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Granule Release of Enterochromaffin (EC) Cells by Cholera Enterotoxin in the Rabbit

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Summary. Crude cholera enterotoxin introduced into the duodenum, either ligated or non-ligated, of the anesthetized rabbit caused marked release of the basal granules in the enterochromaffin (EC) cells. The degranulation was recognized in the tissue fixed as soon as 15 min after administration of the toxin and was still seen in 4 hr specimens. Enlarged granules of conspicuously lowered electron density opened at the basal and lateral cell surface.

A hypothesis was presented that the EC cells are stimulated by cholera enterotoxin at their apical microvilli projecting into the intestinal lumen and they release their products, serotonin and an unidentified polypeptide hormone, which, as "diarrheagenic messengers," cause intestinal hypersecretion characteristic of cholera intoxication.

In our previous reports we demonstrated that the basal-granulated cell or the source cell of gastro-enteric hormones is open to the intestinal lumen with a characteristic tuft of microvilli (KOBAYASHI, FUJITA and SASAGAWA, 1970; SASAGAWA, KOBAYASHI and FUJITA, 1970; FUJITA and KOBAYASHI, 1973). We proposed that intraluminal chemical or physicochemical stimuli, specific for the cell type, may be perceived here and the cell may respond to them by releasing its polypeptide hormone and amine into the connective tissue and blood capillaries. The name receptosecretory cell was thus coined for this cell (FUJITA and KOBAYASHI, 1973).

In man and dog we could demonstrate under the electron microscope that elevated pH in the antropyloic lumen evoked emiocytotic granule release in the G cell, while lowered pH caused it in the D cell (FUJITA and KOBAYASHI, 1971; OSAKA, SASAGAWA and FUJITA, 1973). Our recent study using the electron microscope further confirmed the fluorescence histochemical study by TOBE and his associates (1967) who reported that the serotonin containing granules of the enterochromaffin (EC) cell were markedly decreased after intraluminal administration of a hypertonic glucose solution in the dog duodenum, a treatment which had been known to mimic the human dumping syndrome. We could demonstrate that in several minutes after glucose administration the granules in the EC cell were conspicuously enlarged for release to the basal and lateral cell surface by a clear emiocytotic process (KOBAYASHI and FUJITA, 1973).

In the present study which was undertaken to examine the attitude of the basal-granulated cell in the course of intestinal intoxication with the working hypothesis that a toxin may act upon the apical microvilli of a certain type of basal-granulated cell, we found that the EC cell was sensitive to cholera enterotoxin. As will be demonstrated below, conspicuous degranulation, just exactly comparable with the changes observed in the experimental dumping syndrome above mentioned, occurred

after intraluminal administration of cholera enterotoxin in the rabbit.

Although a parallel study measuring the change in the content of serotonin in the portal blood is still in process of completion, it seems valuable to report the morphological part of the study now, as it is believed to serve to clarify the function of the EC cell which is so widely and numerously distributed in the digestive tract on one hand, and, on the other hand, to elucidate the hitherto unexplained part of the mechanism of diarrhea in cholera (see the reviews by PIERCE, GREENOUGH and CARPENTER, 1971; FINKELSTEIN, 1973) and, more generally, the cell-biological process of intestinal intoxication and diarrhea.

Material and Method

Use was made of young rabbits (600–900 g) of either sex. After fasting for 24–36 hrs the following procedures were carried out under intraperitoneal urethane anesthesia.

1. A duodenal loop was made in 9 rabbits by ligating at a point closely anal to the orifice of the bile duct and at a point 5.5–6.5 cm anal to it. Physiological saline containing 25mg(=5×10⁴ BD)/ml of freeze-dried crude cholera toxin (produced in the Chemo-Sero-Therapeutic Research Institute, Kumamoto) was injected in a dose of 1 ml per kg of animal body weight into the loop. In 1, 2, 3 and 4 hrs thereafter the animals were sacrificed and the mucous membranes in the oral portions of the duodenal loop were taken for fixation.

2. As control I, 2 animals with the same duodenal loop were injected with 2–3 ml physiological saline so that it became swollen to an extent similar to 3 hrs after toxin injection. After 1 hr the mucous membrane was taken for fixation.

3. As control II, 2 rabbits with the same duodenal loop were injected with the above described toxin solution which was previously inactivated by heat (90°C, 3 hrs). The mucous membrane was fixed after 1 hr.

4. To exclude the possible effect of ligation on the intestine, 100 mg/ml/kg of cholera toxin (produced by NIH Cholera Advisory Committee, Bethesda, using B 1307 strain) was injected in 3 rabbits in the uppermost portion of the duodenum which was not ligated. The mucosal tissue was taken for fixation 15 min after.

Specimen preparation and electron microscopy. The tissue pieces were fixed in 2.5% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.4), postfixed in 1.3% OsO₄, dehydrated in ethanol and embedded in Epon 812. Sections made with glass knives were doubly stained by uranium acetate and Millonig's lead, and observed in a Hitachi 125DS electron microscope.

Results

1. Cholera toxin into the duodenal loop

After injection of cholera toxin, the duodenal loop gradually swelled up from the accumulation of mucous content. The swelling was distinct in 1 hr. In 3–4 hrs the extended intestinal wall formed a large transparent bladder. The content then reached 7–10 ml in the original 6 cm long loop. The pH values of the content were 8.2–8.8.

Electron microscope observation of the duodenal mucosa taken 1–4 hrs after toxin

Fig. 1. Apical portion of an EC cell with a tuft of microvilli projecting into the lumen of the duodenal crypt, 2 hrs after administration of cholera toxin into the ligated duodenum. *c* Centriole, *j* junctional complex. $\times 15,000$

Fig. 2. An EC cell 3 hrs after cholera toxin injection into the ligated duodenum. Note the cell apex covered with microvilli directly facing the lumen. The basal granules (*g*) are decreased in number and some large, altered granules (*s*) are shown. The arrow indicates an altered granule widely opening to the intercellular space. *L* a probable lymphocyte. $\times 6,500$

administration showed no cellular changes such as degeneration and exfoliation in the villous and cryptal epithelium, nor such subcellular changes as damaged or hyperfunctioning organelles in the epithelial cells including the goblet cells.

At least five types of basal-granulated cells were found dispersed mainly in the cryptal epithelium of the rabbit. They are: 1) enterochromaffine (EC) cell having basal granules of high electron density and characteristically distorted shapes, 2) L cell with large round granules, 3) S cell with small round granules, 4) M cell with round dark granules of a medium size and long, uniform microvilli densely covering the cell apex, and 5) a cell type which corresponds to the "S cell" of CAPELLA et al. (1969) showing small granules with a small dense core. The EC cell formed the

predominant cell type among these cells. It was most probable that every basal-granulated cell reaches the lumen with its apical process covered by microvilli, though only some of the cells were hit in an optimal direction (Fig. 1, 2).

In all the ligated loops fixed 1–4 hrs after the injection of cholera toxin, conspicuous changes in the EC cell granules were recognized. Many of the granules were enlarged into lake forms of more or less markedly lowered electron density and loosened texture. The dark and homogeneous granules of the moderate size and distorted shapes characteristic of this cell type remained only here and there (Fig. 2, 3)

Some of the enlarged and altered granules were seen to open to the basal or lateral cell surface. The limiting membrane of the granule was connected with the plasma

Fig. 5. A part of an EC cell, 1 hr after toxin injection into the ligated duodenum. Besides typical granules (*g*), an altered granule (*s*) opening to the intercellular space (large arrow) is seen. This granule is associated by a coated vesicle (small arrow). In the Golgi apparatus (*G*) some granules are being formed. $\times 22,000$

Fig. 3. Basal part of an EC cell cross sectioned, 3 hrs after toxin injection into the ligated duodenum. Altered granules (*s*) are gathered to the cell periphery and some of them are seen open to the intercellular space (arrows). $\times 10,000$ The **inset** shows a part of Figure 3 in a closer view. *g* Ordinary granules of the EC cell. $\times 24,000$

Fig. 4. Basal part of an EC cell, 1 hr after toxin injection into the ligated duodenum. Altered granules (*s*) are open to the intercellular space (large arrows). A coated vesicle is shown attached to the altered granule (small arrow). *g* Ordinary granules, *B* basement membrane. $\times 18,000$

membrane of the cell and the granule content was open to the extracellular space, often filling and even widening the space. The opening of the granule was sometimes wide but mostly constricted into a narrow orifice (Fig. 3-5).

In the vicinity of or attached to the altered and opening granules coated vesicles were often found (Fig. 4, 5). Large, empty-looking vacuoles also occurred in some cells but their relation to the altered granules of lowered density was unknown.

Some of the EC cells after cholera toxin administration showed figures indicating an enhanced formation of new granules. In and around the Golgi complex of these cells, small round granules were seen which showed gradations to the ordinary, distorted forms (Fig. 5).

The above described changes of the EC cells seemed to differ inconspicuously between the specimens fixed at 1, 2 and 3 hrs after cholera toxin administration. In the 4 hr specimens some of the EC cells were especially rich in vacuoles and occasionally possessed a pyknotic nucleus. On the other hand, the intensity of granule changes seemed much dependent upon the age of the animals. In the younger ones the more extensive granule alteration and release were recognized.

In the L and S cells no changes in the granules and organelles were found after cholera toxin administration. No figure showing granule release was encountered in these cells.

Fig. 6. An EC cell in the ligated duodenum 1 hr after exposure to the inactivated cholera toxin (control II). Dense granules (*g*) fill the infranuclear cytoplasm. *B* basement membrane. $\times 12,000$

Fig. 7. Basal part of an EC cell in the non-ligated duodenum 15 min after toxin administration. Dense granules (*g*) are few and the infranuclear cytoplasm is mainly occupied by altered granules (*s*) of lower electron density. Vacuoles (*v*) are also mingled. A granule opening to the basal cell space is shown by a large arrow. The small arrow indicates coated vesicles near this granule. *B* basement membrane. $\times 13,000$

2. Control I: physiological saline into the duodenal loop

By injection of 2–3 ml physiological saline into the duodenal loop its wall was distended as was seen in the specimens 3 hrs after cholera toxin injection into the loop. Electron microscopy of the mucous membrane taken 1 hr thereafter revealed few figures of granule alteration and release in the EC cells as well as other cell types.

3. Control II: inactivated toxin into the duodenal loop

After injection of inactivated toxin in a dose of 25 mg/ml per kg body weight, no marked swelling of the loop occurred. The tissues fixed 1 hr after the injection showed few signs of EC cell degranulation. The great majority of the EC cell granules remained dense and unaffected. A few of the granules were loose in texture and slightly enlarged but no figure indicating their release was found.

4. Cholera toxin into the non-ligated duodenum

To avoid the possible effect of ligation and the following distention of the intestinal wall, cholera toxin was injected into the uppermost portion of the duodenum and left to be removed by peristalsis.

As early as 15 min after the toxin administration, when no swelling in the intestine could be recognized as yet, conspicuous alteration and release of granules were found in the EC cells. Although still numerous granules remained unaltered in this early stage of intoxication, the changes in the cells essentially corresponded to those evoked by cholera toxin in the ligated duodenum and they will not be repeated now (Fig. 6, 7).

Discussion

EC cell degranulation

The present study demonstrates that the EC cell in the rabbit undergoes emiocytotic granule release after the mucous surface is exposed to cholera enterotoxin. In precise accordance with the changes of the same cell in dogs intraluminally stimulated with hypertonic glucose (KOBAYASHI and FUJITA, 1973) the degranulation is characterized by the occurrence of enlarged and altered granules and by their emiocytotic opening to the lateral, as well as basal, cell surface. It seems likely that the large, empty-looking vacuoles which were especially numerous in the 4 hr specimens may represent an altered type of granule.

CAPELLA, SOLCIA and VASSALLO (1969) found that in the rabbit some of the granules in the EC cell showed decreased density in their core after intravenous administration of reserpine, and thought that this figure may correspond to the release of serotonin. The present finding evidences that clear emiocytotic release of granules does take place in the EC cell of the rabbit as it did in the dog.

Recently the possibility has been discussed that coated vesicles often found attached to the limiting membrane of a releasing granule in the neurosecretory ending may serve to carry back the used membrane to the cell center (DOUGLAS, NAGASAWA and SCHULZ, 1971). The figures in the present study showing coated vesicles approaching or becoming attached to the membrane of an opening granule are of interest in connection with this possibility.

In previous papers (FUJITA and KOBAYASHI, 1973) we pointed out that the EC cell shares the morphological and cytochemical characteristics of a polypeptide/protein hormone producing cell with other types of basal-granulated cells and with pancreatic islet cells, although only its aminic product, serotonin, has attracted general attention (FORSSMANN and GRUBE, 1973). In this paper it is stressed again that an unidentified polypeptide hormone, together with serotonin, must be released from the EC cell when it undergoes degranulation. This hormone might exert a significant action because its total amount is supposed to be larger than other known gut hormones, if one takes into account the dense and extensive distribution of its source cell.

Mechanism of diarrhea in cholera

It is only a decade since the diarrhea in cholera came to be ascribed not to the destruction of the mucous epithelium as Virchow insisted but to the hypersecretion of mucus and water from morphologically intact intestinal wall (GANGAROSA et al., 1960; GORDON, 1960). Recently research in cholera attracted wide attention as it was demonstrated that cholera enterotoxin alters the intestinal ion transport by increasing the level of cyclic AMP in the gut mucosal cell (FIELD et al., 1969; KIMBERG et al.,

1970; SCHAFER et al., 1970). It is now widely believed that the cholera enterotoxin acts directly upon the luminal surface of some epithelial cells (PIERCE, GREENOUGH and CARPENTER, 1971; FINKELSTEIN, 1973).

It has been reported meanwhile that if one makes two ligated segments in the rabbit intestine and injects cholera toxin into one of them, the other segment swells up to some extent, though less markedly than the injected segment (SEREBRO et al., 1968). Further, if one connects the circulations of two infant rabbits and injects *Vibrio cholerae* in the intestine of one, the non-infected partner also shows increased water in the intestine (VAUGHAN-WILLIAMS and DOHADWALLA, 1969). To explain these phenomena, one may either acknowledge absorption of cholera toxin into the circulation as VAUGHAN-WILLIAMS and DOHADWALLA (1969) did, or hypothesize a "circulating diarrheagenic messenger" (PIERCE, GREENOUGH and CARPENTER, 1971) from a certain cell facing the lumen by the action of the toxin. The first possibility may be denied as it has been evidenced that cholera toxin introduced in the intestine is not or only poorly absorbed into circulation (CURLIN et al., 1970; PIERCE, GREENOUGH and CARPENTER, 1971; FINKELSTEIN, 1973).

In the present study which demonstrated marked degranulation of EC cells specifically caused by intraluminal administration of cholera toxin in the rabbit, it is proposed that the occurrence of diarrhea in cholera is mediated by the EC cells and their products: serotonin and a polypeptide (vide supra). Cholera toxin may act on the apical microvilli of the EC cells to evoke an intensive release of these substances by emiocytosis of the basal granules.

Serotonin is known as a strong secretagogue of gastric mucus in the dog (MENGUY, 1969, THOMPSON, 1971). Although its actions on different glands remain obscure, it seems possible that serotonin alone might be able to increase the secretion of the duodenal gland and some other intestinal secretory elements. The well known activity of serotonin increasing the blood capillary permeability may enhance its secretagogous effect.

Further, it should be remembered that serotonin has been suspected to influence the formation of cyclic AMP (KLAINER et al., 1962). It is presumed that serotonin stimulates mammalian intestinal secretion by elevating the cyclic AMP levels in the target cells, although this action has been evidenced only for blowfly salivary gland (BERRIDGE, 1970) as far as the authors know. The activated cyclic AMP system in the mucosal cell causing intestinal hypersecretion in cholera may, however, possibly be attributed also to the unidentified polypeptide hormone of the EC cell. This postulation may be natural as glucagon, secretin and some other polypeptide hormones are known to stimulate certain glands by activating the cyclic AMP system in the secretory cells (ROBISON, BUTCHER and SUTHERLAND, 1971).

As in man and many animals, the EC cell in the rabbit is most numerous in the upper part of the duodenum. The toxin may cause release of the largest amount of EC cell products or "diarrheagenic messengers" in the upper duodenal portion. As the messengers are believed to act locally in high concentrations before circulating in the body, the diarrheal hypersecretion should take place most actively in the same portion of the intestine. It is thus suggested that the duodenal crypts and duodenal glands may be the main targets of the "diarrheagenic messengers." In accordance

with this, it has been evidenced, in human natural cholera (BANWELL et al., 1970) and in canine (CARPENTER et al., 1968) models, that the greatest rate of fluid secretion occurs in the duodenum and the least from the ileum, whereas the colon produces essentially no fluid.

As stated above, the dumping syndrome, at least in its experimental model using hypertonic glucose in the dog, is now believed to be mediated by the EC cell releasing its products. It may be superfluous to add that explosive diarrhea often occurs in the dumping syndrome, too. A possibility is suggested that the EC cell, with its receptosecretory ability, may also be involved in the occurrence of diarrhea from causes other than cholera.

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コレラトキシンによるウサギの腸クロム親和細胞 (EC 細胞) の果粒放出

藤田 恒夫, 大坂 道敏, 梁取 淑子

麻酔したウサギの十二指腸を結紮し, あるいは結紮せずに, 粗性 コレラ エンテロトキシンを注入すると, 腸クロム親和 (EC) 細胞に基底果粒の顕著な放出像がみられる. この脱果粒は トキシン注入後15分の例に すでにみとめられ, 4時間例でも なお見られる. EC細胞の果粒は膨大し, いちじるしく電子密度が低下し, 細胞の底面と側面に開口する.

EC細胞は 腸内腔に突出する微絨毛によって コレラトキシンの刺激を受容し, セロトニンおよび未確認のポリペプチドホルモンを放出するものと想定される. そして これらの物質が “下痢発症媒介物質” として コレラ中毒に特有の腸液過剰分泌をひきおこすものと考えられる.

References

- Banwell, J. G., N. F. Pierce, R. C. Mitra, K. L. Brigham, G. J. Caranasos, R. I. Keimowitz, D. S. Fedson, J. Thomas, S. L. Gorbach, R. B. Sack and A. Mondal: Intestinal fluid and electrolyte transport in human cholera. *J. clin. Invest.* 49: 183-195 (1970).
- Berridge, M. J.: The role of 5-hydroxytryptamine and cyclic AMP in the control of fluid secretion by isolated salivary glands. *J. exp. Biol.* 53: 171-186 (1970).
- Capella, C., E. Solcia and G. Vassallo: Identification of six types of endocrine cells in the gastrointestinal mucosa of the rabbit. *Arch. histol. jap.* 30: 479-495 (1969).
- Carpenter, C. C. J., R. B. Sack, J. C. Feeley and R. W. Steenberg: Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. *J. clin. Invest.* 47: 1210-1220 (1968).

- Curlin, G. T., J. P. Craig, A. Subong and C. C. J. Carpenter:** Antitoxin immunity in experimental canine cholera. *J. infect. Dis.* 121: 463-470 (1970).
- Douglas, W., W. J. Nagasawa and R. A. Schulz:** Coated microvesicles in neurosecretory terminals of posterior pituitary glands shed their coats to become smooth "synaptic" vesicles. *Nature* 232: 340-341 (1971).
- Field, M., D. Fromm, C. K. Wallace and W. B. Greenough:** Stimulation of active chloride secretion in small intestine by cholera enterotoxin. *J. clin. Invest.* 48: 24a (1969).
- Finkelstein, R. A.:** Cholera. *CRC crit. Rev. Microbiol.* 2: 553-623 (1973).
- Forssmann, W. G. und D. Grube:** Studien über die endokrinen Zellen des Magendarmtraktes. I und II. *Z. Zellforsch.* 140: 535-550, 551-565 (1973).
- Fujita, T. and S. Kobayashi:** Experimentally induced granule release in the endocrine cells of dog pyloric antrum. *Z. Zellforsch.* 116: 52-60 (1971).
- : The cells and hormones of the GEP endocrine system. The current of studies. In: (ed. by) T. Fujita: Gastro-entero-pancreatic endocrine system—A cell-biological approach. Tokyo, Igaku Shoin, 1973 (p. 1-16).
- Gangarosa, E. J., W. R. Beisel, C. Benyajati, H. Sprinz and P. Piyaratn:** The nature of the gastrointestinal lesion in Asiatic cholera and its relation to pathogenesis: a biopsy study. *Amer. J. trop. Med. Hyg.* 9: 125-135 (1960).
- Gordon, R. S., Jr.:** The failure of Asiatic cholera to give rise to "Exudative enteropathy." SEATO Conf. Cholera, Bangkok: 54-57 (1960).
- Kimberg, D. V., M. Field, J. Johnson, A. Henderson and I. Gershaw:** Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. *J. clin. Invest.* 50: 1218-1230 (1971).
- Klainer, L. M., Y. M. Chi, S. L. Freidberg, T. W. Rall and E. W. Sutherland:** Adenylcyclase. IV. The effects of neurohormones on the formation of adenosine 3' 5'-phosphate by preparations from brain and other tissues. *J. biol. Chem.* 237: 1239-1243 (1962).
- Kobayashi, S. and T. Fujita:** Emiocytotic granule release in the basal-granulated cells of the dog induced by intraluminal application of adequate stimuli. In: (ed. by) T. Fujita: Gastro-entero-pancreatic endocrine system—A cell-biological approach. Tokyo, Igaku Shoin, 1973 (p. 49-58).
- Kobayashi, S., T. Fujita and T. Sasagawa:** The endocrine cells of human duodenal mucosa. An electron microscope study. *Arch. histol. jap.* 31: 477-494 (1970).
- Menguy, R.:** Gastric mucus and the gastric mucous barrier. *Amer. J. Surg.* 117: 806-812 (1969).
- Osaka, M., T. Sasagawa and T. Fujita:** Emiocytotic granule release in the human antral endocrine cells. In: (ed. by) T. Fujita: Gastro-entero-pancreatic endocrine system—A cell biological approach. Tokyo, Igaku Shoin, 1973 (p. 59-63).
- Pierce, N. F., W. B. Greenough and C. C. J. Carpenter, Jr.:** *Vibrio cholerae* enterotoxin and its mode of action. *Bacteriol. Rev.* 35: 1-13 (1971).
- Robison, G. A., R. W. Butcher and E. W. Sutherland:** "Cyclic AMP." New York-London, Academic Press, 1971.
- Sasagawa, T., S. Kobayashi and T. Fujita:** The endocrine cells in the human pyloric antrum. An electron microscope study of biopsy material. *Arch. histol. jap.* 32: 275-288 (1970).
- Schafer, D. E., W. D. Lust, B. Sircar and N. O. Goldberg:** Elevation of adenosine 3', 5' cyclic monophosphate concentration in intestinal mucosa after treatment with cholera toxin. *Proc. Nat. Acad. Sci. U.S.A.* 67: 851-855 (1970).
- Serebro, H. A., T. McGonagle, F. L. Iber, R. Royall and T. R. Hendrix:** An effect of cholera toxin on small intestine without direct mucosal contact. *Johns Hopkins Med. J.* 123: 229-232 (1968).
- Thompson, J. H.:** Serotonin and the alimentary tract. *Res. Commun. chem. Pathol. Pharmacol.* 2: 687-781 (1971).
- Tobe, T., C. Kimura and M. Fujiwara:** Role of 5-hydroxytryptamine in the dumping syndrome after gastrectomy. *Histochemical Study. Ann. Surg.* 165: 382-387 (1967).

- Vaughan-Williams, E. M. and A. N. Dohadwalla:** The appearance of a cholera-genic agent in the blood of infant rabbits infected intestinally with *Vibrio cholerae*, demonstrated by crosscirculation. J. infect. Dis. 120: 658-663 (1969).
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