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Immunohistochemical study of cell proliferation and differentiation in epidermis of mice after administration of cholera toxin

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Abstract. Cholera toxin causes reversible epidermal hyperplasia. We observed maximal thickness of the epidermis on the fourth day after treatment and a return to pretreatment values by day 7. The increase in thickness occurred in the basal and intermediate layers, with these layers becoming two to three times thicker than those of normal epidermis. The time sequence of epidermal proliferation was studied using bromodeoxyuridine (BrdU) labelling. We observed a maximum number of labelled basal cells within the first 24 h. Only a few cells were labelled 7 days after toxin injection. *Griffonia simplicifolia*-IB_4 (GSA-IB_4), *Ulex europaeus*-I (UEA-I) and *Griffonia simplicifolia*-II (GSA-II) lectins were used for the analysis of epidermal cell differentiation in the tissue sections. To study keratinocyte differentiation, further immunological staining was performed using two anticytokeratin antibodies, PKK_2 and PKK_3 mouse monoclonal antibodies. From the immunocytochemical results, we conclude that synchronous differentiation of the epidermis occurs after cholera toxin administration.

Key words: Cholera toxin – Epidermal hyperplasia – Bromodeoxyuridine – Lectin – Keratin

Intracutaneous injection of cholera toxin into mice induces synchronous cell division in the epidermis resulting in temporary hyperplasia [6]. Bromodeoxyuridine (BrdU) is a thymidine analogue which is incorporated in to DNA during the S-phase of the cell cycle [10, 15], and single stranded DNA containing BrdU can be detected immunohistochemically [4, 8, 16].

We investigated cell proliferation of the epidermis using BrdU. We also used lectin cytochemical staining to investigate cell proliferation and differentiation, and immunocytochemical staining using cytokeratin antibodies. Lectins are proteins or glycoproteins of plant or animal origin that reversibly bind certain saccharide configurations. Lectins are good markers of epidermal cells, enabling the study of both proliferation and differentiation [1, 3, 5, 11, 17]. The molecular composition of cytokeratin polypeptides differs among epidermal cell layers [9, 13]. The results of these three techniques indicate that synchronous differentiation occurs after the administration of cholera toxin.

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Materials and methods

Reagents

We purchased the following reagents: cholera toxin (Choletox) (Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan), trypsin (Difco Laboratories, Detroit, Mich.), BrdU (Sigma, St. Louis, Mo.), mouse anti-BrdU monoclonal antibody (Becton Dickinson, Mountain View, Calif.), biotinylated goat anti-mouse antibody (Dako, Carpenteria, Calif.), HRP-labelled streptavidin (Bethesda Research Laboratories, Bethesda, Md.), HRP-labelled *Ulex europaeus*-I (UEA-I), biotinylated *Griffonia simplicifolia*-IB_4 (GSA-IB_4) and GSA-II lectins (E.Y. Laboratories, San Mateo, Calif.), mouse monoclonal anti-cytokeratin antibodies PKK_2 and PKK_3 (Labsystems, Helsinki, Finland), 3,3′-diaminobenzidine tetrahydrochloride (Dojin Laboratories, Kumamoto, Japan) and Eukitt (O. Kindler, FRG).

Animals and administration of cholera toxin

Male DDY mice ($n = 20$) aged 6 weeks and weighing approximately 30 g were obtained from Kyudo, Kumamoto, Japan. The dorsal surface of each mouse was shaved with an electric shaver 2 days before the administration of the cholera toxin. Cholera toxin (10 ng) was injected intracutaneously into the back of each mouse. The stock solution of cholera toxin was prepared by dissolving 1 mg/ml in 0.05 *M* Tris buffer, pH 8.0, containing lactose (50 mg/ml) as a stabilizer. Just before injection, the stock solution was diluted with cold phosphate buffered saline (PBS).

Administration of bromodeoxyuridine

Each mouse was injected intraperitoneally with BrdU 50 mg/kg body weight three times at an interval of 20 min and with the third injection 20 min before the mouse was killed.

Specimen preparation

Animals were sacrificed by cervical dislocation always between 3 and 5 p.m., to avoid the effects of circadian variation in the epidermal cell cycle, and 24 h, 48 h, 4 days and 7 days after the administration of cholera toxin. The toxin-injected sites were rapidly excised including control skin injected with PBS. The tissues were fixed with 10% formalin for 2 days and/or ice-cold methanol for 2 h and then embedded in paraffin. Sections of 4 μm thickness were cut and stained with haematoxylin and eosin (H & E) for morphological examination.

Immunohistochemical staining with anti-BrdU antibody staining

After deparaffinization and rehydration, sections for immunohistochemical staining were immersed in 0.3% H_2O_2 in methanol at room temperature for 20 min to block endogenous peroxidase activity. Specimens were treated with 2 *N* HCl for 90 min at 20 °C and further trypsinized with 0.2% trypsin at 37 °C for 30 min. After pretreatment with 1% BSA in PBS for 10 min, the tissue sections were first incubated in anti-BrdU monoclonal antibody diluted 300 times at 4 °C overnight, rinsed with PBS and then incubated for 30 min with biotinylated goat anti-mouse IgG diluted 50 times. Finally streptavidin-HRP was added for 30 min, and then the specimens were stained with DAB solution for 2–5 min. Counterstaining was omitted. After dehydration and clearing, slides were mounted with Eukitt material.

Lectin staining

After deparaffinization and rehydration, the endogenous peroxidase activity of the tissue was blocked as described above. After treatment with 1% BSA in PBS, the sections for lectin staining were incubated with HRP-labelled UEA-I, biotinylated GSA-IB_4 and biotinylated GSA-II lectins at a concentration of 50, 25 and 50 μg/ml, respectively, at 4 °C overnight. The biotinylated lectin-stained slides were further treated with HRP-labelled streptavidin diluted 50 times for 30 mins.

Procedure for anti-cytokeratin antibody immunostaining

Staining with antibodies PKK_2 and PKK_3 was performed using specimens fixed with methanol after deparaffinization, rehydration and blocking of endogeneous peroxidase activity. Sections were first incubated with anti-cytokeratin PKK_2 or PKK_3 antibodies diluted 50 times at 4 °C overnight, rinsed with PBS, then incubated for 30 min at room temperature with biotinylated goat anti-mouse IgG diluted 50 times, and finally incubated with streptavidin–HRP or protein-A colloidal gold sequential processing with physical development.

Results

Morphological observation of the reversible epidermal hyperplasia induced by cholera toxin

Cholera toxin (10 ng) injected intracutaneously into the back of the mice resulted in the formation of a blister 18 h after injection. The blister gradually subsided within 30 h, but induration remained for 7 days. Epidermal hyperplasia was evidenced by a 1–2-fold skin thickening compared with normal skin or the PBS-treated control skin 1 day after the toxin injection, a 2–3-fold thickening 2 days after the injection and a 4–6-fold thickening 4 days after the injection. Seven days after the toxin injection, the thickness of the epidermis returned to normal. The results are summarized in Table 1. Initially, the cholera toxin stimulated mitosis of the basal cells and consequently produced hyperplasia of the epidermis (Fig. 1a–d).

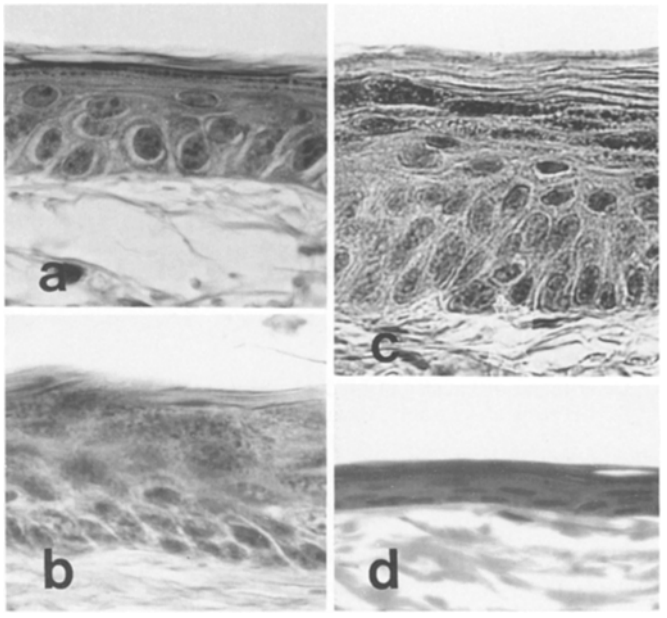

Fig. 1a–d. Histological changes in mouse skin after intracutaneous administration of cholera toxin. H & E staining. × 600. **a** 24 h after toxin injection. Basal cells are cuboidal in shape and the basal zone consists of three layers. Hyperplasia of the epidermis is one to two times more than normal skin. The stratum corneum and granulosum are within the normal range. **b** 48 h after toxin injection. Basal cells are oval in shape and the basal zone consists of three to four layers. Hyperplasia of the epidermis is two to three times more than normal skin. Granular layers have become thicker but the stratum corneum is within the normal range. **c** 4 days after toxin injection. Basal cells are columnar in shape and the basal zone consists of six to seven layers. Hyperplasia of the epidermis is four to six times more than normal skin. The stratum corneum and granulosum have become thickened simultaneously. **d** 7 days after toxin injection. Basal cells are flat in shape and the basal zone consists of two layers. It is similar to that of normal skin. The stratum corneum and granulosum are the same as those of normal skin

Immunohistochemical study by the staining of anti-BrdU antibody

BrdU is only incorporated in the S-phase. The percentage of S-phase cells for each division is given in the Fig. 2. The number of labelled cells reached a maximum (65%) 24 h after the injection and all were localized in the basal layer zone. The labelling tapered off in the following week (Fig. 3a–c).

Cell differentiation investigated by lectin staining

The most pronounced staining was observed at day 4 after the cholera toxin injection, and the staining results

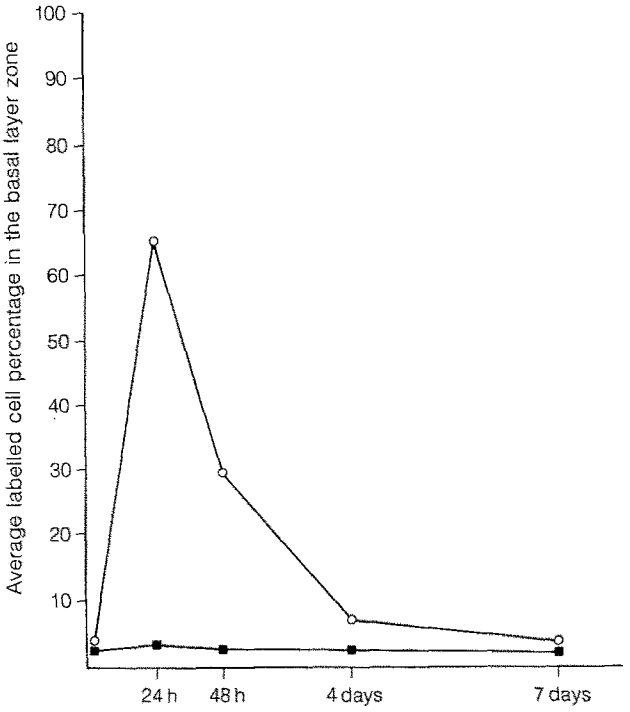

Fig. 2. Labelling curve representing the percentage of labelled cells with anti-BrdU antibody immunostaining in the basal layer zone. ○, administration of cholera toxin 10 ng; ■, administration of PBS (control)

described here are mainly drawn from the observation of this pronounced staining. The GSA-IB_4 lectin is specific for terminal α-D-galactopyranosyl groups. It reacted strongly with basal cells and weakly with spinous cells. Staining was mainly in the cell membrane, but the cytoplasm was also weakly stained (Fig. 4a). The UEA-I lectin is specific for α-L-fucose contained in glycoconjugates. The upper layer of the stratum spinosum and the stratum granulosum reacted strongly with UEA-I. Staining was mainly in the cell membrane, but the cytoplasm was also moderately stained (Fig. 4b). The GSA-II lectin is specific for terminal *N*-acetyl-D-glucosamine. It stained the stratum corneum weakly and some distinct areas of the granular layer. The cell membrane was weakly stained and cytoplasmic staining was possibly present (Fig. 4c). These results are summarized in Table 1.

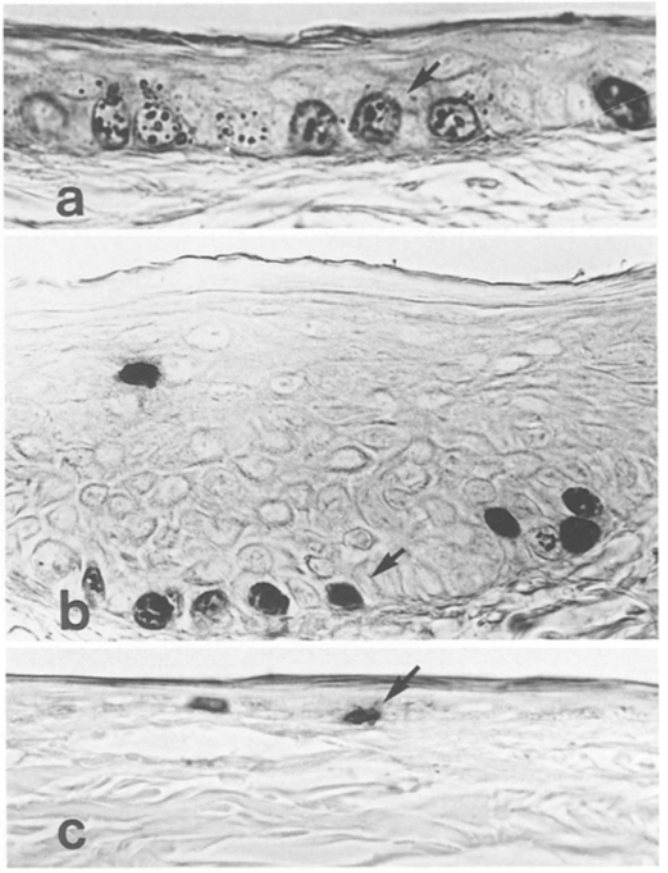

Fig. 3a–c. Immunohistochemical staining of experimental skin by antibromodeoxyuridine (anti-BrdU) antibody. *Arrows* indicate labelled cells. ×600. **a** 24 h after toxin injection. An average of two-thirds of the basal cells are labelled. Stained cells are large and round. **b** 4 days after toxin injection. An average of one-twelfth of the basal cells are labelled. The cells have become smaller and oval. **c** 7 days after toxin injection. Only two cells are labelled in the basal layer. Cells are small and flat

Cytokeratin polypeptide development investigated by anticytokeratin antibody staining

Antibody PKK_2 binds specifically to cytokeratin polypeptides 19, 17, 16 and 7. The basal layer and the lower stratum spinosum were strongly stained with PKK_2

Table 1. Immunohistochemical staining of experimental skin by lectin

Lectin	Basal cells	Spinous cells	Granular cells	Cornified cells	Thickness of different epidermal layers				
					1 day after toxin injection	2 days after toxin injection	4 days after toxin injection	7 days after toxin injection	7 days after PBS injection
GSAI-B4	+++	+			2–3 layers, basal zone	2–3 layers, basal zone	4–6 layers, basal zone	1–2 layers, basal zone	1–2 layers, basal zone
UEA-I		++	+++		2–3 layers, intermediate zone	2–3 layers, intermediate zone	4–6 layers, intermediate zone	1–2 layers, intermediate zone	1–2 layers, intermediate zone
GSA-II			±	+	corneal layer, nd	corneal layer, nd	corneal layer, nd	corneal layer, nd	corneal layer, nd

+++, strongly reactive; ++, moderately reactive; +, weakly reactive; ±, very weakly reactive; nd, not distinct

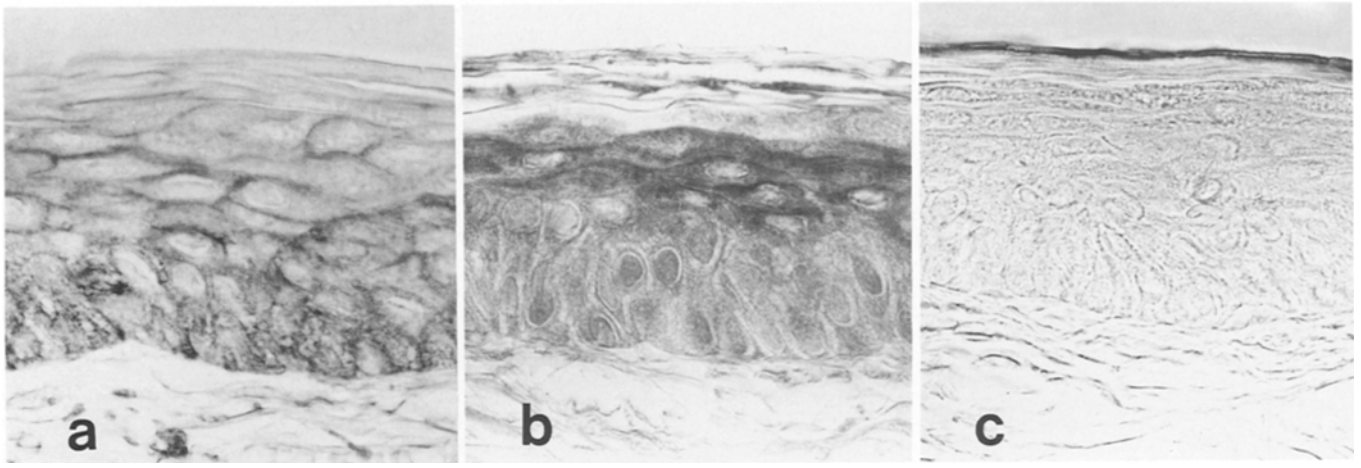

Fig. 4a–c. Lectin binding patterns of the epidermal layer 4 days after the administration of cholera toxin. × 600. **a** GSA-IB_4 staining. Basal cells are strongly reactive and spinous cells are weakly reactive. The staining density amongst these cells is heterogeneous. **b** UEA-I staining. The stratum granulosum and the upper layer of the stratum spinosum of the epidermis are strongly reactive. The cell membrane is strongly stained, and the cytoplasm is moderately stained. **c** GSA-II staining. The stratum corneum is weakly stained and a distinct area within the granular layer of the epidermis is very weakly reactive

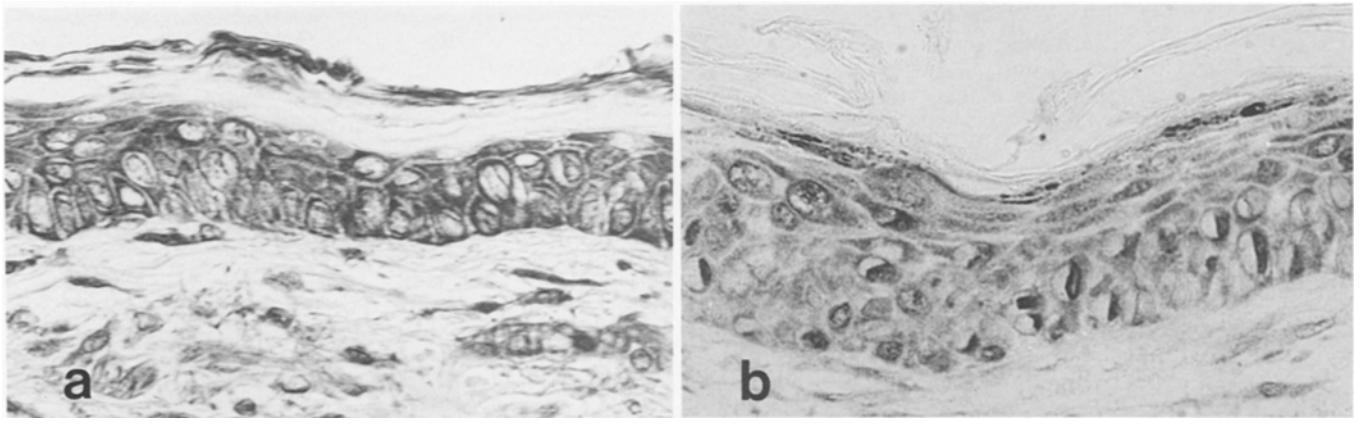

Fig. 5a, b. Distribution of cytokeratin stained with anti-cytokeratin monoclonal antibody 4 days after toxin injection and fixed with cold methanol. × 530. **a** PKK_2 antibody staining. The cells in the basal layer and the lower spinous layer are stained. Other intermediate layers are free of staining. **b** PKK_3 antibody staining. The stratum spinosum and stratum granulosum are shown as strongly stained with silver enhancement. The stratum basale is also weakly stained. The staining shows a granular texture in the cytoplasm

antibody particularly on day 4 after toxin injection (Fig. 5a). Antibody PKK_3 binds only to cytokeratin polypeptide no. 18. Only weak staining of the stratum granulosum spinosum, and basale was observed (Fig. 5b).

Discussion

This experiment was designed to study epidermal cell proliferation and cell differentiation by BrdU immunocytochemistry, lectin staining and cytokeratin immunostaining after the administration of cholera toxin. We confirmed that intracutaneous injection of cholera toxin induces a temporary epidermal proliferation resulting in a 4–6 fold thickening of the epidermis in accordance with the result of a previous experiment [6]. Cholera toxin increases the activity of adenyl cyclase, raising the intracellular concentration of cyclic AMP, which stimulates DNA synthesis of epidermal cells [2, 7]. This toxin is a potent inducer of epidermal hyperplasia, but is not a tumour promoter for mouse skin [7]. The epidermal hyperplasia subsided within a week. DNA replication and cell division occurred only in the basal layer reflecting the pulse effect from the cholera toxin.

Using lectin staining it is possible to show the degree of cell differentiation in relation to the components of the stratified layer. In the rodent epidermis, GSA-IB_4 lectin binds to the basal cells and cells in the lower spinous layer. UEA-I lectin outlines the spinous and granular cells, and GSA-II lectin specifically binds the cornified layer [3, 5]. The differentiation of epidermal cells occurs during the migration from the basal layer to the superficial layer.

Brabec et al. have reported an alteration of the membrane sugar chain accompanying cell differentiation using fluorochrome labelled lectins [3]. In our experiment, the staining patterns of the three lectins, GSA-IB_4, UEA-I, GSA-II, were essentially similar in spite of the different

thicknesses of the epidermis in each group. UEA-I stained diffusely throughout the cytoplasm of suprabasal cells. Similar cytoplasm staining in basal cells was sometimes found. Schaumburg-Lever has observed that keratohyaline granules are stained with colloidal gold-labelled UEA-I [12]. Therefore, it may be considered that the diffuse staining with UEA-I in the cytoplasm of the stratum granulosum is keratohyaline granules. It is known that the fucosylation of glycoconjugates occurs as the final step of glycosylation in the oligosaccharide chain. In the living cell layer of the epidermis, the fucosylation of glycoconjugates indicates the last stage of the intracellular component such as keratohyaline granules in differentiating keratinocytes.

The larger cytokeratin molecules are synthesized in immature keratinocytes or in the basal layer of stratified epithelium [9, 14]. The PKK_2 antibody recognizes the larger cytokeratin molecules. In contrast, the smaller cytokeratin molecules are generated in differentiated cells or suprabasal keratinocytes [14]. The PKK_3 antibody recognizes a smaller cytokeratin, no. 18. This was confirmed by our results. The PKK_3 antibody sometimes stained, not only suprabasal cells, but also basal cells weakly. The results of UEA-I staining and PKK_3 antibody immunostaining suggest that the hyperplasia induced by cholera toxin represents an acceleration of physiological maturation of epidermal cells. The cholera toxin model may be used as an in vivo model for studying epidermal hyperplasia.

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