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Full Papers

The regulatory peptide system of the large bowel in equine grass sickness[1]

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Summary. In recent years, distinct changes in regulatory peptides have been found in a number of gastrointestinal diseases. Grass sickness is a fatal disease of horses for which the etiology has yet to be fully ascertained.
In this study, the peptide-containing nerves and ganglionic and mucosal endocrine cells of the ileum, colon and rectum were investigated in horses with sub-acute or chronic grass sickness and compared with normal controls using immunocytochemistry, at both the light and electron microscopical levels, and radioimmunoassay.
A substantial loss of both peptide-containing cells and nerves was found in all of the sick horses, particularly in the ileum. Electron microscopy revealed marked degeneration of nerves in the gut wall. Fibers containing granules immunostained for substance P or VIP, using the immunogold staining technique, underwent extensive degranulation in grass sickness, with the formation of multiple vacuoles.
Radioimmunoassay of peptide content also showed that the most drastic changes occurred in the ileum. For example, VIP content was significantly reduced from 109 ± 19.8 (mean ± SEM) pmoles/g in controls to 6.8 ± 1.4 pmoles/g in grass sickness ($p < 0.001$) and substance P from 65.9 ± 8.1 to 31.3 ± 9.5 ($p < 0.02$). These results may have applications in the diagnosis and treatment of grass sickness.

Introduction

Grass sickness is a fatal disease of young horses which has drastic effects in terms of both the suffering of the animals and the associated financial loss. The etiology and pathogenesis of the disease remain largely unknown, despite extensive research.
The characteristic features of grass sickness have been described as expressions of 'sympathicotonia'[2] and take the form of sweating, muscular tremors and extensive bowel dysfunction. The main gastrointestinal symptoms at the acute and early sub-acute stages are bowel stasis with no bowel sounds and massive inpouring of fluid into the stomach and small intestine, whilst the colon becomes compacted with dry feces coated in dark mucus. In late sub-acute and chronic cases bowel sounds may return. There is no fluid in the stomach and the contents of the small intestine become semi-liquid. There is no known method of prophylaxis or treatment and, at the moment, the prognosis is hopeless in the acute cases and only a very small number of sub-acute and chronic cases recover[3].
The regulatory peptides form a large and heterogeneous group of biologically active substances present throughout the bodies of all animals so far examined. Their discovery has revolutionized all previous concepts of gut physiology[4]. These peptides are found, in the gut, in mucosal endocrine cells, from which they are released to act as circulating or local hormones, or in nerve fibers, where they may function as neurotransmitters or neuromodulators. The peptide containing nerves form a major component of the autonomic nervous system and

are present along the entire length and breadth of the gut, where they have a predominantly intrinsic origin from intramural ganglia.

An association between abnormalities of the regulatory peptides and pathological conditions of the gut has been found in a number of human diseases[5]. For example, in Chagas' disease, which has certain features, such as severe constipation and bowel stasis, in common with grass sickness, there is a marked reduction in both peptide containing nerves and mucosal cells[6]. In view of this gross derangement of the regulatory peptide system in human gastrointestinal disease, a study was made, using immunocytochemistry at both the light and electron microscopical levels, of peptide containing nerves and cells in the gut of horses with sub-acute and chronic grass sickness. These were compared with normal controls. The quantities of peptides present in the tissue under investigation were measured by radioimmunoassay of tissue extracts.

Materials and methods

Three groups of race-, age- and sex-matched horses were studied (table 1). Specimens of each region of distal gut (proximal and distal ileum, right ventral and right dorsal colon [colon I and IV] and rectum) were removed at post-mortem examination, within 2 h of death, from the horses with grass sickness and the normal controls.

Each sample was processed for conventional histology, immunocytochemistry and radioimmunoassay. Pieces of ileum from horses with sub-acute grass sickness and the normal horses, were, in addition, processed for conventional and immuno-electron microscopy.

Conventional histology. Tissue samples measuring no more than 2 × 2 cm^2 were fixed by immersion in 0.4% para-benzoquinone in 0.01 M phosphate buffered saline (PBS) (pH 7.1–7.4) at 4°C for 4 h[7]. They were then rinsed in PBS containing 7% sucrose and 0.01% sodium azide. Sections (10 μm) were cut in a cryostat, air-dried and stained with hematoxylin and eosin. Mounted sections were examined under a Leitz transmitted light microscope.

Immunocytochemistry. Sections (10 μm), cut from the same cryostat blocks used for conventional histology, were immunostained using the technique of indirect immunofluorescence[8]. The sections were incubated for 16–20 h at 4°C with antisera to a range of regulatory peptides (table 2). A second layer of fluorescein conjugated goat anti-rabbit globulin (1:150) was applied at a dilution of 1:150 for 1 h at room temperature. Sections were mounted in PBS glycerine (1:9 v/v, PBS: glycerine) and examined under a Leitz ultraviolet microscope. Controls for the immunostain included omission of first layer antiserum or replacement with non-immune rabbit serum and lack of immunostaining by absorption of each antiserum with its corresponding peptide (10 nmoles/ml dilution antiserum).

Conventional electron microscopy. Samples of whole wall thickness were taken from the proximal and distal ileum, the area of maximal pathology, of horses with sub-acute grass sickness and normal controls. The samples were fixed by immersion in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2–7.4) for 2 h at 4°C. After washing overnight in 0.1 M phosphate buffer containing 0.1 M sucrose the tissues were postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 4°C. They were then washed, dehydrated and embedded in Araldite. Ultrathin sections were mounted on copper grids and stained with alcoholic uranyl acetate and lead citrate. The sections were examined with an AE1 6B electron microscope.

Immuno-electron microscopy. The tissue was processed as for conventional electron microscopy except that it was fixed in 3% glutaraldehyde for 3 h at room temperature and not postfixed in osmium tetroxide.

Ultrathin 60–90 nm sections were etched in 10% hydrogen peroxide for 8 min. They were then washed with 0.05 M Tris-buffered saline (pH 7.3), blotted and placed in normal goat serum (1:30 dilution) for 5 min. The sections were then incubated in substance P (1:8000) or VIP (1:6000) rabbit antiserum for 1 h. After washing again the sections were incubated with gold- (Au20, 20 nm particle size) labelled goat anti-rabbit serum for 1 h at room temperature. After further washes the sections were counterstained with alcoholic uranyl acetate and lead citrate and examined with a Zeiss 10 electron microscope[9].

Radioimmunoassay. Extraction. Weighed portions of each tissue were placed in boiling 0.5 M acetic acid (10 ml/g wet tissue) and maintained at 100°C for 10–12 min. After cooling, the extracts were stored at −20°C until assay[10].

Assay. The tissue extracts were thawed and duplicate aliquots of 10 μl and 1 μl were assayed for the following peptides: somatostatin (SRIF), mammalian bombesin-like imunoreactivity (BLI) (also known as gastric releasing peptide), VIP, substance P and total glucagon. The values obtained for total glucagon were taken as enteroglucagon since pancreatic glucagon-like immunoreactivity (PG) is effectively limited to the pancreas[11,12]. The

Table 1. 3 groups of race-, age- and sex-matched horses were studied

Horses studied	n
Sub-acute (2–4 days) grass sickness	4
Chronic (14 days) grass sickness	3
Normal controls	7

Table 2. Characteristics of antisera for light microscopical immunocytochemistry

Peptide antiserum raised against	Dilution	Region specificity
Bombesin	1:400	Whole molecule
CCK	1:400	Mid-portion (9–20)
Gastrin	1:400	C-terminal
GIP	1:600	Mid-portion
Glucagon	1:1000	N-terminal
Leu-enkephalin	1:400	Whole molecule
Met-enkephalin	1:400	Whole molecule
Motilin	1:200	C-terminal
Neurotensin	1:800	Whole molecule
Secretin	1:2000	Not known
Somatostatin (14)	1:2000	Whole molecule
Substance P	1:500	C-terminal
VIP	1:2000	C-terminal

Table 3. Summary of the details of each assay and the production of antisera

Assay details	Peptide BLI	Somatostatin	Sub. P.	VIP	Total glucagon
Conjugate	[lys^3]BN-BSA	SRIF-BSA	Sub. P.-BSA	Porcine VIP-BSA	Glucagon-BSA
Method of conjugation	Glut	CDI	Glut	CDI	CDI
Antibody specificity	C-terminal	whole	C-terminal	Mid- to C-terminal	Mid- to C-terminal
^{125}I label	[Tyr^4]-BN	[Tyr^{11}]SRIF	[Tyr^8] Sub. P.	Porcine VIP	Porcine Glucagon
Cross reactivity of antisera with other known peptides	None	2% with SRIF 28	None	None	Fully cross reacts with porcine glicentin and PG
Final antibody dilution	1:640,000	1:240,000	1:8000	1:320,000	1:1600
Sensitivity (fmoles/tube) 95% confidence limit	0.4	0.4	0.3	0.5	0.5

details of each assay and the production of antisera are summarized in table 3. All assays were performed in a total volume of 0.8 ml 0.5 M phosphate buffer, pH 7.4, containing 0.01 M EDTA and 0.3% BSA[10].

After 5 days incubation at 4 °C, the 'antibody-bound' and 'free' labelled antigen were separated by the activated charcoal method.

Treatment of data. All tissue hormone concentrations were expressed in pmoles/g as the mean and standard error of the mean (SEM).

Statistical Analysis. Data from the control and sick group were compared using Student's unpaired t-test.

Results

Post-mortem examinations. Post-mortem examinations revealed no gastrointestinal lesions in the normal control animals. In the sick animals, however, a range of changes were found, varying from no gross abnormalities to very abnormal gastrointestinal tracts. The major pathological features observed, although not consistently present, were sticky, tarry feces in rectum, solid, impacted material in the large colon, instead of the normal liquid material, edema, particularly in the ileum and large colon, and a marked enteritis. Linear ulceration of the esophagus occurred in half of the cases.

There were differences between early sub-acute (2–4 days) and long-standing sub-acute/chronic (< 14 days). The major differences being in the first group the stomach and small intestine were usually greatly distended, with 4.5–18 and 4.5–31.5 liters of fluid respectively, and the large bowel was impacted. In most cases of the sub-acute/chronic group, the large bowel contents were semi-liquid. In chronic cases, the whole alimentary tract appeared, by naked eye examination, to be smaller in size than normal and, although bowel sounds had returned, the whole tract showed marked gastroenteritis.

Conventional histology. The normal control tissues were found to have no histological signs of gastrointestinal disease nor of degenerative changes due to autolysis or fixation.

As in the normal controls, no degenerative changes were observed in the grass sickness tissues which could be attributed to sampling or fixation methods. There was no obvious, consistent pattern of pathological features in the horses with either form of grass sickness. Large numbers of plasma cells, a feature of horses, were frequently found, as in the normal controls. Changes which could be detected included edema, either confined to the mucosa and submucosa or transmural, inflammatory infiltration, infrequently permeating through to the serosa, and fibrosis, mainly of the submucosa. In chronic cases there was sometimes complete detachment of the mucosal epithelium with surface hemorrhaging.

Neural changes ranged from slight central chromatolysis of 1–2 neurons, to vacuolation and shrinkage of most of the nerve cells. There was no apparent relationship between inflammatory changes and ganglion cell damage.

Immunocytochemistry. A pattern of regulatory peptide distribution was seen in the normal horse gut which was similar to that observed in other mammalian species. A vast network of peptide-containing nerves infiltrated each layer of the gut wall. The most abundant peptides found in these nerves were substance P and VIP. Unlike the human gut, where VIP nerves are far more frequent than those containing substance P, in the equine ileum, colon and rectum substance P and VIP nerves were equally numerous. A very minor population of met-enkephalin containing fibers was found in each region of gut, mainly in the myenteric plexus. VIP nerves were most frequent in the circular muscle coat, myenteric and sub-mucous plexuses and in the mucosa, where meshes of fibers could be found in close association with the mucosal epithelium (fig. 1). In the sub-mucosa, VIP fibers surrounded blood vessels and ganglion cells, some of which were immunoreactive, in the main

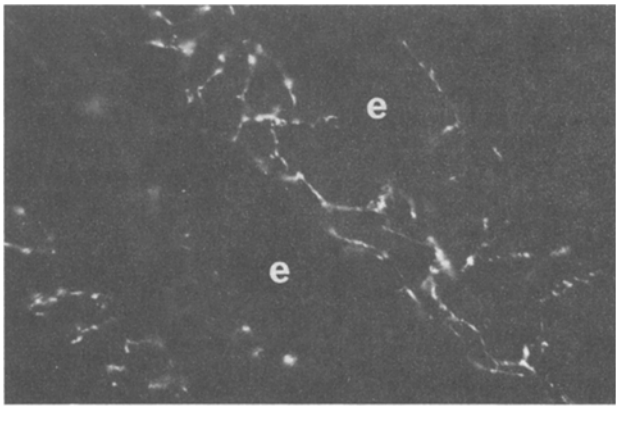

Figure 1. Meshes of VIP-immunoreactive nerves under the epithelium (e) of normal equine ileum. × 320.

plexus. The fibers were all slender and varicose, except where they emitted from ganglion cells. At this site a ribbon-like structure was most typical. The immunoreactive ganglion cells were, generally, round or ovoid with a large nucleus. 2 or 3 immunoreactive ganglion cells could be observed in every group of ganglion cells numbering 6 or more.

Substance P-immunoreactive fibers were particularly prominent in the myenteric plexus and circular muscle. No immunoreactive ganglion cells were found and substance P nerves were less numerous in the sub-mucosa and mucosa than those containing VIP. No other fibers containing peptides were detected. This pattern of distribution of peptide containing nerves was consistent throughout the ileum, colon and rectum.

Peptide containing mucosal cells, on the other hand, showed a variable distribution. In both regions of ileum examined, cells containing neurotensin, somatostatin or enteroglucagon (fig. 2) were present in approximately equal numbers. These all had an apical shape with the somatostatin and enteroglucagon cells sometimes showing luminal or basal elongations. In the colon and rectum enteroglucagon cells were by far the most frequent cell type. Somatostatin cells were also present but no neurotensin cells were found.

The tissues from horses with grass sickness, whether in a sub-acute or chronic form, had undergone a striking change in the level of peptidergic innervation. In the ileum, VIP (fig. 3) and substance P nerves were virtually absent. A few fibers could be found in the myenteric plexus, circular muscle and mucosa. In addition, some VIP nerves were seen around non-immunoreactive ganglion cells in the submucous plexus. Only very occasional met-enkephalin containing fibers were found.

The ileum showed the most drastic loss of peptide containing nerves. Although a marked reduction was also seen in both the colon and rectum, it was not a great. In the large bowel (colon I and IV), as in the ileum, the remaining peptidergic fibers were mainly in the plexuses, circular muscle and mucosa, but no immunoreactive ganglion cells were found. Although the peptidergic fibers were reduced in number throughout, the ones which remained were brightly immunostained.

In the mucosal epithelium, there appeared to be some reduction in the numbers of peptide containing cells. However, this was neither as striking nor as consistent as the alteration in peptide containing nerves. The cells were never completely absent and their distribution and relative proportions did not alter.

Electron microscopy. In the ileal specimens from normal horses, the myenteric and submucous plexuses had a compact structure and nerve bundles of normal size and appearance were present throughout the wall. A great number of nerve profiles were found containing many large (75–95 nm) granular vesicles. Immunogold staining showed that vesicles containing substance P (fig. 4) or VIP immunoreactivity were particularly numerous within the profiles. In comparison, the tissues taken from animals with grass sickness showed dramatic structural changes. In proximal ileum, the general arrangement of the innervation appeared relatively normal. Some individual nerve profiles showed obvious signs of injury. These signs included disrupted mitochondria and slight axonal swelling. In addition, a proportion of ganglion cells in the myenteric and submucous plexuses showed lysis of the mitochondrial matrix, dilation of endoplasmic reticulum and slight crenation of the nucleus. The most striking alteration in this area of gut was, however, the almost total depletion of neuronal vesicles from nerve endings throughout the wall, including those which showed immunoreactivity for VIP and substance P (fig. 5).

In the distal ileum, the cell bodies and degranulated

Figure 2. An enteroglucagon-immunoreactive cell in the mucosal epithelium of normal equine ileum. Note the typical triangular shape of the cell with an elongation reaching to the lumen. × 450.

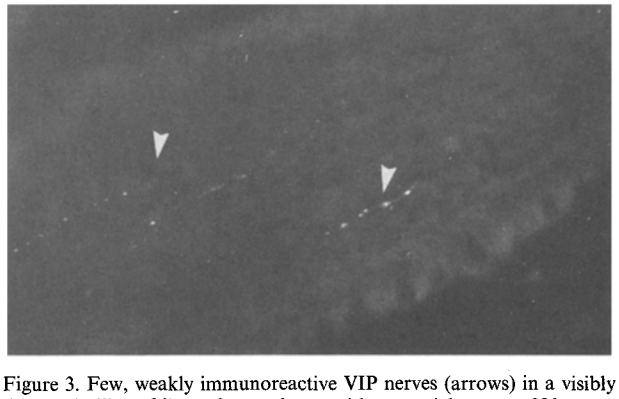

Figure 3. Few, weakly immunoreactive VIP nerves (arrows) in a visibly damaged villus of ileum from a horse with grass sickness. × 320.

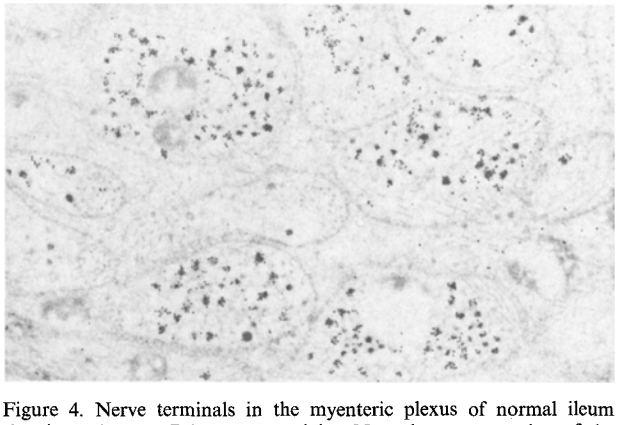

Figure 4. Nerve terminals in the myenteric plexus of normal ileum showing substance P immunoreactivity. Note the concentration of the gold label over the secretory granules. × 27,500.

nerve profiles had undergone extensive degeneration. The nerve bundles contained some nerve profiles with electron dense deposits, swollen, disrupted mitochondria and myelin bodies, while others were enormously dilated and without structural features such as neurotubules and mitochondria. Furthermore, in the myenteric and submucous plexuses, which had lost their normal compact structure, cell bodies contained a highly vacuolated cytoplasm, small electron-dense deposits and a markedly crenated nucleus. Some of the cytoplasmic vacuoles had a double membrane. No other cytoplasmic organelles could be distinguished in these cells.

Radioimmunoassay. The distributions of the peptides as measured by radioimmunoassay were, generally, in close agreement with those shown by immunostaining (table 4). Statistically significant reductions in the concentrations of peptides contained in nerves (i.e. VIP, substance P and bombesin) were found in the ileum of horses with grass sickness. VIP and bombesin were also significantly lower in the colon from the sick horses. Somatostatin and enteroglucagon, which were localized by immunocytochemistry to mucosal endocrine/paracrine cells only, showed no changes.

Discussion

In horses with grass sickness, whether sub-acute or chronic form, there was a consistent loss of regulatory peptide containing autonomic nerves in the distal bowel. In addition, a less striking and more variable change in mucosal endocrine/paracrine cell population was also seen.

These changes, which were particularly marked in the ileum, were detectable at the light and electron microscopical levels. Significant loss of substance P, VIP and bombesin content from the ileum could be demonstrated by radioimmunoassay of tissue extracts. VIP and bombesin were also reduced in the colon. A similar alteration of the enteric regulatory peptide containing system has been noted in human diseases. In both congenital and acquired megacolon, Hirschsprung's[13] and Chagas'[6] disease respectively, both peptide containing nerves and cells are reduced. It is thought that, as both diseases involve absence or degeneration of intramural ganglion cells, the lack of peptide containing nerves is evidence for their predominantly intrinsic origin in the human gut. This intrinsic origin is also shown by the presence of large numbers of peptide immunoreactive ganglion cells in the 2 major intramural plexuses. It had previously been thought that the neuronal damage in grass sickness primarily concerned the extrinsic supply to the gut[14]. However, the findings made in the present study, of peptide immunoreactive ganglion cells, suggest that in horses, as in humans and other mammals, a large proportion of the peptide containing nerves arise from intrinsic cells. Thus, the loss of these nerves in grass sickness indicates a lesion of the intrinsic nervous supply. In humans, it has been found that in an example of pre-ganglionic autonomic degeneration, the Shy-Drager syndrome, the peptide containing innervation of the gut was unaltered[6]. The fact that there is a definite lesion of the enteric peptide containing nerves in grass sickness does not rule out the possibility of further peptide containing nerves arising from extrinsic sources. Immunocytochemical and radioimmunological investigations of relevant ganglia are therefore warranted.

Extensive damage to ganglion cell bodies of the myenteric, and to a lesser extent the submucous, plexus has previously been observed in grass sickness[15]. However, such findings have not been consistently made and, as is shown in the present study, it is not always easy to detect these neural lesions using conventional light microscopy. Straightforward immunostaining, however, reveals clear abnormalities in the enteric nervous system.

The nature, but not the cause, of this damage is further revealed by ultrastructural examination of the nervous supply. However, the most striking feature of the ultrastructure profiles of the enteric nerves in affected ileum is the almost total lack of neuronal vesicles, particularly those shown by immunostaining to contain substance P and VIP. This absence of secretory vesicles explains the lack of immunostaining of peptide containing nerves at the light microscopical level. The most likely reason for the loss of vesicles is the release of peptides from the nerve endings and the almost total absence of vesicles suggests both a massive loss of peptide coupled with a cessation of peptide production within the nerve. The

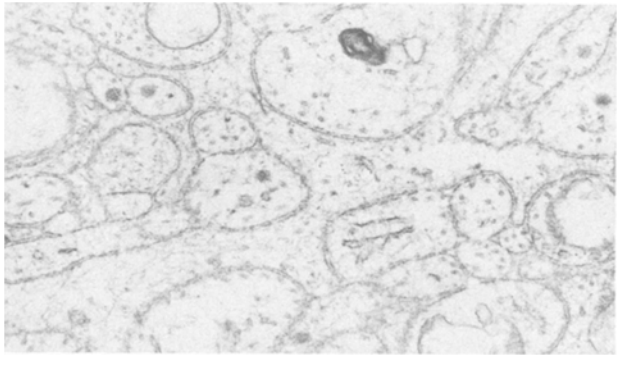

Figure 5. Nerve terminals in the myenteric plexus of ileum from a horse with grass sickness, immunostained for substance P. The terminals are severely disrupted and no immunoreactive secretory vesicles can be seen. × 27,500.

Table 4. Results of radioimmunoassay measurement of peptide content

Peptide	Ileum		Colon		Rectum	
	Control	Grass sickness	Control	Grass sickness	Control	Grass sickness
VIP (pmoles/g)	109.2 ± 19.8***	6.8 ± 1.4	85.5 ± 16.5**	31.1 ± 4.9	121.0 ± 34.9	45.6 ± 11.8
Substance P (pmoles/g)	65.9 ± 8.1*	31.3 ± 9.5	41.7 ± 7.1	27.8 ± 4.4	13.7 ± 3.9	13.6 ± 4.0
Bombesin (pmoles/g)	5.4 ± 1.2**	0.6 ± 0.08	13.2 ± 2.0***	2.6 ± 0.7	5.0 ± 1.4	4.1 ± 1.4
Enteroglucagon (pmoles/g)	78.6 ± 12.6	85.1 ± 16.9	22.2 ± 6.6	29.5 ± 4.5	108.3 ± 27.5	53.6 ± 18.9
Somatostatin (pmoles/g)	39.6 ± 8.5	22.8 ± 2.2	17.9 ± 2.2	22.8 ± 5.4	105.5 ± 35.2	29.7 ± 10.5

*** $p < 0.001$; ** $p < 0.01$; * $p < 0.02$. Data are expressed as mean ± SEM.

fact that peptide containing vesicles were lost from the proximal ileum, without any other degenerative changes, indicates that release of peptides from nerves may form one of the earliest morphologically identifiable events in grass sickness. A massive release of peptides over a short period of time would certainly contribute to a large extent to the development of many of the gastrointestinal features of grass sickness. Both VIP and substance P affect a range of gastrointestinal functions, including, most importantly, secretion, blood flow and motility[4]. The large quantities of fluid found in the stomach and small intestine may be secreted as a direct result of VIP release. This peptide is capable, even in small quantities of stimulating water and electrolyte secretion from the gut[16], an action which is well illustrated by the VIPoma syndrome where over-release of VIP from a tumor results in copious watery diarrhea[17]. Both VIP[18] and substance P[19] cause vasodilation and therefore may be responsible for the congestion which is a frequent post mortem finding in the gut of horses with grass sickness. In addition, nerves containing VIP and substance P appear to form an integral part of the peristaltic pathway[20] , VIP inhibits motility by direct action on smooth muscle cells[21] whilst substance P occurs in excitatory interneurons, stimulating motility by increasing cholinergic activity[22]. Hyperactivity of these nerves with subsequent inaction would therefore severely disrupt bowel motility.

The variable reduction in the mucosal endocrine/paracrine cells may be related to the general loss of bowel activity. It may also be a consequence specifically of the neural degeneration. Reduction of mucosal cell turnover following autonomic denervation has previously been reported[23] and parallel loss of peptide containing nerves and cells appears to be a phenomenon of certain human gastointestinal diseases[5].

Whatever the cause, lack of peptides produced by cells may further contribute to the bowel dysfunction, as each peptide is a potent modulator of gut activity.

Although the exact etiology remains obscure, these findings fit well with previous concepts of absorption, in some way, of a neurotoxin causing grass sickness[14]. If this neurotoxin exists and is ingested by the horse, this may mean that the enteric nerves are first affected, leading to development of the gastrointestinal symptoms, with the generalized neural damage occurring after full absorption of the toxin into the blood. No effective treatment for this disease has been developed. If the loss of regulatory peptides from the gut, whether primary or secondary to the development of the disease, can be compensated for by exogenous administration of peptides, then it may be possible to alleviate the debilitating gastrointestinal symptoms. This may not lead to a full cure, but could feasibly prolong the life of the animal. Early diagnosis of the disease may be made possible by immunocytochemical examination of endoscopic rectal biopsies. Consistent loss of peptides, albeit less marked than in the ileum, could be detected in the rectal mucosa, thus providing the basis for a simple diagnostic test.

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