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

D. BAETENS, C. RUFENER, B. C. SRIKANT, R. DOBBS, R. UNGER,
and L. ORCI

From the Institute of Histology and Embryology, University of Geneva Medical School, Geneva,
Switzerland, the Veterans Administration Hospital, Dallas, Texas 75219, and the Department of
Internal Medicine, The University of Texas, Southwestern Medical School at Dallas, Texas 75219

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 indistinguish-
able 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 Suther-
land 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 immunolog-
ic properties indistinguishable from those of
pancreatic glucagon (24). This polypeptide has
been clearly differentiated from the so-called “glu-
cagon-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 glucu-
gon. Immunocytochemistry was employed in con-
junction with other cytological techniques at both
the light and electron microscope level and with
immunochemical characterization of tissue ex-
tracts.

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 Mar-
tino’s fluid (35) for immunofluorescence and immunocy-
tochemical techniques with the light microscope. Sam-
ple 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 H₂O₂ 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.
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 x 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, 125I-insulin, and 125I-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 gastrointestinal tract.
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. 1a) 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. 1b, c). Sections treated with the peroxidase-labeled antiserum yielded results similar to those obtained with the immunofluorescence technique (Fig. 6a). 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) $\times 23,000$; (b) $\times 23,000$; (c) $\times 23,000$; (d) $\times 22,000$; (e) $\times 22,000$; (f) $\times 22,000$. The bar represents 0.5 $\mu$m.
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. Zamhoni-de Martino's fixation. L = glandular lumen. (b) × 3,500; the bar represents 5 μm. (c) × 37,000, the bar represents 0.5 μm.
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 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. 4e), 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. 4d). 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 peroxidase 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. 4f) in that the deposits were consistently distributed over the entire granule core. Other endothelial...
Endocrine 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 125I-glucagon marker. This peak gave relatively similar measurements both in the highly specific glucagon assay using antiserum 30K and in the GL1 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 GL1 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 GL1 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 antiguacagon 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. 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 immunocytochemistry with a specific anti-GLI serum.

We wish to thank A. Perrelet for critical reading of the manuscript. Mrs. M. Sidler-Ansermet for help with photography. Mrs. I. Fuglister, Mrs. A. Irribara and Mr. M. Baumann for technical assistance.

Parts of this work were presented at the Union Libre des Anatomistes des Universités Suisses, Basel, October 1974, and at the Société Française de Microscopie Electronique, Paris, February 1975.

This work was supported by Fonds National Suisse de la Recherche Scientifique, grant 3.553.75, and by the National Institutes of Health, grant AM 02700—15.

Received for publication 28 October 1975, and in revised form 14 January 1976.

REFERENCES

1. BUSSOLATI, G., C. CAPPELLA, E. SOLCIA, G. VASSALLO, and P. VEZZADINI. 1971. Ultrastructural and immunofluorescent investigations on the secretin cell in the dog intestinal mucosa. Histochemie. 26:218–227.

2. CAPELLA, C., E. SOLCIA, and G. VASSALLO. 1969. Identification of six types of endocrine cells in the gastrointestinal mucosa of the rabbit. Arch. Histol. Japn. 30:479–495.

3. CAVALLERO, C., E. SOLCIA, G. VASSALLO, and C. CAPELLA. 1969. Cellule endocrine della mucosa gastro-enterica ed ormoni gastro-intestinali. Rend. R. R. Gastroenterol. 1:51–61.

4. CAVALLERO, C., E. SOLCIA, G. VASSALLO, C. CAPELLA, and G. BUSSOLATI. 1970. Cytology, cytochemistry and ultrastructure of glucagon-secreting cells. Acta Isot. 10:1961–1969.

5. COONS, A. H., E. H. LEDUC, and J. M. CONNOLLY. 1955. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. J. Exp. Med. 102:49–63.

6. DOBBS, R. H., SAKURAI, H. SASAKI, G. R. FALOONA, I. VALVERDE, D. BAETENS, L. ORCI, and R. H. UNGER. 1975. Glucagon: role in the hyperglycemia of diabetes mellitus. Science (Wash. D. C.). 187:544–547.

7. FALOONA, G. R., and R. H. UNGER. 1974. Glucagon. In Methods of Hormone Radioimmunoassay. B. M. Jaffe and H. R. Behrmann, editors. Academic Press Inc., New York. Chap. 18. 317–330.

8. FORSSMANN, W. G., L. ORCI, R. PICET, A. E. RENOLO, and C. ROUILLER, 1969. The endocrine cells in the epithelium of the gastrointestinal mucosa of the rat. J. Cell Biol. 40:692–715.

9. GRIEBEL, R. C., and M. J. KARNOVSKY. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J. Histochem. Cytochem. 14:291–302.

10. GRIMELIUS, L. 1968. A silver nitrate stain for c₃ cells...
11. Kawaoi, A., and P. K. Nakane. 1973. An improved method of conjugation of peroxidase with proteins. Fed. Proc. 32:840. (Abstr.)

12. Kenny, J. A. 1955. Extractable glucagon of the human pancreas. J. Clin. Endocrinol. Metab. 15:1089–1105.

13. Kubés, L., K. Jirasek, and R. Lomsky. 1974. Endocrine cells of the dog gastrointestinal mucosa. Cytologia. 39:179–194.

14. Luft, J. H. 1961. Improvements in epoxy resin embedding methods. J. Biophys. Biochem. Cytol. 9:409–414.

15. Mashiter, K., P. E. Harding, M. Chou, G. D. Mashiter, J. Stout, D. Diamond, and J. B. Field. 1975. Persistent pancreatic glucagon but not insulin response to arginine in pancreatectomized dogs. Endocrinology. 96:678–693.

16. Matsuyama, T., and P. P. Foa. 1974. Effects of pancreatectomy and of enteral administration of glucose on plasma insulin, total and pancreatic glucagon. Diabetes. 23:344. (Abstr.)

17. Millong, G. 1961. Advantages of a phosphate buffer for OsO4 solutions in fixation. J. Appl. Phys. 32:1637.

18. Muñoz, L., E. Blazquez, and R. Unger. 1975. Gastric alpha-cell function in normal and diabetic dogs. Diabetes. 24:411. (Abstr.)

19. Orci, L., W. G. Forssmann, W. Forssmann, and C. Rouiller. 1968. Electron microscopy of the intestinal endocrine cells. Comparative study. Fourth European Conference on Electron Microscopy, Rome, 1968. S. D. Bocciarelli, editor. Tipografia Poliglotta Vaticana, Rome. 2:369–370.

20. Orci, L., W. G. Forssmann, and R. Pictet. 1967. Mise en évidence des types de cellules à granulations denses dans le système digestif du rat. J. Microsc. (Paris). 6:74 a. (Abstr.)

21. Orci, L., R. Pictet, W. G. Forssmann, A. E. Rennold, and C. Rouiller. 1968. Structural evidence for glucagon producing cells in the intestinal mucosa of the rat. Diabetologia. 4:56–67.

22. Polak, J. M., S. Bloom, I. Coulling, and A. G. E. Pearse. 1971. Immunofluorescent localization of enteroglucagon cells in the gastrointestinal tract of the dog. Gut. 12:311–318.

23. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electronopaque stain in electron microscopy. J. Cell Biol. 17:208–212.

24. Sasaki, H., B. Rubalcava, D. Baetens, E. Blazquez, C. B. Srikant, L. Orci, and R. H. Unger. 1975. Identification of glucagon in the gastrointestinal tract. J. Clin. Invest. 56:135–145.

25. Solcia, E. 1962. Su una nuova tecnica per evidenziare le cellule a granuli alfa delle isole pancreatiche. Boll. Soc. Ital. Biol. Sper. 38:1189–1191.

26. Solcia, E., A. G. E. Pearse, D. Grube, S. Kobayashi, G. Bussolati, W. Creutzfeldt, and W. Gepts. 1973. Revised Wiesbaden classification of gut endocrine cells. Rev. Gastroenterol. 5:13–16.

27. Solcia, E., and R. Sampaio. 1965. Cytologic observations on the pancreatic islets with reference to some endocrine-like cells of the gastrointestinal mucosa. Z. Zellforsch. Mikrosk. Anat. 68:689–698.

28. Solcia, E., G. Vassallo, and C. Capella. 1970. Cytology and cytochemistry of hormone-producing cells of the upper gastro-intestinal tract. In Origin, Physiology and Pathophysiology of the Gastrointestinal Hormones. W. Creutzfeldt, editor. F. K. Schattauer-Verlag, Stuttgart. 3–29.

29. Sternberger, L. A. 1973. Enzyme immunocytochemistry. In Electron Microscopy of Enzymes, Principles and Methods. M. A. Hayat, editor. Van Nostrand Company, Inc., New York. 150–181.

30. Sutherland, E. W., and C. De Duve. 1948. Origine and distribution of the hyperglycemic-glycogenolytic factor of the pancreas. J. Biol. Chem. 175:663–674.

31. Valverde, L. A. 1973. Enzyme immunocytochemistry. In Electron Microscopy of Enzymes, Principles and Methods. M. A. Hayat, editor. Van Nostrand Company, Inc., New York. 150–181.

32. Vassallo, G., C. Capella, and E. Solcia. 1971. Grimalius’ silver stain for endocrine cell granules, as shown by electron microscopy. Stain Technol. 46:7–13.

33. Vassallo, G., E. Solcia, and C. Capella. 1969. Light and electron microscopic identification of several types of endocrine cells in the gastrointestinal mucosa of the cat. Z. Zellforsch. Mikrosk. Anat. 98:333–356.

34. Vranic, M., S. Pek, and R. Kawamori. 1974. Increased “glucagon immunoreactivity” (IRG) in plasma of totally depancreatized dog. Diabetes. 23:905–912.

35. Zamboni, L., and L. de Martino. 1967. Buffered picric acid-formaldehyde: a new rapid fixative for electron microscopy. J. Cell Biol. 35:148 a. (Abstr.)