Intestinal mucosal mast cells in normal and nematode-infected rat intestines are in intimate contact with peptidergic nerves

(substance P/calcitonin-gene-related peptide/neuron-specific enolase)

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ABSTRACT Inflammatory or allergic conditions, as well as situations where healing and repair processes occur, are characterized by the presence of increased numbers of mast cells. Previous work on the effect of neuropeptides on mast cell mediator release showed that only substance P caused such release from intestinal mucosal mast cells [Shanahan, F., Denburg, J. A., Fox, J., Bienenstock, J. & Befus, A. D. (1985) *J. Immunol.* 135, 1331–1337]. Accordingly, we investigated the microanatomical relationship between mast cells and enteric nerves in normal rat intestine and parasite-infected rat intestine, in which mucosal mast cell hyperplasia occurs. Combined immunohistochemistry for neuron-specific enolase and staining with alcian blue at pH 0.5 was employed on paraffin-embedded sections of normal and *Nippostrongylus brasiliensis*-infected rat jejunum. Sixty-seven percent of intestinal mucosal mast cells were touching subepithelial nerves, and an additional 20% were within 2 μm of nerves. Assessment of the proportion of the lamina propria occupied by mast cells (12.5%), the average mast cell area (121 ± 28 μm^2), and the density of enteric nerves (one per 788 ± 151 μm^2) suggested that the association was 5 times greater than would be expected by chance alone ($P < 0.0001$). In consecutive sections, the nerves in contact with mast cells were also shown to contain substance P and/or calcitonin-gene-related peptide. Electron microscopy confirmed this association: 8% of the mast cells in infected rats exhibited membrane–membrane contact with unmyelinated axons containing 70- to 170-nm dense-core vesicles, and an additional 31% were situated less than 250 nm from nerves. Other mast cells appeared to embrace nerve bundles through the projection of lamellopodia. These data provide systematic quantitative evidence that a structural foundation for communication between the immune and nervous systems exists in the rat gastrointestinal tract.

The histochemical and functional heterogeneity of mast cells is well described and accepted, especially in the rat (1, 2). Major functional differences have been shown between mast cells obtained from the peritoneal cavity (PMCs) and intestinal mucosal mast cells (IMMCs) isolated from the normal lamina propria as well as from rats infected with the nematode *Nippostrongylus brasiliensis*, which promotes intestinal mast cell hyperplasia (3, 4). Many secretagogues that affect PMCs have no effect on IMMCs, and similar differential effects are seen with many anti-allergic compounds (5). Indeed, previous reports (J.B. and coworkers, refs. 6 and 7) showed that whereas both endorphins and neuropeptides caused degranulation of PMCs *in vitro*, only substance P promoted histamine release from IMMCs (6, 7).

These *in vitro* observations are in keeping with *in vivo* experiments that strongly suggest that mast cells and nerves may communicate directly, presumably through soluble me-diators. Kiernan (8) and Lembeck and Holzer (9) suggested that mast cells may be involved in the axon reflex responsible for the flare reaction (vasodilation) to noxious stimuli and that substance P-containing nerves are involved. The response to intradermal injection of substance P in rats mimics the response of local injection of histamine or the mast cell secretagogue compound 48/80 (10). This can be mostly inhibited by pretreatment with antihistamine or capsaicin, both of which prevent the flare reaction of the axon reflex in the skin (9, 10). Furthermore, antidromic nerve stimulation causes neurogenic edema (11), mast cell degranulation (12), augmentation of histamine release (13), and histamine-mediated modulation of neurotransmitter release (14).

Since we could not find adequate documentation of a regular structural basis for the apparent functional mast cell–nerve association in any tissue site, we chose to examine normal and *N. brasiliensis*-infected rat intestines to quantitate mast cell–nerve interactions. This allowed us to look for such interactions in a normal state and in disease, when mast cell hyperplasia occurs (15). We report here that a surprising number of mast cells in normal and nematode-infected rat intestines are in contact with peptidergic nerves.

MATERIALS AND METHODS

Animals. Infected and noninfected male Lewis rats of 150–200 g (Charles River Laboratories) were studied. Infection with 3000 stage 3 larvae of *N. brasiliensis* was performed as described (15). All animals were housed under filter-hoods and given food and water ad libitum before being killed on days 0, 22, and 35 by ether anesthesia and cervical dislocation. Pieces of intestine (15–20 cm distal to the pylorus) were promptly removed and fixed by immersion (see below).

Light Microscopy. One-centimeter strips of intestine were gently flushed through with phosphate-buffered saline (PBS: 0.14 M NaCl/0.01 M phosphate, pH 7.2) before the gut was opened. These strips were attached to coverglasses with cyanoacrylate glue (Permabond 910) and immediately immersed in 10% acetic acid in 90% ethanol for 8 hr or neutral buffered formalin for 24 hr. The tissue was carefully removed from the coverglasses and sliced into 2-mm-wide strips, parallel to the longitudinal axis of the gut, before being processed to paraffin and embedded either on edge or with the mucosal face down.

Sections (3 μm) were cut from the acetic acid/ethanol-fixed tissues, dried at 50°C overnight, and stained with 1% alcian blue 8GX (Gurr, BDH, Toronto, ON) in 0.3 M HCl, pH 0.5 (AB 0.5). This was combined with an immunohistochemical method to demonstrate neuron-specific enolase (NSE). In brief, after the sections were dewaxed and rinsed in 100%

Abbreviations: IMMC, intestinal mucosal mast cell; CGRP, calcitonin-gene-related peptide; NSE, neuron-specific enolase; AB 0.5, alcian blue at pH 0.5.
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ethanol, endogenous peroxidase activity was blocked by incubation in 0.5% hydrogen peroxide in methanol for 30 min. Slides were then rinsed in ethanol and 0.3 M HCl before staining in AB 0.5 overnight. After three rinses with fresh 0.3 M HCl, two with distilled water, and two with Tris-buffered saline (TBS: 0.05 M Tris/0.15 M NaCl, pH 7.6), slides were incubated for 30 min in 20% normal swine serum in TBS. NSE was then detected by the peroxidase-antiperoxidase technique of Sternberger *et al.* (16). This involved 30-min incubations in rabbit anti-NSE (diluted 1:50), swine anti-rabbit immunoglobulin (1:25), and rabbit peroxidase-antiperoxidase complex (1:100), with intervening 15-min washes in TBS. All antisera (Dako, Santa Barbara, CA) were diluted in 5% normal swine serum in TBS. To prevent crossreaction with the α and β subunits of the enolase isoenzymes, the anti-NSE was absorbed with acetone-dried normal liver and normal muscle powders (100 mg/ml). Control sections were incubated in parallel with the test slides, substituting similarly absorbed normal rabbit serum. After the final wash in TBS, sections were rinsed in 0.5 M acetate buffer (pH 5.0), and the peroxidase activity was detected with aminoethylcarbazole (30 min) according to the method of Graham *et al.* (17).

For the detection of substance P and calcitonin-gene-related peptide (CGRP), tissues fixed with neutral buffered formalin were employed. Sections were cut as before, endogenous peroxidase activity was blocked in hydrogen peroxide/methanol, and the sections were washed in TBS and transferred to 20% normal swine serum in TBS. The immunohistochemical technique was similar to that described above, except that overnight incubations with anti-substance P (Immuno Nuclear, Stillwater, MN) diluted 1:100 and anti-rat CGRP (Peninsula Laboratories, San Carlos, CA) diluted 1:50 were employed. Control sections, incubated with antisera preabsorbed with specific antigen (100 μg/ml), were run in parallel. The mast cells were then demonstrated by a modification of the prolonged toluidine blue method of Wingren and Enerback (18), using a 1% solution of toluidine blue (Gurr) at pH 0.5, overnight at 37°C. NSE was also demonstrated in adjacent sections, allowing comparison of the distribution of the two neuropeptides with this enzyme.

Quantitation. Visual observations of the AB 0.5/NSE preparations were made at ×800 magnification. Careful evaluation of 25 separate villi selected at random from two animals (35 days after infection) was made to see if NSE-containing nerves were touching or within an estimated half-cell diameter of individual mast cells. A χ^2 test was employed to compare the results with the predicted incidence of contact, extrapolated from the area of the lamina propria occupied by mast cells [degrees of freedom (*df*) = 1: contact or no contact]. The number of mast cell contacts with substance P-containing nerves was also determined visually at ×800 magnification in animals 35 days after infection.

To confirm the visual observations, a Zeiss interactive digital analysis system (ZIDAS) was employed; and limited distance measurements were made from the same AB 0.5/NSE slides at ×500 magnification, with the automatic closure window set at 1.0 mm, by viewing the cursor and digitizing tablet through a camera lucida. If the distance between a mast cell and the closest nerve was less than the threshold set by the closure window, an "invalid measurement" resulted, indicating a separation of less than 2 μm.

The ZIDAS was also employed to determine the area of the lamina propria occupied by mast cells. Using the area parameter with the automatic closure set at 0.1 mm, all alcian blue-stained material, including small fragments of mast cells, was measured at ×200 magnification. The sum of mast cell profiles was then related to the sum of lamina propria areas, after subtraction of the area occupied by vessels.

To assess the distribution of nerves in the lamina propria, photographic transparencies of AB 0.5/NSE preparations were back-projected onto a Zeiss Videoplan image-analysis system, a regular measuring grid was laid over the digitizing tablet, and the number of nerves per square was counted for 280 fields. The software employed (Kontron, Zurich; Version 5.41) provided a histogram and analyzed the distribution with respect to skewness and kurtosis. Two-tailed critical-region analysis (19) with a 5% level of significance was used to evaluate the statistical normality of the distribution.

Nerve density was assessed by measuring the total area of lamina propria in 36 villi and counting the number of nerves, defined as isolated areas of NSE positivity. The average mast cell area was similarly determined on 200 apparently whole cells. A χ^2 test (*df* = 1) was then employed to compare the observed incidence of mast cell–nerve contact with that predicted by the measured nerve density and average mast cell area.

Electron Microscopy. Pieces of jejunum for electron microscopy were opened without flushing, attached to coverglasses with cyanoacrylate glue, and immediately immersed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C for 1 hr. Blocks (1 mm^3) were then taken from the mucosal surface and post-fixed in cacodylate-buffered 1% osmium tetroxide for 1 hr at 4°C before dehydration and embedding in Spurr's resin. Ultrathin sections (60 nm) of villi in both longitudinal and transverse section were stained with uranyl acetate and lead citrate and examined in a Philips 301 transmission electron microscope.

RESULTS

Light Microscopy. In all animals, the sections cut to show intestinal villi in longitudinal plane and stained with AB 0.5/NSE revealed IMMCs associated with a complex nerve network between the crypts. In the villi the IMMCs were predominantly located subepithelially, associated with nerves. In the uninfected animals, only occasional mast cells were seen. At the peak of hyperplasia (day 22) in infected animals, many immature cells occupying the more central region of the villi were prominent. On day 35 after infection, the subepithelial location of IMMCs was pronounced, involving mainly mature cells. The majority of IMMCs were clearly very close to nerves, and the villus mast cell population often appeared as "beads on a string."

In cross-sections of jejunal villi from animals on day 35 after infection, the consistent IMMC–nerve association seen in longitudinal sections was confirmed (Fig. 1). In these preparations it was easy to see that most IMMCs were subepithelial and that the majority were touching one or more nerves. Consecutive sections stained for substance P, NSE, and CGRP showed that either or both neuropeptides were present in many of the NSE-containing subepithelial nerves in contact with IMMCs.

Enumeration of 650 mast cells in AB 0.5/NSE preparations from infected animals indicated that 67% of IMMCs were in contact with nerves and an additional 15% were within an estimated half-cell diameter. With the ZIDAS, 87% of IMMCs were recorded within 2 μm of the nearest nerve. The limited resolution of this system, under the conditions employed in this study, made it impossible to determine the degree of contact by use of the ZIDAS but confirmed that most mast cells were very close to nerves (Table 1). Since the sum of mast cell profiles was found to represent 12.5% of the total area of the lamina propria, the incidence of IMMC–nerve contact was determined to be approximately 5 times greater than would be expected by chance alone ($P < 0.0001$). The visual count of mast cell contacts with substance P-immunoreactive nerves gave similar results, with 61 ± 7%
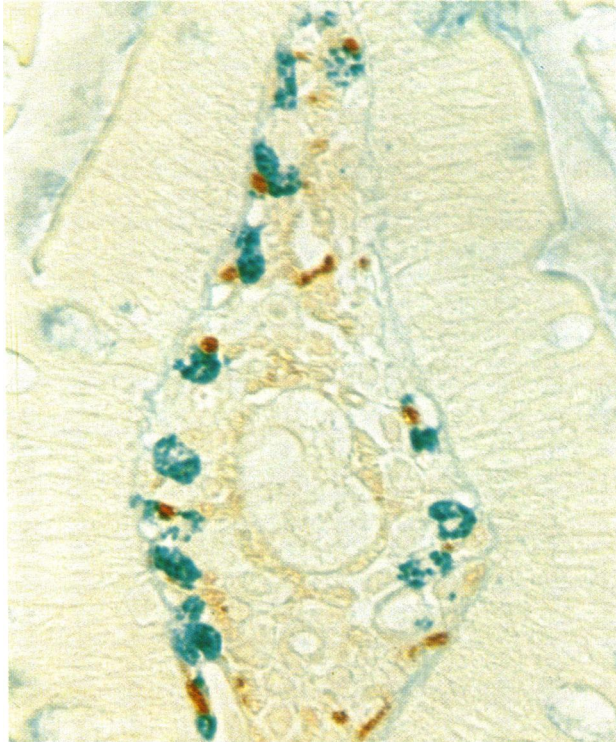
FIG. 1. The close apposition of IMMCs (blue) and enteric nerves (red) can be seen in this cross-section of a rat jejunal villus, 35 days after infection. Note that some mast cells appear to enclose nerves. (AB 0.5/NSE staining; ×425.)

(95% confidence interval, $n = 200$, $P < 0.001$) in close apposition.

In the intestinal lamina propria, the nerve density was determined to be one per $788 \pm 151 \ \mu m^2$, and the average mast cell area was $121 \pm 27 \ \mu m^2$. This indicates a 15% chance of finding a nerve within the area occupied by a mast cell, significantly less than the minimum observed value of 67% ($P < 0.0001$). Furthermore, a histogram of the nerve distribution exhibited a Gaussian curve with skewness of 0.24 ($P = 0.10$) and kurtosis of -0.32 ($P = 0.27$), neither of which led to the rejection of the hypothesis of a normal distribution of nerves in the lamina propria. These data suggest that neither a highly skewed distribution nor a high nerve density was responsible for the number of contacts with IMMCs.

Electron Microscopy. In the electron microscopic preparations, IMMCs were located predominantly subepithelially and close to both nerves and capillaries. The relationship of

Table 1. Percentage of mast cells contacting or close to nerves in *N. brasiliensis*-infected rat intestine

Mode of measurement	Visual	ZIDAS
Total cell count	650	75
Number of contacts	437	65
IMMCs in contact with nerves	67 ± 4%*	NA
IMMCs within estimated half-cell diameter of nerves	82 ± 3%*	NA
IMMCs within 2 μm of nerves	NA	87 ± 8%*

Twenty-five villi were selected at random from six cross-sections of rat jejunum (35 days after infection) stained with AB 0.5 and for NSE, and 650 cells were evaluated at ×800 magnification. Actual nerve–mast cell contact and cells within one-half cell diameter of nerve were recorded. Measurements were also made using a Zeiss interactive digital analysis system (ZIDAS) at ×500 magnification. NA, not applicable.
*Ninety-five percent confidence interval.

mast cells with unmyelinated nerves was striking (Fig. 2). Most of the IMMCs exhibited lamellopodia, which were sometimes short and not numerous but frequently attenuated and branching at the site of contact with nerve, projecting around the latter (Fig. 2). An occasional IMMC contained a nerve within a keyhole invagination in the cell membrane and, in some sections, adjacent mast cells were touching the same nerve. In many sites, the plasma membranes of the IMMCs were in direct contact with axons; however, specific junctions between mast cells and nerves were not seen. Dense-core vesicles ranging in size from 70 to 170 nm were often observed in nerves where they were in contact with mast cells (Fig. 2). Less commonly, vesicles were of two types: (*i*) 95–140 nm and electron dense or (*ii*) pleomorphic and up to 300 nm in diameter.

The ultrastructural observations were quantitated in two animals (days 0 and 35) and approximately one-third of the IMMCs were found to be within 250 nm of nerves, with 8% exhibiting membrane–membrane contact with axons in the infected animals (Table 2).

DISCUSSION

We have demonstrated a consistent mast cell–nerve association in both normal and *N. brasiliensis*-infected intestine. In the light microscopic preparations, 67–87% of IMMCs were observed in close association with s. bepithelial enteric nerves. This is approximately 5 times greater than would be expected by chance alone ($P < 0.0001$), based on the area of the lamina propria occupied by IMMCs (12.5%).

In the pathology literature, it is well known that mast cells are found in benign tumors of nervous tissue (20). Olsson (21)
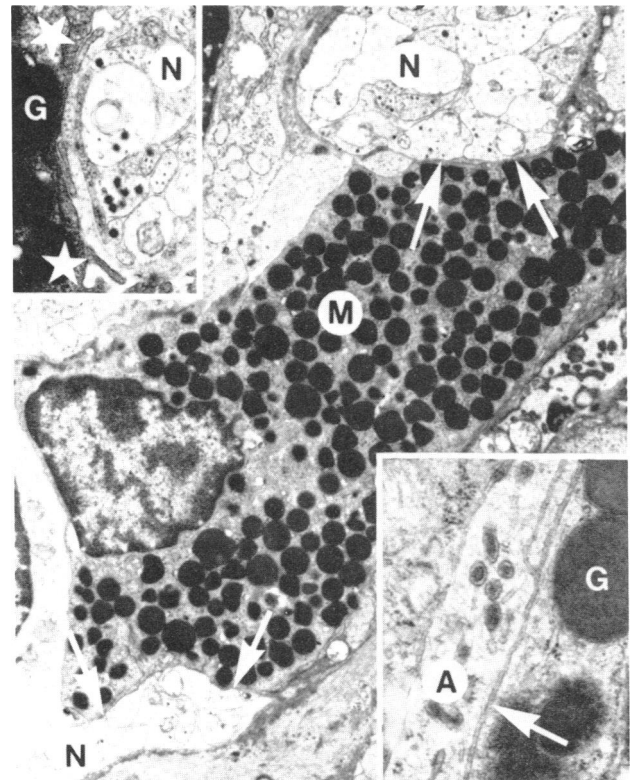

FIG. 2. The intimate association of a mast cell (M) and two unmyelinated nerves (N) is clearly illustrated in this electron micrograph. (×5400.) Upper inset shows an IMMC with its characteristic granules (G) and lamellopodia (*) projecting around a nerve (×14,000.) Lower inset illustrates membrane–membrane contact of an IMMC and an axon (A) containing dense-core vesicles. (×27,000.) Arrows indicate mast cell–nerve apposition.

Table 2. Ultrastructural association of IMMCs with nerves in normal and infected rat jejunum

	No. of cells (out of 100 counted)	
	Normal	Infected
Membrane–membrane contact	4	8
Close to nerves*	28	31
Not touching	68	61

Electron microscopic observations of mast cell–nerve contact were quantitated in one uninfected rat and one rat 35 days after infection.
*Mast cells touching Schwann cells or nerve basement membrane and those which were very close (<250 nm) to nerve but separated by fine cytoplasmic processes of unknown origin.

described an increase in mast cells in the distal portions of sectioned peripheral nerves, and a similar association was observed in glomus tumors (22). Scattered references describe such associations in the skin, myocardium, ileum, mesentery, and diaphragm of a variety of animals (23–26). In the last two sites, occasional substance P-containing nerves were seen in apposition to mast cells (24). However, these anecdotal allusions to mast cell–nerve association have primarily been in other than intestinal tissue. With respect to our study, the most significant of these was the description by Newson *et al.* (25), who observed bouton formation adjacent to only two mast cells in rat intestine.

One objection to the interpretation of our data might be that the frequency of contact depends on the density of both mast cells and nerves in the lamina propria. However, we observed a similar frequency of mast cell–nerve contacts in both normal and infected (day 35) animals at the ultrastructural level (Table 2). Since in the former the number of IMMCs is very low and in the latter, high but not at the peak of the response (15), overcrowding of the lamina propria with mast cells is not the reason for the high incidence of contact with nerves. Additionally, there is a statistically normal distribution of nerves within the lamina propria (skewness, 0.24; kurtosis, −0.32), and the frequency of nerves is one per 788 μm^2. This implies a 15% chance of finding a nerve within the area occupied by a mast cell (121 μm^2). Given these values, the probability of observing 67% of mast cells in contact with nerves is highly significant ($P < 0.0001$).

The lower number of contacts counted in our electron microscopic studies are in keeping with our overall observations. Since the sections used in this technique are only 60 nm thick, it would have been surprising to sample a high percentage of actual mast cell–nerve contacts and observe an incidence similar to that seen in the light microscopic preparations, which were 50 times thicker. Nevertheless, 8% of IMMCs exhibited direct contact with nerves in the infected animals, and an additional 31% were within 250 nm. Many of the latter group were touching Schwann cells, nerve basement membrane, or fine cellular processes that could not be classified.

The axons in contact with IMMCs frequently contained dense-core vesicles ranging in size from 70 to 170 nm, in accordance with their definition as "p-type," or peptidergic (27, 28), and possible substance P content (29). This is consistent with our immunohistochemical demonstration of substance P and CGRP in NSE-containing nerves adjacent to mast cells. Bishop *et al.* (30) observed substance P in NSE-containing nerve cell bodies in rat intestine, and other investigators detected CGRP in enteric nerves in rat (31) and guinea pig (32).

Peripheral nerves are often varicose and do not necessarily form synapses or specialized membrane structures at the points of communication with other cells (33). In the gastrointestinal mucosa, such varicosities have been observed

200–300 nm from smooth muscle and gland cells (33), and synaptic contact with enteroendocrine cells was not observed by Wade and Westfall (34) or Lundberg *et al.* (35). It is now presumed that enteric nerves exert their influence on these mucosal cell types by *en passant* release of neurotransmitters from varicosities (36), which may also serve as the site for uptake of other mediators (37). Substance P is colocalized with other neurotransmitters in varicose fibers in the enteric nervous system (38). Thus, the close proximity of mast cells and substance P-containing nerves seen in our study suggests a humoral form of communication, as was proposed for smooth muscle by Lever *et al.* (39).

We have no direct explanation for the physiological basis for the association between mast cells and nerves. However, proteoglycans containing heparan sulfate (which is similar to heparin, the major constituent of PMC granules) have a significant neurotrophic effect (40–42). It is not known if the predominant proteoglycan of IMMCs, which contains chondroitin sulfate di-B (43), has the same effect.

It is interesting that Crohn disease, in which an increase in enteric nerves has been shown (44), is also characterized by a significant mast cell hyperplasia (45). In this respect, both mast cells and nerves may be influenced locally by nerve growth factor. This substance, in addition to its role in sustaining and promoting nerve growth, has been shown to cause mast cell degranulation *in vitro* (46) and to promote mast cell hyperplasia *in vivo* (47).

Our observations indicate an almost invariable cell–cell contact between IMMCs and enteric nerves in normal and nematode-infected rats. Whether the implied communication is one-way or two-way, and in which direction it occurs, is not known. Mast cells could release constituents that communicate with the enteric nervous system, which in turn regulates intestinal physiological function. In fact, Perdue *et al.* (48) showed that abrupt and dramatic changes of physiological functions in rat intestine occur when IMMCs are sensitized with IgE and challenged with specific antigen (48). This is in keeping with suggestions that mast cells and substance P-containing nerves may form a functional unit involved in the axon reflex in other sites (13, 14) and that CGRP might be involved in the axon reflex in the gut (32). Our data provide a structural basis for a similar communication pathway in the normal and the inflamed intestine. In this regard, we believe that mast cell–nerve interaction may also occur in humans. We think it likely that human diseases where mast cells have been incriminated in the pathogenesis, such as asthma or inflammatory bowel disease, may turn out to be extensively influenced by mast cell–nerve interaction.

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