IDENTIFICATION OF GLUCAGON-PRODUCING CELLS (A CELLS) IN DOG GASTRIC MUCOSA

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ABSTRACT

An immunocytochemical technique using specific antiglucagon serum reveals the presence of glucagon-containing cells situated exclusively in the oxyntic glandular mucosa of the dog stomach. Electron microscope examination of the mucosa demonstrated endocrine cells containing secretory granules with a round dense core surrounded by a clear halo, indistinguishable from secretory granules of pancreatic A cells. Like the alpha granules of pancreatic A cells, the granules of these gastric endocrine cells exhibited a peripheral distribution of silver grains after Grimelius silver staining. Moreover, the granules of these cells were found to be specifically labeled with reaction product, using the peroxidase immunocytochemical technique at the ultrastructural level. Accordingly, these cells were named gastric A cells.

These data suggest that the gastric oxyntic mucosa contains cells indistinguishable cytologically, cytochemically, and immunocytochemically from pancreatic A cells. It is believed that gastric A cells are responsible for the secretion of the gastric glucagon.

The glucagon-like activity first observed by Sutherland and De Duve in alcohol extracts of the upper gastrointestinal tract of the dog (30) is now attributed largely to the presence of a polypeptide with physicochemical, biological, and immunological properties indistinguishable from those of pancreatic glucagon (24). This polypeptide has been clearly differentiated from the so-called "glucagon-like immunoreactivity" (GLI) (24), which is most abundant in the postduodenal small intestine (31). Since the oxyntic glandular mucosa of the canine stomach has been reported previously to contain cells resembling pancreatic A cells (19, 3, 4, 28, 13), the present study was designed to determine, by immunocytochemistry, whether these gastric cells are the source of gastric glucagon. Immunocytochemistry was employed in conjunction with other cytological techniques at both the light and electron microscope level and with immunochemical characterization of tissue extracts.

MATERIALS AND METHODS

Gastrointestinal tissue was obtained from 12 dogs. Under Nembutal anesthesia, the animals were subjected to laparotomy and samples of mucosa were taken from the following regions: cardia, fundus, corpus (these two latter forming the so-called oxyntic part of the stomach), pylorus, duodenum, jejunum, ileum, and colon. Pieces of each region were fixed in Bouin's and Zamboni-de Martino's fluid (35) for immunofluorescence and immunocytochemical techniques with the light microscope. Samples of the above-mentioned tissues were also fixed in 4% glutaraldehyde solution in 0.1 M phosphate buffer at pH 7.4 for conventional electron microscope examination.

For cytochemical and immunocytochemical investigations at the ultrastructural level, tissues were fixed in a mixture of 2.5% formaldehyde (vol/vol), 2.5% glutaraldehyde (vol/vol), and 15% (vol/vol) of a saturated aqueous solution of picric acid in phosphate buffer or in Zamboni-de Martino's solution. Tissue from one animal was frozen immediately in liquid nitrogen, and cryostat sections were prepared for dark-field microscopy (25). After removal of tissue fragments for morphological studies, the dogs were killed by an overdose of Nembutal and the gut was removed for glucagon extraction.

Light Microscopy

After fixation and embedding in paraffin, 5-μm tissue sections were processed for immunocytochemical studies. For immunofluorescence investigations, the indirect method of Coons et al. was used (5). Sections were incubated for 1 h with rabbit antiglucagon 15K serum, equivalent to antiserum 30K with respect to specificity for true glucagon,[1] washed in phosphate-saline buffer (pH 7.4), and then incubated for 1 h with fluorescein-labeled sheep anti-rabbit gamma globulin (Pasteur Institute, Paris). In addition, the following controls were used: (*a*) normal rabbit serum followed by fluorescein-labeled antiserum; (*b*) fluorescein-labeled antiserum alone; (*c*) antiglucagon serum, preincubated with an excess of porcine glucagon, followed by the fluorescein-labeled antiserum. In a parallel investigation, sheep anti-rabbit gamma globulin was conjugated to horseradish peroxidase (Sigma, type VI) according to the method of Kawaoi et al. and used as marker for immunocytochemical studies (11). Peroxidase was revealed according to Graham and Karnovsky (9). The controls were as follows: (*a*) normal rabbit serum followed by peroxidase-labeled antiserum; (*b*) antiglucagon serum, preincubated with an excess of porcine glucagon, followed by the peroxidase-labeled antiserum.

Electron Microscopy

After fixation in 4% glutaraldehyde, part of the tissue was postfixed in 2% buffered osmium tetroxide (17), dehydrated in ethanol, and embedded in Epon (14). Tissue pieces fixed in the mixture of formaldehyde, glutaraldehyde, and picric acid were cut into 120-μm thick sections with a Smith-Farquhar tissue sectioner (Du Pont Instruments, Sorvall Operations, Newtown, Conn.) and subsequently impregnated with a silver nitrate solution, according to Grimelius (10), modified for electron microscopy by Vassallo et al. (32). After silver staining, the sections were postfixed in 1% buffered osmium tetroxide and processed as usual for Epon embedding.

The peroxidase immunocytochemical technique was performed as described by Sternberger (29). The tissue fixed in Zamboni-de Martino's fluid was cut into 30-μm thick sections with a Smith-Farquhar tissue sectioner; sections were subsequently incubated with rabbit antiglucagon serum for 15 h, sheep anti-rabbit globulin serum for 8 hr, peroxidase-antiperoxidase complex for 12 h, diaminobenzidine and H_2O_2 for 30 min. Postfixation was performed in 2% buffered osmium tetroxide followed by embedding in Epon. Control tissue was incubated with the specific antiserum, previously adsorbed with an excess of porcine glucagon. Semithin sections of Epon-embedded tissue were studied with a phase-contrast microscope in order to identify the exact localization of the mucosal area to be cut for ultrastructural examination. Ultrathin sections of suitable areas were obtained

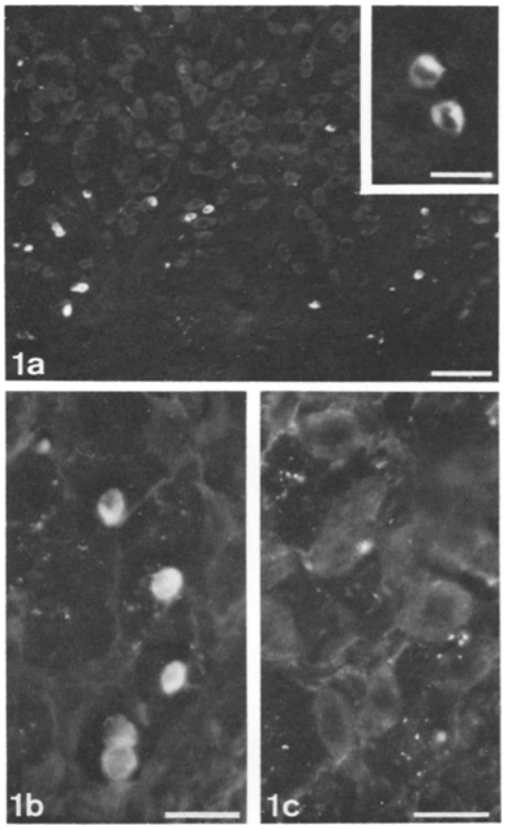

FIGURE 1 Dog oxyntic mucosa. (*a*) Immunofluorescent staining with 15K antiglucagon serum shows several positive cells in the gastric glands. *Inset* displays positive cells at higher magnification. Note the absence of reaction in the nucleus. × 160, the bar represents 50 μm. *Inset*, × 400, the bar represents 20 μm. (*b*,*c*) Two serial sections of a gastric gland showing that immunofluorescent staining with 15K antiglucagon serum (*b*) is inhibited by incubation of the serum with excess of native glucagon before treating the section (*c*). (*b*) × 500; (*c*) × 500; the bar represents 20 μm.

[1] 15K and 30K antisera gave similar results in immunofluorescence (unpublished observations).

with an LKB ultramicrotome and stained with uranyl acetate and lead citrate (23), whereas those sections previously impregnated with silver were treated with uranyl acetate only. The ultrathin sections were observed in a Philips EM 300 electron microscope.

Biochemical Procedures

The mucosa of the gastrointestinal tract was scraped away from the musculature, frozen immediately in dry ice, and stored at −20°C until the time of extraction. Extracts were prepared according to Kenny (12). 20 mg of extract of the canine oxyntic mucosa and of the ileal mucosa were dissolved in 4 ml of a 50 mM ammonium bicarbonate buffer, pH 8.8, and subjected to gel filtration on a Biogel P-10 column (0.9 cm × 115 cm) equilibrated previously with 50 mM ammonium bicarbonate buffer, pH 8.8. Elution was carried out with the same buffer at a flow rate of 8 ml/h and fractions of 1.6 ml were collected. Fractions were kept frozen until the time of assay for glucagon immunoreactivity using antiserum 30K which is highly specific for pancreatic glucagon, and for GLI with antiserum 78J, which cross-reacts avidly with GLI (7). Blue dextran, ^{125}I-insulin, and ^{125}I-glucagon were used as molecular weight markers in the gel filtration studies.

RESULTS

Light Microscopy

Cryostat sections of the glandular oxyntic mucosa observed under dark-field microscopy showed cells containing granules with a brightness comparable to that of alpha granules of pancreatic A cells (27). These cells were scarce and scattered throughout the glands. Cells with bright granules were not encountered elsewhere in the gastrointes-

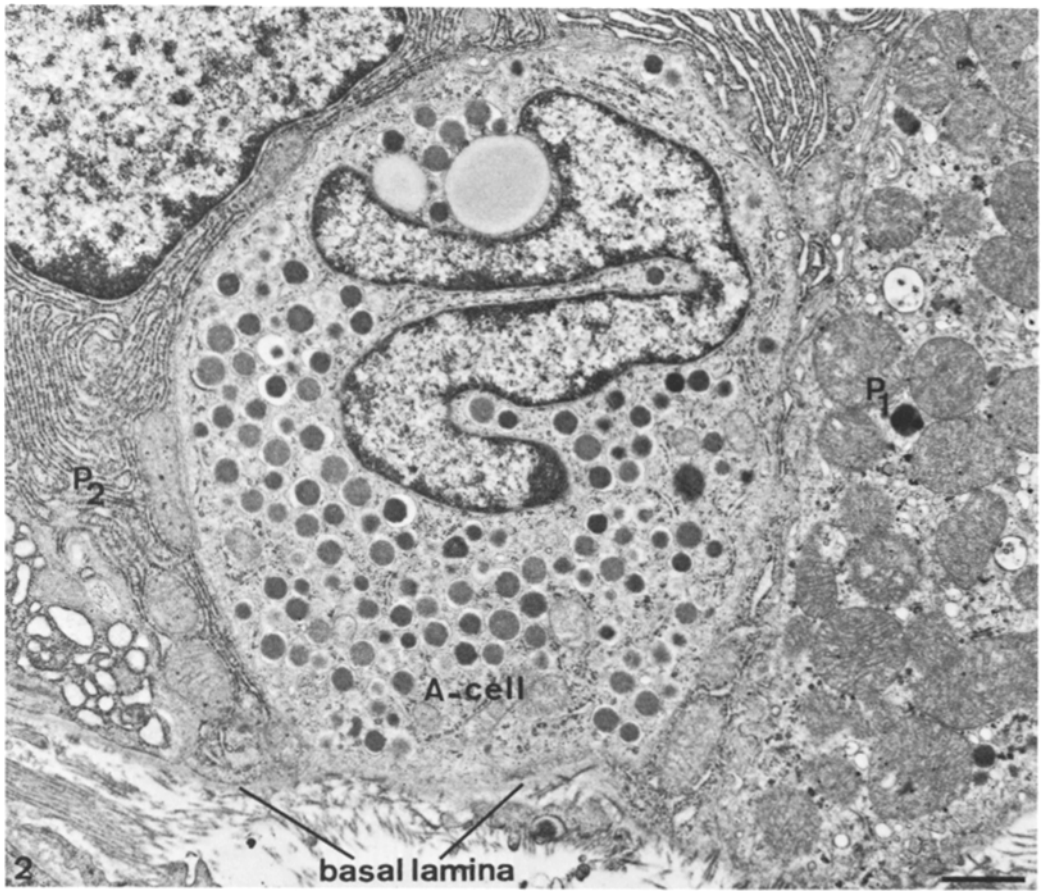

FIGURE 2 Typical gastric A cell of the dog oxyntic mucosa, surrounded by a parietal (P_1) and principal (P_2) cell. Glutaraldehyde fixation. Note the pleomorphic nucleus as compared to that of the gastric A cell shown in Fig. 5. We attribute this difference to the different types of fixative used (Zamboni-de Martino's fixed tissue in Fig. 5 is highly swollen and could explain the roundish form of the nucleus) × 11,000.

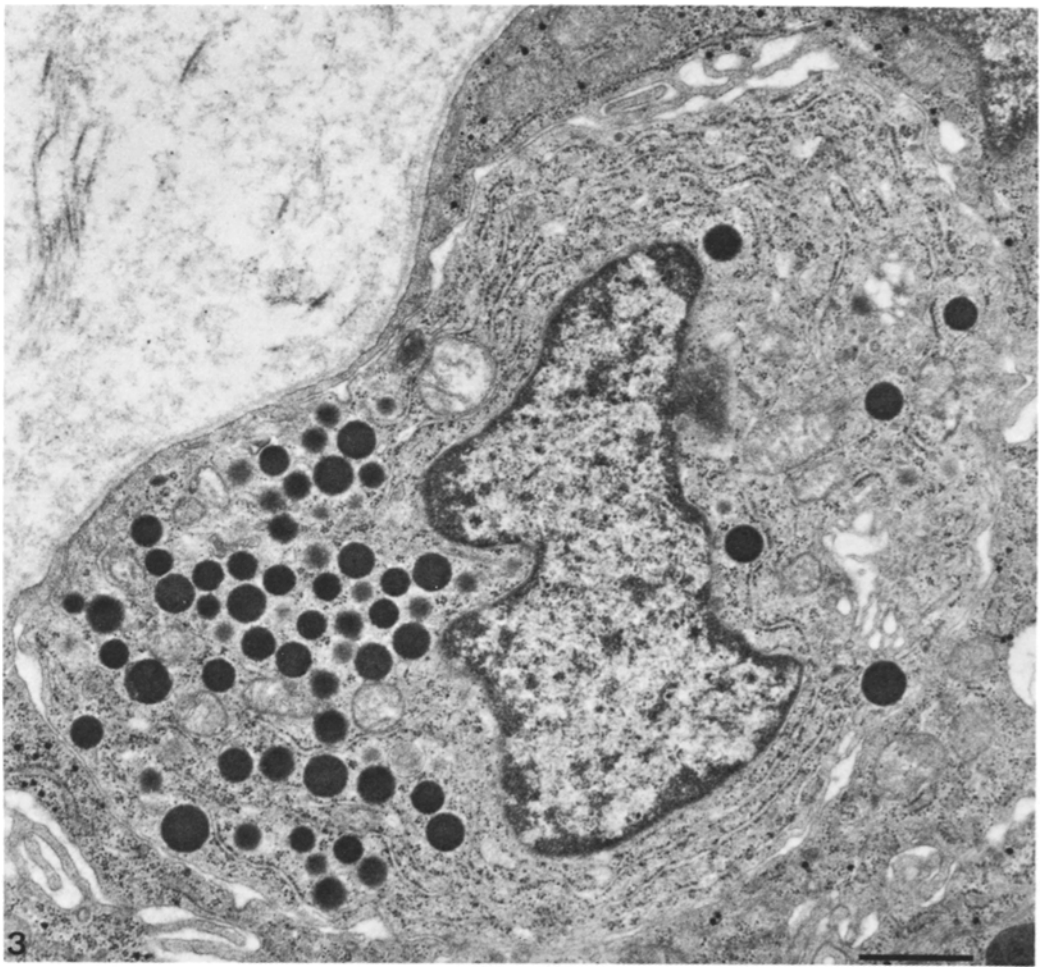

FIGURE 3 Typical A-like cell of the dog oxyntic mucosa. × 15,000.

tinal tract. In paraffin sections, cells showing immunofluorescence with the specific antiserum for pancreatic glucagon were found after both Bouin's and Zamboni-de Martino's fixation. Such cells were present only in the glandular oxyntic mucosa (Fig. 1 *a*) and were not observed in the antrum or in the pyloric region. The immunofluorescent cells, generally of small size, were situated in the middle and deeper portions of the glands, close to the basement membrane. Their apical pole did not seem to reach the glandular lumen. None of the control sections showed specific immunofluorescent staining (Fig. 1 *b*, *c*). Sections treated with the peroxidase-labeled antiserum yielded results similar to those obtained with the immunofluorescence technique (Fig. 6 *a*). Positive cells showed a brownish-colored granular cytoplasm, contrasting with the cytoplasm of the surrounding epithelial cells and of the other endocrine cells which was not stained. Control sections showed no specific immunocytochemical reaction. Sections of the small and large intestine treated for immunofluorescence contained no positive cells.

Electron Microscopy

Electron microscope examination of the glandular oxyntic mucosa of the stomach revealed the presence of endocrine cells with a cytoplasm lighter than that of the neighboring epithelial cells and containing numerous secretory granules morphologically indistinguishable from those of pancreatic A cells (Fig. 2). The granules were located mostly in the basal part of the cell and were very

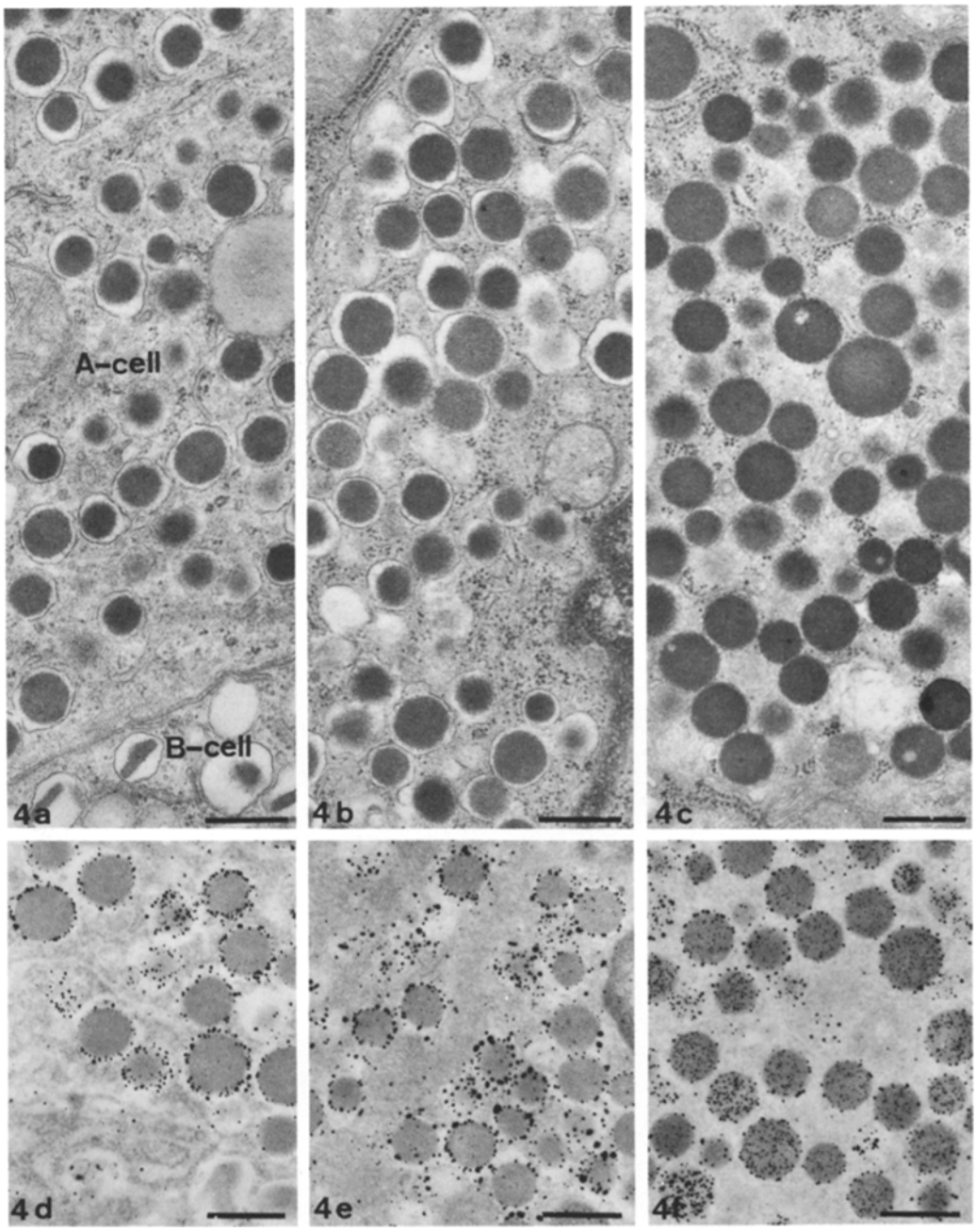

FIGURE 4 Comparison between an A cell from the endocrine pancreas (*a*), a gastric A cell (*b*), and a gastric A-like cell (*c*) showing the similarities and the differences among respective secretory granules. A similar comparison is presented with respect to reactivity with the Grimelius silver staining in Fig. 4 (*d*) (pancreatic A cell), (*e*) (gastric A cell), and (*f*) (gastric A-like cell). (*a*) × 23,000; (*b*) × 23,000; (*c*) × 23,000; (*d*) × 22,000; (*e*) × 22,000; (*f*) × 22,000. The bar represents 0.5 μm.

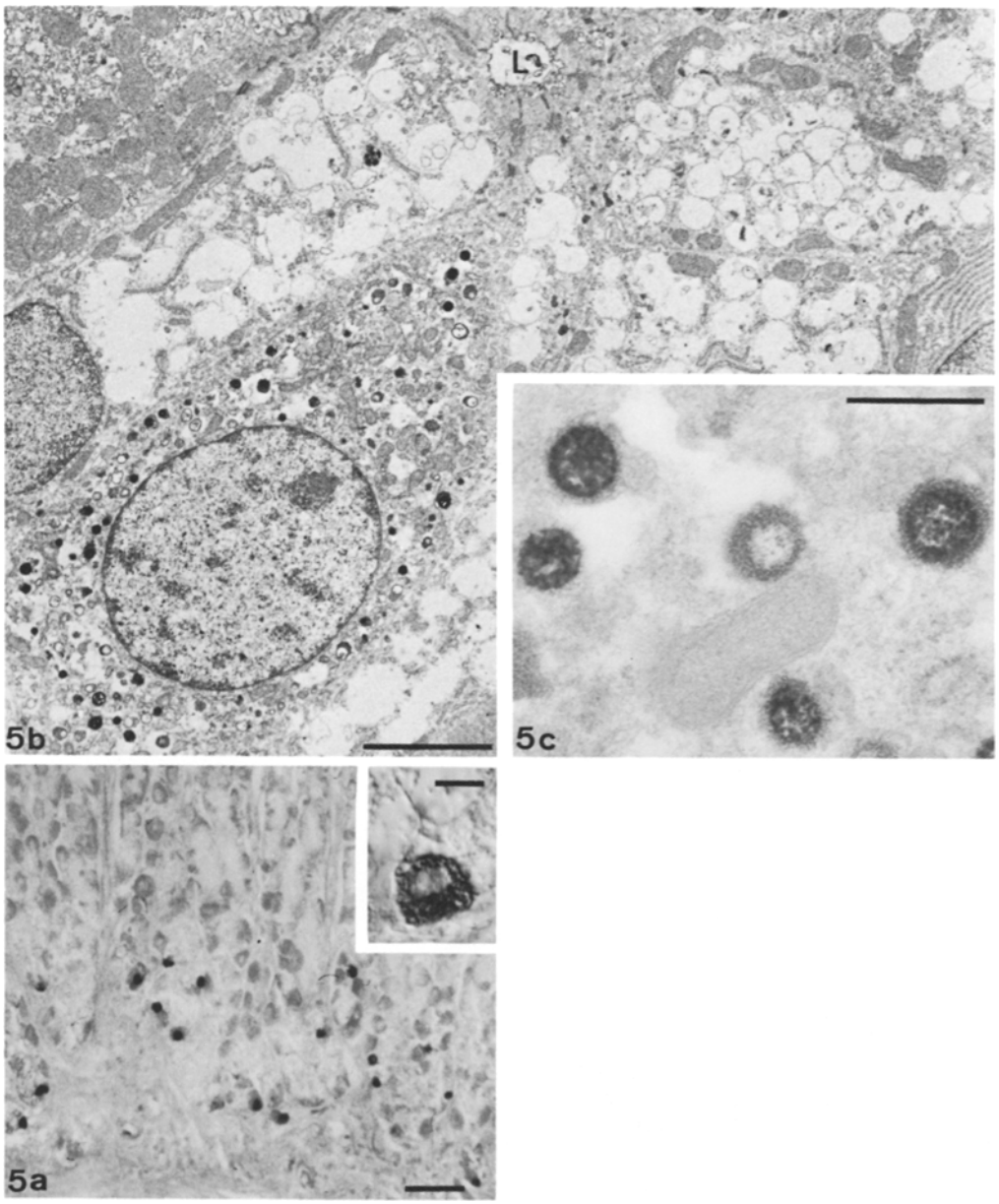

FIGURE 5 Dog oxyntic mucosa. (*a*) Immunoperoxidase staining with 15K antiglucagon serum of a paraffin section, showing several positive cells in the gastric mucosa. *Inset* illustrates a positive cell at higher magnification. ×160; the bar represents 50 μm. *Inset*, × 1,300; the bar represents 5 μm. (*b*, *c*) Thin section from material treated by immunoperoxidase technique for antiglucagon serum (15K). (*b*) Endocrine cell with positively stained granules (A cell); (*c*) high magnification of the immunoperoxidase-stained granules showing a fine-grained reaction product. Note the presence of reaction product in the core and in the halo of the secretory granules. Zamboni-de Martino's fixation. *L* = glandular lumen. (*b*) × 3,500; the bar represents 5 μm. (*c*) × 37,000, the bar represents 0.5 μm.

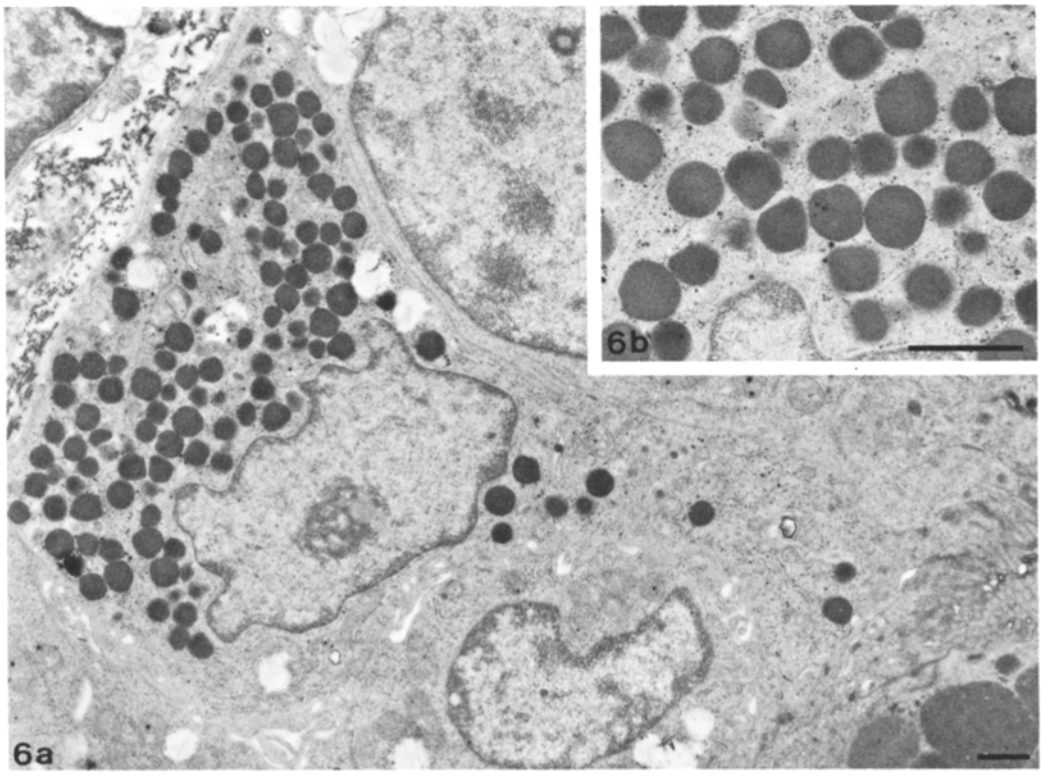

FIGURE 6 Dog ileum. (*a*) Typical L (or EG) cell with dense secretory granules. (*b*) The shape of individual granules is somewhat irregular and there is no halo between the core and the limiting membrane. (*a*) × 7,000; (*b*) × 15,000.

scarce at the apical pole. Granules appeared uniformly round and showed a rather dense and homogeneous secretory product. A feature strikingly similar to the secretory granules of pancreatic A cells was the presence of a clear halo separating the limiting membrane from the dark granule core. The diameter[2] of the secretory granules in the gastric cells was 430 nm as compared to that of the pancreatic A cells which was 440 nm.

In Grimelius silver preparations (Fig. 4 *e*), the silver deposits were selectively located in the clear space between the granule core and the limiting membrane and in the periphery of the granule core, a pattern identical to that found in alpha granules of the pancreatic A cells (Fig. 4 *d*). In view of these profound similarities, these gastric cells will henceforth be called gastric A cells. After incubation with the unlabeled antibody-peroxidase antiperoxidase technique, fine-grained reaction product was found over the secretory granules of endocrine cells recognized above as gastric A cells (Fig. 5). The surrounding parietal and principal cells, as well as other endocrine cells of the oxyntic mucosa, were free of perioxidase staining. The controls were negative. In addition to the gastric A cell, an endocrine cell resembling but not identical to the pancreatic A cell was found in the oxyntic mucosa. Its round, rather electron-dense secretory granules lacked the clear space between the limiting membrane and the granule core (Fig. 3). The diameter of the secretory granules (360 nm) was smaller than that of the gastric A cells. In Grimelius silver preparations, the granules of this cell also showed a difference (Fig. 4 *f*) in that the deposits were consistently distributed over the entire granule core. Other endo-

[2] Diameter was estimated by measuring the width of 500 granular profiles taken at random. Values then obtained were averaged and the resulting mean (d) was corrected to account for the sectioning artifact with the formula: $D = (4/\pi)\cdot\bar{d}$. D value obtained by this method was similar to that calculated by averaging the 100 largest diameters found in the 500 randomly measured granular profiles.

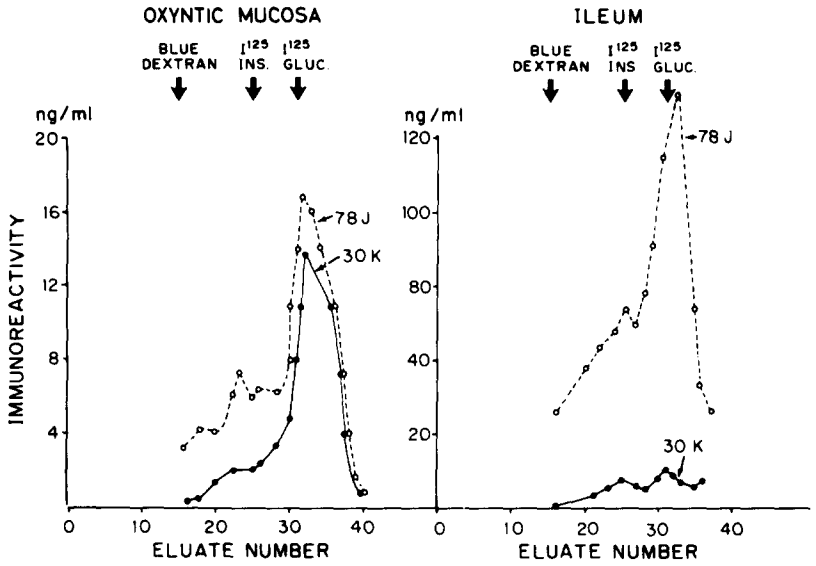

FIGURE 7 Elution patterns of immunoreactivity of extracts of canine oxyntic and ileal mucosa as measured with highly specific antiglucagon serum 30K and GLI-crossreactive antiserum 78J. Chromatography on Biogel P-10 columns.

crine cells were found along the intestinal mucosa. Among these latter were the so-called L (or EG) cells (26, 28) present from the duodenum to the colon (Fig. 6).

Molecular Size and Immunometric Patterns of Glucagon Immunoreactivity and Glucagon-Like Immunoreactivity in Fundus and Ileum

Earlier studies have revealed 30K reactive immunoreactive glucagon (IRG) in the canine oxyntic mucosa but only traces in other areas of the gastrointestinal tract, most notably the duodenum (24). However, the 30K-reactive IRG of the oxyntic mucosa has not been characterized as to its molecular size, nor have immunometric comparisons using antisera of widely differing specificity of the various immunoreactive components been made. Gel filtration of extracts of oxyntic mucosa and small intestine was, therefore, carried out on P-10 Biogel columns, and eluates were assayed with both 30K and 78J antisera. Patterns typified by those of Fig. 7 were observed. Extracts of the oxyntic mucosa contained a large peak of immunoreactivity which consistently eluted with the ^{125}I-glucagon marker. This peak gave relatively similar measurements both in the highly specific glucagon assay using antiserum 30K and in the GLI assay using cross-reacting antiserum 78J, and thus resembled pancreatic glucagon. By contrast, extracts of ileum contained a major peak of immunoreactivity that gave a much higher measurement in the nonspecific 78J assay than in the highly specific 30K assay, suggesting that it represents GLI rather than true glucagon.

DISCUSSION

In the present investigation, a specific antiglucagon serum has been used for the first time in immunocytochemical studies and has revealed the presence of positively stained endocrine cells in the oxyntic mucosa of the dog stomach at both light and electron microscope levels. With this antiserum, which measures less than 3% of the GLI assayed with nonspecific antiglucagon serum 78J, and which does not react at all with secretin, pancreozymin, gastric inhibitory peptide (GIP), or vasoactive intestinal peptide (VIP), no immunofluorescent and immunoperoxidase-positive cells were present outside the oxyntic mucosa. These results, coupled with those of the conventional thin-section electron microscopy and of the silver impregnation technique, thus indicate that at least one endocrine cell type of the stomach, the gastric A cell, is similar in all respects to the pancreatic A cell and might be the source of gastric glucagon.

Accordingly, the immunoreactivity in extracts

of the gastric oxyntic mucosa resembled that of pancreatic glucagon with respect to both molecular size and comparative measurements with antisera of widely contrasting specificity, while that of ileal extracts resembled that of GLI in that measurements with the two antisera differed sharply, reacting only very weakly with the highly specific antiserum. These findings are in accord with data suggesting that the major source of the extrapancreatic glucagon of depancreatized dogs is the gastric oxyntic mucosa (34, 16, 15, 6, 18).

Ultrastructural evidence for the presence in the gastrointestinal tract of cells resembling pancreatic A cells was first reported in 1967 (20). In the original descriptions (21, 8), the endocrine cells with round, electron-dense secretory granules were classified as A-like cells, whether or not there was a clear space between the granule core and the granule-limiting membrane. Further, Capella et al. (2), Vassallo et al. (33), Cavallero et al. (3), and Bussolati et al. (1) reported the presence of cells with large granules (L cells) in the intestine of several mammals, including the dog. These cells were prevalent in the jejunum and ileum and rare in the duodenum.

Using an antiglucagon serum which cross-reacts with GLI, Polak et al. (22) found immunofluorescent cells in those parts of the gastrointestinal tract where A-like and L cells were formerly described as being found. Accordingly, but in spite of the GLI cross-reactivity, these authors proposed the term enteroglucagon cells, or EG cells, for the immunofluorescent cells; these latter were assumed to embody the former A-like cells and the L cells. In view of our experimental results, the situation could be restated as follows: the former A-like cells of the oxyntic mucosa (26, 28) should be divided into two subgroups: (*a*) the gastric A cells identified here ultrastructurally, cytochemically, and immunocytochemically; (*b*) the cells resembling but not identical to the pancreatic A cells. It is to these latter cells only that we propose to restrict now the term A-like cells.[3] Whether the A-like cells in our restricted sense, the formerly defined EG cells (except the gastric A cells), and the L cells represent a single cell type, possibly responsible for the secretion of GLI, awaits now the use of ultrastructural immunochemistry with a specific anti-GLI serum.

[3] In our view, these A-like cells include the formerly defined X cells (27).

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