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The Paneth cell: A source of intestinal lysozyme[1]

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SUMMARY An antiserum prepared against lysozyme isolated from mucosal scrapings of mouse small intestine was used to stain sections of mouse small intestine with the indirect fluorescent antibody technique. Mucosal fluorescence was confined to the base of the crypts of Lieberkühn, where Paneth cells are located. After the intravenous administration of 4 mg of pilocarpine fluorescence was no longer found in the Paneth cell but in the crypt lumen. Perfusion studies confirmed these findings. The basal lysozyme output of 0·1 to 0·4 μg/ml was raised to peak rates of 1·8 to 6·5 μg/ml after the intravenous administration of 1 mg of pilocarpine.

Our results demonstrate that the lysozyme of the succus entericus is, at least in part, derived from the Paneth cell, and is probably present in the Paneth cell granules. Its secretion is stimulated by pilocarpine. Our model could be very useful for studying the function of the Paneth cell, which probably forms part of an intestinal defence system.

The characteristic features of the Paneth cell, which is located at the base of the crypts of Lieberkühn in man and some other animal species, is the presence of large granules in the apical part. Paneth himself recognized their secretory function as early as 1888 (Paneth, 1888). However, the contents of these granules and their function are still largely unknown.

Speculative reasoning led Creamer (1967) to suggest that Paneth cells regulate crypt cell turnover, and experimental evidence for the secretion of free amino acids by the Paneth cell has been presented (Gent and Creamer, 1972). According to Riecken and Pearse (1966), the Paneth cell granules fulfil the enzymatic criteria of lysosomes; they suggested that Paneth cells are intestinal glands participating in the breakdown of intestinal contents. However, Lewin (1969) was unable to find proteolytic enzymes in the Paneth cell.

Speece (1964), who used an indirect histochemical method, was the first to suggest that lysozyme is present in the Paneth cells of mice. This finding has been confirmed by cell fractionation studies (Deckx, Vantrappen, and Parein, 1967), histochemical studies using substrate films of chitin (Ghoos and Vantrappen, 1971), and recently by the unlabelled antibody technique (Erlandsen, Parsons, and Taylor, 1974).

In this paper the presence of lysozyme in the Paneth cell of mice, its secretion into the crypt lumen and its ability to serve as a marker of Paneth cell function are demonstrated by the indirect fluorescent antibody technique and by perfusion studies.

Materials and Methods

FLUORESCENCE MICROSCOPY

Lysozyme was isolated from mucosal scrapings of mouse small intestine by combined ammonium sulphate precipitation, ion exchange, and molecular sieve chromatography. The purity of the isolated product was established by cellulose acetate electrophoresis, polyacrylamide electrophoresis, isoelectric focusing and gel filtration. The amino acid composition resembled that of hen's egg white lysozyme although basic residues were more abundant. (Details of the isolation procedure and the characteristics of this lysozyme will be published elsewhere.)

Rabbits were immunized by the injection of 1 mg of pure lysozyme dissolved in 1 ml of NaCl 0·9% and emulsified with 1 ml of complete Freund's adjuvant (Difco Laboratories, Detroit, Mich, USA). The antigen was injected in the hind footpads and into several subcutaneous sites. This procedure was repeated one week later and the rabbits were bled one week after the second injection. The monospecificity of the antisera was tested by immunodiffusion and immunoelectrophoresis in agarose.

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The goat antirabbit immunoglobulin antiserum labelled with fluorescein isothyocyanate was obtained from Nordic Pharmaceuticals and Diagnostics, Berchem (Belgium).

Mice were killed with a sharp blow on the head, the small intestine was quickly removed and washed with ice-cold 0·9% NaCl. Small sections were snap frozen and cut in a cryostat at 4 μg. The tissue slices were fixed in cold ethanol for 20 minutes and air-dried. After rehydratation they were covered successively with a drop of the antilysozyme antiserum and, after being washed with Coons buffer (pH 7·2), with the fluorescent antirabbit immunoglobulin. After a final washing they were mounted in aquamount (Gurr, Ltd, London, UK). Incubation was carried out in a humid chamber, at room temperature, for 30 minutes. The antisera were spun at 4000 *g* for 15 min and their optimal dilutions determined from a checkerboard titration. For both antisera a 1:8 dilution provided the most satisfactory results.

The preparations were observed in a Wild microscope (Heerbrugg, Switzerland) equipped with an ultraviolet light source (Osram HB0 200), a high aperture, dark field condenser (N.A.: 1·4, Wild), excitation filters BG 23, GG 23 (Leitz) and FITC (Wild), and a secondary filter FITC/313742 (Wild). Photographs were taken on Ektachrome EH-135 film with the aid of a photographic chamber with automatic exposure (Photomat Mka 5, Wild).

PERFUSION TECHNIQUE

Female mice of the NMRI-strain, weighing approximately 20 g, were anaesthetized with urethane (0·1 mg/g body weight, injected intraperitoneally). A laparatomy was performed and the jejunum was incised 4 cm and 24 cm distal to the pyloric sphincter. Soft plastic tubes were ligated in place, and the inlet tube was connected to a roller pump. The 20 cm jejunal segment was perfused with physiological saline at a rate of 4·0 ml/hr. The outlet tube was connected to a fraction collector. Fractions of 1 ml were collected, and the sampling rate was recorded. In all experiments the output rate deviated little from the input rate, so that one fraction corresponded to a perfusion of 15 minutes. Pilocarpine was dissolved in physiological saline and injected in one of the caudal veins. After each experiment the position of the inlet tube relative to the common pancreatic bile duct was checked stereoscopically. In all experiments contamination of the perfusion with bile or pancreas fluid could be excluded.

ENZYME ASSAYS

Lysozyme was assayed turbidimetrically using hen egg white lysozyme as a standard (Gorin, Wang, and Papapavlou, 1971). The method of Caraway (1959) was used to determine amylase activity. The enzyme activites are expressed in μg/ml hen egg white lysozyme equivalents for lysozyme and as Somogyi units per ml perfusate (SU/ml) for amylase.

Results

FLUORESCENCE MICROSCOPY

A typical result obtained when sections of mouse small intestine were stained immunochemically for lysozyme is shown in figure 1. Fluorescence is found at the bottom of the crypts of Lieberkühn, where the Paneth cells are located. In the other cells of the mucous membrane the nuclei are seen as dark spots surrounded by faint cytoplasmic fluorescence. In the connective tissue there is also some fluorescence which is probably due to the presence of leucocytes. Fluorescence was absent when normal rabbit serum was used instead of the specific antilysozyme antiserum or when the specific antiserum was preincubated with the pure antigen. The intravenous

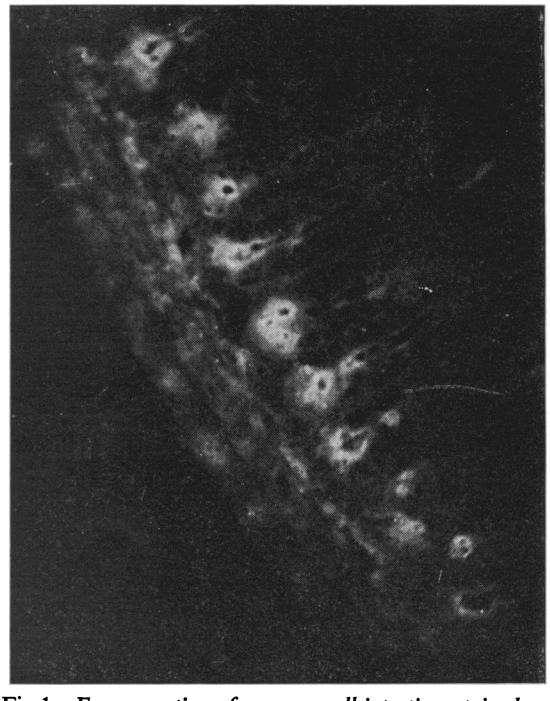

Fig 1 *Frozen section of mouse small intestine, stained with rabbit antilysozyme antiserum and FITC-conjugated goat antirabbit gamma globulins. The structure of the villi and the muscle layers are easily seen as a faint background fluorescence. The FITC-fluorescence is largely confined to the base of the crypts, where the Paneth cells are located* (× *1000*).

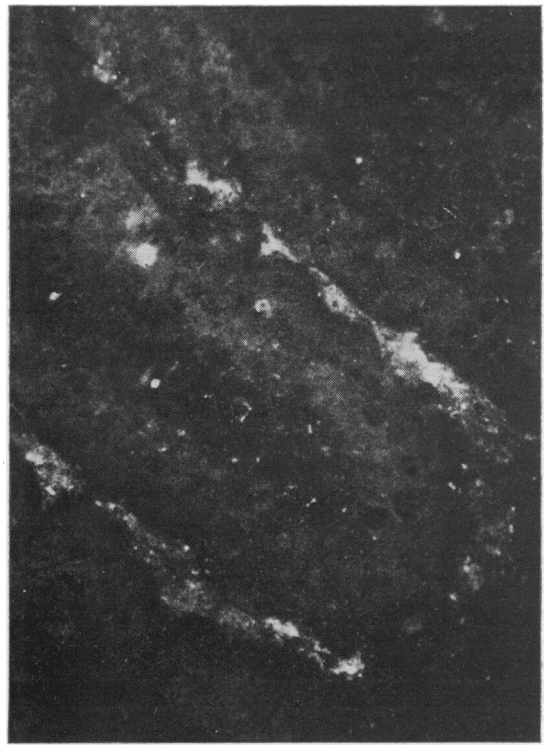

Fig 2

Fig 3

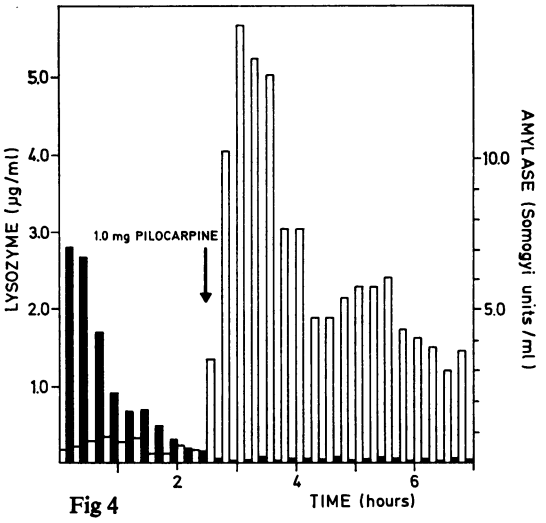

Fig 4

Fig 2 *Frozen section of mouse small intestine, prepared five minutes after the intravenous administration of 4 mg pilocarpine, and stained with rabbit antilysozyme and FITC-conjugated goat antirabbit gamma globulins. The lumen is at the lower right corner. One villus occupies the centre and the spaces between this villus and the two adjacent ones are filled with a stream of fluorescent material, granular in appearance* (× *2000*).

Fig 3 *Frozen section of mouse small intestine, 10 minutes after pilocarpine administration, stained as described. The strongly fluorescent material is located at the tops of the villi* (× *2000*).

Fig 4 *Effect of pilocarpine upon the release of lysozyme (open bars) and amylase (closed bars) into the perfused small intestine of mouse. Perfusion was done with physiological saline at a constant input rate of 4·0 ml/hr. Fractions represent 1 ml collections, ie, time intervals of approximately 15 minutes. After 10 fractions (arrow) 1·0 mg pilocarpine was injected intravenously.*

injection of 4 mg of pilocarpine five minutes before the dissection of the mice resulted in the disappearance of all fluorescence from the crypts, while a stream of fluorescent material appeared between the villi (fig 2). Ten minutes after the administration of pilocarpine fluorescent material, granular in appearance, was found on top of the villi (fig 3).

PERFUSION STUDIES

The results of a typical experiment are presented in figure 4. The initial fluid contained high levels of amylase, which decreased progressively over a 'washing-out' period of approximately 2·5 hours to a stable low basal value. In contrast, the lysozyme level in the perfusion fluid was low from the beginning of the experiment and remained low during the basal period. The intravenous injection of 1 mg of pilocarpine provoked a sharp rise in the lysozyme level, whereas the concentration of amylase was unaffected. Subsequently the lysozyme output decreased but remained at higher than basal values until the animal was sacrificed.

Initially the animals were not starved before the experiment. Later, when it became apparent that the emptying of the intestine before the start of the perfusion was often difficult, the animals were starved for 24 hours, although they were given water. On analysis of the data it was found that the basal values for lysozyme (mean lysozyme concentration in the perfusate before pilocarpine stimulation) were lower in starved animals ($\bar{x}$ = 0·16 μg/ml; SD = 0·086; n = 12) than in unstarved ($\bar{x}$ = 0·31 μg/ml; SD = 0·19; n = 10). The difference between these two values is significant at the 5% level. The data obtained in five mice before and after stimulation by 1 mg of pilocarpine are summarized in table I. The peak rate for lysozyme was computed from the highest value for 45-minute collections, described as the basal value. Because of the 'wash-out' phenomenon observed with amylase both these values are mmeaningless for this enzyme. In the first fraction the amylase concentration was always 5-6 SU/ml and the amylase became undetectable after two to three hours. It will be seen from the table that a significant rise in lysozyme secretion was observed in all five mice studied, the mean peak rate being 3·9 ± 1·9 SD.

Experiment No	*Lysozyme*		*Amylase*	
	Basal (μg/ml)	*Peak (μg/ml)*	*Initial Level (SU/ml)*	*Wash-out (hours)*
1[1]	0·27	5·1	6·9	2½
2	0·12	3·7	6·5	2¾
3	0·21	6·5	7·5	2¾
4	0·06	1·8	5·7	3
5	0·07	2·6	6·1	2¼

Table *Summary of lysozyme and amylase determinations in the perfusate of the small intestine of five mice before and after stimulation by 1 mg of pilocarpine*

[1]Unfasted mouse

In their electron microscopical study of Paneth cell secretion in mice, Trier *et al* (1967) found that secretion in Paneth cell granules was stimulated by the injection of 4 mg of pilocarpine intraperitoneally. Few mice survived the intravenous injection of such a dose for more than 30 minutes, and although a strong increase in lysozyme concentration was found, these results were discarded.

Discussion

Our results confirm and extend previous observations concerning the presence of lysozyme in the Paneth cell. They also demonstrate that the lysozyme present in the succus entericus is, at least in part, derived from the Paneth cell, which secretes this enzyme into the crypt lumen.

Immunofluorescence has been used to demonstrate lysozyme activity in human bone marrow (Briggs, Perille, and Finch, 1966), blood cells (Asamer, Schmalz, and Braunsteiner, 1969), the parotid gland (Kraus and Mestecky, 1971), distinct epithelial cells of the oral cavity (Raeste, 1972), and in mouse and rat macrophages (Glynn, 1964). Evidence for the presence of lysozyme in the Paneth cell was until recently based mainly upon histochemical methods using substrate films of *Micrococcus lysodeikticus* (Geyer, 1973) or chitin (Ghoos and Vantrappen, 1971). In cell fractionation studies (Deckx *et al*, 1967) particle-bound lysozyme activity has been found in a fraction containing Paneth cell granules from mouse small intestine. Recently Erlandsen *et al* (1974) used the unlabelled antibody technique to localize lysozyme in Paneth cells at the ultrastructural level. Their findings are in complete agreement with those presented in this paper. Erlandsen *et al* (1974) found specific staining for lysozyme over secretion granules in the apical cytoplasm, within the region of the Golgi apparatus, and within some lysosomes. They suggested that in substrate film studies, lysozyme from a lysosomal origin may have contributed to a considerable extent to the localization of enzyme. Although the same argument could be used against our method, the staining pattern after pilocarpine administration, combined with the results of the perfusion studies, suggest that staining is due to lysozyme in secretory granules. The absence of fluorescence in the crypt region shortly after secretory stimulation, when the lysosomal apparatus

is presumably still intact, indicates that immunofluorescence is not sensitive enough to detect lysozyme within the lysosomes. Therefore, the results obtained with the extremely sensitive unlabelled antibody technique and our observations taken together suggest that in the Paneth cell most of the lysozyme is located within the granule itself.

Trier *et al* (1967) demonstrated that the number of granules remains constant during pilocarpine stimulation and concluded that pilocarpine results not only in a stimulation of the secretion but also in an increased synthesis of the granules. Our observations indicate that pilocarpine stimulation leads to a complete disappearance of fluorescent granules from the Paneth cells and to the appearance of fluorescent material in the crypt lumen. These two observations are not necessarily contradictory because our histochemical data relate to the first 10 minutes of stimulation whereas those of Trier *et al* (1967) bear on a much later period (three and six hours after the administration of pilocarpine). On the other hand, the high lysozyme level which was still found in our perfusion experiments, for a period of up to three hours after stimulation, may be due to an increased rate of synthesis. However, it may also reflect the time taken for the secreted material to reach the intestinal lumen and the time needed to solubilize a highly viscous product. It should be noted in this respect, that the 'wash-out' period for amylase was already two to three hours. In earlier studies (Klein, 1906; Miram, 1912; Cordier, 1923) a 'secretory cycle', consisting in the accumulation of granules and sectretion after feeding, has been described. Trier *et al* (1967) found no evidence for such a cycle, although our observations of higher basal lysozyme levels in non-fasting mice support the hypothesis of some stimulation by feeding. Our model provides the means to investigate this and other factors or agents responsible for Paneth cell secretion in a relatively simple way.

The action of pilocarpine is of course not limited to the Paneth cell. In this study attention was paid to a possible contamination of the perfusate with material originating from the pancreas. Such contamination could be excluded on two grounds: the position of the inlet tube and the absence of a rise in amylase concentration after stimulation by pilocarpine.

It seems unlikely that lysozyme should be the only secretory product of the Paneth cell. The investigations of Gent and Creamer (1972) provide evidence for the secretion of free amino acids. This point should be reinvestigated in the light of our present knowledge. In view of the bactericidal properties of lysozyme, the Paneth cell probably forms part of a defence system in the small intestine. Already the phagocytosis of an intestinal parasite, *Hexamita muris* (Erlandsen and Chase, 1972a), and of spiral microorganisms (Erlandsen and Chase, 1972b) by the Paneth cell has been demonstrated. Although the bactericidal properties of lysozyme itself are limited, they are enhanced in the presence of other substances such as immunoglobulins and complement (Bladen, Hageage, Harr, and Pollack, 1973; Glynn, 1968). It would be interesting to find out whether the Paneth cell granules themselves do not contain agents which act synergystically with lysozyme.

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