DIARRHEA-INDUCED CHANGES
IN CHEMICAL PHENOTYPES
OF ENTERIC NEURAL STRUCTURES IN THE PIG
ARE NOT SUFFICIENT TO DISCRIMINATE BETWEEN
THE VIRAL AND BACTERIAL ETIOLOGY OF THE DISEASE

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The present study was aimed at disclosing whether changes in the chemical coding of
neural structures observed within the intestinal wall may be of diagnostic importance for
distinguishing between the viral vs. bacterial etiology of a naturally acquired diarrhea in piglets.
Sections from the jejunum of pigs with clinically and microbiologically diagnosed viral (V
group) and bacterial diarrhea (B group) were labelled by means of indirect immunofluorescence
technique. Antibodies against galanin (GAL), somatostatin (SOM), substance P (SP) and
vasoactive intestinal polypeptide (VIP) were used to compare the chemical coding of enteric
nervous structures in age-matched, clinically healthy control animals (C group) with those
observed in specimens from the diarrhea-suffering littermates. While in the healthy animals, the
distribution pattern of nerve fibres/intrinsic neurons exhibiting substances studied virtually
resembled that described elsewhere, both, viral and bacterial diarrhea induced a dramatic
increase in the number of neural structures (both nerve fibres and neuronal cell bodies) exhibiting
GAL and SP. An increase observed in the number/density of elements containing SOM or VIP
was less pronounced. It should be stressed that both kinds of changes observed as well as their
intensity were very similar in V and B groups of animals making a distinction between the viral
or bacterial diarrhea virtually impossible. Thus, although the procedure described may be of
importance for better understanding the neural aspects of the diarrhea-induced changes in, e.g.,
intestinal motility, water resorption or changed epithelial secretion, this technique per se appears
to be not sufficient for clear-cut diagnosis of the diarrhea etiology.

Key words: pig, enteric nervous system, neuronal plasticity, galanin,
somatostatin, substance P, vasoactive intestinal polypeptide,
diarrhea.
Catarrhal inflammation of the intestine is one of the most often observed
diseases in young piglets that can result in a considerable loss of animals at fattening
farms. Diarrhea is not only accompanied by severe disturbances of the animal
organism’s homeostasis, but also induces an activation of the immune (4, 19) and
nervous systems of the intestine (for a review see (26)) due to the direct and indirect
action of the pathogenic factors.

A great number of extrinsic and intrinsic neurons supplies the
gastrointestinal tract. These neurons are involved in numerous circuits controlling
particular gut functions. For example, a large population of enteric neurons is engaged
in the control of intestinal motility forming both ascending and descending neural
pathways essential for proper co-ordination of the activity of particular segments of the
gastro-intestinal tract (24, 29). On the other hand, intrinsic sensory neurons provide
other enteric neurons with information that is required for autonomic control of
digestion, whereas extrinsic afferents transmit to the brain information about processes
that are relevant for energy and fluid homeostasis and the sensation of discomfort and
pain (for a review see (12)). It should be stressed that due to the complexity of
functions controlled by these neurons, each subset of functionally different enteric
perikarya express diverse, highly specific pattern of co-existence of biologically active
substances called “neurochemical coding” (for a review see (3)).

Recently, it has been shown that the enteric nerve system is deeply involved
in inflammatory processes of the intestinal wall, being responsible for pain and altered
motility of the inflamed intestine (26). For example, the extrinsic afferent nerve fibres
have recently been suggested to mediate the effects of bacterial toxins in the course of
intestinal diseases (16, 17). Moreover, SP and/or calcitonin gene-related peptide, their
primary mediators, have been thought to be of importance for an axon reflex mediating
neurogenic inflammation in the intestine (6, 11, 15).

Recently, it is getting more evident that the neuronal plasticity is an
important homeostatic mechanism in the intestine, both during, as well as following
inflammatory processes of the organ (for a review see, (31, 35). It has been shown that
different kinds of injury (i.e. mechanical, like axotomy, or chemical, like inflammation)
are able to induce different changes in chemical phenotypes of affected sensory (13),
sympathetic (for a review see (14) or enteric neurons (2, 26). Furthermore, pathological
mechanisms involved in the induction of viral and bacterial intestinal inflammation are
also thought to be different (i.e. mechanical impairment of the enterocytes by viruses
vs. bacterial toxin-induced damage). It is therefore reasonable to assume that also
chemical phenotypes of nerve fibres/intramural neurons vary not only between health
and sick animals but also between animals suffering from diarrhea of different, i.e.
viral or bacterial etiology.

Therefore, in this study changes in the expression pattern of GAL, SOM, SP
and VIP in neural elements within the bowel wall of health and diarrhea-suffering pigs,
in order to establish the diagnostic value of alterations observed as a supportive tool for
distinguishing between viral and bacterial diarrheas in the pig were investigated.

**Material and Methods**

**Animals.** Altogether sixty Polish Large White piglets were included in the
study and divided into two main groups consisting of healthy animals, serving as a
control, age-matched group (C group, n=30) and animals suffering from naturally
acquired diarrhea of unknown etiology. The appropriate microbiological samples were taken from all the animals of the latter group and examined by means of routine microbiological methods as described previously in details (22). Based on results of microbiological tests applied, diarrhea-suffering animals were divided into two groups, according to the main pathogen found:

a) Piglets suffering from viral diarrhea (V group; pathogen – Rotaviridae, n= 9)
b) Piglets suffering from bacterial diarrhea (B group; pathogen – Clostridium perfringens type C and E. coli with fimbrial antigen F4; n=9).

Three animals were randomly chosen from both control and experimental groups.

**Preparation of experimental material.** According to the guides of Local Ethical Commission, the animals from each group were pretreated with propionylpromasine (Combelen, Bayer, FRG; 0.4 mg/kg body weight) 30 min before the main narcotic (sodium pentobarbital, Biowet, Poland) was overdosed (90 mg/kg body weight, i.v.). After cessation of breathing, the animals were transcardially perfused with 4% buffered paraformaldehyde (pH 7.4). Collected segments of the jejunal loops (3 cm long, three from each animal) were dissected out and postfixed in the same fixative for 20 min after a short clean of their content in warm Krebs solution (37°C). After postfixation they were washed with phosphate buffer (pH 7.4) and finally transferred to 18% buffered sucrose (pH 7.4) where they were stored until sectioning at 4°C.

**Immunohistochemistry.** Immunohistochemistry was performed on 12-μm-thick transverse cryostat sections of the jejunum mounted on chromealum-gelatine-coated glass slides. Fifteen sections from each jejunal fragment (taken in triplicate at intervals of 0.5 cm) were stained for each of biologically active substance studied (Table 1) by means of single-labelling immunofluorescence technique. Thus, after being air-dried at room temperature (rt) for 45 min, the sections were incubated with a solution containing 1% Triton X-100, 0.1% bovine serum albumin, 0.01% sodium azide and 10% of normal goat or donkey serum in 0.01 M phosphate-buffered saline (PBS) for 1 h at rt, rinsed in PBS (3 x 10 min) and incubated with one of the primary antibodies. Two different protocols were used for the incubation: a commonly used overnight incubation in a humid chamber (approximately 16 h, rt) and incubation in an oven (30 min at 37°C). After washing with PBS, the sections were then incubated (30 min, rt) either with appropriate biotinylated goat anti-rabbit IgG or with FITC-conjugated IgG. In case of biotinylated IgG, the reaction was then visualized by incubation of sections with CY-3 conjugated streptavidin (30 min, rt). All primary antibodies and secondary reagents used are listed in Table 1. Standard tests, i.e. preabsorption, replacement by non-immune sera or omission of primary or secondary antibodies were applied to control the specificity of immunofluorescence. The immunostained sections were studied and photographed with a Zeiss Axioskop microscope equipped for epi-illumination and an appropriate filter blocks for FITC or CY3.

**Evaluation of the density of nerve terminals/number of cell bodies.** Since the main goal of the present study was to elucidate whether the immunolabelling may be useable as a supporting tool for fast laboratory evaluation of a putative pathological factor responsible for the etiology of the illness (i.e. to discriminate between the viral or bacterial etiology of the diarrhea), the density of intramural nerve fibres and/or the number of neuronal profiles within the ganglionated enteric plexuses was estimated and rated according to subjective scale (Table 2) in each of the sections studied. The differences in the density of nerve profiles and/or in the number of neuronal somata
expressing particular antigens in the sections from the animals belonging to particular groups were then classified as unimportant or important for distinguishing between pathogens responsible for the disease.

Table 1
Specification of immunoreagents

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antigen code/clone</th>
<th>Host species</th>
<th>Dilution</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL</td>
<td>RIN 7153</td>
<td>rabbit</td>
<td>1:1600</td>
<td>Peninsula, St. Helens, UK</td>
</tr>
<tr>
<td>SOM</td>
<td>11180</td>
<td>rabbit</td>
<td>1:600</td>
<td>ICN Pharmaceuticals, Aurora, USA</td>
</tr>
<tr>
<td>SOM</td>
<td>YC7</td>
<td>rat</td>
<td>1:100</td>
<td>Biogenesis, Poole, UK</td>
</tr>
<tr>
<td>SP</td>
<td>11192</td>
<td>rabbit</td>
<td>1:800</td>
<td>ICN Pharmaceuticals, Aurora, USA</td>
</tr>
<tr>
<td>SP</td>
<td>34HL</td>
<td>rat</td>
<td>1:400</td>
<td>Biogenesis, Poole, UK</td>
</tr>
<tr>
<td>VIP</td>
<td>11428</td>
<td>rabbit</td>
<td>1:1000</td>
<td>ICN Pharmaceuticals, Aurora, USA</td>
</tr>
<tr>
<td>VIP</td>
<td>VIP-001</td>
<td>mouse</td>
<td>1:800</td>
<td>Biogenesis, Poole, UK</td>
</tr>
</tbody>
</table>

Table 2
Arbitrary evaluation of the number of neuronal cell bodies/density of intramural nerve fibres in the sections from the jejunal wall in the control animals (C) and pigs suffering from viral (V) or bacterial (B) diarrhea

<table>
<thead>
<tr>
<th>Structure:</th>
<th>Group</th>
<th>Substances:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>GAL</td>
</tr>
<tr>
<td>Mucosa and submucosa</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>V</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inner submuco-us plexus</td>
<td>V</td>
<td>+++</td>
</tr>
<tr>
<td>B</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Outer</td>
<td>C</td>
<td>+</td>
</tr>
<tr>
<td>Submucous plexus</td>
<td>V</td>
<td>+++</td>
</tr>
<tr>
<td>B</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myenteron</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Myenteric plexus</td>
<td>V</td>
<td>+</td>
</tr>
<tr>
<td>B</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Subjective rating of the density of nerve fibres/number of neuronal cell bodies: –, not observed; +, single fibres/cell bodies; ++, few fibres/cell bodies; ++++, moderate number of fibres/cell bodies; ++++, many fibres/numerous cell bodies; ++++, very dense plexuses of nerve fibres/very numerous cell bodies.
**Figs 1a-d.** Distribution of GAL-IR structures in the wall of inflammed jejunum in the pig suffering from viral (a, b) and bacterial (c, d) diarrhea. Note a moderate number of GAL-IR nerve terminals within the submucosa and mucosa (a) as well as a very dense nerve plexus in myenteron (d). Both kinds of pathogens were able to up-regulate also GAL in the neuronal somata (b) and nerve terminals (c) in submucosus ganglia.

**Fig. 2.** Viral (a) and bacterial (b) jejunitis in the pig. Both kinds of pathogens induced a moderate increase in the number of SOM-IR nerve fibres within the myenteric plexus, while the number of SOM-IR neurons resembled that observed in the control animals.

**Fig. 3.** Distribution of SP-IR nerve fibres in the myenteric (a) and submucous (b) plexus of animals suffering from diarrhea of viral (a) and bacterial (b) etiology. Note relatively numerous SP-IR terminals in myenteron in the animal of group B (b). However, the density of these fibres was comparable with that observed in the control tissues.

**Fig. 4.** VIP-immunoreactive structures in the porcine jejunum in the course of virally (4a) and bacterially (4b, c) induced diarrhea. In both groups, an increase in the number of VIP-IR nerve fibres and VIP-IR perikarya was observed in both the submucous plexus (a, b) and in myenteron (c). Magnification for all pictures: x100.

**Results**

The quality of immunolabelling obtained with both protocols, i.e. incubation of sections in a humid chamber for 16 h at rt and for 30 min at 37 °C in an oven was comparable (c.f. Figs 1a and 1d).

All the substances studied were found in enteric neurons and/or intramural nerve plexuses. Their distribution resembled that described earlier in a number of reports with regard to neurochemical organization of enteric nerve system in the pig (for details see (1, 21, 29, 30). Therefore, this part of the results is not described and the density of particular classes of neuronal cell bodies/intramural nerve fibres is only shown in Table 2 as semiquantitative, arbitrary values.

In both experimental groups, irrespective of the putative inflammatory agent diagnosed microbiologically, diarrhea-induced changes in the chemical coding of the affected intramural neural elements were pronounced, correlating well with the clinical picture of the illness, thus being more prominent in the animals suffering from more exacerbated diarrhea. However, no clear-cut differences in the activation of particular synthesis pathway for the individual substance studied in the present experiment could be observed between specimens taken from the animals of group V and B.

In both experimental groups GAL was observed in nerve fibres and cell bodies in all layers of the intestinal wall (Figs 1a-d). While in the animals of both V and B groups, numerous GAL-positive nerve fibres were present in the mucosal membrane (often close to the intestinal glands (Fig. 1a)) and virtually all neurons in the submucous plexuses were GAL-IR (Fig. 1b), such coded neurons were sporadically found within the myenteric plexus (Fig. 1c). However, very dense, brightly fluorescing nerve terminals surrounding them were observed in both groups studied (Fig. 1c). While viral diarrhea was able to induce only moderate increase in the GAL-IR nerve fibres in the muscle layers (not shown), diarrhea of bacterial etiology caused somewhat
SOM was observed in dense to very dense plexuses surrounding mainly SOM-immunonegative perikarya of the myenteric and submucous plexuses in both groups of the animals studied (Figs 2a, b). These nerve fibres were relatively less numerous within the submucosa and mucosa, as well as sporadically encountered within the muscle layers. SOM-IR neurons were only sporadically observed (Fig. 2b).

SP was present in dense to very dense plexuses supplying myenteric ganglia in both V and B groups (Fig. 3a). Furthermore, axons containing this peptide were observed in the muscle layers in both groups (Figs 3a, b). Within the submucous plexuses, SP-IR nerve terminals were also numerous, but their number was lower than that observed in the myenteric plexus (compare Figs 3a and b). A low to moderate number of SP-IR neurons was observed in the submucous ganglia, while larger population of enteric perikarya expressed this peptide in the myenteric ganglia.

VIP-IR neurons were found in each of the enteric ganglia studied (Figs 4a-c), however, they were much more numerous in the inner submucous ganglia (Fig. 4b). VIP-exhibiting nerve fibres were especially numerous in the muscle layers (Fig. 4c) of both studied groups of the animals.

Discussion

It has recently been proposed that during intestinal inflammation (and, especially, during its phase of exacerbation) not only cells of the immune system are engaged. There are now some evidences that this pathological process may also activate a number of other cellular components of the intestinal wall, first of all the enteric neurons located in intramural ganglionated plexuses. However, attention has also been paid to cells other than those in the immune or nerve system that may acquire the ability to direct participation in inflammatory response or that may constitute previously unknown source(s) of inflammatory changes, e.g. smooth muscle cells (13). The latter cells have been recently shown to be able to present antigens to activated immune cells (T lymphocytes) as well as to be a prominent source of various cytokines that, apart from other roles, can also directly influence functions of intramural enteric neurons (4, 5). Thus, a particular challenge lies in understanding the interactions between various components of the intestinal wall that may be involved in the inflammatory response to acting pathogenic factor.

As mentioned earlier, it is getting more and more evident that the neuronal plasticity may be an important homeostatic mechanism in the intestine, both during, as well as following inflammatory processes of the organ (for a review see, (23, 26). The exact mechanism(s) responsible for an increase in the number of enteric neurons immunoreactive to particular neuropeptides, observed in the present study, has not been revealed as yet. However, it is tempting to assume that the pro-inflammatory agents released at the site of injury by activated immune, smooth muscle or even neuronal cells themselves may play a crucial role in these adaptive responses. For example, interleukin-1 and interleukin-3, cytokines present in the wall of inflamed bowel segments are known to acutely alter the release of several neurotransmitter(s) (6). This activation of biochemical pathways in affected neurons may thus be of importance for triggering the phenotypic changes observed during the present study in the enteric perikarya located within the impaired parts of the porcine jejunum.

In the present study, we observed that both kinds of the pathogens,
diagnosed microbiologically as factors responsible for the diarrhea, evoked a similar increase in the density of nerve fibres within the myenteron of the inflamed ileum. This phenomenon may be explained in two ways. First, the arrest in the axonal transport and the inhibition of the release of the molecules from varicosities may be responsible for accumulation of a particular neurotransmitter substance in nerve fibres, what allows to visualize them easier by the technique used. However, axonal sprouting should also be taken into consideration. This latter possibility appears to be even more probable because the similar response to inflammation was observed in the distal bowel. For example, extensive changes were observed in the distribution pattern of SP- or VIP-immunoreactive nerve fibres during chemically induced colitis with an initial decrease in their number followed by an increase in their density (18). It has been shown that intestinal inflammation leads to an increase in the number of smooth muscle cells (9). This may thus suggest that intrinsic neurons undergo axonal proliferation to maintain the density of the smooth muscle cell innervation. During the present study we also observed an increase in the number of mucosal nerve fibres in inflamed segments of the porcine jejunum. It appears to be possible that neuronal plasticity is an essential part of reparative mechanisms also in the mucosal membrane. This is strongly confirmed by observation that parasite-evoked inflammation of the jejunum in the rat (27), guinea-pig (20) and pig (1) induces a distinct decrease in the number of mucosal axons that is, however, followed by a further regeneration of the nerve processes.

In the present study, we found that in the intestinal segments with signs of inflammation not only nerve terminals, but also neuronal cell bodies themselves underwent profound changes in respect to their chemical phenotypes. Interestingly, the most prominent up-regulation in the synthesis rate of the substances studied in affected neurons concerned GAL and VIP. Both these substances have been thought to be of crucial importance for survival and regeneration of injured autonomic neurons (14, 25, 31). Thus, an increase in the number of neuronal cells containing these antigens may be indicative for the involvement of these neuropeptides in repairing processes accompanying the catarrhal inflammation in the porcine jejunum. Furthermore, these neuropeptides have been shown to contribute to inhibitory pathways in both the intra-intestinal regulatory circuits (7, 8) and in those connecting the intestinal nerve system with extrinsic autonomic ganglia (for a review, see (28). Thus, in addition to a putative relevance of these substances as neuroprotective cues, a possibility that the up-regulation of their synthesis rate may be representative for the neurally driven inhibition of the motility of the irritated jejunal loop should also be taken into consideration.

Nonetheless, further functional studies are necessary to answer the question whether the above-mentioned mechanisms are involved in the diarrhea-induced changes in the neurochemical organization of the porcine jejunum.

In conclusion, although immunohistochemistry has been proved to be an excellent tool to investigate changes in the distribution pattern of neuroactive substances (both in nerve terminals and ganglionated plexuses harbouring their parental perikarya) during catarrhal inflammation of the porcine small intestines, this technique alone, however, is not sufficient as a tool for clear-cut differentiation between clinically diagnosed diarrhea of putative viral or bacterial etiology on the basis of the adaptive changes observed.
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References


