Gastric endocrine cells share a clonal origin with other gut cell lineages

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Summary

There has been considerable debate about the ontological origin of gut endocrine cells as being either from the neural crest (or primitive epiblast) or from the endodermal stem cell. We have attempted to define the ontological origin of endocrine cells by applying an experimental system that uses a marker to identify one of the two phenotypes present in chimaeric mice as suggested by Ponder et al. (1985).

This study involved two separate experiments. The first made use of the unique staining properties of Dolichos biflorus agglutinin (DBA), a lectin that binds to the N-acetyl galactosamine sugar residues present on the surface of C57Bl mouse gut, but absent from RoRIII mouse gut, in C57Bl↔RoIII mouse chimaeras at the ultrastructural level. A four-stage procedure for staining at the EM level was developed. Although mature villous endocrine cells stained for DBA, immature endocrine cells did not, either in the positive crypts of chimaeric mouse gut or in gut from C57Bl positive controls.

Thus a second marker was chosen. This experiment combined immunocytochemistry (to identify gastric antral gastrin cells chosen as a representative neuroendocrine cell) with in situ DNA hybridization for the mouse male chromosome repeat sequence PY 353 (to identify XY cells) in XX↔XY chimaeric mice. This study showed that the sex chromosomal pattern in the gastrin cells parallels that of other cells in the same gastric gland and therefore are clonal with them. This suggests that gut endocrine cells share a common stem cell with other epithelial cell lineages in the antrum and are endodermally derived.

Key words: enteroendocrine cells; in situ hybridization; Y probe; lectin–gold staining; electron microscopy; immunocytochemistry; gastrin; chimaeras.

Introduction

The controversy concerning the origin of gut endocrine cells has been hotly debated since the early part of this century. Danisch (1924) suggested a neural origin with enteroendocrine cells migrating from the solar plexus into the intestinal epithelium. At a similar time Masson (1928, 1932) strongly championed an endodermal origin on the basis of embryological studies and observations of carcinoid tumours. More recently Pearse, going against the ground swell of opinion, which was moving towards a common enterocyte origin, proposed the neural crest hypothesis, incorporating it into his APUD (amine precursor uptake and decarboxylation) concept (Pearse 1966a,b) which took note of the remarkable cytochemical similarities between apparently unrelated 'neuroendocrine' cells. In its original form, the neural crest theory of Pearse has now been shown to be wrong, principally by the extirpation experiments in chick embryos of Andrew (1963, 1974, 1976) and by the use of quail–chick chimaeras in which the nuclei from each species are easily distinguished (Fontaine and Le Douarin, 1977).

Accepting that the neural crest itself is not the source of gut endocrine cells, Pearse has recently modified his neural crest theory. In 1977 he proposed that endocrine cells are derived from cells of the epiblast that have been 'neuroendocrine-programmed' from the outset and later expanded on this hypothesis (Pearse, 1982, 1984). The implication is that endocrine cells have a stem cell pool separate from the other cell lineages in the gastrointestinal tract. Scothorne (1988) has suggested support for the concept of preprogramming, at least at the level of organogenesis, in his interpretation of the work of Svajger et al. (1981). However, the latter authors were much more cautious in the conclusions they drew from their own data.

Although Kirkland (1988) has shown in an elegant experiment that, by repeated single cell cloning of human colonic carcinoma cell lines, endocrine cells and other epithelial cell lineages can have a common origin in tumours, it is not justified to extrapolate from this to the normal situation.

The other line of scientific investigation that is relevant is the interesting experiment using the differ-

ential staining properties of *Dolichos biflorus* in chimaeric mice as carried out by Ponder *et al.* (1985); this work showed that all the cells in each crypt were always stained the same way, either positively or negatively, by *Dolichos biflorus* agglutinin, indicating that crypts were monoclonal and that all cell lineages had a common stem cell, at least in the case of Paneth, mucous and columnar cells. However, they were not able to extend their observations in a light microscopical study to endocrine cells because of their very small luminal surface area. A similar study in G6PD deficient mice using enzyme histochemistry gave an identical result (Griffiths *et al.* 1988) but again the resolution in frozen sections was inadequate to comment on the clonality of endocrine cells.

None of the experimental evidence to date, with the single potential exception of a marker in chimaeric mice, can definitively disprove the modified neural crest theory. The aim of these experiments was to take two markers and use the Ponder model in order to try and prove whether endocrine cells are indeed clonal with other gut epithelial cells. If endocrine cells share a common origin with epithelial cell lineages, they will mark the same way as adjacent cells in the same crypt or gland. If they have a separate 'endocrine programmed' stem cell, the pattern will be random, and as often as not, at variance with adjacent cells.

Materials and methods

Experiment 1

The mouse gut used was from C57Bl↔RoRIII chimaeras obtained from Dr B. Ponder (Sutton) and either fixed in formalin and paraffin-embedded, or fixed in 0.25 % glutaraldehyde, since higher concentrations abolish lectin staining (B. Ponder *via* G. Wilson, personal communication) and embedded in Araldite.

Lectin staining

The paraffin was removed with CNP30. The resin was removed in semithin sections by a saturated solution of sodium hydroxide in absolute ethanol and ultrathin sections were etched with 10 % hydrogen peroxide for 15 min before staining. Sections were subsequently washed in Tris buffer 0.01 M pH 7.4 three times. They were then stained with *Dolichos biflorus* agglutinin (DBA), obtained from Sigma, at a concentration of 1:60 for light microscopic sections and 1:300 for EM sections overnight at 8°C, followed by rabbit anti-lectin antibody (Sigma) 1:20 at light level and 1:300 for EM. Subsequently, biotinylated goat anti-rabbit antibody (Vector) was applied at 1:150 or 1:300 at light and electron microscopy, respectively. Lastly, streptavidin–fluorescein conjugate (Amersham) 1:100 for light microscopy or streptavidin–gold 1:5 (Janssen with 10 nm particles) for EM sections was used. Each of the last three stages was incubated for 30 min at room temperature. Between each stage sections were rinsed three times with Tris. Sections prepared for light microscopy were then mounted in Hydromount. Sections for EM were washed in filtered distilled water, dried in air and negatively stained by aqueous uranyl acetate followed by Reynold's lead citrate, rinsed in distilled water and dried. Sections for light microscopy were viewed on a Leitz Dialux

ultraviolet microscope; ultrathin sections were examined on an AEI 801 electron microscope.

Initially some of the staining on ultrathin sections was found to be uneven but it was found that, if the grids were wiped gently on the side opposite to the sections on fibre-free absorbent paper every 60 s during the etching process, the evenness of staining improved considerably. Negative staining with alcoholic uranyl acetate abolished all traces of lectin binding but aqueous uranyl acetate did not have the same effect.

Controls

(1) A negative control with the appropriate buffer for the first layer was always included.

(2) Positive controls. When chimaeric sections were being stained, a C57Bl positive control section was always included. In C57Bl mouse gut, all cells in every crypt should be positive, since all cells have a common origin with respect to positivity for lectin.

(3) Absorption control. Preincubation of the DBA with *N*-acetyl galactosamine (i.e. the appropriate sugar) or *N*-acetyl glucosamine (i.e. the *in*appropriate sugar) at 0.1 M concentration was done and gave appropriate results.

Experiment 2

Mouse aggregation chimaeras were prepared at the Institute of Animal Physiology and Genetics Research by Dr Fundele as previously described (Fundele *et al.* 1989). One mouse was a CFLP↔F_2 chimaera and the other was a BALB/c↔F_2 chimaera (where F_1=C57Bl/6 female×CBA male). Pieces of stomach were fixed in formalin, processed routinely and embedded in paraffin. 3 μm sections were mounted on silane-coated slides.

Immunostaining for gastrin

The paraffin was removed with CNP 30 and the slides rehydrated through graded alcohols. The sections were blocked with normal swine serum 1:20 for 10 min and incubated at room temperature for 4 h with a polyclonal antigastrin antibody (1198) 1:4000 kindly supplied by Professor J. Polak, RPMS. After washing three times with 0.01 M phosphate-buffered saline (PBS) pH 7.0, 5 min each, they were then incubated with biotinylated swine anti-rabbit antibody 1:500 (Dakopatts) for 45 min. Washing in PBS×2 as before was followed by one 5 min wash in Tris 0.05 M pH 7.4. Then avidin–biotin–alkaline phosphatase complex (Vector ABC alkaline phosphatase kit), which had been made up according to manufacturer's instructions, was applied to the sections for 30 min. The sections were washed twice in Tris and once in 0.2 M Tris–HCl buffer pH 9.0 for 5 min and developed in 2.5 % naphthol ASTR (Sigma) in Tris–HCl buffer with 0.05 % sodium nitrite and 0.05 % new acid fuchsin for 15 min at 37 °C. Sections were left at 4 °C in PBS overnight before *in situ* hybridization was carried out.

In situ *hybridization for the Y chromosome repeat sequence*

The probe used, pY 353, was made by Dr C. Bishop. It was nick translated with digoxigenin-labelled UTP (Boehringer) in the manner previously described for biotinylation by Chan *et al.* (1985). The probe size was found to be approximately 180–400 base pairs long by gel electrophoresis and Southern blotting. For *in situ* hybridization, the slides were first digested with either 0.1 % pepsin (Sigma) in 75 mM NaCl with 0.5 % triton (Sigma) and 50 mM HCl at 37 °C for 15 min, or predigested in DNAse buffer (50 mM Tris with 10 mM $MgCl_2$) for 20 min at 55 °C then digested in proteinase K at 50 μg ml^{-1}

in 300 mM sodium acetate. 200 mM Tris, 50 mM EDTA, 1 % SDS and 1 % *N*-lauroyl sarcosine also at 55 °C for 15 min on a shaker. They were washed in half-strength PBS pH 7.2 for 10 min, rinsed in distilled autoclaved water and allowed to dry. The probe was added at 500 ng ml^{-1} to a hybridization mix of 10 % dextran sulphate (Pharmacia), 2×PBS, 50 % deionised formamide (Sigma), 2.5 mM EDTA pH 7.0, and 400 μg ml^{-1} sheared herring sperm DNA (Sigma). 10 μl was placed on 22×22 mm glass coverslips and the sections lowered to contact, sealed with high vacuum grease (Dow Corning), and denatured at 95 °C for 25 min. Hybridisation was performed at 37 °C for 1.5 h. After removal of the coverslips, the sections were washed in prewarmed PBS pH 7.2 with 0.5 % triton on a shaker three times 5 min and then in prewarmed half-strength PBS three times 5 min at 65 °C. The sections were blocked for 20 min at 37 °C in PBS containing 5 % bovine albumin (Sigma) and 0.5 % triton. This was followed by a 30 min incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer) 1:750 at 37 °C. Three 5 min washes in PBS/0.5 % triton were performed at room temperature, followed by three 3 min washes in PBS and two 3 min washes in 0.1 M Tris/MgCl$_2$ buffer pH 9.0. The sections were then developed in a nitro blue tetrazolium (NBT)/bromo-chloro-indolyl phosphate (BCIP) solution.

Results

Experiment 1

Sections were stained for light microscopy in order to check that the technique would work before proceeding to electron microscopy, and this showed crypts to be either all positive or all negative as described by Ponder *et al.* (1985). In the sections prepared for ultrastructural examination, there was clear outlining of the luminal surface by the lectin–gold complex, which was visible even at low power. Staining was strong and reproducible. On high power, gold particles could be seen sticking to the tips of the microvilli, and also in small vesicular structures (at the apex of the cells), which are probably bits of Golgi apparatus transporting the sugar to the surface. The search for endocrine cells was confined to the crypts, since villous epithelial cells are polyclonal and are known to be derived from four or five crypts (Ponder *et al.* 1985).

Chimaeras

Unfortunately crypt endocrine cells did not show lectin–gold binding on the surface even in C57Bl **positive control** sections in which all cells would be expected to be positive regardless of which theory for endocrine cell origin was correct (Figs 1 and 2). Epithelial cells on each side were positive for lectin and gold was seen being transported up to the surface in these cells but not in the endocrine cells. It was noted that the microvilli of the crypt endocrine cells were very rudimentary. One positive endocrine cell was seen low on a villus but this cell had well formed microvilli. It was concluded that crypt endocrine cells do not bind lectin but villous ones do have this capability. The most likely explanation for these observations would seem to be that the binding sugar, *N*-acetyl galactosamine, is manufactured by columnar and mucous cells, trans-
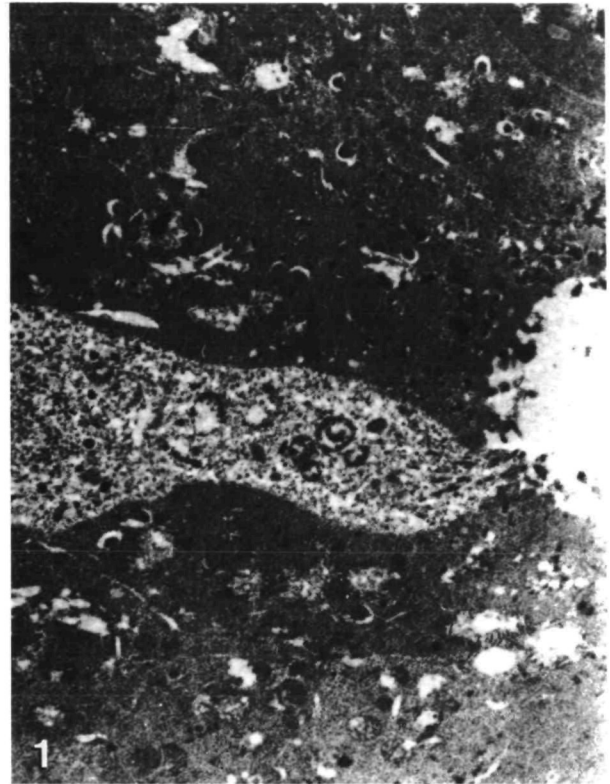

Fig. 1. Low-power electron micrograph from a crypt of a C57Bl (positive control) animal stained with DBA. Endocrine cell arrowed. (×6400).

ported to the surface and largely present in the mucus which then sticks to the tips of the microvilli. This would account for the observation that endocrine cells with well-developed microvilli can bind DBA, and would also fit with the absence of gold seen packeted near the cell surface of endocrine cells.

Experiment 2

The two XX↔XY mice showed patches of glands that were positive for the Y spot as judged by *in situ* hybridization alternating with patches that were negative (Fig. 3). A normal non-chimaeric control male mouse was uniformly positive. If there was a separate stem cell for gut endocrine cells, the distribution of XX and XY gastrin-positive cells in the chimaeras should be random with respect to the other cells in the gastric glands. A total of 409 and 129 antral gastrin cells were analysed in the XX↔XY chimaeras and a control non-chimaeric male mouse respectively, the results of which are shown in Table 1. This shows that 105 out of 160 gastrin cells were positive for the Y chromosome in XY patches; that is there was 66 % concordance (Fig. 4), which was the same as in the male control mouse for gastrin cells (86 out of 129, Fig. 5) and for other epithelial cells (95 out of 150 were positive). Presumably 100 % concordance was not obtained in all male cells because the relevant part of the chromosome did not always appear in the plane of section. There were no Y spots seen in a female control mouse. In the

chimaeras, two gastrin cells were Y spot positive in apparently negative gastric glands, but in one of these there was another Y spot positive cell in the same gland. This may imply that there are occasional mixed glands in the gastric antrum. However, in view of previous work (Ponder *et al.* 1985; Griffiths *et al.* 1988), a much more likely explanation is that the hybridization or digestion had been focally uneven.
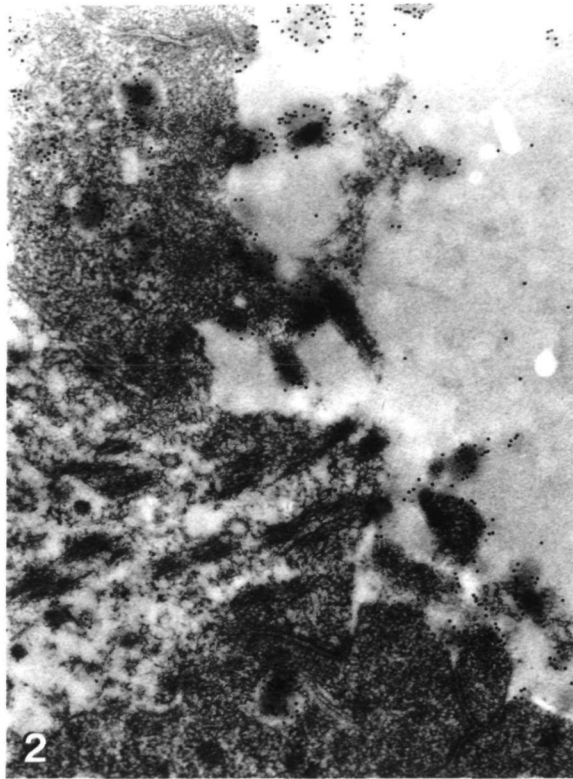

Fig. 2. High-power of the luminal surface of the cells shown in Fig. 1 shows that the absence of lectin–gold staining on the microvilli or in the apical cytoplasm of the endocrine cell. Note the gold in mucin droplets in the two adjacent cells (×35 000).

Table 1. *Distribution of Y spot positive and Y spot negative gastrin cells (G cells) in XY animals and XX↔XY chimaeras*

XY mouse		XX↔XY chimaeras			
Positive gastric glands		Positive gastric glands		Negative gastric glands	
Positive G cells	Negative G cells	Positive G cells	Negative G cells	Positive G cells	Negative G cells
86	43	105	55	2	246

In the control male mouse about two thirds (86/129) of gastrin cells were positive for Y spots. A similar result was found in positive gastric glands in the chimaeras (105/160).

The distribution of positive and negative gastrin cells in the chimaeric mice was significantly different from that expected by chance (Chi square=208, d.f.=1, $P<0.001$).

Discussion

There is a considerable body of evidence favouring an endodermal origin for gut endocrine cells. In addition to the experiments already cited, evidence comes from kinetic studies in mice (Cheng and Leblond, 1974*a,b,c*), the culture of tumour cell lines (Goldenberg and Fisher, 1970; Cox and Pierce, 1982), and the observation of endocrine cells in gut adenocarcinomas (Azzopardi and Pollock, 1963; Tahara *et al.* 1982; Bonar and Sweeney, 1986; Smith and Haggitt, 1984; Iwafuchi *et al.* 1987). In teratomas, the frequent presence of endocrine cells in gut-type epithelium in the absence of nervous tissue also suggests a common stem cell (Bosman and Louwerens, 1981; Brodner *et al.* 1980). Other supportive evidence comes from (1) the observation of amphicrine cells, which show dual differentiation (Melmed *et al.* 1972; Cheng and Leblond, 1974*a,b,c*; Melmed, 1979; Eusebi *et al.* 1981; Bonar and Sweeney, 1986; Tahara *et al.* 1982), (2) the finding that metaplasias and heterotopias appear to be single step changes (Slack, 1986), and (3) several experimental systems using regeneration as a model for studying endocrine cell production (Matsuyama and Suzuki, 1970; Hattori *et al.* 1982; Boquist, 1968).

However, the only experimental system that could disprove Pearse's modified neural crest theory is the use of a marker that identifies cells from one of the two strains present in a chimaeric mouse. If endocrine cells share a common stem cell origin with the other epithelial cell lineages in the gastrointestinal tract, they will always have the same staining pattern as adjacent cells; that is, they will be monophenotypic. Our conclusion from the *in situ* hybridization experiment is that antral gastrin cells stain the same way as adjacent epithelial cells. Therefore the stem cell is common to all epithelial cell lineages in the stomach, and it is no longer tenable to postulate a separate neuroendocrine stem cell. The only other possible explanation of our results is local co-segregation of cells that have migrated from separate sites during embryogenesis. This seems intrinsically unlikely and is made even less plausible in view of the observation that there is no correlation in the staining pattern for Y spots in the epithelium and the underlying lamina propria (Fig. 3).

Previous attempts to demonstrate the clonal origin of gut endocrine cells and other gut cell lineages, using chimaeric mice (Ponder *et al.* 1985) or mice heterozygous for an abnormal X-linked G6PD gene (Griffiths *et al.* 1988) have been unsuccessful because the resolution of the marker was insufficient to allow recognition of the endocrine cell lineage. It is clear that a combined immunocytochemical and *in situ* hybridisation study in XX/XY chimaeric mice provides a novel method with sufficiently high resolution to make definitive conclusions about the clonal origin of specific but less abundant cell lineages.
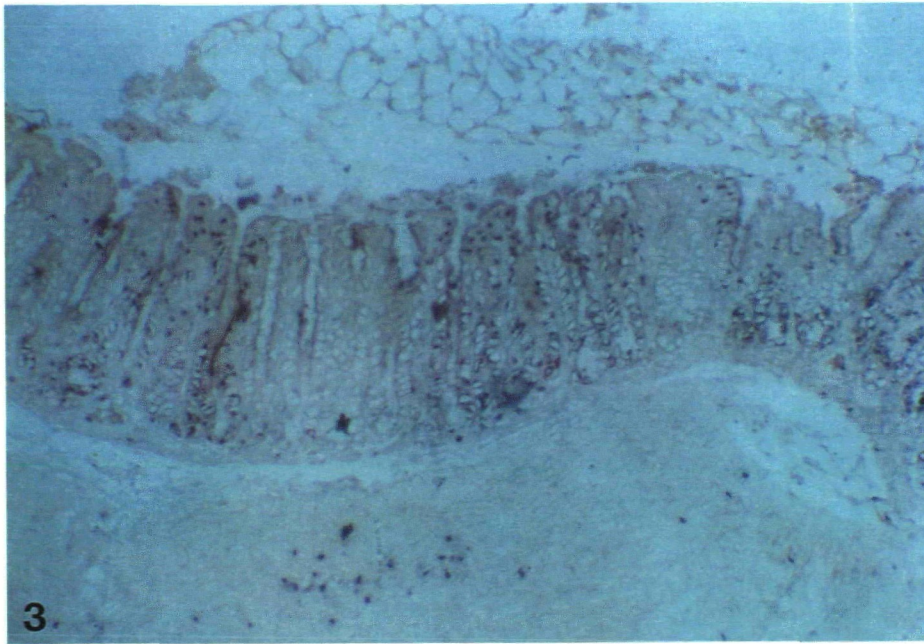
Fig. 3. Low-power photograph (×112) in an XX↔XY chimaera showing the Y spot pattern in the mucosa and the underlying tissue. Note that they do not coincide, an expected finding in view of their different embryological origins.
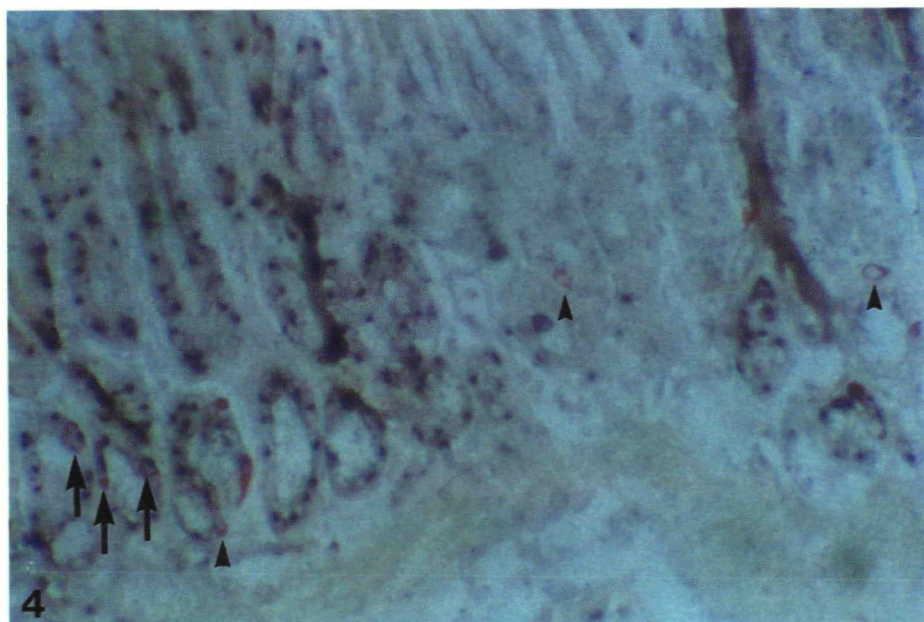

Fig. 4. Photograph of antrum (×260) from an XX↔XY chimaera stained immunocytochemically for gastrin and by *in situ* DNA hybridization for the Y chromosome repeat sequence. Y negative gastrin cells (small arrows) are present in both Y positive and Y negative gastric glands, but Y positive gastrin cells (large arrows) are seen only in Y positive glands (with rare exceptions – see Results).
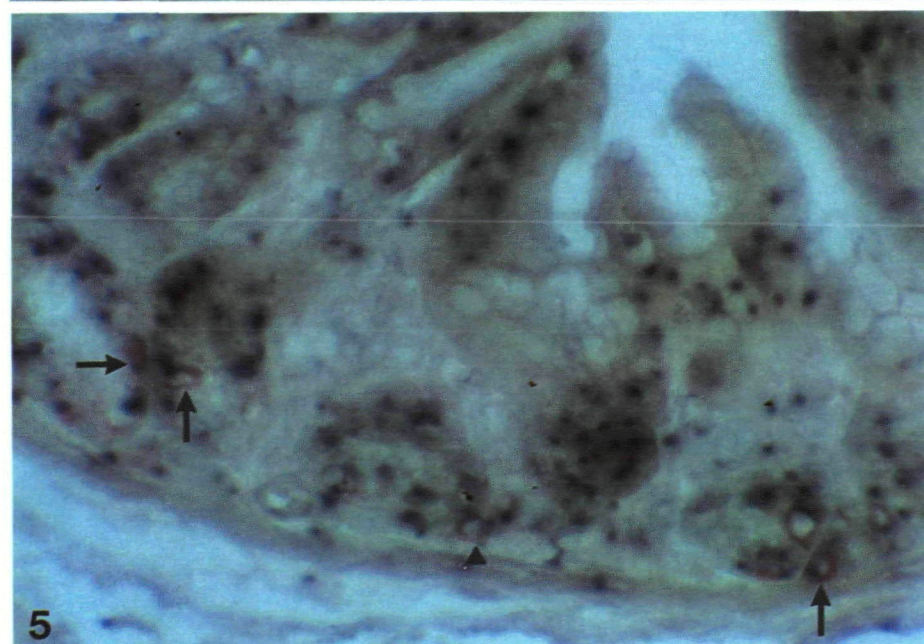

Fig. 5. Photograph showing antrum (×325) from a normal non-chimaeric male mouse stained for gastrin cells and the Y spot. All the glands are Y spot positive and although the majority of gastrin cells are Y positive (large arrows) there are still some that do not show this (small arrows).

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