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INDUCTION OF METAMORPHOSIS BY THYROID HORMONE IN ANURAN SMALL INTESTINE CULTURED ORGANOTYPICALLY IN VITRO

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SUMMARY

We have developed an organ culture system of the anuran small intestine to reproduce in vitro the transition from larval to adult epithelial form which occurs during spontaneous metamorphosis. Tubular fragments isolated from the small intestine of *Xenopus laevis* tadpoles were slit open and placed on membrane filters in culture dishes. In 60% Leibovitz 15 medium supplemented with 10% charcoal-treated serum, the explants were maintained in good condition for at least 10 days without any morphologic changes. Addition of triiodothyronine (T_3) at a concentration higher than 10^{-9} *M* to the medium could induce cell death of larval epithelial cells, but T_3 alone was not sufficient for proliferation and differentiation of adult epithelial cells. When insulin (5 μg/ml) and cortisol (0.5 μg/ml) besides T_3 were added, the adult cells proliferated and differentiated just as during spontaneous metamorphosis. On Day 5 of cultivation, the adult cells rapidly proliferated to form typical islets, whereas the larval ones rapidly degenerated. At the same time, the connective tissue beneath the epithelium suddenly increased in cell density. These changes correspond to those occurring at the onset of metamorphic climax. By Day 10, the adult cells differentiated into a simple columnar epithelium which possessed the brush border and showed the adult-type lectin-binding pattern. Therefore, the larval epithelium of the small intestine responded to the hormones and transformed into the adult one. This organ culture system may be useful for clarifying the mechanism of the epithelial transition from larval to adult type during metamorphosis.

Key words: small intestine; organ culture; metamorphosis; *Xenopus*; thyroid hormone.

INTRODUCTION

The anuran small intestine dramatically changes from larval to adult form during a short period of spontaneous metamorphosis. At the onset of metamorphic climax, larval epithelial cells begin to undergo cell death, while adult ones rapidly proliferate and then differentiate (1,6,9,17). The control mechanism of these epithelial changes is an interesting problem from the standpoint of organogenesis, but has not yet been clarified. This is probably due to the absence of an in vitro system where the metamorphic changes can be reproduced and analyzed at the cellular level. Under culture conditions reported by previous authors, the larval cells degenerated, but the adult cells proliferated insufficiently and never differentiated into a simple columnar epithelium possessing the brush border which is specific to the small intestine (20,21). Therefore, the purpose of the present study is to establish an organ culture system which permits the larval epithelium of the small intestine to transform just as it does during spontaneous metamorphosis. Previously, we studied normal development of the small intestine in *Xenopus* tadpoles both morphologically and histochemically. We found that the epithelial metamorphic changes are closely related to development of the connective tissue (12,13), and that the lectin-binding pattern of larval epithelial cells is different from that of the adult cells, especially for *Ricinus communis* agglutinin I (RCA-I) and soybean agglutinin (SBA) (14). In the present paper, we have compared morphologic and histochemical properties of the small intestine cultured in vitro with those during spontaneous metamorphosis.

MATERIALS AND METHODS

Animals. Tadpoles of the South African clawed frog (*Xenopus laevis*) were purchased from a commercial source in Hamamatsu. One day before the start of cultivation, they were immersed in tap water containing 100 U/ml of penicillin (GIBCO, Grand Island, NY) and 100 μg/ml of streptomycin (GIBCO) and were kept there overnight.

Organ culture. Tubular fragments, about 3 mm in length, were isolated from the anterior part of the small intestine just behind the bile duct junction of NF stage 57 (18) tadpoles and slit open lengthwise with forceps. Culture medium was based on 60% Leibovitz 15 medium diluted by sterile water supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% fetal bovine serum (GIBCO) which had been treated with activated charcoal (Sigma, St. Louis, MO) according to the method of Yoshizato et al. (27) to remove endogenous thyroid hormones (CTS medium). 3,3',5-Triiodo-L-thyronine (T_3), insulin, and hydrocortisone (Sigma) were added to CTS medium at 10^{-8} *M*, 5 μg/ml, and 0.5 μg/ml, respectively (Exp. medium), unless otherwise noted. CTS medium and Exp. medium deprived of T_3 were treated as control media. For the dose-response curve, T_3 concentrations were varied from 10^{-10} to 10^{-7} *M*. The explants were washed several times in the culture medium and cultured by a modified Trowell's technique (25). They were placed on membrane filters (pore size 1.2 μm, type HAWP; Millipore Corp., Bedford, MA) laid on stainless steel grids in 30-mm culture dishes containing 3 ml of the culture medium and were incubated for 0 to 10 days at 26° C. The culture medium was changed every other day, and the explants were transplanted to new membrane filters on Day 5 of cultivation.

Histology and measurement of mitotic index. The explants were treated with 2 μg/ml of Colcemid (Sigma) for 4 h as described by Simnett and Balls (24) and fixed with Bouin's solution. They were then embedded in paraffin, serially sectioned at 4 μm, and stained with hematoxylin and eosin. The mitotic index (%) was calculated as the average of the ratio, metaphase-arrested nuclei/total epithelial nuclei, in more than five sections randomly selected from each explant. The number of connective tissue cells just beneath the epithelium in each explant was counted with the aid of an ocular micrometer in at least three randomly selected unit areas (0.002 mm^2). At least three explants were examined for each experimental time point.

Histochemistry. The binding of two biotinylated lectins, RCA-I and SBA, to the epithelium of the explants was examined as described in our previous paper (14). Briefly, the explants were fixed for 2 h in Carnoy's fixative, embedded in paraffin, and sectioned at 4 μm. The sections were incubated with the lectins (25 μg/ml), rinsed, and then incubated with avidin-biotin-peroxidase complex (ABC kit; Vector, Burlingame, CA). They were reacted with a mixture of 0.05% 3,3'-diaminobenzidine-4HCl (Wako, Oosaka, Japan) and 0.01% H_2O_2. As a control procedure, to confirm the specificity of the histochemical reactions, several sections were preincubated with appropriate hapten sugars and then incubated with biotinylated lectins in the presence of hapten sugars (8). Sugars used were 0.2 *M* lactose (Wako) for RCA-I and 0.2 *M* *N*-acetyl-D-galactosamine (Aldrich, Milwaukee, WI) for SBA.

Transmission electron microscopy. The explants were fixed in 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) at 4° C for 2 h and postfixed with 1% osmium tetroxide in the same buffer for 2 h at 4° C. They were then stained en bloc with uranyl acetate and embedded in epoxy resin. Ultrathin sections were stained with lead citrate and examined with a JEOL 200CX electron microscope.

Results

At the beginning of cultivation, the layer of connective tissue was very thin except in a longitudinal fold, called the typhlosole (Fig. 1 *A*), where a few fibroblasts, slender or irregular in shape, were scattered at random (Fig. 1 *B*). A larval epithelium was simple columnar and consisted mainly of absorptive cells, which possessed the brush border on the luminal surface and small lysosomelike granules in the supranuclear region, and a small number of goblet cells, which contained mucous granules (Fig. 1 *B*). As to the lectin-binding pattern of the epithelium, SBA stained only lysosomelike granules, and RCA-I intensely stained only the brush border of absorptive cells (Fig. 2 *A*).

Maintenance of explants in control media. There was no appreciable change in morphologic or histochemical properties of the larval epithelium throughout the cultivation in both CTS medium and Exp. medium deprived of T_3 (control media). A simple columnar form of the larval epithelium was maintained in good condition. The absorptive cells retained the brush border and the lysosomelike granules, and the goblet cells retained the mucous granules until Day 10 of cultivation (Fig. 1 *C*). The mitotic index of the epithelium was almost constant (Fig. 3), and cell death was not remarkable. The lectin-binding pattern of the epithelium remained the larval type (Fig. 2 *B*). The connective tissue remained undeveloped and did not increase in cell density throughout the cultivation (Fig. 1 *C*).

Chronological changes of explants in Exp. medium. By Day 2 of cultivation the lysosomelike granules of the larval epithelium increased in number and size. The brush border of the absorptive cells and the mucous granules of the goblet cells became less prominent. On Day 3 many lysosomelike granules of various sizes were observed in almost all of the epithelial cells, mainly in their supranuclear region (Fig. 1 *D*). On Day 5 the explants became globular in shape with the epithelium outside and the connective tissue and the muscle inside. The brush border or the mucous granules of the epithelium were no longer observed. Many degenerated larval epithelial cells were sloughed off from the explants. The remainder of the epithelium now consisted of degenerating larval cells possessing the lysosomelike granules and basal cells which were basophilic and proliferating rapidly. The latter cells formed clusters, called islets, among the larval cells (Fig. 1 *E*). The islets continued to increase in size by rapid cell proliferation and protruded into the connective tissue, which now has increased in thickness and is prominent in every region (Fig. 1 *F*). At this time, mitotic connective tissue cells were often found, and the cell density of the connective tissue became high, especially just beneath the epithelium. Thereafter, the mitotic index of the epithelium decreased (Fig. 3), and the islets began to form a simple columnar epithelium, which gradually replaced the larval epithelial cells. On Day 10 the larval cells were no longer observed, and the simple columnar epithelium, originating from the islets, consisted mainly of absorptive cells and a small number of goblet cells (Fig. 1 *G*). The absorptive cells developed the brush border, about 1.0 to 1.2 μm in length, on the luminal surface (Fig. 1 *H*) and showed the adult-type, lectin-binding pattern. Their supranuclear region was preferentially stained by SBA and RCA-I (Fig. 2 *C*) just as in adult absorptive cells during spontaneous metamorphosis (Fig. 2 *D*).

Effects of T_3. The relationship between the dose of T_3 and its effects on the explants cultured for 5 days is summarized in Fig. 4. Addition of 10^{-10} *M* T_3 to CTS medium did not affect the epithelial mitotic index nor the connective tissue cell density. In the medium with 10^{-9} *M* T_3, the mitotic index of the epithelium slightly increased and the connective tissue beneath the epithelium partially increased in cell density. Degenerating larval epithelial cells possessing large lysosomelike granules were often observed. At a concentration higher than 5×10^{-9} *M*, both the epithelial mitotic index and the connective tissue cell density were high. The larval epithelial cells decreased in number, whereas the basal cells proliferated rapidly to form typical islets. These results indicate that 10^{-9} *M* T_3 is a critical concentration for metamorphic changes of the epithelium.

Effects of other hormones. Epithelial differentiation of the explants cultured for 10 days in CTS medium supplemented with 10^{-8} *M* T_3 and various concentrations of insulin or cortisol is summarized in Fig. 5. The epithelium cultured in CTS medium supplemented with T_3 alone could form small islets, but did not differentiate into a simple columnar epithelium possessing the brush border even if cultured longer. The epithelial mitotic index on Day 5 was much lower than that in Exp. medium (Figs. 3 and 4). An insufficient epithelial cell number caused the adult epithelium to become simple squamous or cuboidal. Insulin was more effective than cortisol for differentiation into a simple columnar epithelium. The maximum effect was obtained at 5 μg/ml of insulin. Moreover, addition of 0.5 μg/ml of cortisol besides insulin increased the ratio of cells showing the adult-type, lectin-binding pattern to total epithelial cells.

Discussion

There have been only a few studies in vitro on metamorphic changes of the anuran intestine (20,21). They reported on cell death of the larval epithelium and proliferation of the adult one within a week of cultivation but not on differentiation of the adult one. Under culture conditions described in the present study, the explants could be cultured longer and undergo full differentiation of

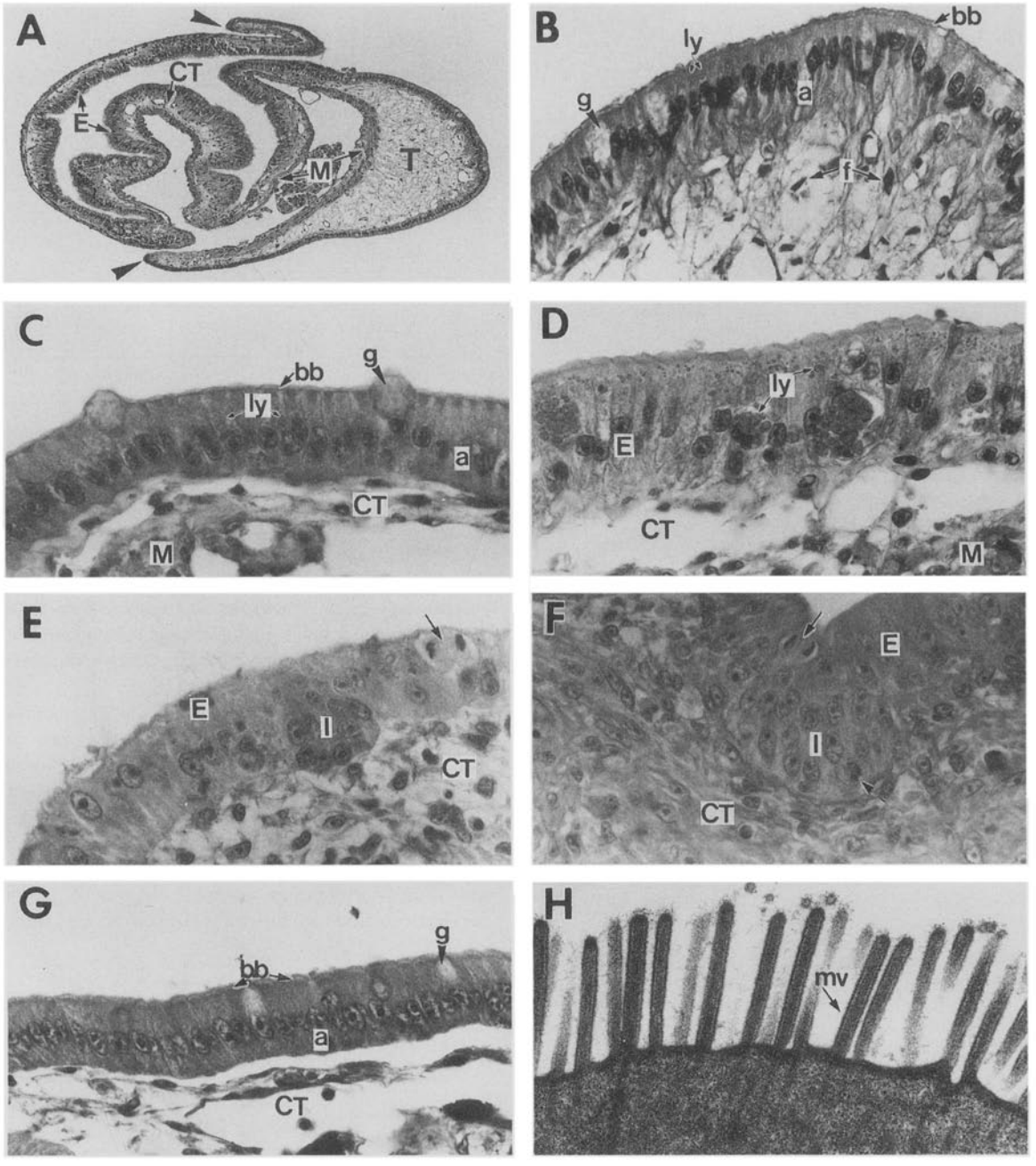

FIG. 1. Photomicrographs (*A–G*) and electron micrograph (*H*) of the explants of the small intestine. *A*, whole explant at the beginning of cultivation. The intestinal wall was opened at *arrowheads*. *CT*, connective tissue; *E*, epithelium; *M*, muscle; *T*, typhlosole, ×90. *B*, higher magnification of the typhlosole in *A*. Larval epithelium consists of absorptive cells (*a*) and goblet cells (*g*). *bb*, brush border; *ly*, lysosomelike granules; *f*, fibroblasts. ×610. *C*, control explant cultured in CTS medium for 10 days. The larval epithelium is maintained in good condition, and the connective tissue remains undeveloped. ×610. *D*, explant cultured in Exp. medium for 3 days. Lysosomelike granules are numerous in the epithelium. ×610. *E*, explant cultured in Exp. medium for 5 days. The connective tissue is thicker and its cell density is higher than on Day 3. Adult epithelial cells rapidly proliferate (*arrow*) and form an islet (*I*). ×610. *F*, explant cultured in Exp. medium for 5 days. A typical islet develops by rapid cell proliferation (*arrows*) and is surrounded by densely packed connective tissue cells. ×500. *G*, explant cultured in Exp. medium for 10 days. A simple columnar epithelium of adult cells develops. ×610. *H*, higher magnification of an absorptive cell in the same explant as in *G*. Brush border consisting of microvilli (*mv*), about 1.0 to 1.2 μm in length, develops. ×25 000.

the adult epithelium. This may be because the degenerated larval epithelial cells, which were sloughed off from the luminal surface of the explants by Day 5 of cultivation and would thereafter cause necrosis of the whole explant, could be easily removed by the procedures used in the present study: Tubular fragments of the intestine were slit open to prevent the degenerated cells from remaining inside the lumen of the explants, and furthermore the explants were transplanted to new membrane filters on Day 5 to prevent the con-

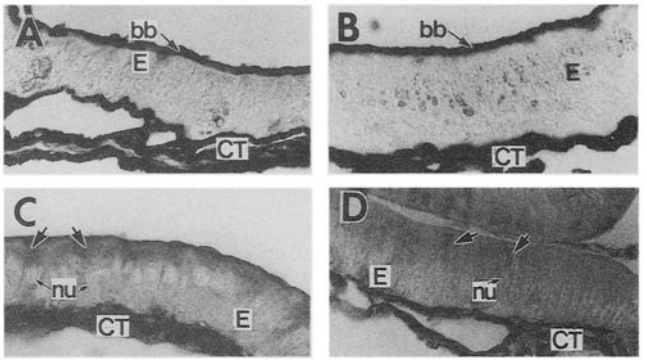

FIG. 2. Binding to RCA-I of the intestinal epithelium in the explants. *A*, larval epithelium at the beginning of cultivation (NF stage 57). Only the brush border (*bb*) is intensely stained. *CT*, connective tissue; *E*, epithelium. ×610. *B*, epithelium of the control explant cultured in CTS medium for 10 days. Binding-pattern remains the larval-type. ×610. *C*, epithelium of the explant cultured in Exp. medium for 10 days. Supranuclear region is preferentially stained (*arrows*). *nu*, nuclei. ×610. *D*, adult epithelium at the completion of spontaneous metamorphosis (NF stage 66). Supranuclear region is preferentially stained (*arrows*) as in *C*. ×610.

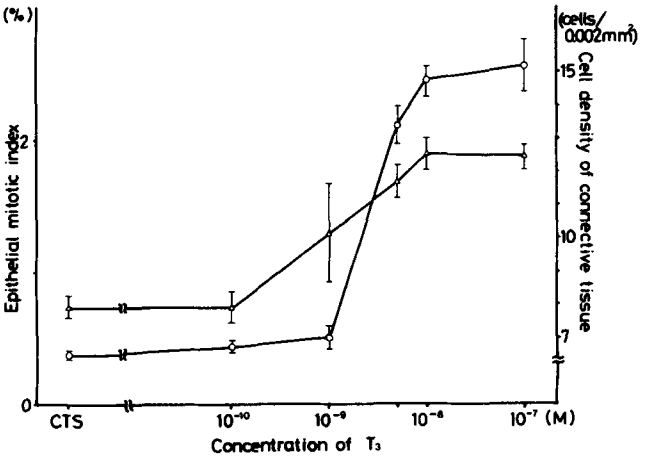

FIG. 4. Effects of T_3 at various concentrations on the mitotic index of epithelial cells (*open circles*) and on the cell density of the connective tissue beneath the epithelium (*open triangles*) in the explants cultured for 5 days. *Each point* represents the mean ± SE of more than three explants.

tamination by debris of the degenerated cells. When these procedures were omitted, almost all explants in Exp. medium were contaminated on and after Day 7, whereas those in control media were free from contamination because larval cell death was not remarkable (our preliminary data). By Day 10 a simple columnar epithelium possessing the brush border, which is characteristic of differentiated absorptive cells, developed. The brush border, about 1.0 to 1.2 μm in length, was shorter than that of the larval epithelium, which is about 1.7 μm (7) or 2.0 μm (our unpublished data), and was almost the same as that of the adult one which is about 1.2 to 1.4 μm (2,7) in *Xenopus laevis*. The epithelium cultured for 10 days also showed the adult-type, lectin-binding pattern. Therefore, the present study demonstrated that the transition from the larval to the adult epithelial form of the anuran small intestine, which is indistinguishable from that occurring during spontaneous metamorphosis, can be induced in vitro.

Thyroid hormones have been considered to induce metamorphic changes in the anuran small intestine (3,9). However, in previous in vitro studies (20,21), serum containing a relatively high and uncontrollable amount of thyroid hormones was inevitably added to the medium. Thus, it is difficult in these experiments to discuss dose-dependent effects of the hormones on the metamorphic changes. In contrast, we have used thyroid hormone-deprived serum (27), and demonstrated that the metamorphic changes of the intestine are clearly dependent on the concentration of exogenous T_3. The

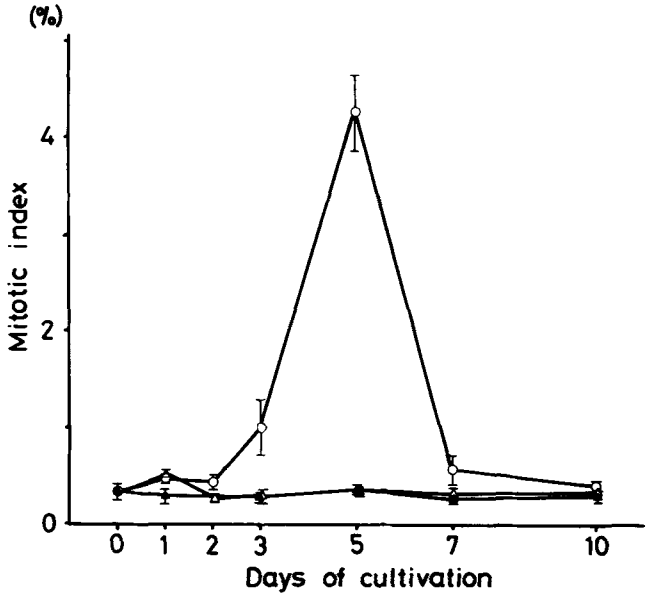

FIG. 3. Chronological changes in the mitotic index of epithelial cells of the explants cultured in Exp. medium (*open circles*), in Exp. medium deprived of T_3 (*open triangles*), and in CTS medium (*solid triangles*). *Each point* represents the mean ± SE of more than three explants.

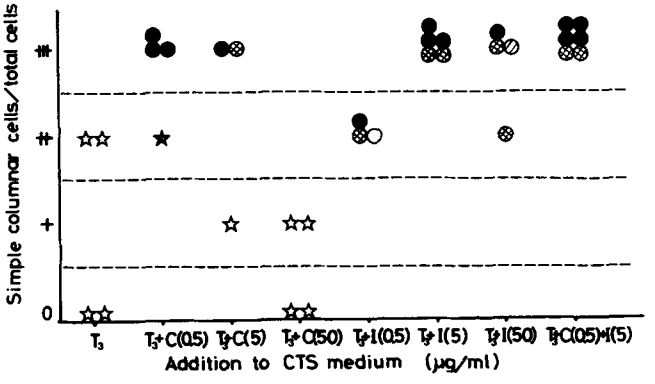

FIG. 5. Differentiation of a simple columnar epithelium of the explants cultured for 10 days in CTS medium supplemented with T_3 (10^{-8} *M*) and various concentrations (0.5 to 50 μg/ml) of cortisol (*C*) and insulin (*I*). One symbol indicates one explant possessing the brush border (○) or none (☆). The ratio of simple columnar cells to total cells was scored as 0 (0%), + (<50%), ++ (≧ 50%; <80%), and +++ (≧ 80%). In each explant, the lectin-binding pattern was examined on several sections. ▨, ▧, and ⊠, only some cells show the adult-type binding pattern for SBA, RCA-I, and both, respectively; ▩, ▧, and ■, more than half of cells show the adult-type binding pattern for SBA, RCA-I, and both, respectively.

changes occurred slightly at 10^{-9} *M* T_3 and intensely at 10^{-8} *M* T_3. These concentrations of T_3 are physiologic levels (16) and are comparable to those that caused a metamorphic change (cell death) in anuran tail epidermis in vitro (19,26). However, for full epithelial differentiation, T_3 alone was insufficient. It has been reported that during metamorphic climax, the anuran adrenal glands rapidly develop (10), and circulating levels of glucocorticoids increase (15). In the small intestine, cortisol generally stimulates the activities of brush border enzymes in the adult epithelium in vivo (4) and in vitro (22). Also in the present study, cortisol enhanced cytodifferentiation of the adult epithelium. On the other hand, circulating insulin levels are maximum just before the onset of metamorphic climax (11) and its effects vary according to the kind of brush border enzymes (4,22). In the present study, insulin increased the number of adult epithelial cells and, as a result, gave the adult cells good condition for differentiation into a simple columnar epithelium. In the media containing T_3 but not insulin, a small number of the adult epithelial cells covered the whole surface of the explant, and thus they tended to become squamous. Therefore, this hormone seems to be more effective than cortisol for differentiation of adult epithelial cells, and may function primarily as a factor that stimulates proliferation of the adult cells at the onset of metamorphic climax.

Development of the intestinal connective tissue during thyroid hormone-induced metamorphosis has so far been ignored both in vivo (9) and in vitro (20,21). We found in the present study that the connective tissue cell density just beneath the epithelium was high whenever adult epithelial cells were rapidly proliferating. This phenomenon corresponds to that observed at the onset of spontaneous metamorphic climax (12). On the contrary, in the control explants, the connective tissue remained undeveloped, and neither rapid proliferation nor differentiation of the adult epithelial cells occurred. These results strongly support our earlier assumption that fibroblasts just beneath the epithelium play some role in rapid proliferation at the onset of metamorphic climax and subsequent differentiation of adult epithelial cells in the anuran small intestine (13,14). Some kind of growth factor(s) produced by the stromal fibroblasts may influence proliferation and differentiation of the epithelial cells, as has been suggested in mammalian epidermis (23) and mammary gland (5). The culture system reported in the present study may be useful for clarifying the mechanism of epithelial proliferation, differentiation, and cell death, which occur in harmony during a short period of spontaneous metamorphosis in the anuran small intestine.

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