Preparation of Human Epidermal Tissue for Functional Immune Studies

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A trypsinization technique was optimized for preparation of human epidermal single-cell suspensions from suction blisters. The conditions which provided the highest epidermal cell yield and viability was Sigma trypsin 0.25% (Type II, crude; cat. no. T8128). The incubation time should be 45 min at 37°C with continuous shaking of the epidermal tissue. The use of suction blisters was evaluated for in vivo determination of epidermal thymocyte activating factor (ETAF/Il-1). Trypsinization of epidermis diminished ETAF/Il-1 activity. Dialysis of epidermal homogenates improves determination of ETAF/Il-1-like activity due to removal of low-weight inhibitors. Determination of ETAF/Il-1-like activity in homogenates of suction blisters constitutes a reliable model with acceptable reproducibility. *Key words: Epidermis; ETAF; Interleukin-1; Trypsin.* (Received April 28, 1988.)

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Human epidermis is an important source of material for the study of inflammatory disorders in the skin. We have found considerable variations in skin preparation procedures (1–6) and have therefore sought to establish the most favorable experimental conditions for isolation and preparation of epidermis with respect to cell yield and later functional studies.

We have also evaluated the use of suction blisters for in vivo determination of epidermal thymocyte activating factor identical with interleukin-1 (ETAF/Il-1) with regard to pretreatment of epidermal tissue and reproducibility of results.

PATIENTS AND METHODS

Epidermis

Human epidermal cells (ECs) were obtained from normal-looking skin in patients admitted to our Department because of various dermatological disorders (psoriasis, hand eczema, leg ulcers) and from skin following plastic surgery.

Epidermis was separated from dermis in vivo by the suction blister technique (7–9). The instrument consists of a compressor that gives a negative pressure. A magnetic valve ensures a suitable constant decompression. Tubes and cups are of acrylic material, and all is built into a small cabinet on wheels. The suction cups have a diameter of 4.5 cm with slightly concave, exchangeable plastic discs perforated with from seven to seventeen holes with a diameter of 0.5 cm. A negative pressure of 300–350 mmHg is applied and blisters are formed after 60 to 75 min. The blister area is approximately 0.2 cm^2 per blister. The fluid within the blisters contains no erythrocytes. Blisters are removed using a pair of sterile scissors, and transferred to a sterile plastic vial containing RPMI 1640 (Gibco) and kept at 4°C before use, never exceeding 2 h.

Trypsinization

The epidermal sheets were washed once in Hanks' balanced salt solution (HBSS) in order to remove inhibitors of trypsin (i.e. plasma in suction blister fluid). Conditions such as source of trypsin, trypsin concentration, incubation time, temperature and mechanical influence were investigated. In all instances, trypsinization was stopped by adding 5 ml of RPMI 1640 with 10% fetal calf serum (FCS). The visible cell debris was carefully removed and the single-cell suspension washed twice in HBSS with 10% FCS

and centrifuged for 10 min at 650 *g*. Cell yield was determined by counting in a Bürker-Turk counting chamber. Viability was judged by the trypan blue exclusion test. The experimental conditions for the study of cell yield using the trypsinization technique are listed in Table I.

Homogenization of epidermal sheets

The epidermal content of ETAF/Il-1 was measured in homogenized epidermal sheets from suction blisters, which were kept in PBS at −20°C and homogenized within 4 days. One ml of phosphate buffer solution (PBS) was added to the thawed sheets and processed in a glass homogenizer (type H 103/31/91, Bie & Berntsen, Denmark). The homogenate was decanted, spun for 20 min at 650 *g* and dialysed overnight in 20× volume RPMI 1640 in a dialysis tube with a pore size of 15 nm (Struers Denmark) in order to remove low-weight inhibitors (PGE_2) of Il-1 activity. The dialysis tube was boiled before use to remove other inhibitory factors. The dialysed homogenate was ultrafiltered in a Filtron dispersable chamber (Filtron Corp; cut-off at 3 kD) to any suitable volume. All procedures were carried out at 4°C and samples were stored at −20°C and examined within a week.

ETAF/Il-1-like activity

Epidermal homogenates were tested for their enhancement of phytohemagglutinin (PHA) induced proliferation of thymocytes from 6–8-week-old female C3H/SSI mice (Statens Serum Institut, Copenhagen) as described earlier (10). Single-cell suspensions of thymocytes were prepared, washed once in medium and cultured with epidermis homogenates for 72 h in flat-bottomed tissue culture plates (NUNC) (1×10^6 cells/ml) together with 10 µg/ml of PHA (Difco) and 2×10^{-5} M mercaptoethanol. The epidermal homogenate was incubated (37°C, 5% CO_2) using a $\log_2$ dilution row. 0.5 µCi of [^{3}H]thymidine was added after 48 h and harvesting took place 24 h later using a Titertek harvester. Radioactivity was measured in a liquid scintillation counter. The results are expressed as the arithmetic mean of counts per minute (cpm). Each experiment included a standard supernatant of Il-1 and by comparison, ETAF/Il-1-like activity of the sample could be determined (11). Activity is expressed as units of Il-1 per cm^2 of epidermis, since the approximate surface from each suction blister area could be determined.

Statistical evaluation

The non-parametric Wilcoxon test for pair differences was used. A *p*-value below 0.05 was considered significant.

RESULTS

Trypsinization of epidermal sheets

Five different experiments (I–V) were carried out in order to determine the optimal conditions for trypsinization of epidermal sheets.

I. *Comparison of two different brands of trypsin:* Epidermis (suction blisters) from 8 patients was divided into two equally sized portions and incubated for 45 min at 37°C (see Table I) with 5 ml trypsin solution from Sigma (Type II: crude, catalogue no. T8128) or Gibco (catalogue no. 043-5090).

Table I. *Experimental conditions for the experiments where trypsinization was included in procedures*

Var. refers to the parameter which was varied in the experiment concerned (see text)

Exp. no.	Trypsin origin (manufact.)	Trypsin conc. (%)	Temp. (°C)	Incubation time (min)	Mechanical influence (− or +)
I	Var.	0.25	37	45	+
II	Sigma	Var.	37	45	+
III	Sigma	0.25	Var.	45	+
IV	Sigma	0.25	37	Var.	+
V	Sigma	0.25	37	45	Var.
VIII	Sigma	0.25	37	45	+

Trypsin from Sigma provided a significantly higher yield of EC and a higher cell viability ($p \leq 0.01$) (Table II *a*). In preliminary experiments (data not shown), trypsin from Novo, Bagsværd, Denmark, and Difco gave much smaller yields than either Sigma or Gibco trypsin. Trypsin from Sigma was used in all subsequent experiments.

II. *Comparison of four different trypsin concentrations:* Four different concentrations of trypsin were used: 0.015%, 0.06%, 0.25% and 1.00%. Each was applied to epidermal sheets as described above and in Table I. Table II *b* shows that the yield of viable cells was negligible at 0.015%, and 0.06% compared with 0.25% and 1.0% ($p \leq 0.01$). The 1.00% trypsin solution did not improve the cell yield compared with the 0.25% solution ($p > 0.1$). EC viability was identical, using the three highest concentrations.

III. *The significance of mechanical influence:* Epidermal sheets were separated into two equally sized portions and incubated with trypsin for 45 min in a water bath at 37°C with or without shaking the vials. In all instances continuous shaking of the vial provided a higher yield of ECs ($p \leq 0.01$) (Table II *c*). Viability was not affected ($p > 0.1$). In a preliminary study we used a magnetic stirrer, but this procedure reduced the number of viable EC (<50%).

Table II (a–e). *Results from five experiments (I–V), testing different experimental conditions during trypsinization when preparing single-cell suspensions of ECs*

Two different trypsin preparations (from Sigma and Gibco, see text) were tested; parameters such as trypsin concentration, mechanical influence during incubation, temperature and duration of trypsinization were also studied. EC yield is given as number of cells obtained per skin area (EC/cm^2). Viability of cells in a single-cell preparation is given as percentage (%). SD is standard deviation and *n* is number of experiments

	n	EC yield	SD	Viability (%)	SD (%)
Table II a. Expt I					
Manufacturer					
Gibco	8	1.10×10^5	0.8×10^5	68	16
Sigma	8	2.50×10^5	1.0×10^5	90	7
Table II b. Expt II					
Trypsin concentration (%)					
0.015	8	0.15×10^5	0.20×10^5	–	–
0.06	8	0.58×10^5	0.38×10^5	81	10
0.25	8	2.90×10^5	1.35×10^5	82	10
1.00	8	2.50×10^5	1.10×10^5	79	9
Table II c. Expt III					
Mechanical stimulation					
–	8	0.86×10^5	0.60×10^5	81	5
+	8	3.71×10^5	2.21×10^5	81	5
Table II d. Expt IV					
Temperature (°C)					
25	6	0.62×10^5	0.44×10^5	88	4
37	6	3.89×10^5	0.15×10^5	88	6
Table II e. Expt V					
Trypsinization time (min)					
15	8	0.19×10^5	0.38×10^5	–	–
30	8	1.72×10^5	1.67×10^5	80	9
45	8	4.20×10^5	1.90×10^5	78	11
60	8	3.29×10^5	2.46×10^5	70	12

IV. *The influence of temperature on trypsinization:* Epidermal sheets were trypsinized at 25°C or at 37°C. The cell yield is strongly dependent upon the incubation temperature ($p \leq 0.05$), since in all 6 patients cell yield was 3-fold higher at 37°C than 25°C (Table II *d*). No difference was observed in viability ($p > 0.1$). Trypsinization at 4°C gave less than 1% of EC compared with 37°C.

V. *The influence of time on trypsinization:* Epidermal sheets were trypsinized for 15, 30, 45 and 60 min, respectively. The ECs yield was negligible after 15 min (Table II *e*). 45 min of incubation produced a significantly higher cell yield than 30 min ($p < 0.05$), while no difference was detected on comparison of 45 min vs. 60 min with respect to cell yield and viability ($p > 0.1$).

Determination of ETAF/Il-1 in human epidermal sheets

VI. *Reproducibility of ETAF/Il-1 determinations of epidermal homogenates:* Epidermal homogenates were prepared from suction blisters on normal abdominal skin from 8 patients. ETAF/Il-1 was determined at each side of the abdominal wall. The results showed that the ETAF/Il-1 activity (units/cm^2) for the left side was 660±323 units/cm^2 and for the right side, 662±363 units/cm^2 (mean ± SD).

VII. *ETAF/Il-1 activity in untreated versus dialyzed epidermal homogenates:* Epidermis was isolated from skin obtained from mammoplastic surgery using a dermatome (disposable dermatome cutting head, Davol Inc., USA). The separated epidermal flake was floated on RPMI 1640 and after estimation of surface area homogenized as described above. The ETAF/Il-1 activity was higher in the dialysed sample (655±318 units/cm^2) than without dialysis (200±127 units/cm^2).

VIII. *ETAF/Il-1 activity in untreated versus trypsinized human epidermis:* Human epidermis was trypsinized using six different trypsin concentrations (0%, 0.03%, 0.06%, 0.12%, 0.25% and 0.5%, respectively) before homogenization. The experiment revealed that ETAF/Il-1-like activity declined when trypsin was added to epidermis. The decline was dose-dependent. Ratios between activity in epidermis from untreated epidermis and epidermal tissue exposed to 0.5% trypsin were in 3 persons 4.7, 6.4 and 3.8, indicating that a large part of the epidermal ETAF/Il-1 is membrane-bound.

DISCUSSION

Optimal trypsinization was found to be dependent upon a variety of factors. The best conditions were trypsinization for 45 min at 37°C in a water-bath with continuous shaking of the vials using 0.25% trypsin from Sigma (Type II: crude, catalogue no. T8128). Yield was 3.4×10^5 ECs/cm^2 $\pm 1.7 \times 10^5$ ECs/cm^2.

Two of the most frequently used trypsin products were compared in this study. The reason that Sigma seems better than Gibco may be that the optimal concentration for Gibco lies between 0.25% and 1.00% and that our results reflect a difference in potency. We did however experience batch differences and therefore every batch should be carefully checked before taken into use.

The present study emphasizes the importance of mechanical shaking of the vials during the trypsinization process, which leads to a significantly higher cell yield. This is in agreement with earlier studies on chick embryo epithelia (12), rat buccal epithelium (13) and newborn rat epidermis (14), which revealed that additional mechanical action further disperses trypsinized tissue.

Trypsin disperses epidermis through an attack on the desmosomes and also removes cell

surface material, leading to a loss of normal cell surface topography (2). The cells in the basal and spinous layers exhibit a polyhedral shape and are highly convoluted at lower temperatures (2). Therefore trypsinized epidermal cells from normal or diseased skin should not be damaged further and exposure to trypsin should be as limited as possible. Even though cell viability is unchanged at 60 vis-à-vis 45 min of trypsinization (Table II *e*), damage to surface receptors cannot be excluded. Todd revealed a 50% loss of immunoprecipitable ^{125}I-H-2 tissue antigen on mouse EL-4 cells using 0.5% trypsin for 60 min (6), which emphasizes the importance of not increasing trypsin concentration more than necessary. Gromkowski et al. (15) found that Sigma trypsin in concentrations of 0.1–0.3% did not affect the HLA-DR surface receptors on human lymphocytes trypsinized for 30 min. Another study, on mouse T lymphocytes, demonstrated that trypsinization (0.05 mg/ml) could uncover immune-associated surface tissue antigens on approximately 20% of the T cells, whereas 2 mg/ml of trypsin removed all surface antigens (16). Tanaka & Sakai (5) have observed that rat skin epidermal cells are less stimulatory in a mixed skin lymphocyte reaction following trypsinization. This lack of antigenicity disappears if the cells are left in culture medium for 3 h, showing that the cells, at least partially, can repair their damage from the contact with trypsin.

The suction blister technique provides sufficient epidermal tissue and reproducible results concerning ETAF/Il-1 determination. We confirmed the necessity of removing low-weight inhibitors from epidermal homogenates (17). Trypsinized epidermis was not suitable for determination of ETAF/Il-1. This could be due to a loss of membrane-bound ETAF/Il-1 during trypsinization. Trypsin may also have a toxic effect on the murine thymocyte proliferation assay, if small amounts of trypsin are not inactivated by the addition of FCS.

In conclusion, the experimental conditions for single-cell preparation of epidermis such as trypsinization time, trypsin concentration, temperature and the influence of mechanical influence is important for obtaining maximum EC yields. Also, considerable differences exist between different trypsin products. We prefer and recommend trypsin from Sigma (Type II, crude; cat. no. T8128) in a concentration of 0.25% for 45 min at 37°C and with continuous shaking of the vials.

Suction blisters are well suited for obtaining epidermal tissue for determination of ETAF/Il-1 activity and a 95% significance limit is acceptable. Dialysis of epidermal homogenates improves the measurable ETAF/Il-1-like activity.

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