Immune Reactions in Mucous Membranes

IV. Histochemistry of Intestinal Mast Cells During Helminth Expulsion in the Rat

H. R. P. Miller, BVMS, PhD and R. Walshaw

The histochemistry of intestinal (IMC) and connective tissue mast cells (CTMC) in the normal rat is compared. Acid mucopolysaccharide appears to be less strongly sulfated and the granule content of monoamines is lower in IMC. After infection with the intestinal helminth, *Nippostrongylus brasiliensis*, the mucosal content of IMC is altered. During the early phase of immunologic expulsion of this parasite (self-cure) the IMC proliferate and differentiate; the histochemical properties of the granules appear to reflect this process. Very large numbers of mature IMC are found in the mucosa during the later, rapid phase of worm expulsion. Both acid mucopolysaccharide and monoamines are depleted from the granules of these cells; this is consistent with previous studies suggesting that IMC discharge occurs at this stage. These findings lend further support to the hypothesis that the biogenic products of IMC discharge are responsible for the increase in mucosal permeability which occurs during self-cure and which may facilitate the rapid translocation of antiworm antibody into the intestinal lumen (Am J Pathol 69:195–208, 1972).

IF RATS ARE INFECTED with the intestinal helminth *Nippostrongylus brasiliensis*, the numbers and distribution of mast cells in the intestinal mucosa (IMC) fluctuate.[1–3] During immunologic expulsion of the parasite from the intestine (self-cure), a new population of IMC differentiates and proliferates in the mucosa,[2,4,5] and many of the cells migrate into the epithelium and discharge to become globule leukocytes (GL).[2,6,7] A clear-cut temporal relationship exists between the increase in IMC numbers, IMC discharge and worm expulsion.[1–3,8]

The IMC in the normal rat differs not only morphologically but in its fixation and cytochemical properties from the connective tissue mast cell (CTMC).[9–11] Moreover, during self-cure, IMC discharge involves the lysis of cells without extrusion of their granules,[8] a process which may be mediated by reaginic antibody and allergen.[12,13] It was of interest, therefore, to further characterize the histochemical properties of IMC and to compare the results with histochemical changes among proliferating and discharging IMC at the time of self-cure. Three as-

From the Department of Veterinary Pathology, Veterinary School, University of Glasgow, Glasgow, Scotland.

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Address reprint requests to Dr. H. R. P. Miller, Unité de Immunocytochimie, Departement de Biologie Moleculaire, Institut Pasteur, Paris 15e, France.

pects were studied: a) the nature and fate of the acid mucopolysaccharide in the IMC granules; b) the presence or absence of basic protein in IMC and c) the granule content and fate of the biogenic amines, 5-hydroxytryptamine and histamine.

Materials and Methods

Fifty-five of 70 female hooded Lister rats, weighing 170 to 220 g, were infected subcutaneously in the region of the groin with 3000 *N brasiliensis* larvae; the remaining 15 were kept as controls. Groups of 4 to 5 rats were anesthetised on days 10, 11, 12, 14, 16, 19 and 35, and several segments of jejunum were taken from an area 12 to 15 cm behind the pylorus;[2] the rats were then killed by cervical dislocation. The tongues and an equivalent region of jejunum were taken from control rats. In addition, 2 rats from each group, on days 10 to 12, 14 and 19, one rat on day 35 and 5 controls, were given 3 intraperitoneal injections of 40 mg/kg L-Dopa (L-β-3, 4-dihydroxyphenylalanine, Koch-Light Laboratories Limited, England) at hourly intervals and were killed 1 hour after the third dose.[2,11]

Fixation and Tissue Preparation

The intestinal mucosa was orientated as described previously [2] and the tissues were fixed in either Carnoy's fluid or 4% phosphate-buffered formaldehyde, prepared from paraformaldehyde, for 24 to 48 hours, then dehydrated and cleared in an alcohol-amyl acetate-chloroform series and embedded in paraffin wax. Sections were cut at 6 μ.

To demonstrate monoamines,[14,15] small pieces of tissue were quenched in isopentane cooled in liquid nitrogen, transferred directly to a freeze-drying apparatus maintained at −40 C and dried *in vacuo* with a phosphorus pentoxide water vapor trap for 24 hours. Some of the tissue was treated with paraformaldehyde for 1 hour at 80 C and was embedded either in degassed molten paraffin wax at 57 to 60 C or in an Epon-Araldite mixture, using a Pearse-Edwardes resin-embedding accessory. The rest of the unfixed tissue was embedded immediately in paraffin wax.

Staining Procedures

Demonstration of Acid Mucopolysaccharides

Toluidine blue was used in a 0.5 aqueous solution at pH 4 (McIlvaine's citric acid-disodium phosphate buffer); staining time was 45 seconds.[6,7,10] A 0.1% aqueous solution of toluidine blue was made by diluting the dye in 0.7 N HCl. Sections were stained for 10 minutes after which they were rinsed in 0.7 N HCl for 10 minutes.[6,7,10]

Astrablue (G. T. Gurr Ltd, London) was used in a 0.5% aqueous solution at pH 0.3;[6,7,10] staining time was 30 minutes. Sections were also stained with 0.5% Alcian blue 8GX (E. Gurr Ltd, London and I.C.I. Ltd, Manchester), following the same procedure used for Astrablue.[10] Some sections stained with either Astra or Alcian blue were counterstained with 0.5% Safranin O (Hopkin and Williams Ltd) in 0.125 N HCl (approximately pH 1) for 30 seconds.[7,10]

Alcian blue 8GX was also used for the critical electrolyte concentration technic.[16] Serial deparaffinized sections were immersed in upright Coplin jars filled with 0.04% dye solution (buffered with calcium acetate at pH 5.8) containing increasing concentrations of electrolyte (0.1 M increments of $MgCl_2$). Molarities ranged from 0.0 to 1.4. After rinsing in distilled water, the sections were dehydrated, cleared and mounted.

Demonstration of Basic Proteins

Biebrich scarlet was used as a 0.04% solution in glycine buffer at pH 8, 9 and 10; staining time was 30 to 90 minutes.[17] Some sections were transferred directly to 95% alcohol, dehydrated and cleared; others were rinsed in distilled water prior to dehydration.

Demonstration of Monoamines

Catecholamines and 5-hydroxytryptamine fluorescence were detected in both paraffin and 1-μ plastic sections of freeze-dried paraformaldehyde-treated tissues. Sections of tissues which had not been treated with paraformaldehyde were used as controls. In addition, 1-μ plastic sections were examined for fluorescence, and the adjacent sections, stained by the method of Richardson *et al*,[18] were examined in the light microscope.

Intracellular histamine was detected by staining sections of freeze-dried tissues with *o*-phthaldialdehyde in ethyl benzene, using the method of Shelly, Ohman and Parnes.[15] Similar results were obtained with tissues fixed briefly in Carnoy's fluid and rapidly dehydrated.[19] Control sections were treated with ethyl benzene to which no *o*-phthaldialdehyde was added.

Sections were examined in a Leitz Ortholux fluorescence microscope using a mercury vapor lamp (Wotan HBO22OW) as a light source and, for catecholamines, a BG12 3-mm exciting filter with a Leitz K530 barrier filter. For histamine, UG1 and BG12 exciting filters and K410 and K530 barrier filters were used.

Results

Acid Mucopolysaccharide

Normal Rats

The histochemical properties of the acid mucopolysaccharides of the IMC and tongue CTMC fixed by different methods are shown in Table 1. Formaldehyde-fixed IMC were only demonstrable with the critical electrolyte concentration (CEC) technic at molarities between

Table 1—Histochemistry of the Acid Mucopolysaccharides in IMC and CTMC in Normal Rats

	Alcian or Astra blue/safranin		Toluidine blue (pH 0.3)		Toluidine blue (pH 4)		CEC*	
Fixative	IMC	CTMC	IMC	CTMC	IMC	CTMC	IMC	CTMC
Carnoy	B+++	R+++ (B+)†	B++	P+++	RP+	P+++	1.2	‡
Formaldehyde	—	R+++ (B+)†	—	P+++	—	P+++	0.9	‡

* Molarity of $MgCl_2$ at which Alcian blue staining is extinguished.

† A few cells stained blue and others had red and blue granules.

‡ Still staining in 1.4 M $MgCl_2$.

B = blue; R = red; P = purple. Staining intensity: — = none; + = weak; ++ = moderate; +++ = strong

0.4 to 0.9 M. Apart from the IMC, no other cell type in the intestinal mucosa was stained by any of the technics used.

Parasitized Rats

The elimination of the worm burden from female hooded Lister rats follows a recognizable pattern.[3,20] Ten to twelve days after infestation, there is a period during which the worms are expelled at a slow rate; [3] IMC differentiation and proliferation take place during this time.[2,4] During the second phase, 12 to 16 days after infection, the worms are expelled very rapidly,[3] and at this stage there is evidence of massive IMC discharge.[2,8]

The results shown in Table 2 summarize the histochemistry of the acid mucopolysaccharides in Carnoy-fixed IMC during the different phases of worm expulsion. The toluidine blue metachromasia of acid mucopolysaccharide in infected rats and its affinity for thiazine and copper phthalocyanine dyes at low pH are indistinguishable from those of the normal rat. However, application of the CEC technic shows that the maturing IMC and GL, 11 days after infection, have a slightly reduced alcianophilia (Table 3). In addition the majority of GL on days 14 and 16, as well as many IMC, are cut out at low CEC when compared with IMC in normal rats (Table 3, Figures 1 and 2).

Basic Protein

Normal Rats

The CTMC did not stain with Biebrich scarlet with either of the fixatives employed. When fixed in Carnoy's fluid, the IMC stained

Table 2—Histochemistry of IMC and GL during the Immunologic Expulsion of **Nippostrongylus brasiliensis**

	Normal Rats	Infected Rats					
		Day 11		Days 14 and 16		Day 19	
	IMC	IMC	GL	IMC	GL	IMC	GL
Toluidine blue (pH 0.3)	B++	B++	B++	B++	B++	B++	B++
(pH 4.0)	RP+	RP+	RP+	RP+	RP+	RP+	RP+
Astra or Alcian blue/safranin	B+++	B+++	B+++	B+++	B+++	B+++	B+++
Biebrich Scarlet (pH 10.0)	++	+	+	++	+(+)	++	++

B = blue; R = red; P = purple. Staining intensity: + = weak, ++ = moderate; +++ = strong

Table 3—The Critical Electrolyte Concentrations for the Staining of IMC and GL with Alcian Blue 8GX During Expulsion of **N brasiliensis**

Molarity of $MgCl_2$	Normal Rat	Infected Rats					
		Day 11		Days 14 and 16		Day 19	
	IMC	IMC	GL	IMC	GL	IMC	GL
0.4	+++	+++	+++	+++	+++	+++	+++
0.5	+++	+++	+++	+++	+++	+++	+++
0.6	+++	+++	+++	+++	+++	+++	+++
0.7	+++	+++	+++	+++*	+++*	+++	+++*
0.8	+++	+++*	++*	+++*	++†	+++*	++†
0.9	++	++*	+†	++†	+†	++*	+†
1.0	++*	+†	+†	+†	+†	++*	+†
1.1	+†	+†	+†	+†	+†	+†	+†
1.2	+†	+†	+†	+†	+†	+†	+†
1.4	—	—	—	—	—	—	—

* Staining of a few cells cut out.
† Staining of many cells cut out.
Staining intensity: — = none; + = weak; ++ = moderate; +++ = strong

strongly and, if the sections were dehydrated without prior rinsing, their nuclear morphology distinguished the IMC from other granule-containing cells, such as eosinophils and polymorphonuclear leukocytes, whose granules did not stain.

Parasitized Rats

Basic protein was present in the granules of both IMC and GL, although the staining intensity was reduced in both on days 10 to 12, and was slightly reduced in GL on days 14 and 16 (Table 2). A cell type resembling the basophil was also present in the mucosa during the period 10 to 14 days.[5] Its granules were slightly smaller and stained less strongly than those in the IMC; it was distinguished from the IMC by its polymorphous nucleus.

Monoamines

Normal Rats

CATECHOLAMINES AND 5 HYDROXYTRYPTAMINE. The IMC in the villi and upper crypt regions fluoresced a weak, dull green. Those in the basal mucosa had a yellow fluorescence characteristic of 5-hydroxytryptamine, but were smaller and fluoresced less intensely than the CTMC in the tongue. Intestinal mast cells were distinguished from enterochromaffin cells by their different morphology, the larger

size of their granules and their location in the lamina propria (Figure 3). Nonspecific fluorescence was recognized because it did not diminish with prolonged exposure to ultraviolet light and was present in freeze-dried tissues not pretreated with paraformaldehyde.

In rats treated with L-Dopa the IMC fluoresced a bright green, except the IMC in the basal mucosa, which remained yellow. Connective tissue mast cells and enterochromaffin cells continued to fluoresce yellow. Intestinal mast cells in 1 to 1.5-μ plastic sections had a bright green fluorescence after treatment with L-Dopa and were identified in the adjacent section stained with Azure II—methylene blue (Figures 5 and 6).

Parasitized Rats

Days 10 to 12. The IMC and GL contained relatively few granules which, in the IMC, had a moderately bright but dirty yellow fluorescence; small numbers of GL had a similar bright fluorescence (Figure 3) and the remainder fluoresced dull green. After treatment with L-Dopa, both IMC and GL fluoresced a bright apple green (Figure 3, inset).

Days 14 and 16. IMC and GL were more fully granulated than on days 10 to 12, but the intensity of their fluorescence varied. In some areas there were IMC which fluoresced a moderately bright dirty yellow, but the majority either were not fluorescent or were a dull green. Globule leukocytes, although as numerous as IMC,[2] mostly did not fluoresce and the few that were detected were a faint, dull green. Some IMC, including occasional fragmented cells (Figure 4), fluoresced bright green in L-Dopa-treated rats; in contrast, very few GL were detected and they contained fewer, more weakly fluorescent granules than the IMC (Figure 4).

Days 19 and 35. As in the normal rat, the IMC in the basal mucosa fluoresced yellow and those in the villi were a faint dull green. Globule leukocytes exhibited little or no fluorescence.

In the rats given L-Dopa the IMC of the villi and upper crypt region were bright green, well granulated and compact (Figure 5). In contrast, many GL failed to fluoresce (compare Figures 5 and 6) and others showed, at best, a weak green fluorescence.

Histamine

Normal Rat

Mast cells in both freeze-dried and Carnoy-fixed tissues were demonstrable with *o*-phthaldialdehyde. The CTMC fluoresced bright yellow,

indicating the presence of histamine. Intestinal mast cells also had a yellow fluorescence but of lower intensity than the CTMC.

Parasitized Rats

Days 10–12. The numbers and morphology of the IMC and GL treated with *o*-phthaldialdehyde were similar to those seen in intestine treated with paraformaldehyde. Their granules also fluoresced yellow.

Days 14 and 16. Although IMC and GL were numerous at this time,[2] only a small proportion of IMC and an occasional GL fluoresced yellow. Fluorescent cells were more fully granulated than on days 10 to 12.

Day 19. Yellow, fluorescent, fully granulated IMC were detected at all levels in the lamina propria. Their numbers were only slightly increased above the normal levels although, at this time, four times as many IMC were present in the mucosa.[2] Fluorescent GL were rare and contained fewer granules than IMC.

Discussion

Normal Rat

In the normal rat, the IMC is considered atypical because cells are smaller, more irregular in size and contain fewer granules than CTMC.[9] The histochemical properties of the granules are different from those of the CTMC because, in the mature CTMC, the majority of granules stain red in the Alcian blue/safranin sequence,[21] whereas IMC granules stain blue.[10] The present results confirmed these observations and, in addition, showed that the alcianophilia of IMC granules was extinguished at lower CEC than that of the CTMC granules. This would suggest that the acid mucopolysaccharides of IMC granules is less strongly sulfated than that in the CTMC.

A further striking difference was that formaldehyde-fixed IMC were not demonstrable with thiazine or copper phthalocyanin dyes at low pH. It has been suggested that this may be due to dissolution of the granules [9] but, using the CEC method, the IMC stained at molarities between 0.4 to 0.9. This would suggest that the polyanions are blocked by cationic proteins, a phenomenon known to be reversed by staining with Alcian blue in salt solutions.[22] The fact that neither acid mucopolysaccharides nor basic protein [5] were demonstrable after formaldehyde fixation, whereas both were detected after Carnoy fixation lends further support to the possibility that basic protein blocks the basophilia of the acid mucopolysaccharides.

The IMC contains relatively small amounts of catecholamines and 5HT [11,23] which, because of the low intensity of the fluorescence in the majority of cells, could be dopamine or low concentrations of 5-hydroxytryptamine.[23] The IMC in the basal mucosa of the hooded Lister rat did have relatively high concentrations of 5-hydroxytryptamine; these cells, unlike the IMC in the villi, did not appear to take up L-Dopa. After treatment with *o*-phthaldialdehyde, the fluorescent intensity of the IMC was always lower than that of the CTMC, suggesting that the IMC had a smaller histamine content.

These results provide a basis for studies of the IMC changes during infection with *Nippostrongylus brasiliensis*. Previous work has shown that two phenomena are associated with worm expulsion: a) IMC proliferation and differentiation,[2,4] and b) discharge of the granule contents of IMC at the same time they migrate into the epithelium to become GL.[2,6–8]

Parasitized Rats

Proliferation of IMC

New IMC arise from blast cells, probably of lymphoid origin, approximately 10 days after infection.[4] The granules are elaborated from the Golgi complexes,[4] and the cells proliferate and differentiate [2,4] so that, by day 14, very large numbers of mature cells are found in the mucosa.[2] On days 10 to 11, the IMC are immature and contain few granules; [2,4] this study shows that these immature cells contain acid mucopolysaccharide, basic protein and monoamines.

There were several histochemical differences between maturing IMC in infected rats and mature IMC in normal rats. The acid mucopolysaccharide in the maturing cell had reduced alcianophilia and the staining intensity of the basic protein was also lower than in the mature IMC. This would be consistent with the accumulation and/or sulfation of the acid mucopolysaccharide and the addition of basic protein to the maturing granules because, on days 10 to 11, there is little evidence of granule discharge.[8] An analogous situation exists in the maturing CTMC, although, in this cell, the sulfation of the heparin precursors is accompanied by a shift from alcianophilia to an affinity for safranin.[21] The granules of the maturing IMC also contained monoamines and were able to take up L-Dopa.

Ultrastructurally, the maturation of IMC differs from that of CTMC because, in the latter, marked changes in granule structure accompany the shift in staining properties attributed to N-sulfation of heparin precursors.[24] No equivalent change in granule morphology is seen

in the maturing IMC,[4] although histochemical comparison with the normal mature IMC suggests that the acid mucopolysaccharide of the IMC does become more fully sulfated during maturation. There are, therefore, clear cut differences, not only with regard to the morphology and histochemistry of IMC and CTMC, but also in the maturation of these two cells. This would suggest that the IMC and CTMC are different cell-types, rather than the same cell modified by different environments.

Discharge of IMC

Mature GL are IMC which have migrated intraepithelially and discharged their granule contents [6,7] so the proportion of mature GL present is a useful measure of the extent of IMC discharge.[2] During the rapid phase of worm expulsion 14 and 16 days after infection, 50% of the IMC population are GL[2] and, ultrastructurally, many IMC in the lamina propria also appear to be partially discharged at this time.[8]

Intestinal mast cell discharge could not be demonstrated by standard histochemical technics because the altered granules retained their affinity for basic dyes even at very low pH. However, application of the CEC method showed that the majority of GL and many IMC had a markedly reduced alcianophilia and were cut out at much lower electrolyte concentrations than in the normal rat. It would seem, therefore, that there is a degree of acid mucopolysaccharide depletion from these cells; this would account for the reduced basophilia of their granules in 1-μ plastic-embedded sections.[8]

Basic proteins were consistently present in both IMC and GL, although their staining intensity was slightly reduced in GL. Different technics would be necessary to determine any changes in the nature or amount of the basic protein. In the electron microscope, paracrystalline structures were found in the granules of discharged cells, and these were probably remnants of basic protein.[7,8]

Although monoamines were present in maturing IMC and GL between days 10 to 12, the mature cells on days 14 and 16 contained little or no detectable monoamines. A further striking difference was that maturing GL and IMC both took up L-Dopa, whereas GL, on day 14 and subsequently, were unable to do so. Since, ultrastructurally, the majority of granules in the maturing IMC and GL were intact, whereas those in the mature cells, on days 14 and 16, were disrupted,[4,8] it is likely that the decreased ability of the latter to store and to take up monoamines was due to the loss of integrity of the perigranular membranes.

These results clearly demonstrate that, during the rapid phase of worm expulsion, the GL and many IMC are unable to store and have also released monoamines. In addition, there appears to be a reduction of their content of acid mucopolysaccharide and, possibly, of basic protein. This is consistent with kinetic and ultrastructural studies which point to extensive mast cell discharge during self-cure.[2,8]

It is known that, during this rapid phase of expulsion, mucosal permeability increases [3,12] and is associated with extensive mucosal damage.[5] Furthermore, experiments with antagonists [25,26] and depletors [27] of monoamines suggest that both histamine and 5-hydroxytryptamine are involved in worm expulsion. The present findings thus provide further support for the hypothesis that biogenic products released from the mast cell granules could be partly responsible for increased mucosal permeability.[3,5,8,12] This latter event is thought to enhance the passage of antiworm antibodies into the intestinal lumen [3,7,12,28] and effect the rapid expulsion of the parasites.

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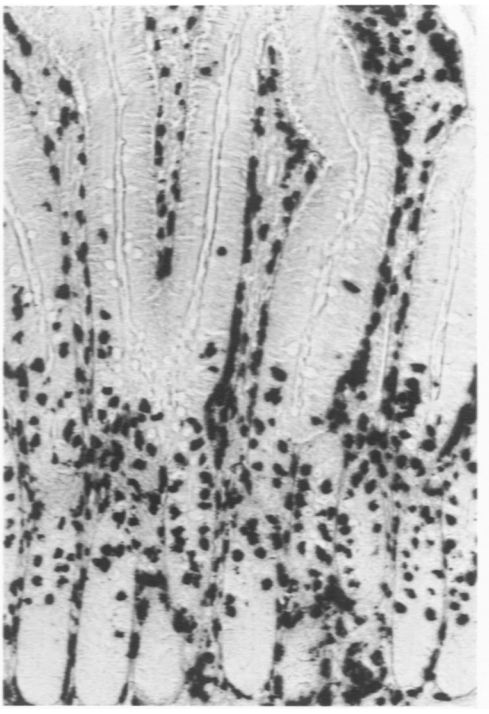
[Illustrations follow]



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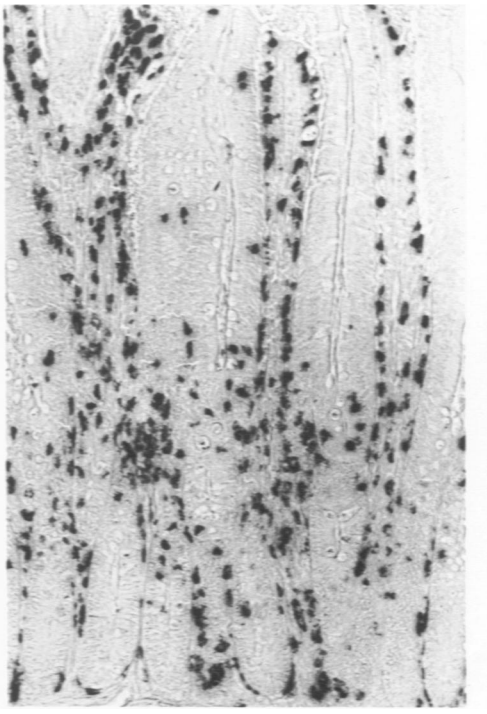

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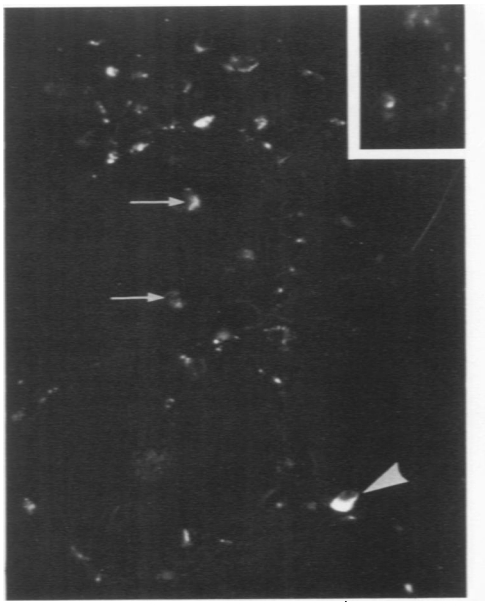

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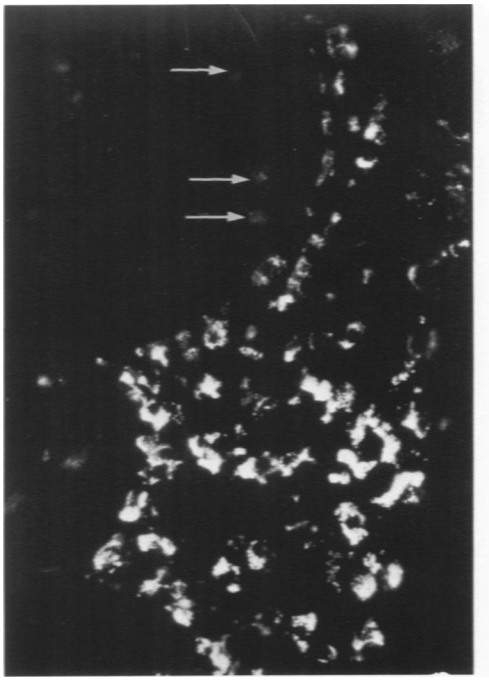
Figs 1 and 2—Intestinal mast cells and GL in the mucosa 16 days after infection with *N brasiliensis* and stained with Alcian Blue 8GX in electrolyte. Note the reduced staining and disappearance of cells in 0.8 M $MgCl_2$ (1, 0.4 M $MgCl_2$; 2, 0.8 M $MgCl_2$, × 150). **Fig 3**—IMC and GL (*arrows*) are poorly granulated but are brightly fluorescent in the jejunal mucosa 12 days after infection. Enterochromaffin cell (*arrowhead*) (× 350). **Inset**—GL in an L-Dopa treated rat 12 days after infection (× 1500).

Fig 4—Intestinal mast cells and fragmented IMC, fluoresce a bright green in the lamina propria of a L-Dopa treated rat 14 days after infection. In contrast, GL (*arrows*) have a weak fluorescence (× 350). **Fig 5**—One micron Epon-Araldite section from L-Dopa treated rat 19 days after infection. The intestinal mast cells are compact and have bright green fluorescence. The location of GL (*arrows*) is confirmed in Figure 6 (× 1300). **Fig 6**—One-micron section adjacent to that in Figure 5 and stained with Azure 2–Methylene Blue illustrates the distribution of intestinal mast cells and GL (*arrows*) present in Figure 5 (× 1300).



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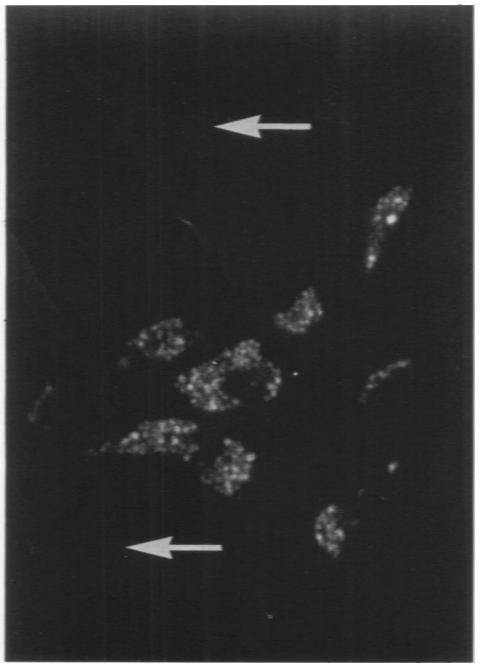

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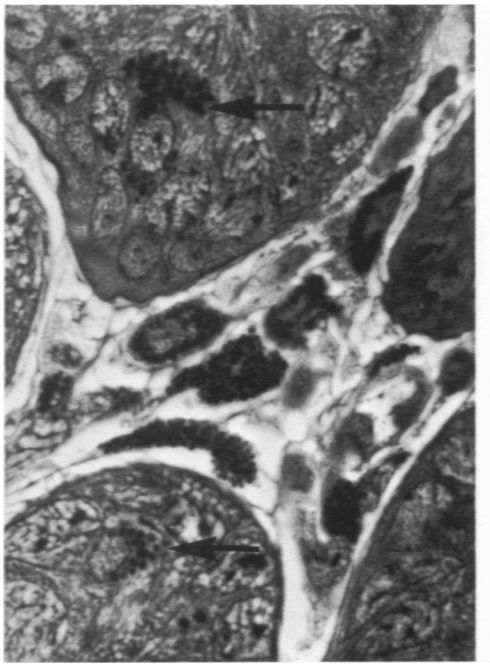

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