VIP-, substance P- and met-enkephalin-immunoreactive innervation of the human gastroduodenal mucosa and Brunner's glands

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SUMMARY VIP-, substance P- and met-enkephalin-containing innervation of the human gastroduodenal mucosa and Brunner's glands was studied by immunocytochemistry on whole mount tissue preparations. A dense VIP-containing nerve supply was found around fundic and pyloric glands, while the few and scattered substance P-immunoreactive fibres tended to run across the full thickness of the gastric mucosa. In the duodenum, both VIP and substance P were present in a striking nerve network in the villi as well as in the muscularis mucosae and around blood vessels. Both peptides were also immunostained in nerve bundles and neuronal perikarya between the lobules of Brunner's glands, while only very few fibres reached the proximity of acinar cells. Met-enkephalin-immunoreactivity was detected in a small number of nerve fibres, virtually confined to the basal parts of the mucosa and to the duodenal submucous plexus.

Several regulatory peptides are known to be present in nervous structures in the mammalian gut. Of these, VIP,¹ substance P² and enkephalin³ have been demonstrated in the human.

We have recently studied in detail the distribution of nerve fibres immunoreactive for these three peptides in the human lower gut mucosa, using microdissected and whole mounted tissue preparations.⁴ We report here a similar investigation on the proximal human gut. In view of the rich supply of VIP-immunoreactive nerves recently shown in Brunner's glands of the rat duodenum,⁵ the duodenal submucosa was also studied.

Methods

MATERIALS

Fresh samples of macroscopically normal body fundus of the stomach (oxyntic area, n=7, at least 4 cm below the oesophagogastric junction), gastric antrum (n=8, 3 cm above the pyloric canal) and duodenal bulb (n=8, 0.5 cm below the pyloric canal)

were obtained during total or partial gastrectomy for gastric (n=6) or extragastric carcinoma (one of pancreas and one of oesophagus). For immunocytochemistry, samples were fixed in p-benzoquinone solution (0.4% in 0.01 mol/l phosphate buffered saline, pH 7.2-7.3)⁶ for two hours, washed in phosphate buffered saline and stored in phosphate buffered saline containing NaN₃ (0.02%) at 4°C. Adjacent samples were fixed in formalin or Bouin's fixative and used for routine histology, in order to exclude neoplastic infiltration or other changes in the vicinity of the specimens used for immunocytochemistry.

Samples of mucosa (and of Brunner's glands with the surrounding submucosa, for the duodenal samples) were removed from the benzoquinone fixed specimens by cutting through the submucosa with fine scissors. They were dehydrated in graded alcohols, cleared in xylene (2×45 min), rehydrated and divided into strips (0.5-1 mm wide). From the strips, thin slices of mucosa (containing approximately three to five rows of glands or one row of villi), or thin slices of Brunner's glands with the surrounding submucosa, were cut with a scalpel under a dissecting microscope. Preparations of full thickness muscularis mucosae were also removed

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from the remainder of the mucosa with watchmaker's forceps and a scalpel. This one step microdissection, completed before immunostaining, was quicker to carry out than the previous two step method⁴ and was therefore routinely used.

Microdissected preparations were immunostained *in toto* by immunofluorescence,⁷ using the antisera: anti-VIP 652 (dilution 1:500), anti-substance P 768 (C-terminally directed, not cross reacting with bombesin, 1:250) and anti-met-enkephalin 773 (1:250). These antisera have previously been characterised.⁴⁻⁸ Prolonged incubations (40 h for the primary antiserum; overnight for the FITC-conjugated second layer, 1:50) and washing times (six to eight hours) were used. Immunocytochemical controls included preabsorption of each antiserum (at the working dilution) with the specific peptide (10 $\mu\text{mol/l}$), the use of non-immune rabbit serum as first layer and the substitution of each layer in turn with phosphate buffered saline. After immunostaining, samples were carefully laid on slides in glycerine-phosphate buffered saline (5:3) under a dissecting microscope, coverslipped and gently pressed, in order to make them as flat as possible. A fluorescence microscope was used for examination and photography.

In view of the immunocytochemical method used, the results described should be taken to imply peptide-like immunoreactivity throughout.

Results

The approach used consistently allowed the full three dimensional demonstration of peptide-containing nerve networks. No differences in the density of immunostaining were detected along the course of the single nerve bundles across the mucosa, thus confirming the homogeneous penetration of the antibodies used.

In the stomach, intensely VIP-immunostained nerve bundles formed a tight network around oxyntic glands (Figs. 1, 2), while more delicate fibres circumscribed the pyloric glands. In both the oxyntic and antral stomach, these fibres anastomosed extensively at the junction between gastric glands and pits (Fig. 1). Above this level, they abruptly decreased in number in the luminal direction, only a small number of scattered fibres running towards the luminal epithelium in the pit region (Fig. 1).

Substance P-immunoreactivity was shown in the oxyntic area in few, thin fibres, which appeared to follow a rather direct course across the mucosa. Close to the luminal epithelium, they occasionally formed delicate loops around the upper portion or the opening of a pit (Fig. 3). Fibres containing this peptide were more numerous and interconnecting in

the antrum (Fig. 4).

In the duodenum, VIP-containing fibres formed a striking, extensively anastomosing network in the villi (Fig. 5), while the crypt region was comparatively less innervated. At the crypt-villus junction a large number of VIP-immunoreactive nerve fibres ran horizontally towards the adjacent mucosa (Fig. 6). The substance P-immunoreactive nerve network was less abundant and did not show this pattern at the crypt-villus junction (Fig. 7).

VIP-immunostaining of whole mount preparations of muscularis mucosae showed numerous nerve fibres throughout the stomach and a striking network in the duodenum. At this level, several different planes of branching and anastomosing fibres were present (Fig. 12). Substance P-immunoreactivity was revealed in a small number of fibres in this area.

In the duodenal submucosa, numerous VIP- and substance P-containing nerve fibres were revealed in the internodal strands and in the ganglia of Meissner's plexus (Figs. 8, 11). It is interesting to note that some of the nerve bundles present here were composed of smooth fibres (Fig. 10), not showing the innumerable varicosities which are commonly found in peptide-containing nerve fibres in the gut mucosa. Numerous neuronal perikarya of the submucous plexus showed VIP-immunoreactivity (Fig. 8), while fewer contained substance P. VIP-immunostaining also revealed a rich supply of fibres at the periphery of Brunner's glands (Fig. 9). Only very few of these fibres, however, were seen to enter the glands and reach the proximity of acinar cells (Fig. 9).

In both stomach and duodenum, VIP- and substance P-containing nerve fibres frequently followed closely the course of blood vessels, both in the mucosa and submucosa (Fig. 13).

In the stomach, met-enkephalin-immunoreactivity was shown in a few scattered nerve bundles in the basal part of the mucosa and in the muscularis mucosae. In the duodenum, nerve bundles brightly immunostained for met-enkephalin ran in the internodal strands for a relatively long way and formed a dense network around neuronal perikarya in occasional ganglia (Fig. 14).

Discussion

The method used in this study allowed the detailed investigation of peptide-containing nerves throughout the human gastroduodenal mucosa and duodenal submucosa, by revealing the different nerve networks in their full extent. The complete penetration of the antibodies, and thus the reliability of the immunostaining technique, was

Fig. 1, 2 Gastric oxyntic mucosa. VIP-immunostaining showing numerous anastomosing nerve bundles along glands (Fig. 2) and at the junction between glands and pits (Fig. 1), while only a single bundle runs vertically in pit region towards lumen (Fig. 1). a: luminal epithelium, p: pit, g: oxyntic glands, $\times 290$, $\times 250$ (original magnification).

Fig. 3, 4 Substance P-immunostaining in the stomach. In oxyntic area a thin fibre forms delicate loops around upper portion of pit (Fig. 3). In antral mucosa, fibres are more numerous and more frequently interconnecting (Fig. 4). a: luminal epithelium, g: pyloric gland, $\times 240$, $\times 150$ (original magnification).

Fig. 5–7 Duodenal mucosa. Rich VIP-containing network in a villus (Fig. 5) and at the villus-crypt junction (Fig. 6). In the latter, note numerous fibres running horizontally to nearby mucosa. Substance P-immunoreactive nerves are less numerous and do not show the above pattern (Fig. 7). v: villus base, c: crypts, $\times 240$ (original magnification).

confirmed by the uniform staining intensity found along the course of the single nerve bundles across the mucosa.

In the stomach, the VIP-immunoreactive innervation was richer around oxyntic glands than pyloric ones and only sparse in the pit area. Thus, fibres containing VIP were most numerous in the acid secreting portion of the stomach and almost confined to the glandular part of the mucosa throughout. Vasoactive intestinal polypeptide has an inhibitory effect on gastric acid secretion in man and in various animals.⁹ In addition, VIP has been reported to activate cAMP synthesis in mucous and peptic cells in both fundic and oxyntic glands of

man.¹⁰ This finding may fit well with the presence of VIP-containing nerves throughout the glandular part of the gastric mucosa.

Only a very sparse VIP-immunoreactive innervation was revealed in contact with the luminal epithelium in the gastric mucosa. Conversely, in the duodenum the lamina propria adjacent to the lumen appeared to be very richly supplied. Interestingly, this latter pattern is very similar to that observed in the distal ileum and distinctly different from the sparser innervation observed in the nearby jejunum.⁴ It is of interest that acid instillation into the human duodenum causes a considerable release of VIP.¹¹

Fig. 8, 9 VIP-immunostaining in the duodenal submucosa. Numerous bundles (Figs. 8, 9) and neuronal perykaria (Fig. 8) are revealed in submucous plexus and around Brunner's glands. Only few, thin fibres enter parenchima and reach proximity of acinar cells (Fig. 9). *m*: muscularis mucosae, *b*: Brunner's glands, *s*: surrounding submucosa, $\times 240$, $\times 260$ (original magnification).

Fig. 10 Submucosal nerve bundle composed of smooth nerve fibres (top) and several varicose fibres (bottom). Duodenum, VIP-immunostaining, $\times 300$ (original magnification).

Fig. 11 Numerous substance P-immunoreactive fibres in a ganglion of the submucous plexus. *b*: Brunner's glands, $\times 250$ (original magnification).

Fig. 12 Striking VIP-immunoreactive nerve network present in duodenal muscularis mucosae. $\times 240$ (original magnification).

Fig. 13 Delicate, but tight VIP-immunoreactive nerve network around a blood vessel (*v*) in the duodenal submucosa. $\times 150$ (original magnification).

Fig. 14 Brightly stained met-enkephalin-containing nerve bundle branching in duodenal submucous ganglion. *b*: Brunner's glands, $\times 240$ (original magnification).

In the stomach, absorption is very limited and the mucosa acts as a barrier to water and ion movements. Luminal contents emptied from the stomach are osmotically equilibrated in the duodenum, nutrients are absorbed from an isotonic chyme in the jejunum and active ion absorption follows in the ileum and colon.¹² Thus, it is in the regions of the gut most concerned with the control of ionic and osmotic strength of the luminal contents that VIP-immunoreactive innervation is richer near the luminal epithelium. Such a distribution may fit well with the known effects of VIP infusion on intestinal functions (inhibition of absorption and stimulation of active secretion).¹³

It should be noted that substance P, too, is well represented in nerve fibres in the duodenal mucosa and has been reported to affect intestinal ionic movements.¹⁴

In the rat, Brunner's glands have been reported to receive a rich supply of VIP-containing nerves.⁵ From the results of the present study, this does not appear to be the case in man.

It has been suggested that the muscularis mucosae affects absorption and secretion by altering the unstirred layer adjacent to the villi and compressing mucosal glands.¹⁵ When specifically tested on preparations of muscularis mucosae, VIP has been found to produce relaxation, and substance P

contraction, of this thin muscular layer.¹⁶ The VIP- and substance P-containing innervation of intramucosal blood vessels of the stomach is of interest. In fact, both peptides are well known vasodilators.^{17 18}

It has been suggested that endogenous opiates play a role in the normal control of gastric acid secretion in man.¹⁹ The presence of only a small number of met-enkephalin-immunoreactive nerve fibres in the gastric mucosa suggests that either other opiate-like substance(s) are involved or an indirect mechanism is operating.

In conclusion, a distinct differential pattern of distribution of peptide-containing nerves has been shown in the mucosa of the human stomach and duodenum.

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