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Selective killing of Paneth cells by intravenous administration of dithizone in rats

Mitsutaka Sawada, Kiyoyuki Takahashi*,
Shinji Sawada and Osamu Midorikawa
*Department of Pathology, Faculty of Medicine, Kyoto University, and *College of Medical Technology, Kyoto University, Japan*

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Summary. Paneth cells are zinc-containing cells widely distributed in Lieberkühn's crypts of intestine in a variety of species. We found that rapid selective killing of Paneth cells took place after the intravenous (i.v.) injection of diphenylthiocarbazone (dithizone), a chelator forming a zinc dithizonate complex, in the rat. As soon as 5 min after the i.v. injection of dithizone, degeneration of Paneth cells occurred. At this stage, zinc dithizonate complexes were observed as purple-red granules in bright field microscopy. Thirty to 60 min later, Paneth cells were detached from the basement membrane and shed into the cryptic lumen. After 6 h, the cell debris in the crypts was no longer seen and the crypts once housing Paneth cells were now occupied by neighbouring crypt base columnar cells. Histochemically demonstrable zinc totally disappeared. After 12–24 h, however, definite Paneth cells began to resume. Histochemical staining for zinc was again positive at the apex of these cells. One week after dithizone administration, the number of Paneth cells increased twice as much as in uninjected control and histochemical staining for zinc was highly positive. After 2 weeks, Paneth cell hyperplasia subsided. X-ray microanalysis revealed that zinc was the most abundant metal in Paneth cells. We concluded that chelation of zinc and formation of zinc–dithizone complexes in Paneth cells' cytoplasm would be responsible for the selective degeneration observed after dithizone administration.

Keywords: Paneth cells, zinc, diphenylthiocarbazone, rat, electronmicroscopy, X-ray microanalysis

Since the earliest description of Paneth cells in the bottom of Lieberkühn's crypts by Schwalbe (1872) and Paneth (1888), their function has been a subject of considerable controversy but no definitive role has been ascribed to them (Sandow & Whitehead 1979). Speece (1964) speculated that Paneth cells have a bacteriostatic function, since their secretory granules contain lysozyme, an antibacterial enzyme. Erlandsen *et al.* (1976) also showed Paneth cells have immunoglobulin A. Others have suggested that Paneth cells play an important role in eliminating heavy metals (Millar *et al.* 1961;

Correspondence: Dr Mitsutaka Sawada, Department of Pathology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

Elmes 1976; Mottet & Body 1976; Dinsdale 1984; Dinsdale & Biles 1986). For example, zinc-containing cytoplasmic granules disappear on a low zinc diet, but zinc becomes concentrated in Paneth cells by intraperitoneal (i.p.) administration of zinc salt in rats (Millar *et al.* 1961; Elmes 1976).

Histochemical demonstration of zinc in Paneth cells was first reported by Stampfl (1959) after the i.v. injection of dithizone, a metal chelator which has a high affinity for zinc, into rats. The finding indicated that dithizone penetrates Paneth cells and entraps the metal *in vivo*.

We investigated the effect of zinc dithizonate complex formation within Paneth cells and their morphological changes after a single injection of dithizone in Wistar rats and discuss possible functions of Paneth cells.

Materials and methods

Animals

Male SPF Slc: Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing 150–250 g (5–7 weeks of age) were used. They were kept in stainless steel cages and fed commercial rat chow (Funabashi F-2, Chiba). Deionized water was given *ad libitum*.

Dithizone administration

A 10 mg/ml dithizone solution was prepared by dissolving Dithizon (Merck, Darmstadt) in a saturated alkaline solution of Li_2CO_3 (pH 11.8) (WAKO, Osaka) under magnetic stirring over a warm water bath. The solution was filtered and used immediately.

Under pentobarbital anaesthesia, each rat was injected with 100 mg of dithizone per kg of body weight through the tail vein over a period of 3 min. Immediately after injection of dithizone, skin colour of ears and feet was changed to brown, indicating rapid and systemic distribution of the chemical. Rats were killed by severing the subclavian artery 5 min, 30 min, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h, 1 week, 2 weeks and 3 weeks (three animals in each group) after the dithizone injection. Ten rats injected with physiological saline served as controls.

Histochemical procedures

Immediately after sacrifice, the entire small intestine was removed. Two 2-cm-long samples were used; one was from the duodenum, 2 cm distal from the pylorus, and the other was from the ileum, 2 cm proximal to the ileocaecal junction. Each sample was divided into two equal parts. One part was fixed with 70% ethanol saturated with hydrogen sulphide and another part was fixed with 10% neutralized formalin. After an hour, the samples were cut into 1-mm-long segments and further fixed for 24 h. All the fixed samples were then dehydrated, embedded in paraffin, and sectioned to 3.5 μm in thickness. The formalin-fixed sections were stained with haematoxylin and eosin and used for counting the number of Paneth cells. One hundred crypts well oriented with the plane of section passing vertically down the crypts were viewed in each tissue. Counting was done for the cells with visible nucleus and more than half of the cytoplasm, allowing identification of the cell type (Elmes 1976). The sections fixed by ethanol saturated with hydrogen sulphide were used for the detection of heavy metals by Timm's method (Timm 1958a, b) and by hydrogen sulphide–Grimelius' silver reaction (H_2S–Grimelius' method). The method was according to Takahashi *et al.* (1989); tissues were fixed in 70% ethanol saturated with hydrogen sulphide for 24 h to yield insoluble sulphated metals. After fixation, paraffin-embedded sections were made and stained by Grimelius' silver reaction. This H_2S–Grimelius method is convenient and reproducible and gives more stable and accurate staining of heavy metals than the original Timm's method.

Intracellular zinc was examined 5 min, 2 h, 6 h, 12 h and 24 h after the injection of dithizone. Two 1-cm-long samples were har-

vested from the remaining stumps of the duodenum and ileum. They were frozen in liquid nitrogen for 15 s and embedded in optimum cutting temperature compound (Miles, Elkhart, IN.) and sectioned at −20°C. Eight-μm thick sections were layered on glass slides and fixed with cold 10% formalin for 5 min. They were then stained with Mayer's haematoxylin, mounted in Apathy's gum syrup and immediately observed under a light microscope with a bright and dark field device (CH2-PCD, Olympus, Tokyo).

Electronmicroscopy and X-ray microanalysis

For the ultrastructural observation and the X-ray microanalysis of metals, two normal rats and two rats in each groups (5 min, 30 min, 1 h, 2 h, 6 h, 9 h, 12 h, 24 h, 48 h, 72 h, 1 week, 2 weeks, 3 weeks) were killed under pentobarbital anaesthesia. Two 1-cm-long samples were taken 2 cm proximal to the ileocaecal junction. They were divided into two equal parts and cut into 1 mm lengths. One part was immediately immersed in 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4, 345 mOsm/kg) for 1.5 h at 4°C and the other in 2% glutaraldehyde in 0.05 M phosphate buffer saturated with hydrogen sulphide (2% H_2S–glutaraldehyde) for 30 min at 4°C. Subsequently, the latter samples were transferred to 2% glutaraldehyde in the same buffer for 1.5 h at 4°C. The former samples were then further post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4, 251 mOsm/kg) for 1 h at 4°C. We used both for the electron microscopic observation and the latter for X-ray microanalysis. These samples were dehydrated in a series of ethanol and embedded in Epon 812 (Oken, Tokyo).

Ultrathin sections (80 nm) for electron microscopic observation were selected after toluidine blue staining, further stained with uranium acetate and lead citrate, and examined under a Hitachi H-700 electron microscope (Hitachi, Tokyo). Sections (200 nm) for X-ray microanalysis were also stained with toluidine blue for selection, and were examined with a Kevex energy-dispersive X-ray detector (Kevex, Foster City, CA).

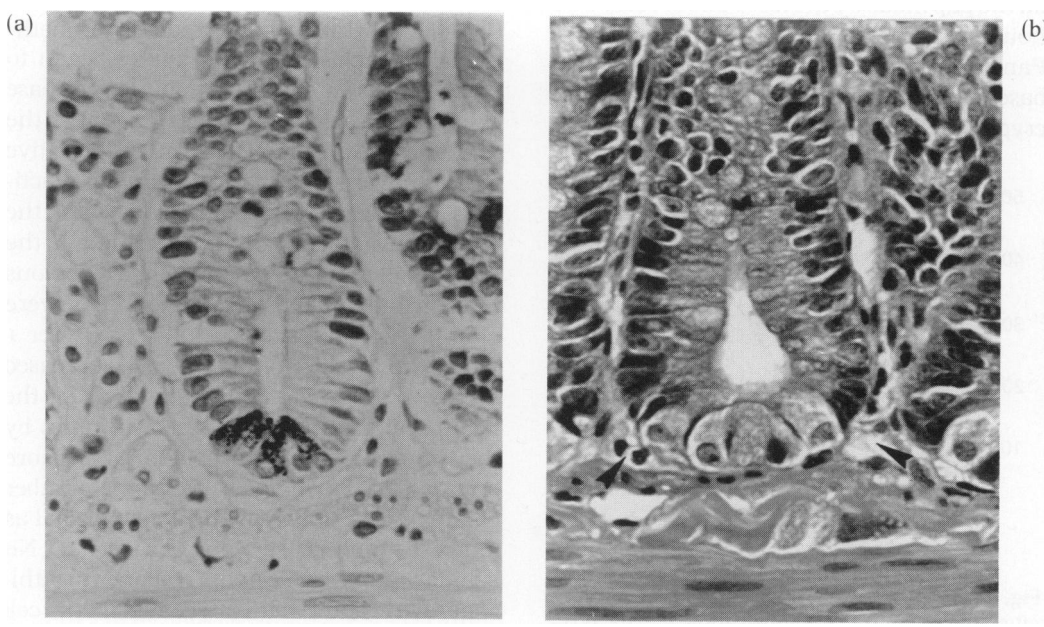


Fig. 1. Paneth cells at the base of Lieberkühn's crypt in the ileum of a control rat. a, H_2S–Grimelius' method. ×400. b, H&E (between arrowheads). ×400.

Results

Rapid selective killing of Paneth cells after dithizone injection

Paneth cells were pyramidal cells at the bases of Lieberkühn's crypts of the small intestine. They had many apically located characteristic large eosinophilic granules which were stained blackish brown by Timm's method and H_2S–Grimelius' method (Fig. 1a). An irregularly shaped nucleus with a prominent nucleolus was located at the cell base (Fig. 1b). The number of Paneth cells in each crypt ranged from 0 to 6, and was greater in the ileum than in the duodenum (Fig. 2).

Five minutes after the dithizone injection, the nuclei of Paneth cells became pyknotic and the granules of some Paneth cells became obscure (Fig. 3). But goblet cells and other columnar cells appeared unaffected by this procedure. Thirty minutes to an hour following the dithizone injection, degeneration and necrosis of Paneth cells became obvious. They were finally shed into Lieberkühn's crypts (Fig. 4a, b). At the same time adjacent crypt base columnar cells began to fill the gap made by the lost Paneth cells. Two hours after the dithizone injection, all the Paneth cells died and detached from the basement membrane into Lieberkühn's crypts. Six hours later, almost all the necrotic masses in the crypts were cleared and only columnar cells without detectable heavy metals were found at the bases of the crypts (Fig. 5a, b). Using the sulphide–silver method, faint blackish brown granules began to reappear in the apical portion of crypt base columnar cells in some crypts 12 h after the injection of dithizone. The number of positive granules was small, and the staining reactivity was weak in comparison with the control group (Fig. 6a, b). After 24 h the eosinophilic granules became more obvious than before. Apparently Paneth cells were almost recovered after 72 h (Fig. 7). After 1 week, the number of Paneth cells increased from 0–6 to 5–8 cells in each crypt of the ileum and the staining of their granules by the sulphide–silver method became more intense (Fig. 8a, b). By 2 weeks, the number of Paneth cells returned to almost normal as much as uninjected control (Fig. 2). No significant difference in the response to dithizone was observed between Paneth cell populations residing in the duodenum and ileum.

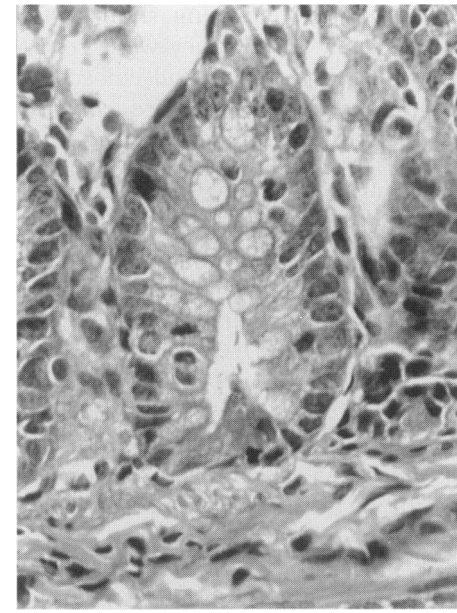


Fig. 3. Paneth cells 5 min after dithizone injection. H&E. ×400.

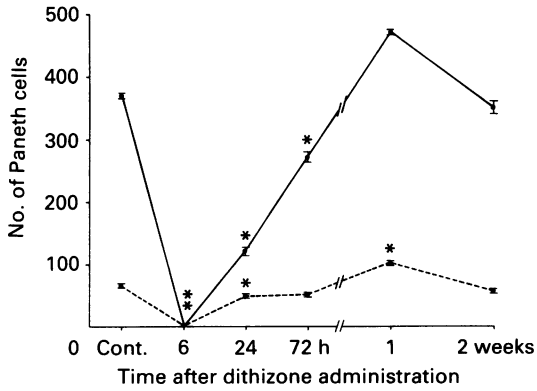


Fig. 2. The number of Paneth cells per 100 crypts after dithizone administration. *$P<0.05$, **$P<0.001$, Wilcoxon's test. —, Ileum; - - -, duodenum.

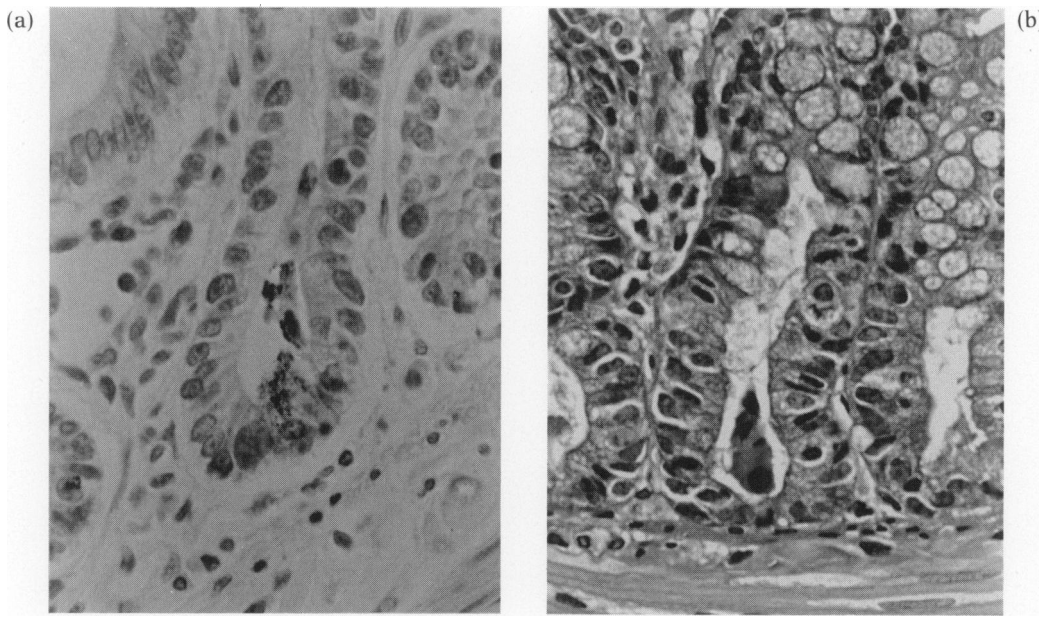


Fig. 4. Paneth cells 30 min after dithizone injection. a, H_2S–Grimelius' method. ×400. b, H&E. ×400.

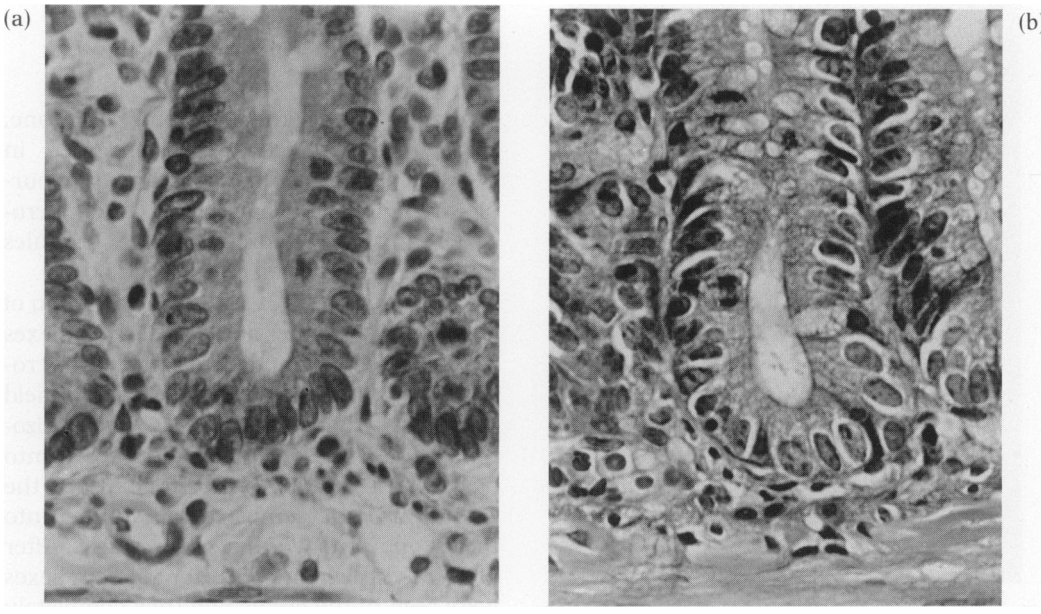


Fig. 5. Paneth cells 6 h after dithizone injection. a, H_2S–Grimelius' method. ×400. b, H&E. ×400.

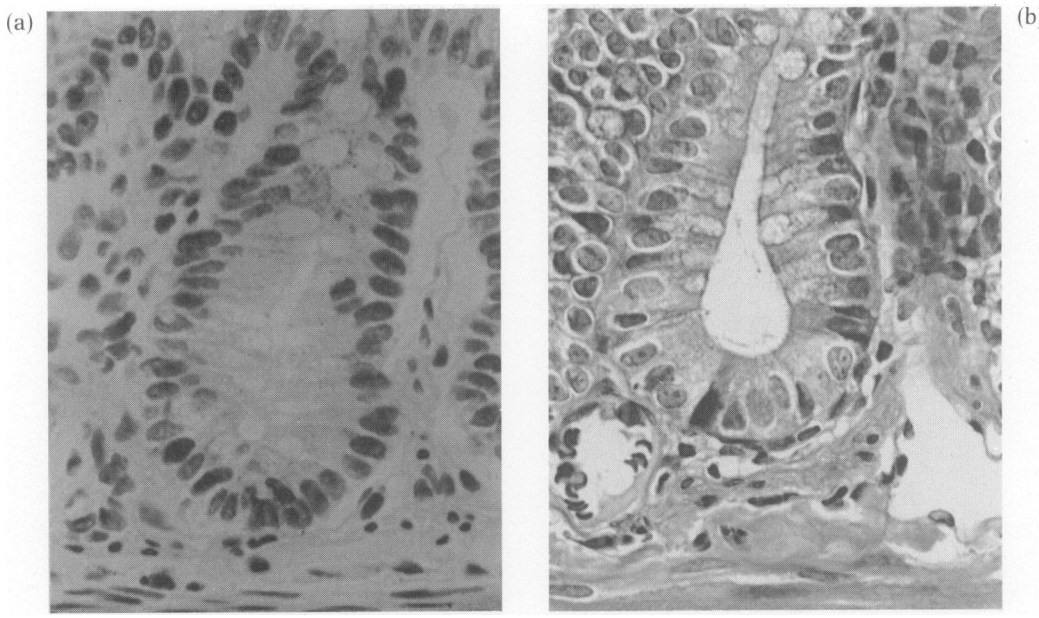


Fig. 6. Paneth cells 12 h after dithizone injection. a, H_2S–Grimelius' method. ×400. b, H&E. ×400.

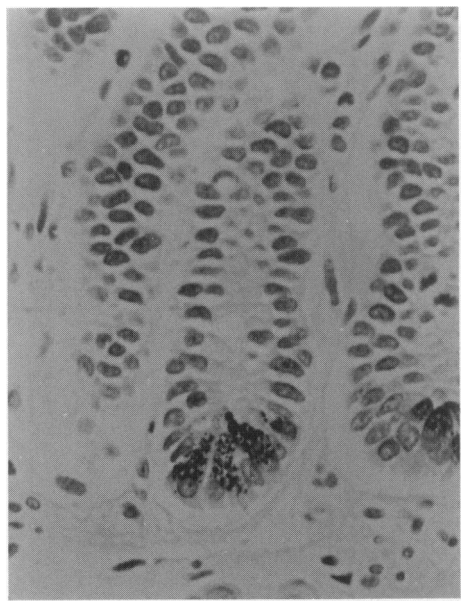


Fig. 7. Paneth cells 72 h after dithizone injection. H_2S–Grimelius' method. ×400.

In-vivo chelation of zinc in Paneth cells by dithizone

Five minutes after the injection of dithizone, zinc dithizonate complexes appeared in Paneth cells as granules with intensely purple-red colour under a bright field microscope and as intensely orange granules under a dark field microscope (Fig. 9).

Thirty minutes to 1 h after the injection of dithizone, the zinc dithizonate complexes appeared deep red under a bright field microscope and bright orange under a dark field microscope. Furthermore, the zinc dithizonate complexes were seen discharged into Lieberkühn's crypts. After 2 h almost all the zinc dithizonate complexes discharged into the cryptic lumen with necrotized cells. After 6 h, only a few zinc dithizonate complexes were seen in the crypts and they completely disappeared after 12 h.

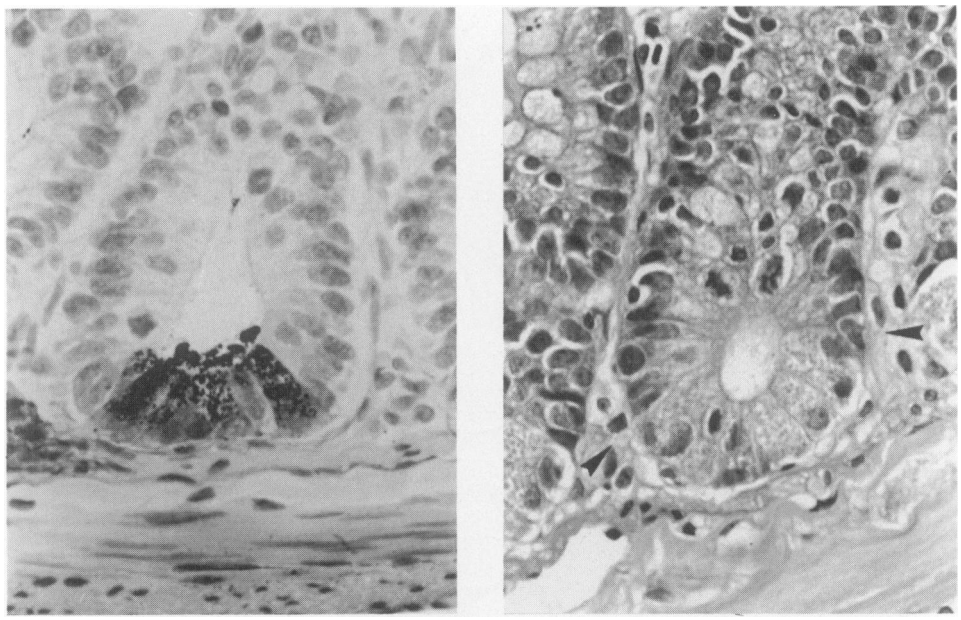


Fig. 8. Paneth cells 1 week after dithizone injection. a, H_2S–Grimelius' method. ×400. b, H&E (between arrowheads). ×400.

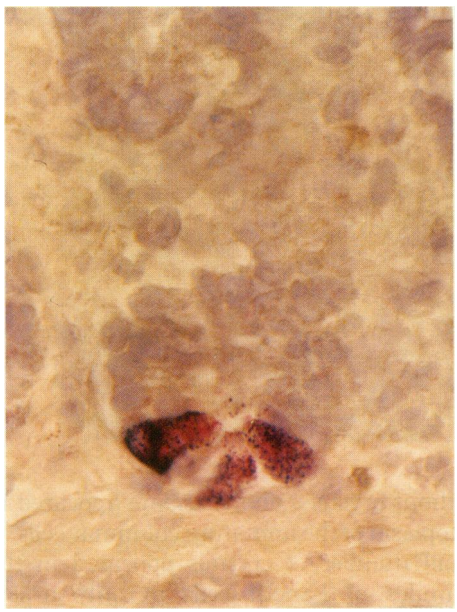


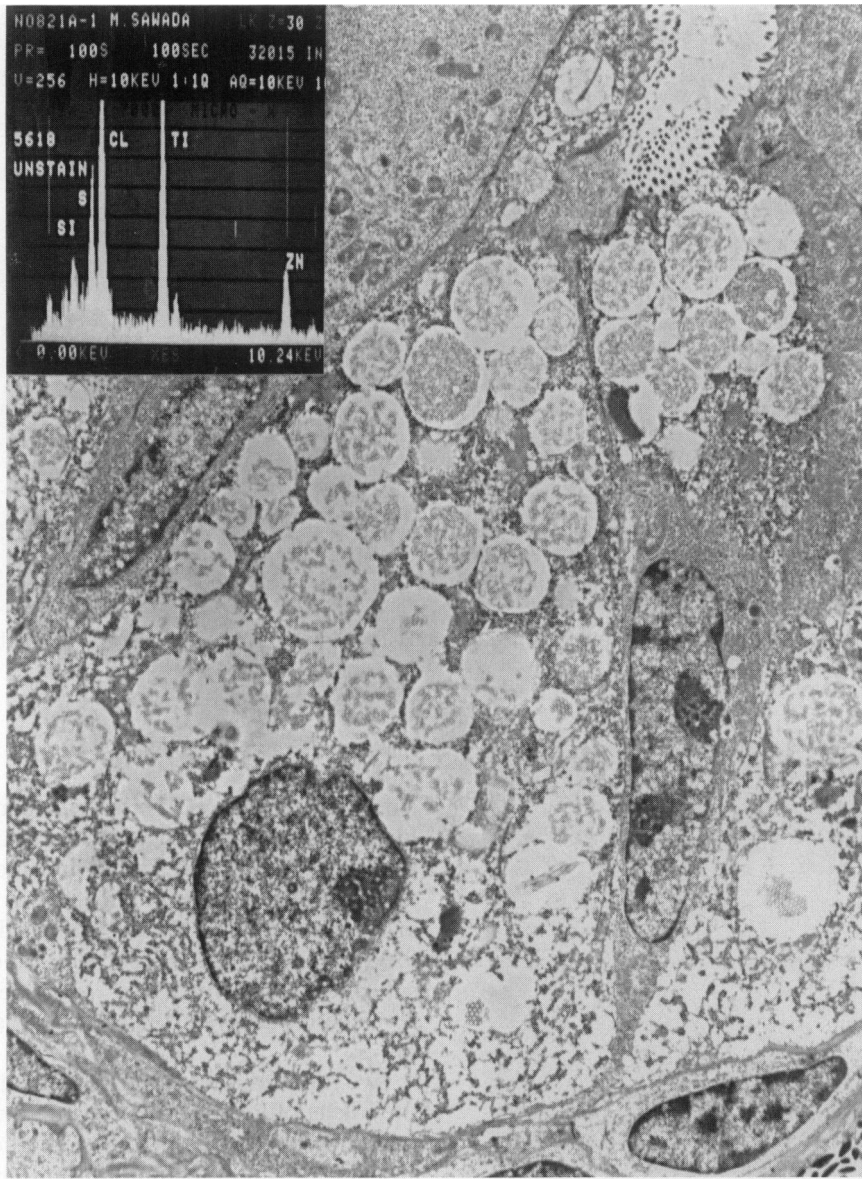
Fig. 9. Paneth cells 5 min after dithizone injection. Bright field microscopic observation of a freshly frozen section, counterstained with Mayer's haematoxylin. ×400.

Electronmicroscopic findings

Normally mature Paneth cells contained large numbers of cytoplasmic granules. In this study, the characteristic granules (2–5 μm diameter) revealed homogeneous matrix or obscure crystals in 2% glutaraldehyde-fixed sections, but in 2% glutaraldehyde saturated with hydrogen sulphide, they revealed clear crystals like fibrils (Fig. 10a, b).

Five minutes after the dithizone injection, the nuclei of Paneth cells became pyknotic, part of the granules fused to each other, and swelling of the mitochondria and cisternal dilatation of the rough endoplasmic reticulum were discerned in the cytoplasm. In a few crypts of Lieberkühn, crypt base columnar cells adjacent to Paneth cells revealed mitotic figures. Some Paneth cells started to detach from basement membrane (Fig. 11). Thirty minutes to an hour following the dithizone injection, Paneth cells died and finally were shed into Lieberkühn's crypts. At this time there appeared immature col-

(a)



umnar cells having low-electron-dense cytoplasm and prominent mitochondria in the bases of some Lieberkühn's crypts (Fig. 12). It is likely that they are columnar cells adjacent to Paneth cells. After 6–12 h, many immature columnar cells containing Paneth cells debris in their phagosome appeared in the base of Lieberkühn's crypts. After 12 h, rough endoplasmic reticulum, granules without crystals, and lysosome appeared in cytoplasm of the immature columnar cells. A small number of crystals appeared in the granules of Paneth cells in only a few Lieberkühn's crypts (Fig. 13). These cells seemed to be immature Paneth cells. After 72 h, Paneth cells contained a large number of granules with crystals and well developed rough endoplasmic reticulum in cytoplasm

(b)

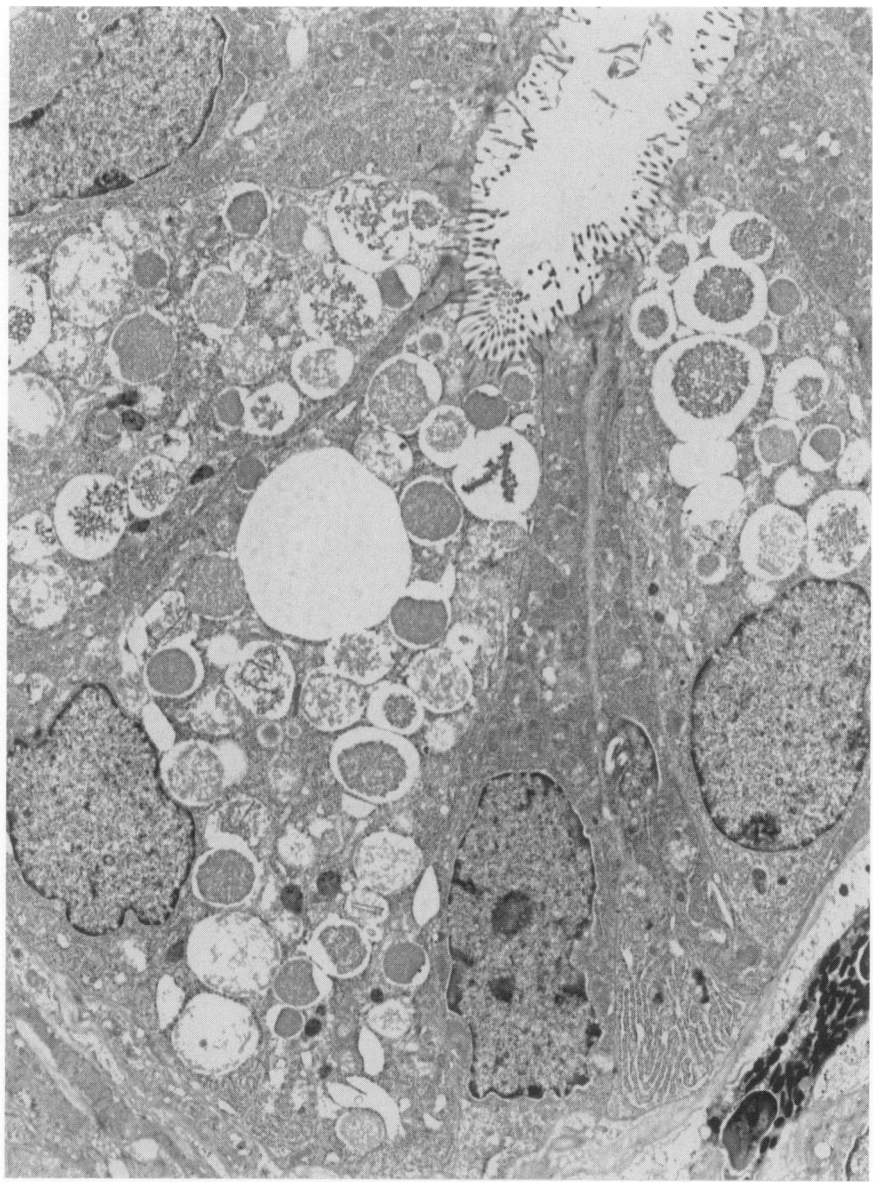


Fig. 10. Electron photomicrograph of the ileum of a normal rat. a, Energy-dispersive X-ray spectra (inset) of one of the granules of a Paneth cell, 2% H_2S–glutaraldehyde fixed. × 2000. b, 2% glutaraldehyde fixed. × 2000.

as well as control groups. One week after the dithizone injection, Paneth cells revealed hyperplasia, but the appearance of individual Paneth cells was similar to that of normal resting Paneth cells.

X-ray microanalysis of Paneth cells

The X-ray microanalysis of the Paneth cell granules showed a high zinc peak (Fig. 10a), but iron, copper and other heavy metals

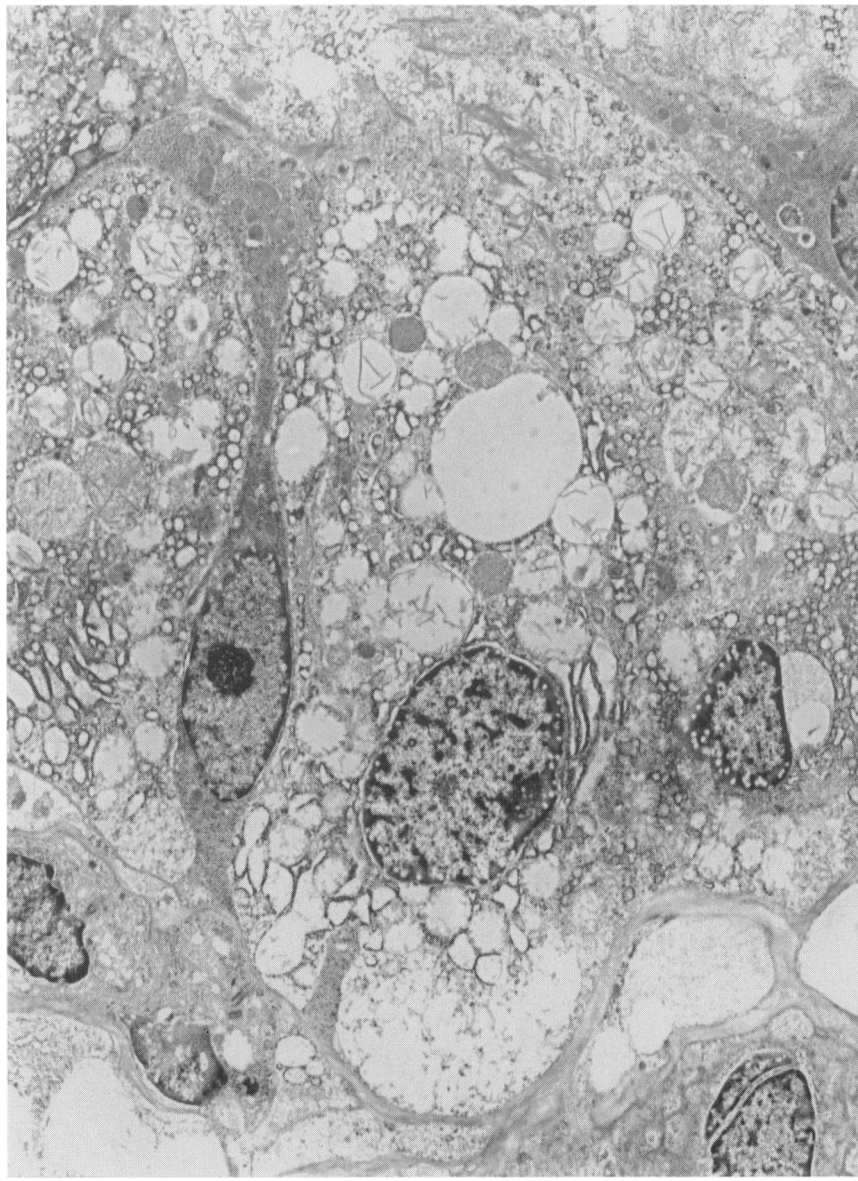


Fig. 11. Electron photomicrograph of the ileum of rat after 5 min following the dithizone injection. 2% glutaraldehyde fixed. × 2000.

were not detectable by our method in control groups. Zinc in the cytoplasmic granules of Paneth cells disappeared with Paneth cell shedding, but reappeared after 12 h following the dithizone injection.

Discussion

Dithizone forms a water-insoluble chelate with several kinds of metals, e.g. silver, mercury, lead, copper, cobalt, nickel and

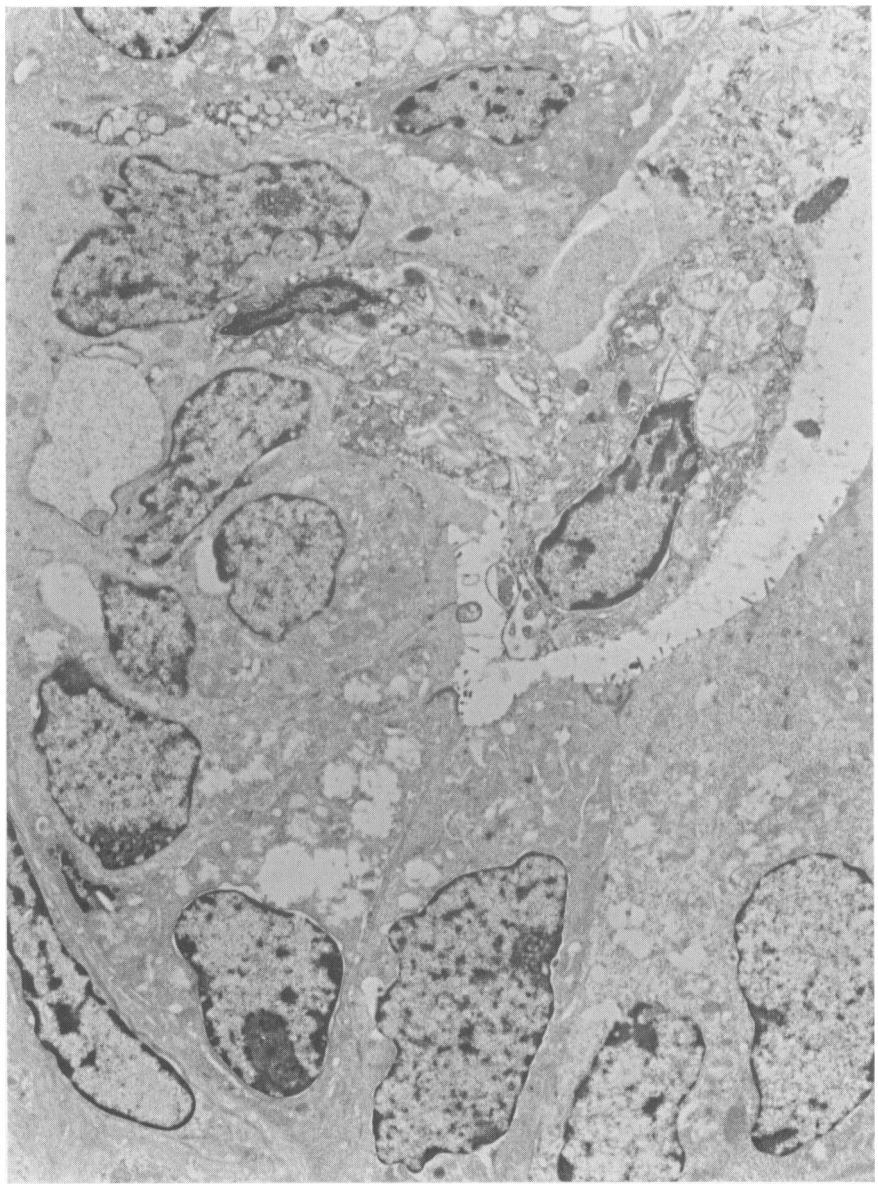


Fig. 12. Electron photomicrograph of the ileum of rat after 30 min following the dithizone injection. 2% H_2S–glutaraldehyde fixed. × 2000.

cadmium. With zinc ions, it forms a characteristic deep-red salt. Formation of zinc chelate *in vivo* has been shown to induce selective killing of zinc-containing cells in certain tissues. Using diphenylthiocarbazide, dithizone, and other chelating agents as histochemical reagents, Okamoto (1942, 1955) demonstrated the presence of zinc in rabbit pancreatic islets beta-cells. He also showed that administration of these chemicals destroys beta-cells by chelating intracellular zinc and thus induces diabetes mellitus.

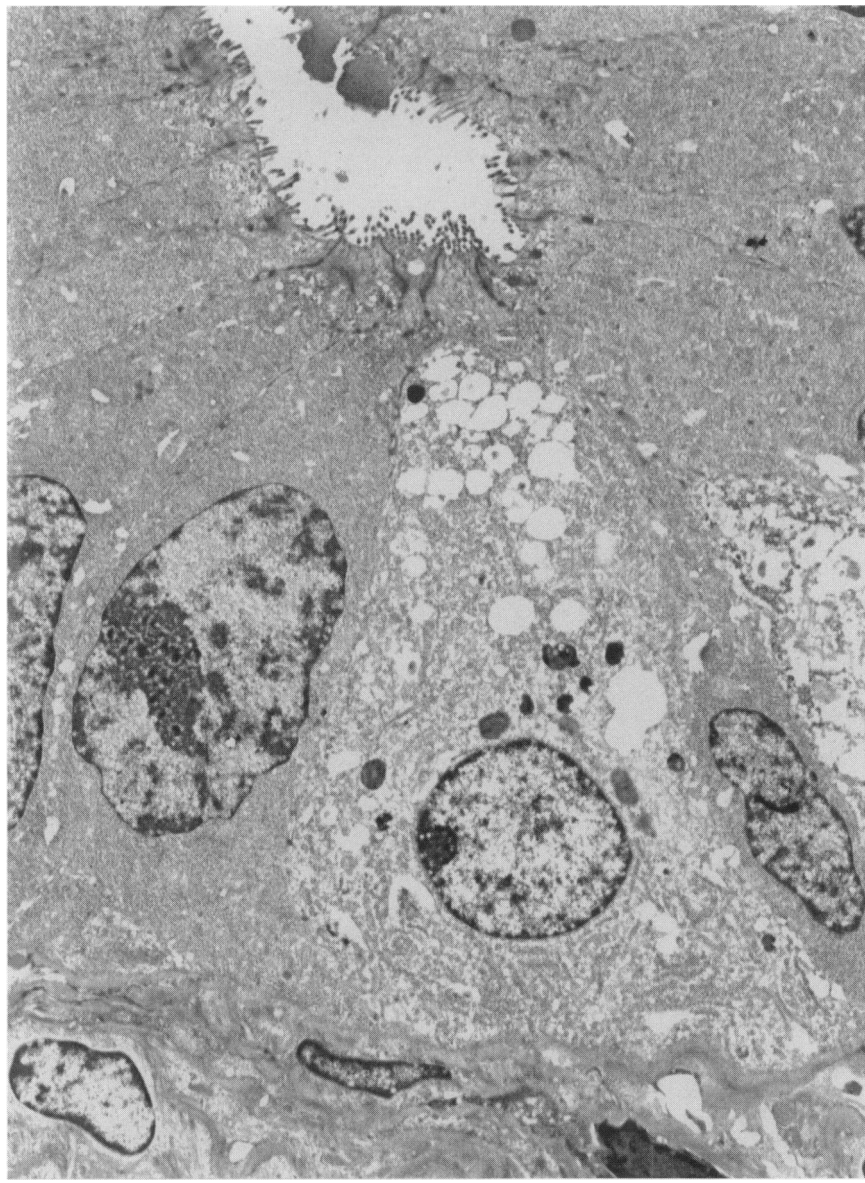


Fig. 13. Electron photomicrograph of the ileum of rat after 12 h following the dithizone injection. 2% H_2S–glutaraldehyde fixed. × 2000.

Kadota and Midorikawa (1951) also showed elimination of beta-cells by sodium diethyldithiocarbamate. A similar approach was used for prostate by Logothetopoulos (1960) and Sternberg *et al.* (1965), who observed necrosis of the prostate acinal epithelium within 3 h after dithizone injection and subsequent regeneration. Szerdahelyi and Kasa (1987) demonstrated decreased histochemical reactivity of zinc in the hippocampus 40–80 min after treatment with sodium diethyldithiocarbamate and its recovery 6 h

after treatment. In their report, however, significant necrosis or degeneration are not described.

Midorikawa and Eder (1962) demonstrated the presence of zinc in intestinal Paneth cells in rat, rabbit and guinea-pig using Timm's method, dithizone staining, and intra-vital staining with dithizone. With X-ray microanalysis, Dinsdale (1984) showed that the granules of Paneth cells contain a high amount of zinc. In the present study, we confirmed their findings and also found that single i.v. injection of dithizone specifically destroyed all Paneth cells in duodenum and ileum and shed them into Lieberkühn's crypts. There are some differences in Paneth cell distribution in the small intestine of some species and the number of Paneth cells increases from duodenum to terminal ileum in rat (Mols 1930; Hertzog 1937; Windisch 1966; Elmes 1976). No significant difference in the response to dithizone was observed between Paneth cell populations residing in the duodenum and ileum in this study. We observed similar killing of Paneth cells by sodium diethyldithiocarbamate, another zinc chelator. Such an effect is not specific to the rat: elimination of Paneth cells was observed in rabbits injected with dithizone, but unlike the rats, no definite debris of Paneth cells was detected in the lumen of Lieberkühn's crypts (data not shown). The exact mechanism of Paneth cell death induced by dithizone has remained obscure. Considering the highly selective effect on Paneth cells but not on adjacent crypt base columnar cells, nonspecific toxicity of the chemical is unlikely. Formation of zinc–dithizonate complexes in Paneth cell immediately after administration and subsequent death of the cells indicates that the change in the chemical state of zinc, presumably complexed with some essential proteins in the Paneth cells, would be the most likely explanation. Mottet and Body (1976) showed similar Paneth cell degeneration following methylmercury hydroxide ingestion in monkeys. However, their report indicates that desquamation of Paneth cells into Lieberkühn's crypt occurred only occasionally. It remains obscure whether dithizone and methylmercury share the same mechanism in killing Paneth cells.

Cheng and Leblond (1974) have suggested that Paneth cells may originate from crypt base columnar cells. Bjerknes and Cheng (1981) claimed that stem cells localizing at position 5 or higher of Lieberkühn's crypts provide the complete lineage of differentiated intestinal epithelium. Mathan *et al.* (1987) also suggested that maturation of Paneth cells started at position 7 or 8. We observed that Paneth cells reappeared as early as 12 h after a single injection of dithizone. The time required for regeneration of Paneth cells is much shorter than that being anticipated for recovery by cell division or migration of putative precursor cells. Another possible mechanism would be transformation of adjacent crypt base columnar cells, which are not affected by the chemical. During regeneration, we observed that crypt base columnar cells exhibit several phenotypes indicating transition to Paneth cells. As early as 30 min after dithizone injection, immature columnar cells appeared in the crypt base, the site in which Paneth cells had been localized. Twelve hours later, they had rough endoplasmic reticulum in the infra-nuclear region, and granules without matrix, which are similar to cytoplasmic vacuoles of immature crypt base columnar cells. Over a period 12 h, such granules regained phenotypes of mature Paneth cells granules. These observations strongly indicate that immediate precursors of Paneth cells are among columnar cells in the bottom of crypts. According to this hypothesis, the putative precursors contain little zinc and thus are insensitive to dithizone. However, they can rapidly transform to Paneth cells on request. In the base of Lieberkühn's crypts, an inductive local milieu for Paneth cells may well exist.

During regeneration, the number of Paneth cells transiently overshot normal a week after dithizone injection and resumed normal levels in the next week. Zinc in the

granules of regenerating Paneth cells may be supplied from the blood stream because blood zinc is highly correlated to histochemical staining of Paneth cell granules (Millar *et al.* 1961).

In conclusion, we first showed temporal elimination of Paneth cells by dithizone administration. This experimental procedure will provide a valuable aid for investigation of the Paneth cell function as well as their biology. The present study also suggests a vital role is played by zinc in Paneth cell function. Further biochemical and immunochemical studies to elucidate the function of Paneth cells and the process of their regeneration are now on the way.

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