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# ENZYMATIC LIBERATION OF VIABLE CELLS OF HUMAN SKIN

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*Abstract.* To obtain viable cells from normal human skin, clostridial collagenase was used. Crude collagenase digestion of collagen fibres and basal lamina results in free dermal cells and sheets of epidermis. The collagenase was tested at various concentrations, solvents and incubation periods. The specimens digested were either split or full thickness skin of varying size. The optimal result was obtained by using small (3 mm across) split skin pieces incubated in 2 mg/ml collagenase. The choice of solvent MEM, MEM supplemented with serum, and Tris buffer, was less important. After 3 hours' incubation the epidermis was peeled off in sheets and finally dissociated by trypsin-EDTA. The corium was completely digested after 6 hours. After 6 hours' incubation no viable cells could be seen. The epidermal cells appeared mainly as polygonal cells of various sizes and a few little dendritic cells. The dermal cells had a heterogeneous morphology during the first weeks of cultivation. After 2 weeks the cells appeared as fibroblast-like cells.

*Key words:* Skin Cell culture; Keratinocyte; Fibroblast; Bacterial collagenase

To prepare a cell suspension for primary cell culture, bacterial collagenase has been successfully applied to mammary glands of mice (8, 9), cartilage of chick embryo (1), and vascular smooth muscle cells of umbilical cord (5). Fresh specimens of human skin have been treated with bacterial collagenase (7). In these experiments, the junction structure and collagen fibrils were dissolved, while elastic fibres and desmosomal connections between epidermal cells remained unaffected. Provided the cells retain their viability and growth activity after collagenase incubation, epidermal and dermal cells can be cultivated separately. However, a rather drastic treatment with the enzyme is necessary, as collagen fibrils are arranged in compact masses in the dermis. As yet, the possible toxic effect of bacterial collagenase on live cells is not known.

## MATERIALS

*Normal mammary skin* of young women was supplied by the Department of Plastic Surgery of this hospital. Crude collagenase extracted from *Clostridium histolyticum* (Worthington Co., New Jersey, USA), 125 units per mg, was used. The enzyme was dissolved in various solvents to concentrations of 0.1% and 0.2%.

*Solvents* used for enzyme incubation were made up as follows. (Their abbreviations are given in parentheses.)

1. (MEM) consisted of Eagle's Minimum Essential Medium with Earle's salts 100 ml; 200 mM; dl l-glutamine 2 ml; penicillin 10 000 units; streptomycin 5 mg; and gentamycin 5 mg. The pH was adjusted to 7.4 by 1 mM Hepes buffer.

2. (MEM + S) consisted of MEM supplemented by 10% fetal calf serum. Beforehand, the serum was heat-inactivated at 56°C for 30 min. This medium was also used for further cultivation of established cultures.

3. (Tris) consisted of Tris-hydrochloric acid buffer 50 mM with 5 mM $CaCl_2$, pH 7.4.

*For epidermal cell dissociation*, 0.2% trypsin (Trypure®, NOVO, Denmark), and 0.02% EDTA in a salt solution containing NaCl 8.0 g, KCl 0.2 g, $Na_2HPO_4$ 1.15 g, $KH_2PO_4$ 0.2 g, glucose 0.2 g per litre distilled water were used. The pH was adjusted to 7.6–7.8 with 7.5 w/v % sodium bicarbonate.

## METHODS

### *Trypan blue exclusion technique*

Trypan blue was used for vital staining at a concentration of 1% in normal saline. Equal volumes of trypan blue solution and cell suspension were mixed. The numbers of stained cells after 10 min were counted per 1 000 cells (13).

### *Preparation of skin specimens*

Large skin pieces were separated from the underlying fat with a pair of scissors and were briefly washed in sterile normal saline. The pieces were fixed by pins onto a cork plate, with the epidermal surface upwards. The epidermal surface was cleaned with 70% alcohol and mechanically dried with sterile gauze. Skin specimens of split thickness (STS) were prepared with a 3 mm punch and scissors. The

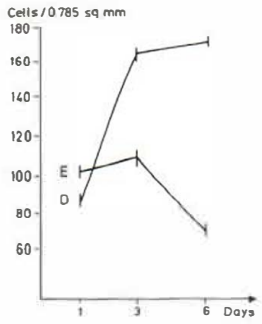


*Fig. 1.* The growth rate of enzyme-liberated skin cells. Epidermal (*E*) and dermal (*D*) cells were grown in separate cultures. On the 1st, 3rd and 6th day, photographs of the cultures were taken, choosing six different areas at random. Each photographed area was 0.785 mm². The total numbers of cells on the photographs were counted.

specimens were incubated in MEM + S at 37°C overnight under constant shaking. Each specimen had an average wet-weight of 11.8 mg and a surface of 7 mm². Full thickness skin (FTS) specimens, each with a surface of 1 cm², were prepared using a knife and kept overnight in the same way as the former.

*Epidermal–dermal separation and dissociation of dermal cells*

The collagenase incubation was performed at 37°C under constant shaking. Two hundred STS specimens were incubated for 6 hours in 10 ml bacterial collagenase 2 mg/ml in the three different solvents. After 3 hours of incubation, the epidermis was peeled off with forceps and kept in MEM + S for later dissociation. The dermal part of the specimens was reintroduced into the incubation medium for further collagenase digestion. After 6 hours, the digested tissue remnants were removed and the cloudy cell solution was centrifuged at 160 *g* for 10 min. The sediment was resuspended in MEM + S and cultivated in Falcon flasks (25 cm²). Before cultivation, the total cell number and the trypan blue stained cells of the suspension were counted.

Incubation with bacterial collagenase (1 mg/ml) in various solvents was also performed. However, at this concentration the epidermis could not be peeled off even after 6 hours.

Full thickness skin (FTS) specimens of 2300 mg wet weight were incubated in 10 ml collagenase solution (2 mg/ml) in the three different solvents for 16 and 24 hours. Sheets of separated epidermis were picked up from the incubation medium with forceps. The undigested tissue was removed and discarded. The cells in the incubation medium were then treated in the same way as the former and cultivated.

*Dissociation of epidermal cells*

The epidermal sheets were washed once with Hanks' balanced salt solution without $Mg^{2+}$ and $Ca^{2+}$ and treated with trypsin-EDTA solution for a few minutes under vigorous shaking. When the solution became cloudy due to dispersion of cells the suspension was transferred into an equal volume of MEM + S. The remaining epidermal sheets were discarded. The cell suspension was centrifuged at 160 *g* for 10 min and the sediment resuspended in MEM + S for further cultivation. The total cell population and those stained by trypan blue were counted before cultivation.

*Cell counting during cultivation*

The unsettled cells were collected and counted after 24 hours of cultivation. Six areas of the cell culture were selected at random and photographs taken after 1, 3, and 6 days of cultivation (Fig. 1). Each photographed area was 0.785 mm². The cells were studied with a Leitz inverted phase contrast microscope and photographs were taken with a Hasselblad camera.

## RESULTS

### 1. *Dissociated cells*

*Epidermal cells.* After the collagenase incubation, the epidermis was released from the corium as a single sheet, the various collagenase solvents tested having shown no remarkable differences (Table I).

Table 1. *Cells dissociated from human skin by treatment with clostridial collagenase*

| | MEM | Tris | MEM + S |
|---|---|---|---|
| *3 hours' incubation* | | | |
| Epidermal cells | | | |
| Cell yield per mm² | 428±202 | 299±152 | 492±329 |
| Trypan blue stained cells (%) | 13± 5 | 30± 17 | 12± 6 |
| Unsettled cells (%) | 63± 36 | 63± 19 | 53± 33 |
| *6 hours' incubation* | | | |
| Dermal cells | | | |
| Cell yield per mg | 1 086±537 | 734±276 | 1 300±718 |
| Trypan blue stained cells (%) | 17± 8 | 32± 18 | 18± 13 |
| Unsettled cells (%) | 59± 21 | 63± 27 | 56± 22 |

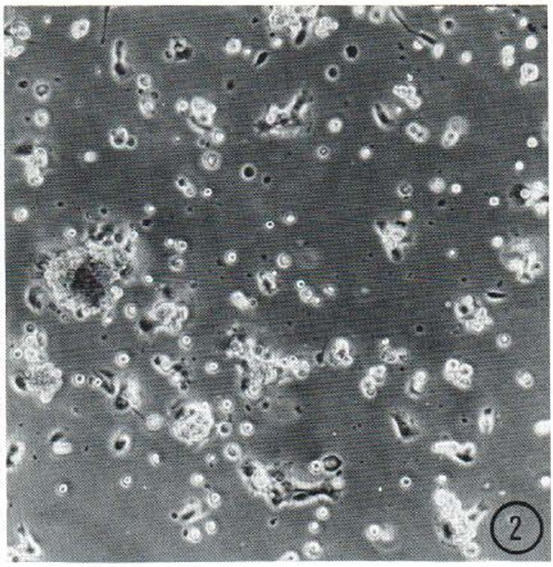

*Fig. 2.* After 1 day, epidermal cells have settled either as single cells or as conglomerates. Unsettled spherical cells float in the medium. ×675.

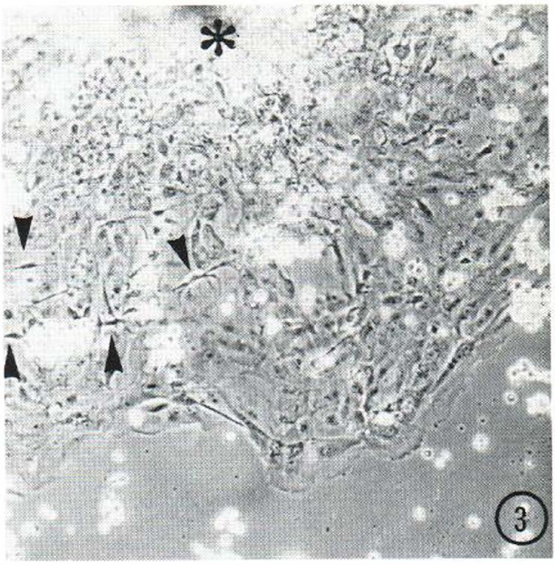

*Fig. 3.* Membranous outgrowth of epidermal cells on the 6th day. A large conglomerate of epidermal cells is seen (asterisk). Small dendritic cells appear among the polygonal keratinocytes (arrow-heads).

After 3 hours' incubation, the epidermis was released at the edges of the STS specimens. Entire sheets of epidermis were easily peeled off at this stage and, hence, unnecessary enzyme treatment was avoided. At 24 hours' incubation, released epidermal sheets floated on the surface of the incubation media. The number of dissociated cells liberated from these epidermal sheets was approximately 300–400 per $mm^2$ after 3 hours' incubation (Table I) and approximately 2000 cells per $mm^2$ after 24 hours. Though trypan blue exclusion tests showed that 70-85% of the cells were unstained, only 40% actually settled to the bottom of the flasks (Table I). After 3 hours of collagenase incubation and subsequent trypsinization, a low percentage of the settled cells were growth active. No growth was obtained from specimens incubated in collagenase for 6 hours or more.

*Dermal cells.* Corium of STS specimens was completely digested for 6 hours. The FTS specimens needed 12 to 24 hours of collagenase incubation, depending on specimen thickness and area. The incubation time needed for complete digestion when using the various solvents revealed no definite differences. At the end of the incubation period, the media appeared cloudy with suspended single cells, but fragments of blood vessels, sweat ducts and elastic fibres were also present. Most growth-active cells were obtained when using collagenase solvent to specimen wet weight was optimal at 1:200. Large FTS specimens gave fewer cells per mg tissue. The yielded cell count after 6, 12 and 24 hours was approximately the same, viz. 1000 cells/mg tissue. However, most growth-active cells were obtained after 6 hours' incubation. The trypan blue test showed that only 20–30% of the cells were stained, but 60% of the cells never settled to the bottom of the flasks (Table I).

2. *Cell growth*

*Epidermal cells.* The suspended cells had a tendency to form conglomerates of varying size. Both spherical single cells and conglomerates settled to the flask bottom (Fig. 2). During the first day, some of the settled cells became polygonal by expanding their cytoplasm in the periphery (Fig. 2). During the next 3–6 days, many migrating cells appeared around larger conglomerates and formed a monolayer of polygonal epidermal cells (Fig. 3). Hence, limited areas of the surface of the flask were covered by epidermal cells, although no mitosis was seen. Some of the polygonal cells increased in size and were finally released from the growth surface during 1 to 2 weeks (Fig. 4*b*). Thick polylayers of

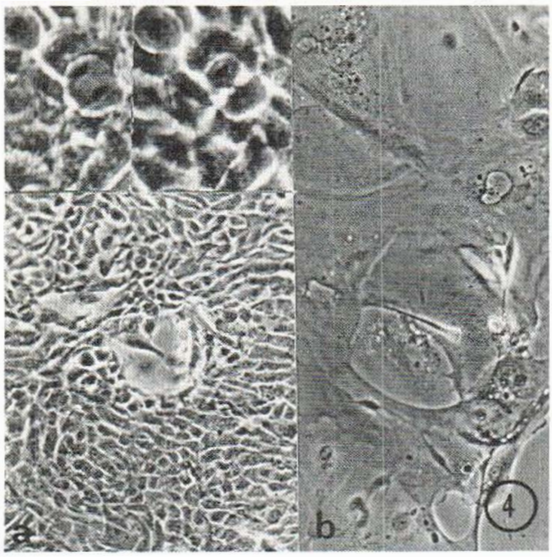


*Fig. 4.* (*a*) After 2 weeks of cultivation, the epidermal cells have formed a confluent layer. ×675. Insets show a mitosis. (*b*) Large keratinocytes with expanding cytoplasm are seen. ×675.

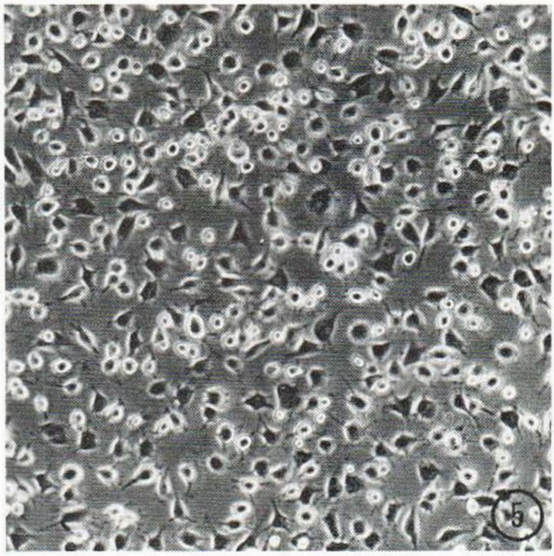


*Fig. 5.* Cells liberated from corium cultivated for 1 day. Note the varying morphology of the cells. ×675.

cells were usually seen in areas with large cells (Fig. 3). Small polygonal cells with numerous mitotic figures were seen mainly in a monolayer (Fig. 4*a*). Small dendritic cells were found between the polygonal keratinocytes (Fig. 3).

*Dermal cells*. Spherical single cells settled evenly all over the flask surface during the first few hours of cultivation. After 24 hours of cultivation, the cells appeared spherical, polygonal and spindle-shaped (Fig. 5). In 3 days, the cell number had doubled (Fig. 1) and formed a confluent layer of cells (Fig. 6). Multi-nucleated giant cells and groups of small cells with dark nuclei were present within the first 2 weeks of cultivation (Fig. 6). These cells gradually disappeared after 2 weeks' culture and were replaced by spindle-shaped cells (Fig. 7). Numerous mitotic figures were seen among the spindle-shaped cells.

## DISCUSSION

To obtain growth-active cells from minced tissue, crude *clostridium histolyticum* collagenase has previously been used at a concentration of 1 mg/ml for rat mammary glands (8) and 2 mg/ml for vascular smooth muscle cells and cartilage (2, 5). However, minced tissue is not suitable for preparation of suspensions of separate dermal and epidermal cells. The present authors have previously applied 2 mg/ml collagenase solution to human split thickness skin specimens and studied the ultrastructural changes of dermal fibres (7). The ultrastructure of the cells was found to be well preserved. In these experiments, the authors noticed that an intact epidermal layer could easily be peeled off, even though the digestion of the corium was not completed. Crude collagenase does not separate the desmosomal connections between epidermal cells (7).

Bacterial collagenase has an optimal pH at 7.2–7.6, both in phosphate and in tris buffer (10). However, tris buffer preserves the enzyme activity longer than phosphate buffer. The presence of Ca ions is obligatory (10). The enzyme activity is lost in the solution for about 24 hours at room temperature (11). The present study showed that short-term collagenase incubation was optimal and resulted in the greatest cell yield and growth activity. Prolonged incubation gave an increased cell yield, but the cell growth decreased proportionally.

Because of the large content of collagen in skin, a much longer period of digestion with collagenase is necessary than that described for other tissues. The prolonged incubation may damage the cell membranes. However, serum supplementation of the incubation solvents may prevent this toxic ef-

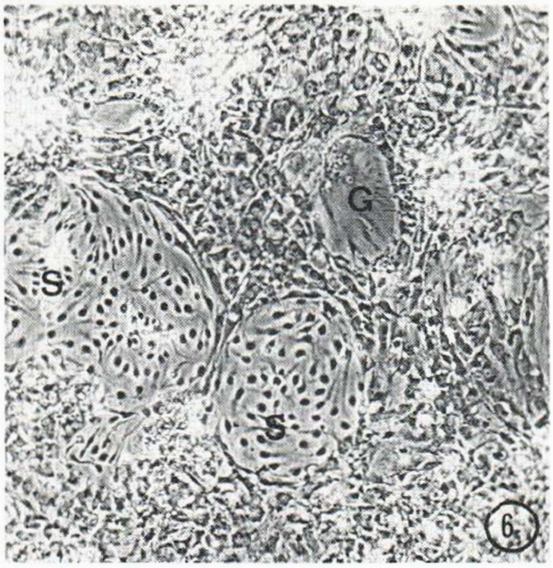


*Fig. 6.* A multinucleated giant cell (*G*) and clustered small cells with dark nuclei (*S*) appearing in dermal cultures in the second week of cultivation. ×675.

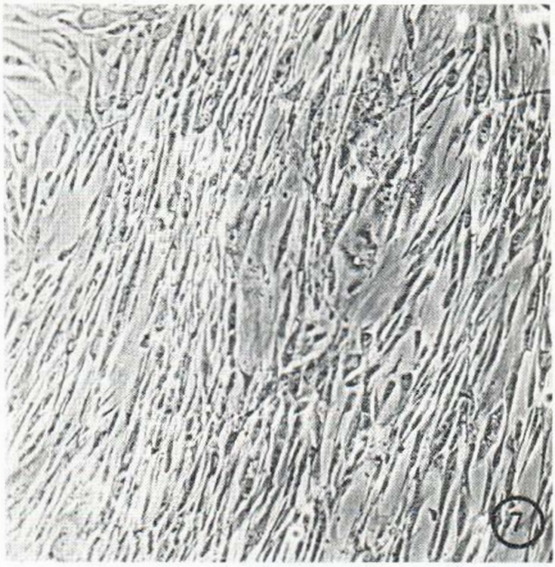


*Fig. 7.* After 2 weeks of cultivation, most dermal cells appear spindle-shaped. ×675.

fect. In contrast to most vertebrate collagenase, bacterial collagenase is not inhibited by serum (12). Coon (1965) reported that a better tissue disaggregation was obtained when serum was present in a mixture of collagenase and trypsin (2). However, the present study showed no significant influence of serum on cell yield and growth. The ratio of tissue weight to collagenase incubation volume should be optimal. Excessive quantities of digestion mixture adversely affect cell growth activity.

It must be emphasized that the trypan blue exclusion test revealed few damaged cells. Nevertheless, significantly more cells remained unsettled. It is therefore concluded that the trypan blue non-stained cells were not identical with the growth-active cells.

An epidermal culture consists mainly of polygonal keratinocytes (3, 6) and few dendritic cells—presumably melanocytes (1). Langerhans cells can be cultivated for few days only (4). The present study demonstrates a focal epidermal growth pattern. During the first week of cultivation, no mitotic figures were present. Hence, the membranous outgrowth around the conglomerates is due to migration. The decrease in epidermal cell number at the sixth day of cultivation is explained by the expanding cytoplasm of the individual cells per photographed area. The two types of epidermal cells—viz. small polygonal cells with many mitotic figures, and large non-dividing cells—probably represent basal cells and keratinizing cells, respectively. In contradistinction to keratinocytes, no growth foci of dermal cells were present. The rapid growth rate of dermal cells differs significantly from that of epidermal cells. The dermal culture is believed to be a mixed population of cells—fibroblasts, macrophages, mast cells, smooth muscle cells, vascular endothelial cells, pericytes, perineural cells, Schwann cells, and cells of eccrine sweat glands. The various cells cannot be identified by phase contrast microscopy. Giant multinuclear cells may be formed by spontaneous cell fusion (11). Spindle-shaped cells seen after 2 weeks of cultivation resemble established fibroblasts (13).

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