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Anatomical Evidence for Enteric Neuroimmune Interactions in Peyer's Patches

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Abstract

Peyer's patches (PP), a key component of the gut-associated lymphoid tissue, serve as the primary inductive sites for intestinal immunity. In the present study, we addressed the hypothesis that the morphological features of PP innervation are consistent with an immunomodulatory role for the enteric nervous system. Laser scanning confocal microscopy was used to collect images through large tissue volumes, yielding a three-dimensional perspective of the neuronal network superimposed on PP follicles from porcine jejunum and human ileum. Peptidergic nerve fibers were found in close apposition to immunocytes within PP subepithelial domes and the adjacent villi. The results suggest that nerve fibers in PP may participate in neuroimmune cross-talk within individual antigen-sampling sites as well as integrate information across multiple antigen-sampling sites.

Keywords

enteric nervous system; mucosal immunity; substance P; vasoactive intestinal peptide; calcitonin gene-related peptide

1. Introduction

The enteric nervous system (ENS) encompasses an estimated 10^8 neurons within the intestinal wall that constitute a complex network of intrinsic primary afferent neurons, interneurons, and effector neurons organized in ganglionated neural plexuses (Furness, 2006). In addition to its well-defined roles in regulating intestinal motility and active transepithelial ion transport, the ENS appears to modulate aspects of intestinal immune function involved in mucosal protection and defense. Enteric neurons innervate the intestinal mucosa, including the gut-associated lymphoid tissue, which carries out the dual tasks of maintaining tolerance to food antigens and commensal microflora while initiating innate and acquired immune responses against luminal pathogens. Peyer's patches (PP), a component of the gut-associated lymphoid tissue, serve as the primary inductive site for intestinal immunity and a major target for infection by food- and water-borne pathogens such as *Salmonella*, *Shigella* and *Yersinia* (Vazquez-Torres and Fang, 2000). The lymphoid follicles of PP are covered by a modified epithelium. Highly specialized

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“microfold” cells (M-cells) within this follicle-associated epithelium capture antigens from the intestinal lumen and transport them into the underlying follicular dome region, where they are processed by antigen-presenting cells (Mowat, 2003; Newberry and Lorentz, 2005).

Enteric ganglia are situated at the base and corona of PP follicles as well as in the interfollicular regions. In murine, porcine and bovine PP, nerve fibers are present within the subepithelial dome but not within the germinal center of follicles (Krammer and Kühnel, 1993; Kulkarni-Narla et al., 1999; Balemba et al., 1998, 1999; Defaweux et al., 2005). Nerve fibers within PP have been hypothesized to modulate intestinal immune function (Pascual et al., 1994) and the cellular uptake of pathogenic bacteria (Green et al., 2003). Enteric nerves also constitute a route for infection of the central nervous system by ingested neurotrophic viruses (Morrison et al., 1991; Narita et al., 1998) and prions (van Keulen et al., 2002). The innervation of PP includes peptidergic fibers, which contain neuropeptides such as vasoactive intestinal peptide (VIP), substance P (SP) and calcitonin gene-related peptide (CGRP). In addition to their role in neuronal communication, these neuropeptides have been shown to alter leukocyte function in lymphoid organs (Mignini et al., 2003; Delgado et al., 2004a). Immune cells express neuropeptide receptors and their activation may influence antigen presentation, cytokine production and lymphocyte proliferation. Therefore, neuropeptides represent a potential signaling interface between enteric nerves and immune cells in PP.

The present study addressed the hypothesis that the morphological features of PP innervation are consistent with an immunomodulatory role of the ENS at this mucosal inductive site. Our objective was to achieve a three-dimensional understanding of the PP neuronal network and to examine the relationship of peptidergic nerve fibers to immunocytes.

2. Materials and Methods

2.1. Porcine intestine

2.1.1. Animals and tissue isolation—Single Peyer’s patches (one to two per pig) were isolated from the distal jejunum of outbred Yorkshire-Landrace pigs ($n = 16$) of either sex that weighed between 10 and 18 kg and were 5 to 9 weeks old. At this age, intestinal immune function has reached a mature stage (Bailey et al, 2001). Animals had continuous access to water and nonmedicated pig feed and were not fasted prior to sacrifice. They were anesthetized by intramuscular injection of tiletamine hydrochloride-zolazepam (Telazol®; 8 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) in combination with xylazine (8 mg/kg), and subsequently euthanized with intravenous Beuthanasia®-D Special (0.5 ml/kg; Schering-Plough Animal Health, Union, NJ) in accordance with approved University of Minnesota IACUC protocols.

2.1.2. Tissue preparation—Jejunal segments containing PP were cut along the mesenteric border, pinned on a Silgard-coated surface mucosal side up, and fixed in modified Zamboni’s fixative (4% paraformaldehyde and 0.2% picric acid) for 2 h at room temperature. After extensive washing in phosphate buffered saline (PBS), the tissue was stored in PBS containing 10% sucrose and 0.05% sodium azide until further use. Three different tissue preparations were used in this study (Fig. 1): 1) whole mounts; 2) microtome sections, thickness 200–300 μm ; and 3) cryostat sections, thickness 20 μm . It should be noted that variability inherent in the tissue processing procedures (e.g. angle of cutting) contributed to slight variations in the appearance of the structures, which were most noticeable in the shape of the domes and the overall thickness of the mucosa.

2.2. Human intestine

Mucosal biopsies of terminal ileum containing PP follicles from one subject were acquired during colonoscopy with subject consent in accordance with an approved University of Minnesota IRB protocol. The tissue was fixed immediately in Zamboni's fixative (2% paraformaldehyde and 0.2% picric acid) for 18 h at 4°C and stored in PBS containing 10% sucrose and 0.05% sodium azide until it was cut into 14 µm slide-mounted cryostat sections. The biopsies were not pinned down before fixation or before freezing which resulted in slight warping of the mucosa around the PP follicles.

2.3. Antisera

The primary antisera used in this study are listed in Table 1. Single-labeled preparations were visualized with Cy3-conjugated secondary antisera raised in donkeys. Double- and triple-labeled preparations were visualized with combinations of Cy3-, Cy5- and Cy2-conjugated donkey antisera, which were matched appropriately to the host species of the primary antisera. All secondary antisera were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and were used at dilutions of 1:300, 1:300 and 1:100 for Cy3-, Cy5- and Cy2-conjugates, respectively. In control experiments on porcine PP, in which incubation with primary antisera was omitted, we noted some non-specific labeling of the tissue with secondary antisera. Most of this non-specific labeling was eliminated by the addition of normal swine serum to the blocking buffer (see below). Normal swine serum blocked the labeling with goat anti-porcine immunoglobulin A. Therefore, in triple-labeling experiments, which included this antibody, secondary antisera were applied sequentially, separated by a blocking step with normal swine serum (i.e., donkey anti-goat secondary antiserum, followed by normal swine serum, followed by remaining secondary antisera).

2.4. Immunohistochemistry

Whole-mount preparations of porcine jejunal PP were cleared by dehydration and re-hydration in a series of ethanol (EtOH) and xylene washes (80% EtOH, 95% EtOH, 95% EtOH, 100% EtOH, 100% EtOH, xylene, xylene, 100% EtOH, 80% EtOH, PBS; 30 min each). The tissue samples were incubated overnight at 4°C in blocking buffer (PBS containing 1% Triton X, 1% BSA, 1% normal swine serum, 0.01% sodium azide), followed by incubation with primary antibodies (Table 1) diluted in blocking buffer for 36 - 48 h at 4°C. Monoclonal antibodies (e.g., rat anti-SP and mouse anti-PGP-9.5) generally produced better staining in this preparation than polyclonal antibodies (e.g., rabbit anti-VIP and goat anti-collagen type IV). After extensive washing in PBS, the preparations were incubated in secondary antisera overnight and washed again. For mounting, the tissues were immersed in molten agar (1.35% w/v Nobel agar; Sigma, St. Louis, MO) and placed on a 22 × 22 mm coverglass mucosa side down. Upon solidification of the agar, the tissue was permanently attached to the coverglass. The coverglasses with agar-embedded tissue were then dehydrated through a series of ethanol and xylene washes and mounted using DPX mounting media (Sigma, St. Louis, MO) onto a 60 × 22 mm coverglass instead of a slide to enable microscopic viewing and imaging of the thick preparations.

The labeling protocol for thick (200-300 µm) microtome sections of porcine jejunal PP was similar to the protocol used for whole-mounts with the exception that the agar-embedded sections were mounted on slides. In addition, microtome sections in some cases were embedded directly in DPX without the use of agar. However, the agar embedding procedure yielded better results.

Slide-mounted cryostat sections of porcine jejunal PP and human terminal ileum biopsies were incubated in blocking buffer (PBS containing 0.03% Triton X, 1% BSA, 1% normal swine serum, 0.01% sodium azide) for 1 h at room temperature, followed by incubation in primary

antisera overnight at 4°C. The slides were then rinsed in PBS, incubated in secondary antisera for 1 h at room and coverslipped using DPX.

2.5. Image collection and processing

Images were collected using a confocal laser-scanning microscope (Bio-Rad MRC 1000 or MRC 1024). The different fluorophores were imaged sequentially. The estimates of imaged tissue volume that are reported in the figure legends were based on the distances between individual optical sections in the z-plane. Due to shrinkage during tissue processing and potential optical stretching in the z-plane, these estimates are expected to deviate from the actual volumes. Stacks of optical sections were projected using Confocal Assistant 4.1 (freeware). All images were adjusted for brightness and contrast using Adobe Photoshop (version 6.0.1, Adobe Systems, San Jose, CA). Color micrographs were generated by digital merging in Adobe Photoshop of corresponding images from double- and triple-labeled preparations.

3. Results

3.1. Overview of immune and neural components of porcine Peyer's patches (PP)

A low magnification survey using immune and neuronal markers revealed some of the main characteristics of the PP mucosa from porcine jejunum (Fig. 2). IgA- and IgM-immunoreactivities (-ir) were used to visualize plasmacyte-rich areas, and CD3-ir was used to detect T lymphocytes. Intense IgA labeling demarcated the crypt areas rich in IgA-positive B cells, whereas diffuse IgA-ir was observed in B cell germinal centers of PP follicles. A similar staining pattern was seen with anti-IgM antisera (data not shown). CD3-positive T lymphocyte-rich areas were located in the interfollicular space. Within the villi, intense CD3-ir was associated with intraepithelial lymphocytes.

The PP neural components were visualized with antisera directed against the pan-neuronal marker protein gene product 9.5 (PGP). PGP staining revealed ganglia near both the abluminal (base) and luminal (corona) aspects of PP follicles. A dense network of nerve fibers was observed in PP domes, which overlie the follicles, as well as in the neighboring villi, which overlie the interfollicular space.

3.2. Ganglion networks associated with porcine PP lymphoid follicles

In whole-mount preparations of swine jejunal PP, enteric ganglia appeared to be clustered at different topographical levels within interfollicular regions (Fig. 3A). At the base of follicles (i.e. abluminal aspect), these ganglia varied considerably in size and shape. Based on their location relative to vascular arcades and the muscularis externa, they appeared to be part of the internal submucosal plexus. Interconnecting nerve strands generally coursed along the perimeter of the follicles, forming a honeycomb pattern. Ganglia situated near the corona (i.e. luminal aspect) of PP follicles were relatively small and either irregularly shaped or ovoid in appearance. They were interspersed within a loose network of nerve fibers in the interfollicular space between domes (Fig. 3B).

Nerve strands projecting in the interfollicular space appeared to connect the ganglion networks at the base and the corona of the follicles (Fig. 3 C, D and 4A). Although the final destinations of these projections were generally indiscernible in the meshwork of fibers, a particular fingerlike termination pattern was noted on multiple occasions (Fig. 3D). Occasionally, these fingerlike nerve endings were seen in close proximity to the processes of large (approximately 25 µm in diameter), often solitary neurons found in the lamina propria of PP villi. The processes of these large neurons appeared to extend towards the tips of the villi and towards the network of ganglia in the corona (Fig. 4D).

Imaging in the transverse plane revealed a dense plexus of nerve fibers in PP domes. Fiber bundles appeared to stream into the dome region from the adjacent villous plexus (Fig. 3C and E, 4A). Imaging of PGP-ir in the horizontal plane of whole-mount mucosal preparations showed that nerve bundles enter the domes in a radial pattern and form a dense meshwork in the dome interior (Fig. 3F).

3.3. Peptidergic innervation of porcine jejunal PP

Neuropeptide labeling was abundant in PP ganglion networks (Fig. 4). VIP-ir was generally seen in smaller neurons and did not overlap with SP- or CGRP-ir. In contrast, there was extensive colocalization of SP- and CGRP-ir, and the majority of SP- and CGRP-ir neurons were also immunoreactive for NF 200 (data not shown). Some of these neurons, including the solitary neurons in PP villi noted above, were very large and manifested morphological characteristics resembling Dogiel type II neurons previously described in the porcine intestine (Hens et al., 2000). Finally, neuropeptide-positive fibers also formed a dense plexus in the PP villi.

The dome innervation was rich in VIP-, SP-, and CGRP-ir fibers (Fig. 4 A-D, Fig. 5A-D). In many nerve fibers SP- and CGRP-ir were extensively co-localized, whereas SP-ir did not overlap with VIP-ir (Fig. 5A-D). To address the possibility that peptidergic nerve fibers in the subepithelial dome may interact with epithelial cells and blood and lymphatic vessels, we examined the relationship of SP- and VIP-ir to markers of these structures in porcine jejunal PP. Inducible nitric oxide synthase (iNOS) was employed as a marker for epithelial cells. Inducible NOS-ir in the jejunal PP was restricted to the follicle-associated epithelium of the domes and the epithelial layer of the adjacent villi (data not shown). Within epithelial cells, intense band of iNOS-ir was present in the region of the microvilli (data not shown). Double labeling experiments indicated that SP-ir fibers terminated in close proximity to the basolateral aspect of iNOS-ir epithelial cells (Fig. 5E). These fibers appeared close to, but did not penetrate the basement membrane, which was visualized by its type IV collagen immunoreactivity (Fig. 5F). Type IV collagen-ir also delineated lymphatic and blood vessels. SP- and VIP-ir (not shown) nerve fibers appeared to course in parallel with collagen-ir vessels, but overall there did not appear to be a close relationship between the two structures (Fig. 5G).

3.4. Relationship of peptidergic nerve fibers to immunocyte subpopulations in porcine PP

To determine whether the observed distribution of peptidergic fibers was consistent with neuro-immune communication in PPs, we performed a series of triple labeling experiments with antisera against neuropeptides and components of the mucosal immune system. An anti-MHC class II antibody was used to visualize antigen-presenting cells; antibody against calprotectin, an inflammatory marker, was used to label macrophages; and anti-IgA and anti-CD3 were used respectively as markers of IgA-positive plasma cells and T lymphocytes.

The relationship of peptidergic fibers and IgA-ir cells in PP domes was not examined because the abundance of IgA-ir cells in the domes varied considerably. Some domes were densely populated with labeled B-cells, whereas others were completely devoid of them. In PP villi, IgA-positive cells were densely packed in the crypt regions and their numbers decreased towards the villous tips (Fig. 6A). In a non-quantitative triple-labeling survey, we observed multiple instances of apparent contacts of peptidergic fibers and IgA-positive cells in both the tips (Fig. 6B) and crypt regions of the villi (Fig. 6C).

Lymphocytes exhibiting intense CD3-ir were generally absent from PP domes. In PP crypt regions, there were many faintly-labeled CD3-positive cells intermingled with fewer, intensely-labeled cells (Fig. 6C). Although nerve fibers appeared to be in close proximity to CD3-ir cells in the crypt region, the relationship of T cells and nerve fibers was difficult to

evaluate due to the low intensity of CD3 staining. In the villous tips, SP-ir fibers were seen within several microns of T cells (Fig. 6B).

Nerve fibers in the subepithelial dome region appeared intermingled with MHC class II-positive cells, which morphologically resembled dendritic cells (Fig. 7A). Imaging at high magnification demonstrated close appositions of these cells to both SP- and VIP-ir nerve fibers (Fig. 7 B and C). SP- and VIP-ir varicosities were observed within 1-2 μ m of MHC class II-immunopositive cells (Fig. 7, insets in B and C). Some of these cells appeared to express cytoplasmic SP-ir. In PP domes, SP-ir fibers and calprotectin-positive cells were also closely apposed (Fig. 7 D and E).

3.4. Peptidergic nerve fibers in PP from human terminal ileum

Porcine jejunal PP are thought to be functionally homologous to PP in the human terminal ileum. Therefore, we examined a biopsy of a human ileal PP for parallels between porcine and human PP innervation. The following similarities were noted: 1) SP-, CGRP- and VIP-positive nerve fibers were seen around the germinal center and within the subepithelial dome (SP-ir shown in Fig. 8A); 2) nerve fibers were generally absent from the germinal center (Fig. 8 A and C); 3) nerve fibers appeared to enter the subepithelial dome from the adjacent villous plexus (Fig. 8 C and E); and 4) in the subepithelial dome, SP- and VIP-positive nerve fibers were seen in close apposition to anti-HLA positive immunocytes which are likely to be antigen presenting cells (Fig. 8H). Due to limited amount of tissue in this study, we did not examine the ganglionated plexuses and the three-dimensional organization of PP innervation in human terminal ileum.

4. Discussion

The present study examined the innervation of porcine jejunal PP by fluorescent immunohistochemistry and laser-scanning confocal microscopy. Imaging of tissue volumes that exceeded 100 μ m in the transverse plane and 200 μ m in the horizontal plane yielded a three-dimensional perspective of the neuronal network associated with the lymphoid follicles. The localization of ganglionated plexuses at different topographical levels relative to the follicles was consistent with previous studies of porcine and bovine PP (Krammer and Kühnel, 1993; Kulkarni-Narla et al., 1999; Balemba et al., 1998, 1999). At the level of the PP corona, previous reports have described several distinct ganglionated plexuses, which were situated close together. We did not attempt to distinguish among these plexuses, but overall our observations of the ganglion network at the corona are consistent with the presence of a mucosal plexus, as proposed by Balemba et al. (1998).

An intriguing observation in this study is the radial projection pattern of nerve bundles in subepithelial dome areas. In the context of the diffuse ganglion network at the corona of PP follicles, the radial projections into a single dome are likely to originate from multiple ganglia interspersed around it and between adjacent domes. This pattern of innervation suggests a novel immunomodulatory role for the enteric nervous system. Nerve fibers in individual domes may be poised to monitor immune activity through nerve-immunocyte communication. The mucosal ganglion network could then rapidly integrate this information, providing means for coordination and modulation of antigen sampling and processing in different subepithelial domes. Furthermore, communication with extramucosal plexuses as well as the potential extramucosal or extrinsic origin of nerve fibers present in PP domes offer additional levels of neuroimmune interactions, including central nervous system processing through input from extrinsic primary afferents.

We observed dense peptidergic innervation in the PP mucosa, extending the findings from previous reports of porcine PP innervation (Balemba et al., 1998; Kulkarni-Narla et al., 1999),

and complementing those from studies of murine and feline PPs (Ottaway et al., 1987; Ichikawa et al., 1994). In the porcine small intestine, enteric neurons projecting to the non-follicular absorptive mucosa that contain both SP- and CGRP-ir are thought to represent intrinsic primary afferent neurons, whereas those that express VIP-ir may play a secretomotor role (Hens et al., 2000; Brown and Timmermans, 2004). Mucosally-projecting enteric neurons that are SP-positive/VIP-negative and VIP-positive/SP-negative may have similar functions in the human small intestine (Hens et al., 2001). It is unknown whether these two distinct subpopulations of nerve cells and fibers serve related roles in porcine and human Peyer's patches. For example, all three neuropeptides have been reported to induce active anion secretion in explants of the small intestinal mucosa or in cultured intestinal epithelial cells (Cox and Tough, 1994; Thorboll et al., 1998), but their effects on the follicle-associated epithelium have not been characterized. Furthermore, the basement membrane of follicle-associated epithelium is porous, allowing access of lymphocytes to the M-cell pocket (Neutra et al., 2001). This porousness may facilitate the diffusion of signaling molecules between epithelial cells and peptidergic nerve fibers, which were seen in close proximity to the basement membrane of follicle-associated epithelium in this study. This anatomical relationship may have pathophysiological implications as communication between enteric nerves and the intestinal epithelium appears to be an important factor in a number of gastrointestinal inflammatory conditions (Sharkey and Mawe, 2002).

There is considerable evidence that SP, CGRP and VIP can alter intestinal immune function. For example, specific binding sites or functional receptors for these and other neuropeptides are expressed on intestinal immunocytes (Pascual et al., 1994; Goode et al., 2000; Hagner et al., 2002) and in Peyer's patches (Ichikawa et al., 1994; Reubi et al., 1998). VIP inhibits the migration of T lymphocytes into PPs (Ottaway, 1984; Miura et al., 1997) and alters B cell-mediated immunoglobulin synthesis (Stanisz et al., 1986). In contrast to VIP, SP stimulates immunoglobulin synthesis in Peyer's patch B lymphocytes (Stanisz et al., 1986, 1987; Pascual et al., 1992). Consistent with these immunomodulatory roles, we observed a number of apparent contacts between IgA-expressing plasma cells and SP- and VIP-containing fibers. The close intermingling of lymphocytes and peptidergic fibers in the crypts of PP villi is reminiscent of the apposition of nerve fibers and mucosal mast cells at immune effector sites in the small intestine (Stead, 1992). Finally, in addition to their direct interactions with lymphocytes, these neuropeptides may alter blood and lymphatic flow in the intestine, and do so in Peyer's patches as well (Wang et al., 1994; Ichikawa et al., 1994; Vanner and Surprenant, 1996).

In the subepithelial dome of both porcine jejunal PP and human ileal PP, peptidergic fibers were seen in close apposition to antigen presenting cells identified based on their MHC class II- or calprotectin immunoreactivities in pig tissue and HLA-DR, DP, DQ immunoreactivity in human tissue. In porcine PPs, immature dendritic cells express MHC class II-ir at high levels; those residing in subepithelial domes additionally express signal regulatory protein *alpha*, but not CD11b (Makala et al., 1998; Bimczok et al., 2005). On the other hand, calprotectin is a well-established granulocyte marker, which has been used to label porcine macrophages (Dell'Angelica et al., 1996; Teifke et al., 2001). A similar, close association between nerve fibers and presumptive dendritic cells has recently been described in the subepithelial (suprafollicular) dome region of murine PPs (Defaweux et al., 2005). These findings are consistent with evidence for the expression of SP, VIP and CGRP receptors in dendritic cells (Carucci et al., 2000; Marriott and Bost, 2001; Delgado et al., 2004b) and macrophages (Goode et al., 2000; Takahashi et al., 2000; Fernandez et al., 2001). These neuropeptides can affect the migration of dendritic cells (Dunzendorfer and Wiedermann, 2001), and alter cytokine production or antigen presentation by macrophages (Nong et al., 1989; Castagliuolo et al., 1997; Ganea and Delgado, 2001; Yaraee et al., 2005). Therefore, the peptidergic nerve fibers observed in the subepithelial PP dome may modulate the induction of the mucosal immune response to antigens through the release of VIP, SP or CGRP. The current understanding of

the effects of neuropeptides on immune cells is largely based on in vitro studies or studies in tissues other than PP. Because immune cell function is dependent on activation status and the specific tissue environment, it will be necessary to investigate further the functional interactions of enteric nerves and antigen-presenting cells specifically within these immune inductive sites.

In addition to their prominent role in adaptive mucosal immunity, PP may transmit through their extensive innervation crucial information about potentially hostile encounters between ingested pathogens, antigens or other exogenous materials and the intestinal mucosa. Afferent nerves in PP might send information to lower segments of the intestinal tract through changes in the activity of enteroenteric reflex pathways as well as communicate with the central nervous system via extrinsic afferents to the spinal cord. Similarly, efferent neural activity may influence immune responses within PP. The bidirectional neuro-immune communication within PP clearly requires further delineation.

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Figure 1.

Schematic representation of porcine jejunal Peyer's patch preparations. Whole mount preparations were imaged in the horizontal plane (indicated by dashed red lines), whereas thick (200-300 μm) microtome sections and thin (20 μm) cryostat sections were imaged in the transverse plane (indicated by dashed red lines).

Figure 2. Cross-section of a jejunal Peyer's patch, showing locations of PGP-immunoreactive (red) nerve fibers (arrows) and ganglia (arrowheads), IgA-expressing plasma cells (blue) and CD3⁺ T lymphocytes (green). M, smooth muscle; S, submucosa; D, subepithelial dome; GC, germinal center of follicle; T, T-cell rich areas. Scale bar: 250 μm.

Figure 3.

Innervation of porcine jejunal Peyer's patches as shown by PGP immunoreactivity (-ir).

A and B. Whole mount mucosal preparations imaged in the horizontal plane. The approximate locations of the imaged planes are indicated on the schematic in G. **A**, Enteric ganglia located within the interfollicular space at the base of follicles. **B**, Innervation at the level of the follicular corona. Dense meshwork of nerve fibers is seen near the base of four domes (arrowheads). Ganglia (arrows) are interspersed in a nerve network within the interfollicular space between the domes. The image is the result of merging of five optical sections collected at 10 μ m intervals. Thus it represents labeling in a 40 μ m volume of PP mucosa, which was located approximately 170 μ m below the villous surface.

C, Image of a thick transverse section, showing a dense plexus of fibers in domes (arrows). Nerve strands (double arrows) from an interfollicular ganglion at the base of the follicles extend towards the region between two domes. The image is the result of merging of 11 optical sections collected at 6 μm intervals and represents labeling in a 60 μm volume of PP mucosa. GC, germinal centers.

D, Higher magnification image of the interfollicular strands in C at the level of the follicular corona. Note the finger-like termination pattern (arrow). The image is the result of merging of 24 optical sections collected at 3 μm intervals, representing a 69 μm volume of PP mucosa.

E and F Innervation of subepithelial domes. **E**, In a transverse section, PGP-ir shows a dense plexus of nerve fibers in the subepithelial dome. The fibers appear to enter the dome from the adjacent villous nerve network (arrow). The image represents a 45- μm volume (10 optical sections collected at 5 μm intervals). **F**, PGP-ir at the base of a dome imaged in the horizontal plane. The dome region appears surrounded by nerve bundles interspersed with small ganglia (arrowhead). Projections to the dome emanate in a radial pattern from these bundles, forming a dense plexus in the dome interior. In the transverse plane, each of the radial projections most likely appears as the nerve bundle indicated by arrow in A. The image represents a 50- μm volume (11 optical sections collected at 5 μm intervals).

G, Schematic representation of the neuronal network in porcine jejunal PP. Scale bar: A, 250 μm ; B and C, 100 μm ; D and E, 50 μm ; F, 100 μm .

Figure 4.

Peptidergic innervation of porcine jejunal PP, visualized by labeling with anti-SP, anti-CGRP and anti-VIP antisera in thick transverse sections.

A-D, Triple labeling with anti-SP (A, D – red), anti-CGRP (B, D – green) and anti-VIP (C, D – blue) demonstrates the distribution of neuropeptides in PP. Neuronal cell bodies that contain both SP- and CGRP-ir appear yellow (arrow). Ganglia are seen at the base of the follicles, in the interfollicular region and at the level of the corona. Solitary neurons (arrow) or small clusters of neurons (double arrows) are also seen in the villous lamina propria. The apparent absence of SP-ir nerve fibers in this image is due to the fact that at this low magnification the labeling intensity of SP-ir nerve fibers appears low relative to the overwhelming intensity of SP-ir cell bodies. The innervation in the subepithelial dome is indicated by an arrowhead. GC, germinal center. The image represents a 50- μ m volume of PP mucosa (11 optical sections collected at 5 μ m intervals).

E - G, Double-labeling with anti-SP (red) and anti-VIP (green) shows that the two peptides are localized in different subsets of neurons; E, ganglion located at the follicular base (2- μ m volume, 2 optical sections collected at 2 μ m intervals); F, interfollicular ganglion (8- μ m volume, 5 optical sections collected at 2 μ m intervals); G, ganglion located at the follicular corona (8- μ m volume, 5 optical sections collected at 2 μ m intervals).

H, Double-labeling with anti-SP (red) and anti-CGRP (green) demonstrates the colocalization (overlap of red- and green-labeled structures) of the two peptides in a ganglion located at the level of the follicular corona (8- μ m volume, 5 optical sections collected at 2 μ m intervals). Scale bars: A - D, 100 μ m; E - H, 20 μ m.

Figure 5.

Peptidergic innervation of the subepithelial dome of porcine jejunal PP.

A and B, Double-labeling with anti-SP (red) and anti-VIP (green) shows that SP- and VIP-ir are localized in different nerve fibers. Arrows indicate the same structures at low (A; 4- μ m volume, 3 optical sections, 2 μ m intervals) and high (B; 16- μ m volume, 9 optical sections, 2 μ m intervals) magnification.

C and D, Double-labeling with anti-SP (red) and anti-CGRP (green) shows colocalization of SP- and CGRP-ir in nerve fibers as indicated by the overlap of red- and green-labeled structures. Arrowheads indicate the same structures at low (C; 6- μ m volume, 4 optical sections, 2 μ m intervals) and high (D; 18- μ m volume, 10 optical sections, 2 μ m intervals) magnification.

E, SP-ir nerve fibers (red) are seen in close proximity to the epithelium visualized by iNOS-ir (red) (6- μ m volume, 7 optical sections, 1 μ m intervals).

F, A SP-ir nerve fiber (red; arrowhead) is seen within several microns of the basement membrane (long arrow) visualized by collagen IV-ir (green).

G, SP-ir fibers occasionally appear to course along collagen IV-ir vessels (examples indicated by arrows; 8- μ m volume, 5 optical sections, 2 μ m intervals).

Scale bars: A, C and G, 50 μ m; B, D, E and F, 20 μ m.

Figure 6.

Relationship of SP-ir (red) nerve fibers to IgA-ir (blue) and CD3-ir (green) in villi of porcine PP (20 µm transverse sections).

A, SP-ir nerve fibers course through the lamina propria of PP villi. IgA-ir cells are most abundant in the crypt region of the villi, whereas CD3-ir is prominent in intraepithelial lymphocytes. Arrow indicates the region shown at higher magnification in B.

B, In a villous tip, an IgA-ir cell is seen in close apposition to a SP-ir nerve fiber (6-µm volume, 7 optical sections, 1 µm intervals). The inset shows the close relationship between the nerve fiber and the cell in a single optical section.

C, The crypt region is densely packed with IgA-ir cells, weakly labeled CD3-ir cells and SP-ir nerve fibers. Many of the labeled cells are found within several microns SP-ir nerve fibers (4-µm volume, 3 optical sections, 2 µm intervals).

Scale bars: A, 100 µm; B and C, 20 µm.

Figure 7.

Relationship of SP-ir (red) and VIP-ir (green) nerve fibers to antigen-presenting cells visualized by MHCII-ir and calprotectin-ir (blue) in subepithelial domes of porcine PP (20 μm transverse sections).

A, MHCII-ir cells and SP- and VIP-ir nerve fibers are intermingled in the dome region and generally absent from the underlying germinal center. The overlap of red and blue structures, seen as pink/purple suggests that some MHCII-ir cells also contain SP-ir.

B and C, MHCII-ir cells are seen in close proximity to peptide-ir nerve fibers (arrows). Insets are digital (rather than optical) magnifications of the cells indicated by arrows, showing the close relationship of varicosities (arrowheads) containing SP- or VIP-ir to MHCII-ir cells. SP-

ir is seen in some (B, purple) but not all (C, blue) MHCII-ir cells. Images in B and C represent a 2- μ m volume (3 optical sections, 1 μ m intervals), while insets were generated from single optical sections.

D and E, Calprotectin (CAL)-ir cells are seen in close proximity to SP-ir nerve fibers. **D**, Calprotectin-ir cells are present in the subepithelial dome and the adjacent villi (V). **E**, A higher magnification image shows the close relationship of calprotectin-ir cells and SP-ir varicosities (6- μ m volume, 7 optical sections, 1 μ m intervals). The inset (single optical section) is a digital magnification of the cell indicated by an arrow in D and E. Scale bars: A and D, 50 μ m; B, C and E, 20 μ m; insets B and E, 5 μ m; inset C, 2 μ m.

Figure 8.

Peptidergic innervation in a PP from human terminal ileum (14 μm transverse sections).

A - F, Double labeling with anti-SP (A, C, and E) and anti-HLA (B, D, and F) of three sections (AB, CD, EF) taken from the same PP follicle and separated by approximately 30 μm. The sections were taken at an oblique angle as illustrated in G. In B and D, note that the intensity of HLA-ir is highest in the germinal center (GC). SP-ir nerve fibers appear to course around the germinal center (A) and enter the dome region from the villous plexus (C and E). The arrow in C indicates the region, which is shown at higher magnification in G.

G, A schematic representation of the likely orientation of the sections shown in A – F.

H, Higher magnification micrograph showing the close relationship (see arrows) of SP-ir (red) and VIP-ir (green) to HLA-ir cells in the subepithelial dome.

Scale bars: A - F, 100 μm; H, 20 μm.

Table 1**Antisera used in this investigation**

Antibody	Host	Dilution	Source
Neuronal markers			
anti-CGRP	Rabbit	1:1000	ImmunoStar, Hudson, WI
anti-CGRP	Mouse	1:2000	Sigma-RBI, discontinued
anti-NF200	Mouse	1:1000	Sigma, St. Louis, MO
anti-PGP	Mouse	1:1000	Biogenesis, Poole, UK
anti-SP	Rat	1:100	Oxford Biotechnology, Kidlington, UK
anti-VIP	Rabbit	1:2000	ImmunoStar, Hudson, WI
Immune cell markers			
anti-Calprotectin	Mouse	1:1000	Serotec, Oxford UK
anti-IgA	Goat	1:2000	Bethyl Laboratories, Montgomery, TX
anti-IgM	Mouse	1:300	Bethyl Laboratories, Montgomery, TX
anti-CD3	Mouse	1:100	Kind gift from Dr. M.D. Pescovitz
anti-MHC class II	Mouse	1:1000	Antigenix America, Huntington Station, NY
anti-HLA (DR, DP, DO)	Mouse	1:3000	BD Biosciences, San Jose, CA
Other markers			
anti-Collagen IV	Goat	1:1000	SouthernBiotech, Birmingham, AL
anti-iNOS	Rabbit	1:300	Upstate, Lake Placid, NY

Abbreviations: CGRP, calcitonin gene-related peptide; Ig, immunoglobulin; iNOS, inducible nitric oxide synthase; MHC, major histocompatibility complex; NF, neurofilament protein; PGP, protein gene product-9.5; SP, substance P; VIP, vasoactive intestinal peptide