Localization of pepsin in the stomach, revealed by fluorescent antibody technique

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Introduction

As commonly known, pepsin is the main constitutions of the gastric juice and play an important role on the digestion of the proteinous materials in the food. Biochemically, pepsin is one of the endopeptidase and splits almost all the peptide bond of the protein, specially attacks the bond next to the aromatic and dicarboxylic amino acids (Akabori, 1956). Holter and Linderstrøm-Lang (1934) have first investigated on the location of pepsin in the stomach and concluded that pepsin is mainly localized in the chief cell and seems to be present in less amount in the mucous neck cell. The method which Holter and Linderstrøm-Lang had used was the combination of the counting of the cell population in the slice of every part of a stomach and the measurement of the peptic activity in the identical slice by biochemical procedure. Though, they found the parallel relationship between the high population of the chief cells and strong biochemical activity of pepsin in the fundus region and made the conclusion as mentioned above, this method was rather indirect identification of pepsin than the direct visualization of the cite of pepsin under the microscope which Bowie (1935 and 1940) had developed; Bowie also noticed the high concentration of pepsin in the chief cell and low in the mucous neck cell. The presence of pepsinogen was also reported in the human semen by Lundquist (1951), but this result is too heterogenic to refer in this study.

The authors have dealt with the localization of pepsin in the stomach, specially in the fundus region of the porcine stomach, by staining the tissue with fluorescein-labelled specific antibody prepared

against porcine pepsin. This method is based on the entirely different principle to visualize the enzymatic sites, from those two other methods used in the above mentioned papers. Special attention was paid to confirm the presence of pepsin in the mucous neck cells, as well as in the chief cells.

Materials and methods

The antigen used in this study was crystalline porcine pepsin of the commercial source.* 20 mg. of antigen, dissolved in the phosphate buffer at pH 8.0 to prevent the autocatalytic activity of pepsin, was mixed well with complete Freund's adjuvant and injected into the foot pads and thigh muscle of a rabbit. Three rabbits received the same treatment for the experiment. 10 mg. of antigen, dissolved in the same buffer as the initial injection, was given intravenously to each animal for the booster injection on the thirty-sixth day from the initial injection of the antigen, together with the preliminary intravenous administration of 10 mg. of restamin.**

A week after the booster injection, the animals were bled either by cardiac puncture or by depletion from the ear artery. The endpoint of the titration, measured by passive hemagglutination (Stavitsky, 1954) using 10 mg. of antigen to sensitize the red cells, was 163840. The sera were pooled, and most of the part was used for the preparation of the conjugate. The remainings were either stored in the refrigerator at 4°C or in the deep freezer at -20°C after a quick freezing.

The fluorescein-conjugated antibody solution was prepared and purified in the same manner as described in the previous paper (Yasuda and Coons, 1966). The molar F/P ratio (McDevitt et al, 1963) of the conjugate was 1.4. The specificity of antigen-antibody reaction was examined by gel-diffusion test (Wilson and Pringle, 1954), to get a clear single precipitin line between porcine pepsin and the homologous antiserum. The antiserum did not show any reaction with porcine pancreatic extract. We did not try to make any precipitin reaction between anti-porcine pepsin serum and pepsin of the other animal source. Therefore, the species specificity of pepsin remained unexamined. The porcine stomachs were obtained at the slaughter house. The alimentary condition of the animals

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before the sacrifice was not clear, since the specimens were taken randomly whenever they were required. It was told in the slaughter house that the animals were fasted for approximately two days before the sacrifice, with the exception of the water supply. But, this did not mean the complete fasting for two days. The only thing which could be imagined was that the animal was relatively hungry when it was sacrificed. Nevertheless, the stomachs, from which the specimens were excised, were always full.

The tissue blocks were quickly frozen with aid of the dry ice-ethanol mixture and then cut at 4μ in the cryostat. The other blocks were fixed with several kinds of the fixatives at 0°C , dehydrated with either ethanol or acetone and processed to the regular paraffin embedding. (Sainte-Marie, 1962). The fixatives used in this study were as follows; absolute acetone, 99 per cent ethanol, 10 per cent ethanol in absolute acetone, 50 per cent ethanol in absolute acetone, 10 per cent acetone in absolute ethanol, 10 per cent formalin in absolute ethanol, 5 per cent formalin in absolute acetone, Carnoy's solution, 10 per cent ethanol in absolute acetone containing 10 per cent formalin, 99 per cent ethanol with 1 per cent glacial acetic acid, and methanol. Among these fixatives, 10 per cent ethanol in absolute acetone was the most satisfactory fixative for the preservation of the antigen.

To prevent the autolysis of the tissue by pepsin, the inhibitors such as hydrazin, phenylhydrazin and semicarbazide (Schales et al, 1948), were mixed with the fixatives at the concentration of 0.02 m.

Among these, semicarbazide was found to be most suitable for the preservation of the tissue, and had no harmful effect on the antigen-antibody reaction on the tissue. Control examinations carried out according to the description by Coons (1950, 1956 and 1958), Cherry et al (1960) and by Yasuda et al (1963 and 1964), results in the presence of no or negligible fluorescence on the sections. Zeiss fluorescence microscope was used exclusively during the whole course of the experiment.

Results

The porcine stomach consists grossly of four regions according to Sisson and Grossman (1956); they are oesophageal region, cardiac gland region, fundus gland region and pyloric gland region. In addition to these regions, Wada (1940) recognized the inter-

mediate zone between fundus and pylorus region, where the histological pattern was the combination of the both regions. These regions are not sharply separated from each other from the histological point of view, be the classification, what it may.

The present study has confined in the observation on the fundus region. The general histology of the porcine stomach, especially the fundus region, is quite similar to the feature of the human stomach. Namely, inner surface of the stomach is covered by the tall columnar epithelial cells which extends into the gastric pit. The bottom of the pit is continuous to the neck portion of the gastric gland where the chief cells, parietal cells, mucous neck cells and argentaffine cells are distinguishable.

Chief cell :

These cells arranged themselves in a single layer along the lumen in the lower two thirds of the glandular tubule. Numerous zymogenic granules are demonstrable in the section fixed with Zenker-formalin and stained with Heidenhain's iron-haematoxylin. The brilliant fluorescence, which indicates the site of the enzyme localization, is observed in the apical cytoplasm of the chief cell, which corresponds to the area of the accumulation of the granules in the normal histological section. (Figs. 2 and 3). As previously expected, the zymogenic granules are proved to have pepsin or pepsinogen; these two proteins are homologous in the immunological aspect. In the tubule located at the bottom of the gastric gland, these are cells which do not show any fluorescence. Often, the cells with pepsin sit next to the cell without fluorescence in the cross section of a single tubule. Occasionally, the cells which line around the glandular lumen of a cross-sectioned tubule are all negative of fluorescence. Moreover, several tubules locating side by side at the bottom of the gastric gland are entirely free from pepsin. These pepsin-negative cells are neither parietal cells nor argentaffine cells, but appear to be the chief cells, judging from their location and cell size under the fluorescence microscopy. In addition, these cells can be observed exclusively at the bottom of the gastric gland.

As illustrated in Figs. 2 and 3, the specific fluorescence appears as granular pattern in the chief cell. Looking at these granules precisely, some of the granules have dark, unstained central cores; this means the center of the granule is devoid of pepsin. This figure seems to be identical with the "vacuolation of the secretory granule" in the light microscopy. As is commonly known, the vacuolation is explained as followings; the mature secretory granules

lose the stainability with iron haematoxylin or other dyes, beginning from the center of the granule towards outer shell. But whether the vacuolation means the loosing of pepsin or the alternation of the location of pepsin within the granules caused by the liquefaction of the granular contents, is still not clear in this immuno-histological study.

In the regular histological sections fixed with Zenker-formalin solution, the zymogenic granules of the chief cell of porcine stomach are PAS-positive and saliva-resistant. This was also confirmed in this study by using fluorescent schiff-type reaction (Kasten, 1959).

The coexistence of PAS-positive substance and the protein in the same granule is recognized in the chief cell near the neck portion of the human gastric gland (Ohishi, 1947) and in the salivary gland (Järvi, 1940 and Lillie, 1950), but not yet found in the body chief cells. Narita (1953) had noticed the PAS-positive mucous granule which are different from the serous granule in the identical cell of the human pyloric gland. In other words, there are two kinds of granules in the human pyloric gland, which are quite different in the chemical nature from each other. Comparing the results with others described above, it seems that the granule which contains pepsin is at the same time positive to PAS-reaction. Namely, the zymogenic granules of the chief cell of the porcine stomach have the amphitropic reaction group, as described by Järvi (1940) in the salivary gland.

Mucous neck cell:

The mucous neck cells, lining scatteringly the lumen of the neck portion of the gastric gland, are also stained strongly with fluorescent antibody solution. (Fig. 4). The specific fluorescence is concentrated in the granular pattern in the apical cytoplasm, but neither around the nuclei nor in the basal cytoplasm. Not like the feature in the chief cells, pepsin-containing granules in the mucous neck cells are small and have no unstained central core in them. Since, most of the typical mucous neck cells lie in the irregular space between parietal cells, they represent elongated triangular shape near the neck portion with their acute angle wedged between the parietal cells. The pepsin-containing granules are encountered in the rather broad apical cytoplasm, where PAS-positive and saliva resistant granules are also present. Though, pepsin and PAS-positive material which might be of mucous nature are unfortunately not demonstrated in a single section simultaneously, the granules in the mucous neck cells seem to react to the both staining methods.

This fact is suggestive of the amphitropic nature of the granules of the mucous cells; this is similarly circumstanced to the situation of the chief cells. But, it is still not clear whether all the pepsin-containing granules are PAS-positive or not, because most of the narrow mucous neck cells have full of pepsin-containing granule, while the PAS-positive granules are occasionally confined in the uppermost area of the apical cytoplasm. Down towards one third of the whole length of the gastric gland, these are obtuse angled triangular cells inserted between the parietal cells. The form of these cells is cubic rather than broad triangular. The broad apical cytoplasm is fully occupied by the pepsin-containing granules (Fig. 5). This cell is quite similar to the chief cell, but is different only because of its particular location, shape, and of less dense stainability by Heidenhain's iron haematoxylin and smaller size of the granules than those in the chief cell. This cell might also fall into the category of the mucous neck cell or the transitional form between the mucous neck cell and the chief cell, and might be identical with the second type cell described by Z i m e r m a n n (1898). The granule of this kind of cell is also PAS-positive, like in the narrow mucous neck cells as well as in the chief cells.

Other cells in and around the gastric gland:

The parietal cells and the argentaffine cells which are also the constituents of the gastric gland have no pepsin in them. The connective tissue around the gastric gland, in other words, the cellular and the fibrous component of the lamina propria mucosae are all negative for the pepsin staining, as well as the wall of the blood vessels.

Gastric pit:

At the bottom of the foveolae, specific fluorescence can be often observed in the diffuse and amorphous pattern along the surface of the mucous cells which cover the foveolae. This kind of the figure is also visible along the lumen of the gastric pit towards the opening of the gland. (Fig. 6). This might be the feature of pepsin which is secreted by the chief cell of the gastric gland into the lumen and then is now on the course of ascending towards the opening. At the bottom of the foveolae, the lumen is slightly enlarged and contain more pepsin than the other part of the foveolae. This part, just above the neck of the gastric gland might play a role as a primitive reservoir for the secreted pepsin for a while before reaching the opening of the gland. It is noteworthy that not every foveola has pepsin in it, namely, each gastric gland may have different secretory

phase.

Surface epithelium :

The surface of the gastric mucosa, as well as the gastric pit, is lined by a tall columnar epithelium. These surface epithelial cells have much mucous material but no pepsin in them. The specific fluorescence is observed in the mucous layer on the surface of the epithelium; sometimes whole the thick mucous layer fluoresces, while occasionally only the surface of the mucous layer contain pepsin.

Discussion

These were indirect proves that pepsin might be localized or produced mainly in the chief cell of the gastric gland (Holter and Linderstrøm-Lang, 1934, Bowie, 1935 and 1940), by using different kinds of the methods. The direct histochemical method to demonstrate the site of pepsin has not been developed yet, because of the difficulties to visualize the end-product of the pepsin-substrate interaction, under the microscope.

In this study, the authors employed immuno-histological method to show the localization of pepsin, basing upon the immunological specificity of pepsin-anti-pepsin system. In short term, this method is an application of antigen-antibody reaction on the tissue, in place of in the test tube. Therefore, this is a technique to demonstrate directly the localization of enzyme protein itself, but not to show the site of the enzymatic activity. By using this method, pepsin is mostly concentrated in the chief cells, as well as in the mucous neck cells.

The presence of pepsin in the chief cells is already suggested (Holter and Linderstrøm-Lang, 1934) or proved histochemically (Bowie, 1935 and 1940), and is also confirmed in this study by using the different kind of method. As described before, there are many chief cells which do not contain any pepsin in them, especially at the bottom of the gastric gland. Most of these cells are filled with unstained vacuoles in the light microscopic sections stained with Heidenhain's iron haematoxylin. Therefore, these cells are now in the vacuolation stage, which is explained to be caused by pre-secretory or liquefied condition of the mature secretory granules, in the conventional cytology. But, it is hard to believe that the mature and liquefied granules which give the appearance of the vacuole in the light microscopy do not contain any pepsin.

It is observed that, as the vacuolation develops from the center of the secretory granules towards periphery, the granules lose the specific fluorescence which represents the location of pepsin from the center to the periphery, leaving the dark, unstained core at the center of the granule. As already being suggested in the electron microscopy (Shibasaki, 1961), the secretion from the chief cell is not necessarily accompanied by the break down of the secretory granules, unlike the case in the pancreatic acinar cell. Then, it is imagined, pepsin might be pushed towards periphery of the secretory granules with the maturation of the granule develops, and eventually diffuses out of the granule to be secreted, losing the stainability for iron haematoxylin from the center of the granule and leaving the shadow of the secretory granule as an appearance of the vacuoles.

One of the exciting results in this study was to show the presence of pepsin in the mucous neck cells. Since Zimmermann (1898) had described on three types of chief cells in the human stomach, a number of papers have been published, reporting on the nature and accordingly on the nomenclature of the mucous neck cells. It was called neck chief cell (Bensley, 1898), "Halshauptzelle" (Oppel, 1896), "Nebenzelle" (Liebert, 1904) and mucous chief cell (Kirk, 1910). Each name represents the nature of the cell. But it is widely accepted that this cell may secrete mucous material and, on the other hand, may play a role as a reproducing center of the cells of the gastric gland, especially the chief cells (Ohishi, 1947 and Shibasaki, 1961). Ohishi (1947) has noticed the transition from the mucous neck cells to the chief cells, in the cytological observation. However, it is only Bowie (1935 and 1940) and Holter and Linderstrøm-Lang (1934) who have referred to the presence of pepsin in the mucous neck cells. Though Bowie recognized that the reaction of pepsin in the mucous neck cells is weaker than in the chief cells, the brightness of the immunofluorescence representing the site of pepsin in this method, is the same in mucous neck cells as in the chief cells. Since, immunohistological method employed in this study is a method to demonstrate the site of the enzyme protein but not to show the reaction site, it might also be considered that the mucous cell has enzyme protein as much as the chief cell does, but the activity of the enzyme in the mucous neck cell is weaker than that in the chief cell.

Summary

The localization of pepsin in the porcine stomach was investigated immuno-histologically, by employing the fluorescent anti-pepsin conjugate as the staining solution.

Pepsin was present in the mucous neck cells as well as in the chief cells of the gastric gland in the granular pattern. The granule in these both cells are also PAS-positive and presumably have amphitropic group in them.

Pepsin was also demonstrated in the foveolae of the gastric pit and along the narrow lumen of the pit. While, the parietal cells, surface epithelial cells and the argentaffine cells have no demonstrable pepsin.

The anti-pepsin antibody was found to be usable in an application of fluorescent antibody method to the enzyme-histochemistry, with the aid of the proper use of inhibitors of the enzymatic reaction and the appropriate treatment of the tissue.

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Plate

Explanation of the figures

- Fig. 1. The gel-diffusion pattern of pepsin-antipepsin system, indicating the formation of single precipitin line between antigen and antibody. The antiserum is placed in the center larger well. The surrounding smaller eight wells are filled with the antigen of several different concentrations, beginning from 10 mg. of pepsin at the top well followed by the counter-clockwise descending gradients of each half dilution.
- Figs. 2 and 3. The cross section of the secretory tubules at the bottom of the gastric gland. The chief cells are full of granules with specific fluorescence which indicates the sites of pepsin. Occasional granules have dark unstained central core, though it is difficult to recognize in these pictures. The nuclei of the chief cells are mostly confined in the basal cytoplasm, showing the image of the negative hollow. There are several tubules which contain the unstained chief cells, sitting next to the pepsin-containing cells. The polygonal cells which locate in or outside of the tubule are the parietal cells, and are negative of pepsin. $\times 400$.
- Fig. 4. The neck portion of the gastric gland. The mucous neck cells are also stained with the fluorescent antibody solution prepared against pepsin. The apical cytoplasm is occupied by the pepsin-containing fine granules. The surrounding blue cells are the parietal cells. $\times 400$.
- Fig. 5. The portion where is slightly down below the neck portion of the gastric gland. The mucous neck cell or the transitional cell between the chief cell and the mucous neck cell contains pepsin in the fine granular pattern. Since, the basal cytoplasm of these cells is inserted in the narrow interspace between two parietal cells and the pepsin-containing granules are mostly localized in the apical cytoplasm, these cells give the impression of arranging themselves inside of the parietal cells and facing the lumen. $\times 400$.
- Fig. 6. The lumen of the gastric pit is often filled with the material which is stained by the labelled anti-pepsin conjugate. The pepsin secreted from the chief-cells and presumably from the mucous neck cells might take an ascending course towards the opening of the gastric pit. The yellow dots in lamina propria mucosae represent the autofluorescence of the red cells in the blood capillaries. $\times 400$.

Fig. 1

Fig. 4

Fig. 2

Fig. 5

Fig. 3

Fig. 6