteristic of such cells.

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Abbreviations:
DDT: ditheothreitol
DMEM: Dulbecco's modified Eagle's medium
EGF: epidermal growth factor
FCS: fetal calf serum
PMSF: phenylmethylsulfonyl fluoride
SCBP: stratum corneum basic protein
TUM: urea in Tris containing β-mercaptoethanol

MATERIALS AND METHODS

Newborn Sprague-Dawley rats were sacrificed by decapitation within 24 h after birth and the skin excised and place on ice. Hair was removed from 6-month-old anesthetized rats using epilating wax. The animals were sacrificed by decapitation and the skin excised and placed on ice. For tissue culture studies the skin was cut into small pieces and these were incubated in a 0.25% trypsin solution at 4°C overnight to separate the epidermis. The trypsin-split epidermis was placed on Dulbecco's modified Eagle's medium (DMEM) containing 50 μg/ml of gentamycin, 0.4 μg/ml hydrocortisone, and 20% fetal calf serum and agitated with a pipette for 5 min. The resulting cell suspension was counted with a Coulter counter and used for culture. To obtain intact epidermis from newborn rats, peices of skin were soaked in 0.24 M NH_4Cl, pH 9.5, at 4°C for 30 min. The epidermis was lifted off and rinsed with distilled water.

Primary cultures and later passages of newborn and adult rat epidermis were obtained by plating the cell suspension on mitomycin-killed murine 3T3 cells as previously described [12]. In some experiments the number of 3T3 feeder cells per 35-mm dish was increased from 140,000 to 200,000 or the cells were omitted. The standard culture medium was DMEM containing 50 μg/ml of gentamycin, 0.4 μg/ml hydrocortisone, 10 μg/ml epidermal growth factor (EGF), 10^{-9} M cholera toxin, and 20% fetal calf serum (FCS). In some experiments the EGF was omitted and in others the FCS concentration reduced.

Cultured epidermis was processed for electron microscopy as described previously [12].

Rat epidermal cells were inoculated into soft agar to determine whether they could form colonies in that medium [13]. Suspensions of cultured cells were also layered on collagen gels impregnated with human fibroblasts (gift of Dr. Paul Ehrlich) [14] which were floated in cultures containing the complete medium. At 2 and 4 weeks the gels were fixed in 10% formalin and processed for light microscopy.

The ammonium chloride-separated rat epidermis was minced and extracted by stirring in 1 M phosphate buffer, pH 7.0, at 37°C for 30 min, centrifuged at 30,000 *g*, and the extraction with 1 M phosphate buffer repeated. The pellet was stirred in 0.05 M phosphate buffer, pH 7.1, with 1% sodium dodecyl sulfate (SDS) for 45 min at 25°C, centrifuged at 30,000 *g*, and the extraction repeated. The pellet was then stirred with 4 M urea in 0.05 M phosphate buffer, pH 7.0, for 18 h at room temperature, centrifuged at 30,000 *g*, and the extraction repeated. Finally the pellet was extracted with 8 M urea in 0.1 M Tris, pH 9.5, containing 0.1 M β-mercaptoethanol (TUM) for 8 h at room temperature and the suspension centrifuged at 30,000 *g*. All the buffers used for extraction contained phenylmethylsulfonyl fluoride (PMSF) at 10 μg/ml.

Confluent cell cultures were homogenized and extracted by the various schemes shown in Table I. The suspensions were centrifuged at 30,000 *g* between steps. PMSF was present in all the buffers at 10 μg/ml. The extract is indicated by a roman numeral and an arabic numeral subscript (e.g., I_1).

Protein was determined by the Bio-Rad method and SDS-polyacrylamide gel electrophoresis (PAGE) was done in tubes using the system described by Neville [15] and in slabs by the Laemmli method [16].

Stratum corneum basic protein (SCBP) was purified from the urea extract of newborn rat epidermis using the technique described by Dale [17]. Antibodies to SCBP were made in rabbits and assayed by the Ouchterlony technique and immunofluorescence as previously described [18].

In order to look for cell envelope precursors, intact epidermis and cultured epidermis were homogenized in 0.05 M Tris, pH 7.5, with 0.001 M EDTA and 0.5 mg/ml dansyl cadaverine. After centrifugation the supernatants were made 0.01 M in calcium and 0.002 M in ditheothreitol (DTT) and incubated at 37°C for 3 h. The reaction mixtures were then prepared for SDS-PAGE.

Incorporation of labeled histidine into cultured epidermal cells was done by adding DMEM with 20% FCS containing 0.4 μCi/ml of L-

TABLE I. *Various procedures for extracting tissue*

Procedure	Extract[a]		
	1	2	3
I	0.05 M Tris, pH 7.5 0.1 M saline 0°C for 1 h	0.1 M Tris, pH 9.5 4.0 M urea 25°C for 4 h	0.1 M Tris, pH 9.5 8.0 M urea 0.1 M mercaptoethanol 25°C for 12 h
II	0.05 M Tris, pH 7.5 0.1 M saline 0°C for 1 h	0.1 M Tris, pH 9.5 8.0 M urea 0.1 M mercaptoethanol 25°C for 12 h	
III	0.1 M Tris, pH 9.5 4.0 M urea 25°C for 4 h	0.1 M Tris, pH 9.5 8.0 M urea 0.1 M mercaptoethanol 25°C for 12 h	
IV	1.0 M phosphate, pH 7.0 37°C for 0.5 h	0.05 M phosphate, pH 7.0 4.0 M urea 25°C for 18 h	
V	2% SDS 0.025 M DTT 0.002 M EDTA 0.1 M Tris, pH 8.6 60°C for 30 min		

[a] The extracts were done sequentially.

[^{14}C(U)]-histidine to cultures 2 days beyond confluence. The concentration of histidine in DMEM was reduced from 80 mg to 0.8 mg/liter. The cultures were incubated for varying periods, rinsed twice with phosphate-buffered saline, and then treated witb one of the buffers described for extracting cell cultures. Autoradiographs were done by placing the dried gels against Kodak X-Omat AR film and storing at −70°C until the film was developed. To measure radioactivity in gels the slices were digested in a 2:1 mixture of 30% H_2O_2 : 70% perchloric acid. Aquasol II was added and the solutions counted in a liquid scintillation counter.

RESULTS

Adult rat epidermal cells behaved very similarly to human foreskin epidermal cells when grown on 3T3 feeder layers with EGF, hydrocortisone, and cholera toxin in the culture medium. They showed a plating efficiency of about 5% between the 2nd and 5th passages, the colonies reached confluency in about 7–10 days with a doubling time of about 20 h, and the cells could be passed 8–10 times. When newborn rat cells were grown under these conditions they grew more slowly and died out after the 2nd passage. Their growth was similar to adult rat cells, however, when the number of killed 3T3 feeder cells per dish was increased from 140,000 to 200,000 and EGF was left out of the medium. Some time between the 3rd and 6th passage the behavior and appearance of the cells changed; the cells assumed a very uniform cuboidal appearance and reached confluency in about 3 days.

Unlike human and adult rat epidermal cells these newborn rat cells showed a plating efficiency of about 40% and a doubling time of 16 h. Newborn rat cells continued to remain attached to the culture dish and replicate for at least 3 months after inoculation, whereas adult rat cells were almost completely detached at 4–6 weeks. Furthermore, newborn rat cells could be passed after remaining attached to the dish for 3 months.

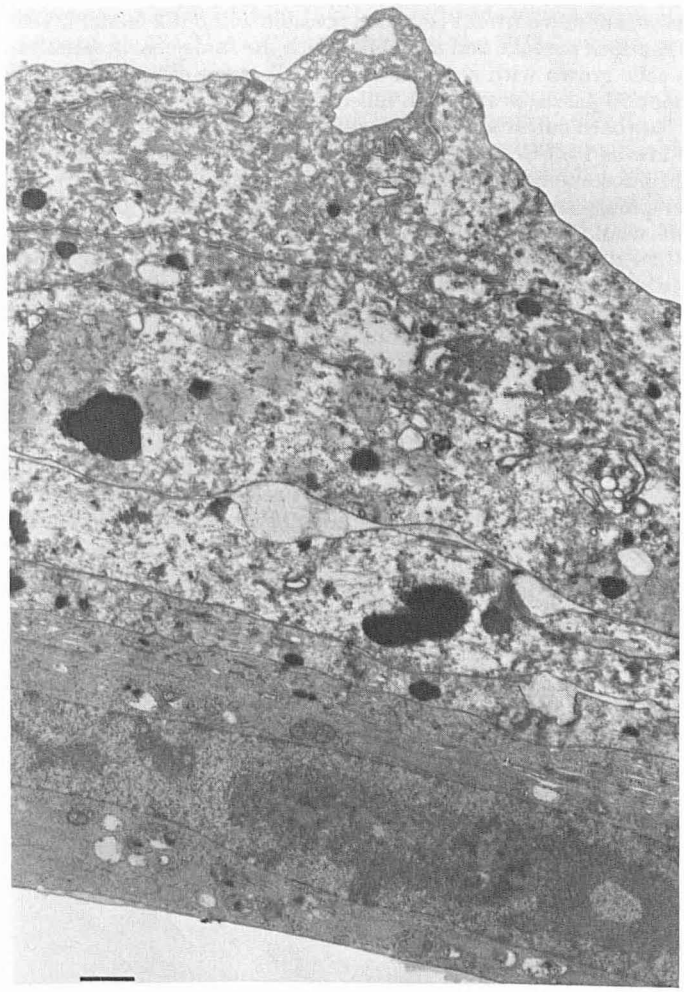

FIG 1. Electron micrograph of cultured, 10th passage newborn rat keratinocytes showing stratification. *Bar* = 1 μm.

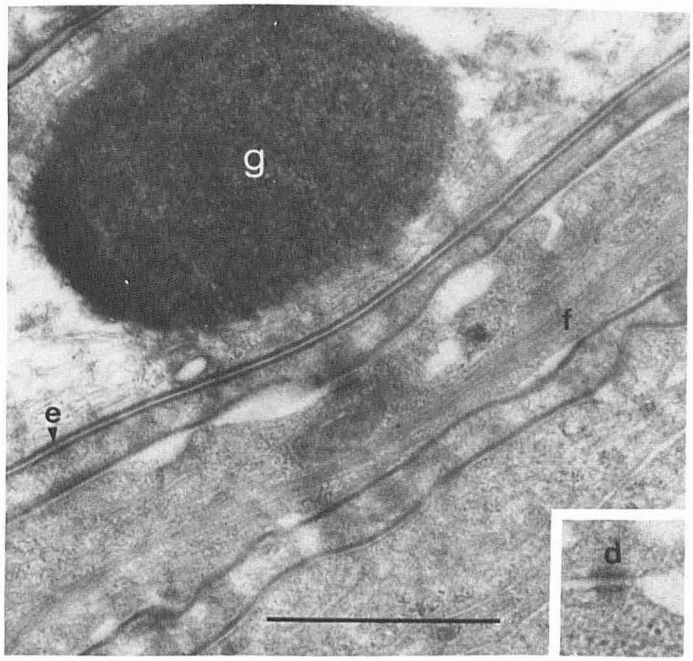

FIG 2. Electron micrograph of cultured keratinocytes showing details of subcellular components. *d* = Desmosome, *e* = cornified envelope, *f* = filament, *g* = keratohyaline-like granule. *Bar* = 1 μm.

Growth of newborn rat cells was possible without a feeder layer at the 10th passage and thereafter with the same characteristics as cells grown with a feeder layer. Cells were carried through about 70 passages and then failed to grow.

Newborn cultured rat cells grew and reached confluency with as low as 1% FCS, although the growth rate was much slower. Chromosome counts of 20th-passage cells showed considerable polyploidy with no cells having the normal number of 42. The cells would not grow and form colonies when plated in soft agar. When the cultured cells were inoculated subcutaneously into newborn rats they did not produce tumors.

The newborn rat cells formed stratified colonies as previously described for human epidermal cells when grown with or without a 3T3 feeder layer (Fig 1). Filaments, desmosomes, and cornified envelopes were observed regularly and there were occasional round, dark bodies of varying size with a granular appearance (Fig 2). The cells were also plated on collagen gels impregnated with human fibroblasts which floated in the tissue culture dish. Histologic examination after 1 month of growth showed a very thick stratum corneum with a large number of blue granules just beneath the stratum corneum when stained with hematoxylin and eosin (Fig 3).

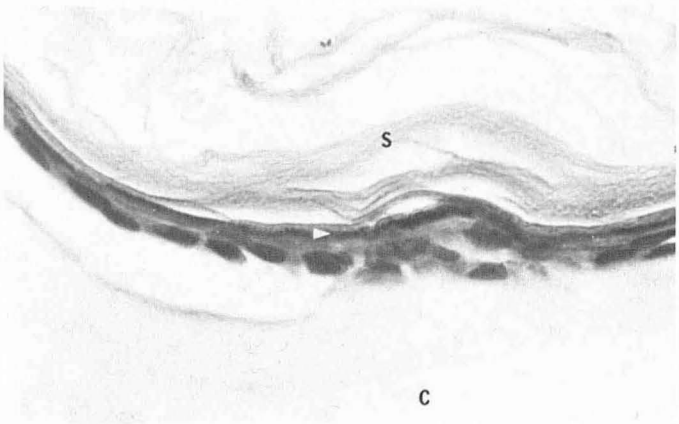

FIG 3. Histologic section of cultured, 20th passage, newborn rat keratinocytes grown for 1 month on collagen gels impregnated with fibroblasts. Keratohyaline-like granules are indicated by *arrowhead*, *S* = stratum corneum, *C* = collagen gel. × 640.

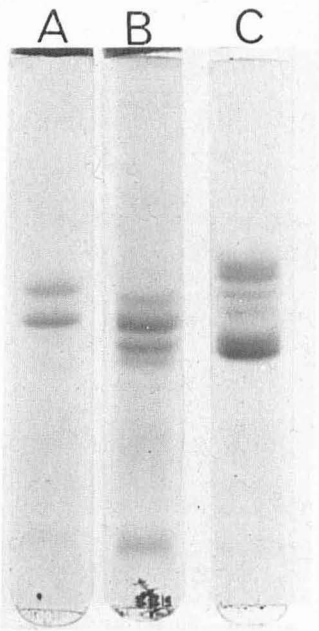

FIG 4. SDS-PAGE patterns of TUM-extracted protein from newborn rat epidermis (*A*) and 10th passage, cultured newborn rat keratinocytes (*B*). *C* is cow snout prekeratin used as a marker, showing M_r 48,000–67,000.

The SDS-PAGE patterns of the urea (I_2) and TUM (I_3) extracts of newborn rat cultured epidermal cells were the same, and the latter (Fig 4) differed from the TUM extract of intact tissue. The pattern of early-passage cells differed somewhat in

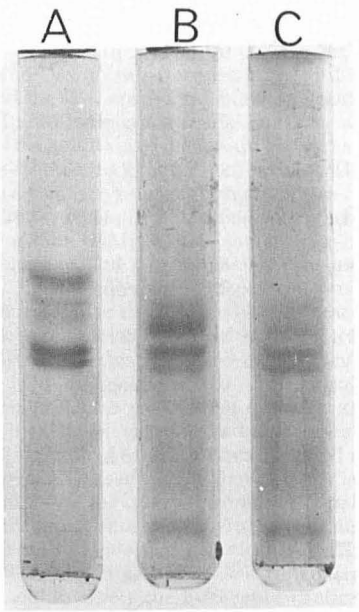

FIG 5. SDS-PAGE patterns of TUM (I_3)-extracted protein from 12th passage (*B*) and 2nd passage (*C*) cultured newborn rat keratinocytes. *A* is cow snout prekeratin.

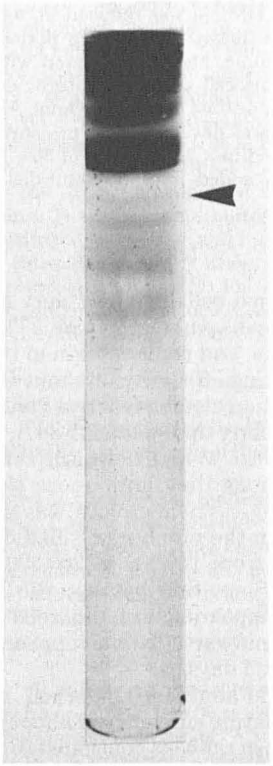

FIG 6. SDS-PAGE pattern of the Tris buffer extract of 10th passage, cultured newborn rat keratinocytes that have been incubated with dansyl cadaverine. The fluorescent band was identified by viewing the 15% acrylamide gel under ultraviolet light and marking with a needle; the gel was then stained. The fluorescent band is indicated by the *arrowhead*.

the relative intensities of the bands from that of later passages (6th and beyond) which then remained quite constant (Fig 5). Patterns of adult rat epidermis showed exactly the same initial pattern and change in pattern, although these cells never developed the growth behavior of newborn rat cells. TUM (I_3) extracts of both early- and late-passage cultured rat epidermal cells reacted with an antibody to epidermal fibrous proteins.

In order to look for precursors of cornified cell envelopes, saline extracts of cultured rat epidermal cells were incubated with dansyl cadaverine and then separated by SDS-PAGE; a fluorescent band of M_r 22,000 was observed (Fig 6). These results were obtained with both early- and late-passage cells. In newborn rat epidermis up to 5 labeled bands were seen in the range of M_r 12,000–45,000, and included the 22,000 component as a major band.

The SCBP purified from newborn rat epidermis showed a single band on SDS-PAGE with a M_r of about 45,000 (in the Neville system) and had an amino acid composition identical to that reported by Dale with a high histidine content [17]. Strong precipitin lines of identity were observed by the Ouchterlony technique when SCBP and the saline (I_1) and 4 M urea (I_2) extracts of cultured newborn rat epidermal cells were tested against the antibody to SCBP (Fig 7*a*); however, the TUM (I_3) extract did not react (Fig 7*b*). A second weaker band was variably seen close to the antibody well; this also was observed when SCBP was purified by being cut out of a SDS polyacrylamide gel and tested against the antibody. The same results were obtained with extracts of 2nd and 30th passage cultured newborn rat keratinocytes as well as with extracts of cultured adult rat keratinocytes. The 1 M phosphate (IV_1) and 4 M urea (IV_2) extracts of cultured cells also reacted with the antibody.

Immunofluorescence studies were done with frozen sections of newborn rat epidermis and 20th passage cultured rat keratinocytes using the antibody to SCBP. As shown in Fig 8, diffuse

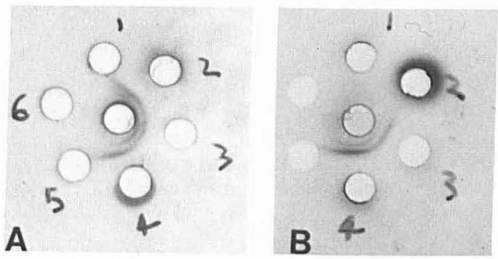

FIG 7. Ouchterlony plates demonstrating reactivity of various extracts to the antibody to SCBP. *a*, Antibody is in the *center well*, *1* = a buffer blank, *2* = the saline (I_1) extract of 10th passage, cultured rat keratinocytes, *3* = SCBP, and *4* = the urea (I_2) extract of the cultured keratinocytes. *b*, Antibody is in the *center well*, *1* = a buffer blank, *2* = the TUM (I_3) extract, *3* = the saline (I_1) extract, and *4* = the urea (I_2) extract of cultured keratinocytes.

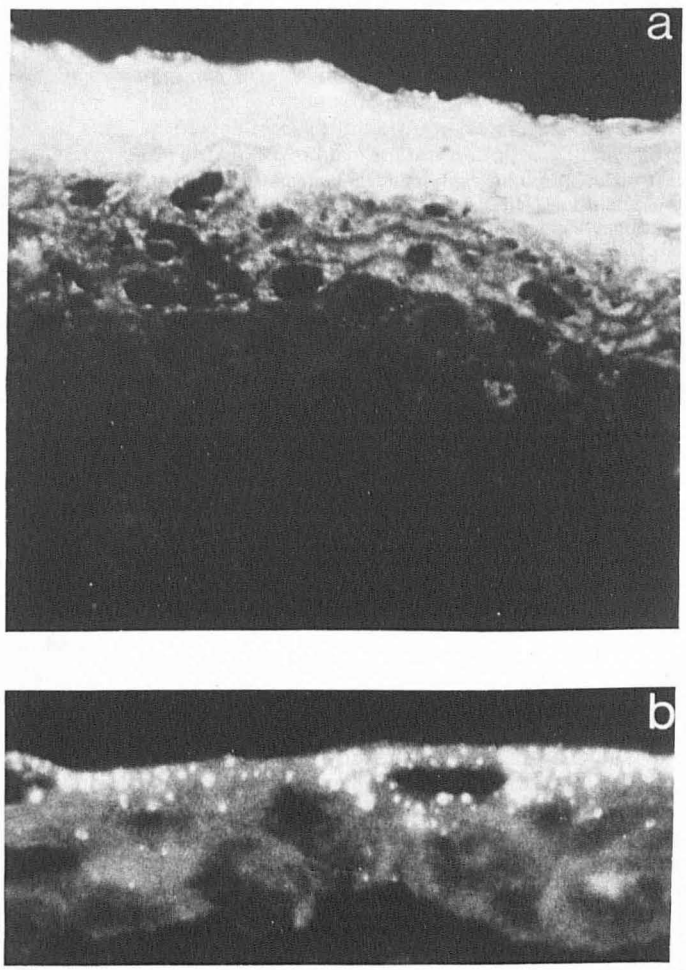

FIG 8. Indirect immunofluorescence studies of newborn rat epidermis (*a*) and cultured newborn rat epidermis (*b*). Frozen sections were stained with the antibody to SCBP and then counterstained with fluorescein-conjugated goat antirabbit γ-globulin.

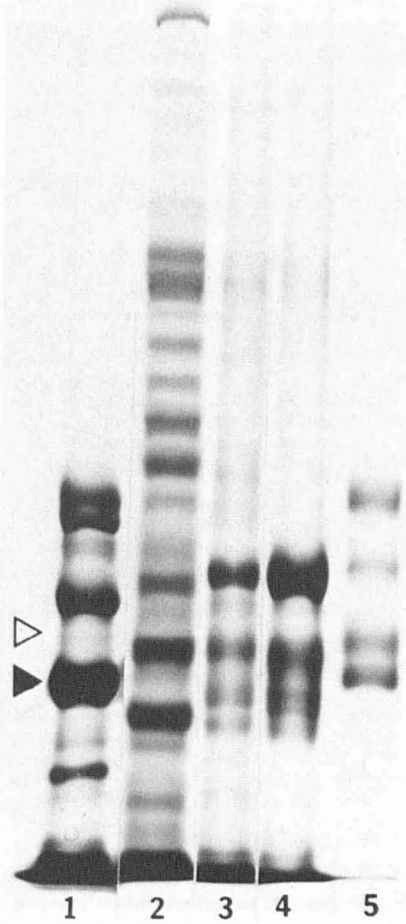

FIG 9. SDS slab gel. *1* = a 4 M urea extract of newborn rat epidermis; *2* = a saline (I_1); *3* = a urea (I_2); *4* = a TUM (I_3) extract of 10th passage, cultured newborn rat keratinocytes; *5* = cow snout prekeratin. The position of SCBP is indicated by the *solid arrowhead* and the phosphorylated derivative (determined on another run and not shown) by the *hollow arrowhead*.

fluorescence was seen in the stratum corneum and granular fluorescence in the granular layer of newborn rat epidermis. Granular fluorescence was observed in the upper layers of the stratified cultures (Fig 8). No fluorescence was observed with serum obtained prior to immunization in both epidermis and cultured cells.

A comparison of the SDS-PAGE pattern of the urea (I_2) extract of cultured cells with the 4 M urea extract of newborn rat epidermis showed several bands similar in mobility to SCBP (Fig 9). However, similar bands appear to be present in the TUM (I_3) extract of cells which did not react with the antibody to SCBP. Another band, also seen in the saline extracts, corresponded very closely to the mobility of the phosphorylated precursor of SCBP. SDS-PAGE was done on a direct 4 M urea (III_1) extract of cultured cells and the various regions of the gel were cut out and the protein extracted. After removing the SDS [19] the various extracts were tested against an antibody to SCBP. Immunoreactivity was found in both SCBP and precursor areas as well as in lower M_r regions (5–8 in Fig 10). There was some reactivity in the M_r range of 70,000–94,000 (*3* and *4* in Fig 10) but these samples broke down to lower M_r material as shown by reelectrophoresis (Fig 10).

Labeled histidine incorporation studies were done with cultured cells in order to identify histidine-rich proteins. Extractions were done with 1 M phosphate (IV_1) and 4 M urea (IV_2) since this is the classical technique for isolating histidine-rich proteins. After 24 h of labeling, autoradiographs of SDS-PAGE patterns of 1 M phosphate (IV_1) and 4 M urea (IV_2) buffer extracts were done as shown in Fig 11*a*. Radioactivity was present in the position of SCBP in the urea extract and in this

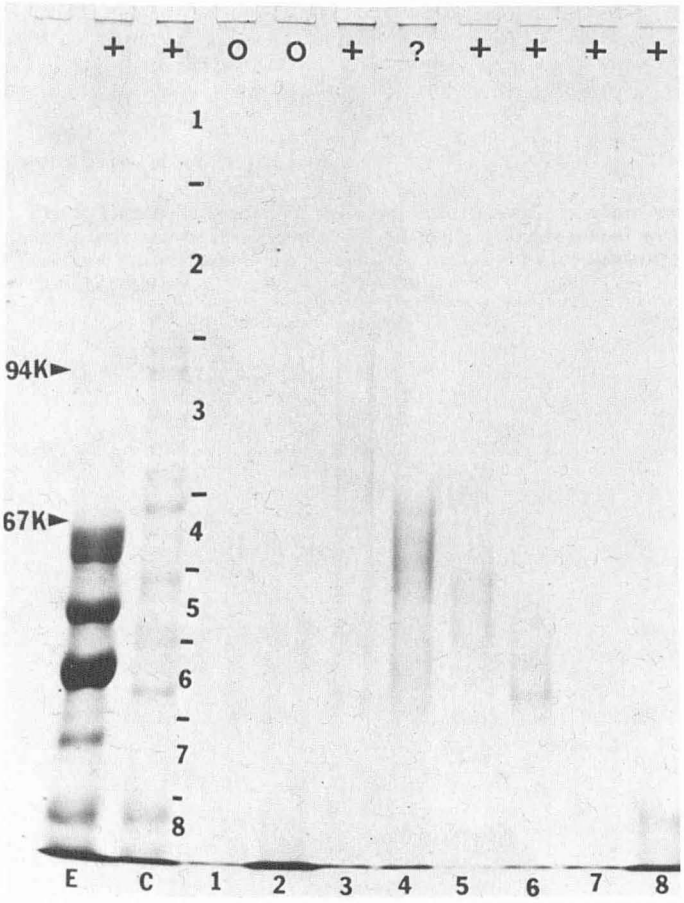

FIG 10. SDS slab gel indicating regions that were tested for reactivity to the antibody to SCBP. *E* = a urea extract of newborn rat epidermis, and *C* = a urea (III_1) extract of cultured rat keratinocytes. The regions cut out of the preparative gel are indicated on *C* and the pattern of the extracted protein is shown in *lanes 1–8*. Those regions reacting to the antibody are indicated by +.

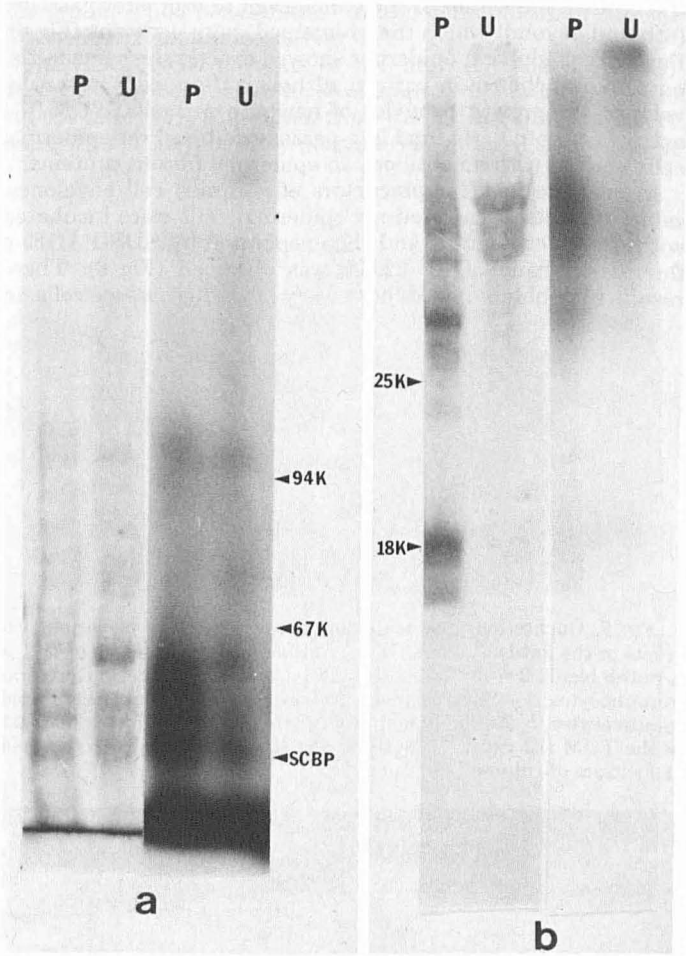

FIG 11. Autoradiographs of SDS-Page patterns of 1 M phospate (*P*) (IV_1) and urea (*U*) (IV_2) extracts of 20th passage, cultured newborn rat keratinocytes incubated with labeled histidine for 24 h. The Laemmli slab technique of electrophoresis was done with 7% (*a*) and 15% (*b*) acrylamide. The stained gels are on the *left* and the autoradiographs on the *right*.

position and above it in the 1 M phosphate extract. There were additional radioactive spots on the gels. The very intense radioactivity at the front must represent small peptides of varying size since it did not appear as a distinct spot on 15% gels (Fig 11*b*). Radioactivity was not present in large amounts in the gel in M_r regions greater than 94,000. Cells were also labeled for 3 h and extracted in the 2% SDS (V_1) buffer. SDS-PAGE was done on a 4%–30% gradient gel and the stained gel was sliced and the pieces counted (Fig 12). Insignificant amounts of radioactivity were found in the M_r range greater than 94,000. Similar experiments were done with 2- and 24-h labeling of cells and extracting with saline (II_1) and TUM (II_2). These also failed to reveal labeling in the high M_r regions.

DISCUSSION

We have been able in this study to obtain a long-lived line of cultured keratinocytes from newborn rat but not adult rat epidermis. Although cultured epidermal cells from only 2 animals were carried for about 70 passages, the change in plating efficiency, growth in the absence of 3T3 cells, and long-term survival were observed in cultured epidermis of a number of newborn animals. This change in the growth of rat keratinocytes required that the initial culturing be done with increased numbers of 3T3 feeder cells and the omission of EGF, which was found to inhibit rat epidermal cells. Although EGF was reported to extend the life of human epidermis [20], it may not have this effect with all epidermal cells.

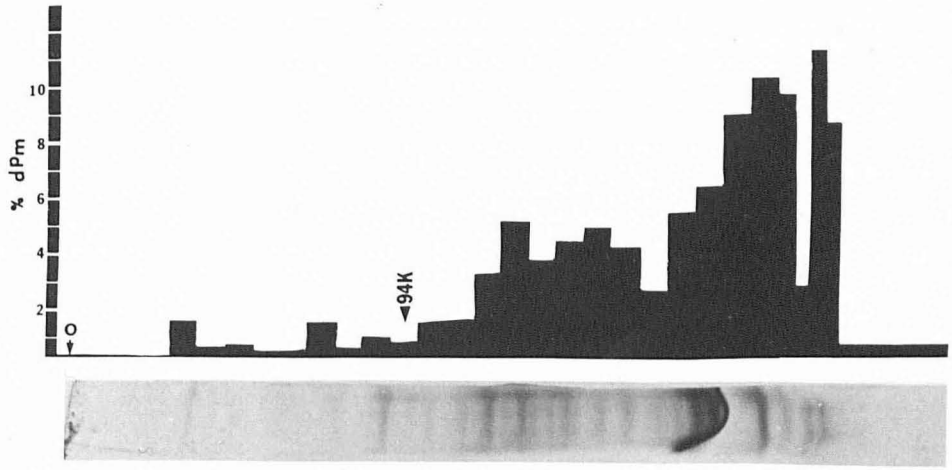

FIG 12. Profile of radioactivity in gel slices cut from a 4–30% SDS acrylamide gradient gel on which was run a 2% SDS (V_1) extract of 20th passage, cultured newborn rat keratinocytes incubated for 3 h with labeled histidine. The gel was sliced as indicated in the *bar diagram* and the d.p.m. per slice is expressed as % of total d.p.m. on the gel. *O* indicates origin of gel.

The results reported by Miller et al [11] on the development of permanent cell lines from newborn mouse epidermis are similar to ours with respect to the need for a feeder layer and colony morphology, but differ in that their cells had a low cloning (plating efficiency). No data were presented in that paper on the biochemical markers of keratinization.

The explanation of why cultured newborn rat keratinocytes grew so well is not apparent and a number of studies were done to determine whether the cells retained their normal characteristics. The cultured keratinocytes would not grow in soft agar, which is used as a criteria for cells that have not undergone malignant transformation. The cells were polyploid, but this has been reported in a number of normal cell lines from rat tissues. Cultured rat lingual epithelium cells, which behave similarly to newborn rat keratinocytes, did show a normal number of chromosomes, but the studies were done using cells from the 5th passage [9]. Implantation of cultured cells into newborn rats did not produce tumor nodules. Furthermore, cultured keratinocytes formed keratohyaline-like granules and a stratified epidermis with a thick stratum corneum when grown on a synthetic dermis.

The SDS-PAGE pattern of fibrous keratin from cultured rat keratinocytes differed from that of intact tissue, which has been reported with other animals [12]. The change in pattern which occurred at the 6th passage was observed with both newborn and adult rat epidermis and is not related to the alteration in growth of newborn rat keratinocytes. The pattern then remained stable and was not altered even when cells were grown in the absence of a feeder layer. In cultured human and bovine epidermal cells the SDS-PAGE keratin pattern does not change as the cells are passed, and the rat cells may be unique in this regard [12]. It will be of interest to investigate the SDS-PAGE keratin pattern of cells grown on artifical dermis using both early- and late-passage adult and newborn rat cultured keratinocytes, since under these conditions a very thick stratum corneum is formed.

The SCBP antibody studies indicate that cultured rat epidermal cells synthesize a keratohyalin-like protein. Furthermore, the immunologic reactivity was observed beyond 30th-passage cells, indicating this characteristic marker of keratinizing epidermis is not lost [21]. A single specific protein species responsible for the reactivity has not been isolated, but the histidine incorporation studies showed labeled proteins with mobilities similar to SCBP [17] and the phosphorylated precursor [22]. However, a labeled high M_r precursor as described by Scott and Harding [23] could not be identified. When the extracts of cultured cells were fractionated on the basis of size by SDS-PAGE, reactivity to an antibody to SCBP was found not only in the SCBP and precursor regions but also at lower M_r regions. This may mean that the basic protein readily breaks down to smaller fragments [23], although great care was taken to prevent this during the isolation procedure.

A soluble precursor of the cell envelope has been identified in cultured cells using the incorporation of dansyl cadaverine, a reaction mediated by the enzyme transglutaminase [24,25]. The identical precursor was observed in cultured newborn and adult rat keratinocytes using both early- and later-passage cells. Although the dansyl cadaverine studies of intact epidermis showed 4 precursor proteins, a major one had the same M_r as that observed in cultured cells.

These studies suggest that a long-lived line of nonmalignant epidermal cells can be obtained using newborn rat epidermis. The cells retain a number of functional, morphologic and biochemical markers of keratinizing cells. Furthermore, these cells appear to exhibit a considerable amount of differentiation when grown on a dermal-like substrate. Considering their ease of growth and long-term survival, these epidermal cells will be useful for studying a wide variety of problems related to keratinization.

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Increased Procollagen mRNA Levels in Scleroderma Skin Fibroblasts

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Procollagen messenger RNA activity in scleroderma and normal skin fibroblasts was measured using a cell-free translation assay. Radioactive translation products were fractionated by electrophoresis and the ratio of procollagen to total incorporation was determined from densitometric scans of gel fluorograms. In 4 scleroderma cell lines 1.78% (± 0.10) of incorporated [^{35}S]-methionine was in procollagen, compared to 1.00% (± 0.20) in 5 normal controls. These values are consistent with previously reported increases in the rates of collagen synthesis obtained with intact cells and show that most if not all of the increase can be explained on the basis of elevated translatable procollagen messenger RNA in scleroderma fibroblasts.

Several studies have shown that the excessive accumulation of collagen in scleroderma skin is associated with an increased rate in collagen synthesis. Incorporation of radioactive proline into hydroxyproline and bacterial collagenase-sensitive polypeptide synthesis was greater in cultured scleroderma skin fibroblasts when compared to normal controls [1–3]. It was later shown that not all skin fibroblasts display the same biosynthetic potential for collagen synthesis. Thus, fibroblasts cultured from the lower levels of the dermis and junction between the dermis and subcutaneous tissue make more collagen than those derived from the upper dermis or subcutaneous tissue [2,4,5]. Collagenase activity of scleroderma fibroblasts was found to be normal [3]. In addition, the ratio of type I/type III collagen was the same as the normal controls [3,5]. It is also noteworthy that higher incorporation rates of labeled proline in scleroderma cell lines could not be attributed to differences in the proline pool [3]. The purpose of this study was to measure procollagen RNA activity from scleroderma and normal skin fibroblasts by translation in an mRNA-dependent reticulocyte lysate. Our results showed that scleroderma fibroblasts contained more procollagen mRNA activity than normal controls.

MATERIALS AND METHODS

Skin Biopsies and Cell Cultures

Excision biopsies were performed under local xylocaine anesthesia from the upper arms or forearms of 4 patients with active systemic scleroderma and 5 normal controls after obtaining consent statements. Clinical information about patients is reported in Table I. The normal controls consisted of 3 females and 2 males, ranging in age from 28 to 56 years. Each specimen was divided into smaller pieces and fibroblasts derived from the explants were allowed to reach near confluency in 60 × 15 mm plastic culture dishes (Falcon) [4]. Further passages were done in 100-mm dishes following trypsinization.

RNA Preparation

The procedure followed was similar to that described by Burnett and Rosenbloom [6]. All solutions and glassware were autoclaved to minimize ribonuclease activity. Cells from 10–20 confluent 100-mm culture dishes were washed twice with Hanks' balanced salt solution or 0.02 M phosphate-buffered saline, pH 7.5. Then, 2 ml of lysis buffer containing 1.0% sodium dodecyl sulfate (SDS), 5 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 65 μg/ml of proteinase K (Beckman) was added to each dish and the suspension was scraped into a Dounce homogenizer and homogenized with 4–5 strokes of a tight-fitting pestle. Following incubation for 1 h at 45°C, an equal volume of 1 M NaCl was added slowly with stirring, and precipitated proteins were removed by centrifugation (10 min at 13,000 × *g*, 25°C).

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Abbreviations:
SDS: sodium dodecyl sulfate