Induction by cholera toxin of synchronous divisions in vivo in the epidermis resulting in hyperplasia
(cyclic AMP/cyclic AMP inducers/GM1 ganglioside)

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ABSTRACT Intracutaneous injection of cholera toxin, exotoxin of Vibrio cholerae, into the dorsal skin of mice, rats, and hamsters at doses of >0.1 ng evoked an acute reaction at the site of injection, which was characterized histologically by an edematous reaction in the dermis and mitotic stimulation in the epidermis. Mitotic and labeling indices of basal cells of the mouse epidermis showed two peaks at 24 and 48 hr after injection, thereby producing epidermal hyperplasia. The thickness of the interfollicular epidermis increased progressively from 32 hr after toxin injection, being greatest on day 4 and decreasing to normal on day 7. The epidermis on day 4 after injection of 1.0 ng of toxin was about 4- to 6-fold thicker than normal or phosphate buffer-treated control skin. This sequence of events indicated that cholera toxin injected two successive synchronous divisions of the epidermal cells and produced temporary hyperplasia without interfering with epidermal differentiation. The complete structure and function of the cholera toxin are required for induction of epidermal hyperplasia: no mitotic stimulation was induced by injection of the A and B units of the cholera toxin molecule or by preincubation of the toxin with anti-cholera toxin antibody and with the membrane receptor, GM1 ganglioside. Five other agents known to increase the level of intracellular cyclic AMP by different means (dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine, theophylline, isoproterenol, and prostaglandin E1) did not produce a skin reaction.

Cholera toxin is a protein exotoxin produced by Vibrio cholerae, which causes diarrhea in cholera patients. The molecular mechanisms of action of cholera toxin have been investigated extensively using intestinal and nonintestinal tissues and are now well understood (1). The toxin molecule has a molecular weight of 84,000 and consists of two types of noncovalently linked subunits, A and B. The pentamer of the B unit binds to the membrane receptor, GM1 ganglioside, and the A unit activates adenylcyclase through mono-ADP ribosylation of GTP-binding protein, thereby increasing the level of cyclic AMP. This ability is not restricted to intestinal cells but has been found in many mammalian cell types tested, and various biological effects of cholera toxin are probably due to this mechanism.

In confirmation of the result of Green (2), during studies on human epidermal cells in culture (3), we found that colonial growth of the cells was markedly enhanced by addition of cholera toxin (unpublished data). This prompted us to examine whether cholera toxin also stimulates growth of epidermal cells in vivo. This paper shows that intracutaneous injection of cholera toxin into the back of mice induced two successive synchronous divisions of basal cells in the epidermis, which resulted in transient hyperplasia without interfering with epidermal differentiation. A preliminary report of this induction of epidermal hyperplasia by cholera toxin was published elsewhere (4).

MATERIALS AND METHODS
Cholera toxin, its A and B units, and its antiserum were obtained from the Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan. Dibutyryl cyclic AMP, theophylline, 3-isobutyl-1-methylxanthine, isoproterenol, and prostaglandin E1 were purchased from Sigma. Demecolcin was purchased from Wako (Tokyo). GM1 ganglioside isolated from bovine brain, was a gift from Y. Nagai of this Department. [methyl-3H]thyminide (25 Ci/mmol; 1 Ci = 3.7 × 1010 becquerels) was obtained from the Radiochemical Centre (Amersham, England). DDD, C57BL, C3H, and hairless (hr/hr) mice were supplied from the breeding house of this institute. CD-1 mice, Sprague-Dawley rats, and Syrian hamsters were purchased from the Japanese Charles River Company (Kanagawa, Japan).

A stock solution of cholera toxin was prepared by dissolving it at 1 mg/ml in 0.05 M Tris buffer (pH 8.0) containing lactose (50 mg/ml) as stabilizer. This solution is stable for 3 wk at 4°C. Immediately before injection, the stock solution was diluted with cold phosphate-buffered saline and a 0.1-ml volume was injected intracutaneously into four sites in the back of male DDD mice, unless otherwise specified, which had been shaved 2 days previously. The mice were killed between 4 and 6 p.m. to avoid the complication of possible circadian variation of the epidermal cell cycle. Demecolcin (2 μg/g of body weight) and [3H]thyminide (2 μCi/g of body weight) were injected intraperitoneally 3 hr and 1 hr, respectively, before the mice were killed. The skin at the site of injection was removed, fixed with 10% (vol/vol) formalin, sectioned in paraffin, and stained with hematoxylin and eosin. The mitotic index (%) was obtained by counts on >400 basal cells in the skin lesion. The thickness of the epidermis and number of nuclei per mm of basement membrane were measured with a video-image processor by courtesy of Olympus Optics (Tokyo). For autoradiography, sections were covered with NR-M2 emulsion (Konzhiroku Photo, Tokyo, Japan), exposed for 3 wk, developed, and stained lightly with hematoxylin. The labeling index (%) was measured by counting >250 basal cells in the skin lesion. Cells with more than five grains were regarded as labeled.

RESULTS
Induction of Synchronous Divisions of Epidermal Cells and Consequently Hyperplasia. Intracutaneous injection of the toxin at a dose of >0.1 ng evoked an acute reaction at the site of injection, which was characterized by formation of a round blister in the skin. This blister, or vesicle, was apparent 18 hr after the injection, reaching a maximum in 24 to 30 hr and then gradually receding, but residual induration of the skin remained for about 7 days. The diameter of the blister was linearly related...
were edematous and the 1 epidermis, um thick, toxin injection. marked edematous which dermis, hr 24 basement membrane, and arrested skin being injection. hyperplasia was seen in both the interfollicular and follicular epidermis. In the hair follicles, hyperplasia was most pronounced in the necks (Fig. 1).

Fig. 2 shows the time-course of events for 2 wk after injection of 1 ng of cholera toxin. The mitotic index of the basal cells increased from 18 hr after the injection and reached a first peak

to the logarithm of the toxin dose within the range of 3–20 mm; 1 and 10 ng of the toxin produced vesicles of about 7- and 12-mm diameter, respectively.

Histologically, the most prominent features of the skin lesion were edematous change of the dermis and subcutaneous tissues and mitotic stimulation of the epidermis. The edematous change developed in parallel with formation of the blister; it became apparent 18 hr after the injection, reaching a peak in 24–48 hr and receding in 3 days (Fig. 1). This edematous reaction is primarily due to increased permeability of capillaries, as evidenced by intravenous injection of pontamine sky blue (0.1 ml of a 5% (wt/vol) solution per mouse), which evoked blueing at the site of the skin lesion. In the epidermis, cholera toxin stimulated mitosis of basal cells and, as a consequence, produced epidermal hyperplasia. As seen in Fig. 1 B and C, abundant mitotic figures were seen in demecolcine-treated epidermis 24 and 48 hr after the injection. Mitoses were also found in the cells of sebaceous glands. Epidermal hyperplasia reached a maximum on day 4 and then gradually decreased in thickness. Hyperplasia was seen in both the interfollicular and follicular epidermis. In the hair follicles, hyperplasia was most pronounced in the necks (Fig. 1).

Fig. 2. Time-course of events induced by injection of 1.0 ng of cholera toxin into the dorsal skin of mice, indicating epidermal hyperplasia resulting from successive synchronous divisions of the epidermal cells. The mice were killed between 4 to 6 p.m. (to avoid the complication of possible circadian variation of the epidermal cell cycle) at various times—i.e., 1, 2, 4, 6, 7, 8, 10, and 14 days after the injection. Demecolcin (2 μg/g of body weight) and [3H]dThd (2 μCi/g of body weight) were injected intraperitoneally 3 hr and 1 hr, respectively, before the mice were killed. (A) Mitotic index (%) of basal cells 3 hr after demecolcin injection. (B) Labeling index (%) of basal cells 1 hr after [3H]dThd injection. (C) Thickness of the epidermis (△) and number of nuclei per 1-mm basement membrane (○). (D) Thickness of stratum corneum (●) and stratum granulosum (●). The shadowed area in panels A, B, C, and D shows values for the phosphate buffer-treated control.
at 24 hr and a second peak at 48 hr; it returned to the normal range after 3 days. The values of the first and second peaks induced by 1.0 ng of cholera toxin were 10.2 and 14.0%, respectively, whereas the mitotic index of control skin after phosphate-buffered-saline injection remained in the range of normal skin, being 0.65% on the average. DNA synthesis, determined by autoradiography as \(^{3}H\)dT incorporation, also showed two sharp peaks at 24 and 48 hr after the injection. The labeling indices at these peaks were about 30%, which was more than 60 times the average of 0.45% for normal skin or phosphate buffer-treated skin. These two peaks of mitosis and DNA synthesis were observed when >1.0 ng of cholera toxin was injected, whereas injection of the toxin at doses of <1.0 ng resulted in smaller and broad peaks (data not included). As a result of this synchronous division, the thickness of the epidermis increased progressively from 32 hr after toxin injection, being greatest on day 4. The interfollicular epidermis on day 4 after injection of 1.0 ng of toxin was about 130 \(\mu\)m thick and contained 500 nuclei on top of a 1-mm basement membrane, consisting of 10 to 12 cell layers (Fig. 1D). This is 4-6 times that of normal or phosphate buffer-treated control skin, which is 15-20 \(\mu\)m thick, has 130-150 nuclei per mm of basement membrane, and consists of two cell layers.

The epidermal hyperplasia induced by cholera toxin was reversible. After day 4, the thickness and number of nuclei decreased linearly and returned to the normal range on day 7 (Figs. 1E and 2). This rapid recovery seemed to be due to terminal differentiation of the stimulated epidermal cells through the stratum granulosum, the layer of cells containing keratin granules, to the stratum corneum, the layer of dead cornified cells. As seen in Figs. 1D and 2D, the stratum granulosum was most developed on day 4, and the stratum corneum was thickest on day 5. Both layers then gradually decreased in thickness, returning to normal on day 7. This sequence of events indicates that cholera toxin induced two successive synchronous divisions of the epidermal cells and produced temporary hyperplasia without interfering with epidermal differentiation.

Fig. 3 shows the dose–response curves of mitotic stimulation and formation of epidermal hyperplasia by cholera toxin at doses of 0.2–10 ng. Epidermal hyperplasia was induced at as low a dose as 0.2 ng. The minimum dose for induction of hyperplasia was 0.1 ng, but at this dose the reproducibility of the response was poor.

All of the above experiments were carried out on male DDD mice. Cholera toxin also induced epidermal hyperplasia in female DDD mice and in other strains of mice—namely, C57BL, C3H/He, CD-1, and hairless (hr/hr) mice. Cholera toxin also induced epidermal hyperplasia in rats and hamsters, although the extent of induction was less than in mice. Hyperplasia was maximal on day 2 in both rats and hamsters (Fig. 4). The epidermis on day 2 after 10 ng of toxin in rats and hamster was approximately 60-70 \(\mu\)m thick and was composed of six to eight cell layers, which is about a 2-fold increase over that in normal skin, suggesting that cholera toxin induced one division of the epidermal cells in rats and hamsters.

It should be noted that all of the above experiments were performed by intracutaneous injection of the toxin, which is essential for induction of epidermal hyperplasia. Subcutaneous
Table 1. Absence of induction of epidermal hyperplasia after modification of cholera toxin

<table>
<thead>
<tr>
<th>Subunit or pretreatment</th>
<th>Dose</th>
<th>Edema</th>
<th>Hyperplasia</th>
</tr>
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<tbody>
<tr>
<td>A unit*</td>
<td>10</td>
<td>-</td>
<td>- (0, 10.9)*</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>-</td>
<td>- (0.24, 11.8)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-</td>
<td>- (0.74, 12.9)</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>-</td>
<td>- (0.63, 12.6)</td>
</tr>
<tr>
<td>B unit*</td>
<td>10</td>
<td>-</td>
<td>- (0.24, 11.5)</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>-</td>
<td>- (0.48, 11.2)</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>-</td>
<td>- (0.94, 15.3)</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>-</td>
<td>- (0, 12.2)</td>
</tr>
<tr>
<td>Preincubation with anti-cholera toxin antibody</td>
<td>1:10</td>
<td>-</td>
<td>- (1.10, 16.9)</td>
</tr>
<tr>
<td></td>
<td>1:50</td>
<td>-</td>
<td>- (0.47, 12.2)</td>
</tr>
<tr>
<td></td>
<td>1:250</td>
<td>-</td>
<td>- (1.12, 12.7)</td>
</tr>
<tr>
<td></td>
<td>1:1250</td>
<td>-</td>
<td>- (2.27, 11.6)</td>
</tr>
<tr>
<td>Preincubation with GM1 ganglioside</td>
<td>1:100</td>
<td>++</td>
<td>- (0.92, 16.6)</td>
</tr>
<tr>
<td></td>
<td>1:10</td>
<td>++</td>
<td>± (4.84, 21.5)</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>++</td>
<td>+ (2.63, 30.3)</td>
</tr>
<tr>
<td>GM1 ganglioside*</td>
<td>22.6</td>
<td>-</td>
<td>- (0.20, 12.2)</td>
</tr>
<tr>
<td>Cholera toxin*</td>
<td>10</td>
<td>+++</td>
<td>+++ (7.79, 121.2)</td>
</tr>
<tr>
<td>Phosphate-buffered saline</td>
<td>-</td>
<td>-</td>
<td>- (0.89, 19.9)</td>
</tr>
</tbody>
</table>

* Dosage in nanograms.
† The first and second values in parentheses indicate the mitotic index (%) 24 hr after injection and the thickness (μm) of the epidermis on day 4, respectively, based on which the degree of hyperplasia (− to ++++) was evaluated.
‡ Preincubation for 60 min in an ice-bath at various dilutions.
§ Preincubation for 60 min in an ice-bath at various molar ratios.

Injection also induced hyperplasia, but results were more variable than on intracutaneous injection. Painting of the back of mice with toxin, a widely used method for application of materials to the skin, evoked no response with up to 50 μg of the toxin per 3 cm² because the surface is protected by the stratum corneum and does not allow penetration of hydrophilic compounds such as cholera toxin.

Requirement of an Intact Molecule of Cholera Toxin for Induction of Hyperplasia. The mechanism of induction of epidermal hyperplasia was investigated by modifying the cholera toxin molecule, (i.e., by preincubating the toxin with anti-cholera toxin antibody and with GM1 ganglioside, the membrane receptor, and separating the A and B units). Results are summarized in Table 1. Preincubation of the toxin (100 ng/ml) with anti-cholera toxin antibody at dilutions up to 1:1250 for 60 min in an ice-bath was found to block completely formation of blisters and induction of hyperplasia, indicating that the cholera toxin molecule itself evokes the skin reaction. Ganglioside GM1 was identified as the membrane receptor that binds rapidly and tightly to the B unit of cholera toxin at a molar ratio of 1:1 (1). As expected, cholera toxin was inactivated by preincubation with GM1 ganglioside for 60 min in an ice bath. Incubation at a molar ratio of monomer of the B unit to GM1 ganglioside of 1:100 completely blocked induction of epidermal hyperplasia, although it did not affect the edematous change. Partial inhibition of hyperplasia induction was observed after incubation at a ratio of 1:1. GM1 ganglioside alone had no effect on the skin. The A and B units of cholera toxin were injected intracutaneously at doses of up to 320 ng. No skin reaction was produced by either unit of the toxin when injected alone.

Effects of Other Cyclic AMP Inducers. Because cholera toxin is known to activate adenylcyclase and, thereby, increase intracellular cyclic AMP, the above observations strongly suggest that cyclic nucleotides are implicated in the control of proliferation of epidermal cells in vivo. Therefore, the effects on the skin of five other agents known to increase the level of cellular cyclic AMP by different means were investigated. None of them induced hyperplasia (Table 2). Intracutaneous injection of dibutyryl cyclic AMP at doses of up to 1 mg did not induce a skin reaction. Two inhibitors of phosphodiesterase, theophylline (up to 1 mg) and 3-isobutyl-1-methylxanthine (up to 100 μg), were also tested in the same way. Neither of these chemicals produced either edematous change or epidermal hyperplasia. Isoproterenol, a β-antagonist, also did not evoke any skin reaction when injected at doses of up to 1 mg. Although marginal increase of the mitotic index was observed 24 hr after injection of prostaglandin E1 at doses of up to 10 μg, no hyperplasia was detectable on day 4 after its injection.

DISCUSSION

A number of physical and chemical agents are known to induce hyperplasia by changing the equilibrium of the steady state of cell gain and cell loss. These agents are useful in investigating control mechanisms of tissue homeostasis and their possible role in cancer development. Extensive studies have been carried out on induction of hyperplasia of the skin by various chemicals, including 12-O-tetradecanoylphorbol 13-acetate (TPA), a tumor promoter in two-stage carcinogenesis of mouse skin. As far as we know, however, cholera toxin has not been reported to induce epidermal hyperplasia. This seems most surprising because intracutaneous injection of cholera toxin into guinea pigs and rabbits has been used for quantitation of cholera toxin since the report by Craig in 1965 (5)—i.e., the blunting dose (BD), a biological unit of the toxin, is measured as the least dose caus-
ing increased permeability at the site of the skin lesion after intravenous injection of pontamine sky blue. It is interesting that the bluing dose is almost equivalent to the dose causing epidermal hyperplasia (i.e., 0.1 ng).

Almost all synchronous divisions of cells have been achieved using cultured cells in vitro because growth in culture can be controlled easily, and there are only a few reports of synchronous division in vivo. Barka (6) reported that intraperitoneal injection of isoproterenol into rats induced a marked increase in DNA synthesis in the salivary glands, which reached a peak about 28 hr after the injection and was followed by a peak of mitosis. Topical application of 12-O-tetradecanoylphorbol 13-acetate and ionophore A23187 to mouse skin evoked a peak of DNA labeling of the epidermal cells after 18 hr and a peak of mitosis after 26 hr, which resulted in epidermal hyperplasia after 2–4 days (7). In the present study, intracutaneous injection of cholera toxin into mice induced two successive synchronous divisions, which resulted in a 4- to 6-fold increase in epidermal cells. This sequence of events seems very similar to that of synchronous cells in culture.

The mechanism by which cholera toxin induced epidermal hyperplasia is of particular interest. The notion that the edematous reaction itself triggered the mitotic response seems unlikely because these two reactions could be separated: preincubation of the toxin with GM1 ganglioside reduced mitotic stimulation but did not affect the edematous reaction. Another possible mechanism is that mitotic stimulation results from binding per se of the toxin to GM1 ganglioside in the cell membrane. This possibility was suggested by the experiment of Sela et al. (8) in which DNA synthesis of lymphocytes was stimulated by treatment with anti-GM1 antibody. However, this mechanism also seems unlikely because no mitotic stimulation was induced by injection of the purified B unit, which binds to GM1 ganglioside with high affinity. The most probable mechanism is an increase in the cellular level of cyclic nucleotides in epidermal cells, as suggested by the fact that cholera toxin is a potent inducer of adenylcyclyase in intestinal and nonintestinal tissues. Measurement of cyclic AMP and cyclic GMP in the skin lesion is now being carried out with the hope of elucidating whether cyclic nucleotides have a role in epidermal proliferation. The reason why five other agents that increase the cyclic AMP content by different means did not stimulate mitosis of epidermal cells should also be explained by measurements of the levels of intracellular cyclic nucleotides.

The possible implications of cyclic nucleotides in the regulation of cell division is a subject of much controversy. In most studies using fibroblastic cells, an increase in the cellular cyclic AMP content by various means has usually been thought to result in inhibition of cell division (9). Cholera toxin has been used to raise the level of intracellular cyclic AMP. Hollenberg et al. (10, 11) reported that cholera toxin inhibited DNA synthesis of human fibroblasts and transformed mouse epithelial cells; in the latter, the degree of inhibition was parallel to the ganglioside composition of the cells. Similar inhibitory effects have been observed with lymphocytes of mice and humans (12–15). In contrast to these observations, treatment with cholera toxin and other procedures to increase the cyclic AMP level were reported to stimulate DNA synthesis. Pawelek et al. (16) isolated a variant of melanoma cells, the growth of which was markedly stimulated by cholera toxin and other agents. Ruff et al. (17) reported that rat Schwann cells were stimulated to divide by addition of cholera toxin. Human mammary epithelial cells were also reported to be stimulated in the presence of cholera toxin and epidermal growth factor (18). Recently, Davison and Karese (19) reported that growth of human microvascular endothelial cells was stimulated by cholera toxin added together with 3-isobutyl-1-methylxanthine.

In earlier studies on cyclic nucleotides in epidermis, mouse epidermis was incubated for a short time and increase in the cyclic AMP level was found to inhibit cell division (20–22). On the other hand, cholera toxin and other agents have been found to stimulate growth of cultivated epidermal keratinocytes. Green (2) reported that colonial growth of human epidermal cells was enhanced by four agents known to increase the level of cyclic AMP, among which cholera toxin was the most effective. Marcello and Duell (23) found that both the toxin and dibutylc AMP stimulate DNA synthesis of the cells. These results are in accordance with the present investigation in vivo.

The present experimental system provides a unique model for investigating not only the mechanism which controls functional and mitotic homeostasis in normal tissues but also the possible association of various agents (e.g., carcinogens) with a specific cell cycle. Cholera toxin could be used as a tumor promoter in the two-stage carcinogenesis of mouse skin.

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