Immunology, 1971, **20**, 277.

Immune Reactions in Mucous Membranes

I. INTESTINAL MAST CELL RESPONSE DURING HELMINTH EXPULSION IN THE RAT

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(*Received 3rd June* 1970)

Summary. Measurement of the mast cell response in the jejunal mucosae of rats infected with *Nippostrongylus brasiliensis* was carried out at the time of worm expulsion (self-cure). Just prior to the start of self-cure, a new mast cell population differentiated from cells with large nuclei and prominent nucleoli. Beginning on the 10th day of infection, mast cell numbers increased in an exponential fashion and reached a peak in the middle of the self-cure reaction 14 days after infection. At first the cells had few granules but their granule content subsequently increased and by day 14 some of them appeared to be mature. Mitoses were observed in granulated cells at all stages of the population expansion.

Differential counts showed that an increasing proportion of the mast cell population migrated intraepithelially to become globule leucocytes so that by day 14 the ratio of globule leucocytes to mast cells was 1/1. On subsequent days of infection, this ratio and the total population of granulated cells gradually diminished.

The results suggest that cell differentiation and division are responsible for the population increase. The high globule leucocyte/mast cell ratio points to an extensive release of amines from the granules during self-cure. The significance of this reaction in relation to antibody release from the mucosa and to worm expulsion is discussed.

INTRODUCTION

In recent years there has been increasing interest in the manner in which antibodies are released at mucous surfaces. Dramatic examples of such phenomena may be seen when nematode parasites are expelled from the gastro-intestinal tracts of their hosts.

As early as 1953, Stewart (1953) suggested that immediate-type hypersensitivity reactions, localized in the mucous membrane, could play a part in the expulsion of helminths from sheep. In *Nippostrongylus brasiliensis* infection of the rat, a localized anaphylactic shock can be generated in the intestinal mucosa by intravenous injection of worm antigen (Urquhart, Mulligan, Eadie and Jennings, 1965). Experiments using transplanted worm burdens, a heterologous shocking system and hyperimmune serum suggest that 'intestinal anaphylaxis' facilitates the passage of anti-worm antibody into the intestinal lumen (Barth, Jarrett and Urquhart, 1966).

Natural *N. brasiliensis* infections are terminated towards the end of the second week by a reaction known as self-cure (Africa, 1931) when the worms are expelled from the intestine over the course of several days (Jarrett, Jarrett and Urquhart, 1968b). During this

period there is an increase in the number of mast cells in the intestinal mucosa (Jarrett, Jarrett, Miller and Urquhart, 1968a) and globule leucocytes (GL cells) also become very numerous (Whur, 1966; Jarrett *et al.*, 1968a). The latter are intraepithelially located and partially discharged mast cells (Murray, Miller and Jarrett, 1968). The likelihood that immediate-type hypersensitivity plays a part in the expulsion of *N. brasiliensis* prompted us to examine the changes occurring among mast cells during self-cure. A study of the growth of the mast cell population has been carried out to throw more light on the derivation of these cells. The work described here has been done in conjunction with ultrastructural and histochemical studies which are reported elsewhere (Miller, 1971a, b, c).

MATERIALS AND METHODS

Seventy female hooded Lister rats weighing 170–220 g were used; fifty-five were infected subcutaneously in the groin with 3000 larvae of *N. brasiliensis*. Groups of at least five rats were anaesthetized on days 8, 10, 11, 12, 14, 16 and 19 and four rats on the 35th day after infection. Tissues were removed for histological examination before the animals were killed by cervical dislocation. Ten uninfected rats were used as controls.

Two rats from each group on days 10, 12, 14 and 19 and one rat on day 35 as well as five uninfected controls were given intraperitoneal injections of 3×40 mg/kg DL DOPA (DL-β-3,4, dihydroxyphenylalanine Koch-Light Laboratories Ltd) at hourly intervals and were killed 1 hour later (Enerbäck, 1966b).

A segment of jejunum where the worms are found, approximately 1 cm in length, was removed unopened from a site 12–15 cm behind the pylorus. This was immersed for 1–2 minutes in fixative to prevent muscle contraction, before being opened longitudinally. An approximate estimation was made of the worm burden in that segment and in the remainder of the intestine. Tissues were allowed to fix for 24–48 hours in Carnoy's fluid before being dehydrated, cleared in an alcohol-amyl acetate–chloroform series and embedded in paraffin wax. Blocks were trimmed and oriented to obtain sections along the long axis of the jejunum, at right angles to the surface of the mucosa, and parallel to the long axis of the villi. Sections were cut at approximately 6 μ thickness and were stained with Astra blue/safranin (Enerbäck, 1966a; Murray *et al.*, 1968).

Method of counting cells

The villi of the jejunum of the rat tend to be tongue-shaped with their broader axes at right angles to the long axis of the intestine (Reynolds, Brim and Sheehy, 1967; Nordstrom, Dahlqvist and Josefsson, 1968). In longitudinal sections of the mucosa the majority of villi appear to be finger-shaped and regularly spaced, being separated basally by the gland crypts.

For the purpose of counting cells, the mucosa was divided into 'villus-crypt' units (VC). The number of mast cells lying between two gland crypts and in the lamina propria of the villus above was counted. Globule leucocytes were counted in the length of epithelium outlining that area of lamina propria. The whole area comprised a VC unit and was delimited basally by the muscularis mucosae. Cells were counted only in those units which were sectioned longitudinally.

The granules of mast cells and GL cells were stained by Astra blue and the nuclei were outlined by safranin, but mitotic figures and nucleoli were strongly safranin positive. Nucleated mast cells and GL cells were counted separately in each of twenty villus crypt

units and the number for each rat was expressed as a mean value per VC unit. During the period of population expansion, growth was computed by plotting the log of the geometric mean of the combined mast cell and GL counts of each group against time.

In order to estimate the degree of mucosal expansion during infection, the length of the villus was measured from its tip to the muscularis mucosa and the width from midway between the tip and the neck of the gland crypt. The product of villus length and villus width, the villus area, was recorded for ten villi in each of five rats in a group, except on day 35 when only four rats were examined. The measurements were made with the aid of a Leitz micrometer eyepiece.

RESULTS

WORM EXPULSION

Self-cure followed the pattern described by Jarrett *et al.* (1968b). Worms were present in large numbers on the 10th day and no obvious decrease could be observed until 12 days after infection. By day 14, the worm burden was reduced in all regions of the small intestine. Few parasites were found in rats on day 16 and on day 19 worms were difficult to find. 35 days after infection, only a very small residual population remained.

NUMBERS OF MAST CELLS

Normal rats

Mast cells were present at all levels in the intestinal lamina propria and were evenly distributed amongst the VC units although they tended to be more numerous in the crypt region than in the lamina propria of the villus (Fig. 1). Intraepithelial GL cells were rarely observed. Treatment with L-DOPA, which was carried out for cytochemical purposes (Miller, 1971c), did not affect the number or morphology of mast cells (Table 1).

Parasitized rats

8 days after infection mast cells were virtually absent from the mucosae of the majority of rats examined (Table 1). None was found at sites where the parasites were lying close to the mucosa (Fig. 2) or where there was extensive oedema of the lamina propria. The few cells that remained were located in the tips of the villi and had clumped granules and pyknotic nuclei.

10 days after infection, the villi had assumed a more normal shape although their area was greater than it was in controls, but between days 10–14, during the expansion of the mast cell population, there was no significant change in villus area (Table 2). Small numbers of mast cells and GL cells were found in the lamina propria and epithelium respectively on the 10th day. Both cell types had large, often reniform, pale-staining nuclei with one or two prominent nucleoli and characteristically contained only one or two Astra blue positive granules in their cytoplasm (Fig. 3): an occasional cell had a larger complement of granules, but it could be readily distinguished from the effete cells seen on the 8th day of infection. Mast cell and GL cell distribution was irregular, some VC units contained few, if any, whereas others had as many as five to ten granulated cells. Occasional mitotic figures were observed in both mast and GL cells.

The population of granulated cells increased on the 11th, 12th and 14th days after infection. The plot of the logarithms of the geometric means of the combined cell numbers

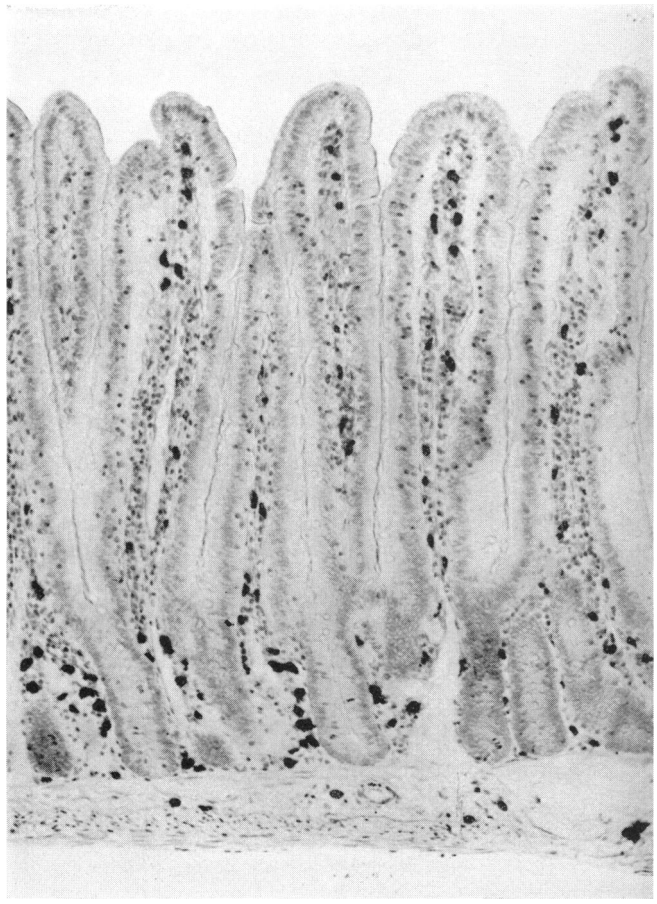

FIG. 1. Jejunal mucosa of a control rat. Mast cells are evident at all levels in the lamina propria. Astra blue/safranin. × 150.

TABLE 1

NUMBERS OF MAST CELLS AND GL CELLS IN THE INTESTINAL MUCOSAE OF RATS DURING THE IMMUNE EXPULSION OF *N. brasiliensis*

Day of infection	No. of rats in group	Mast cells per VC unit	GL cells per VC unit	Total cells* per VC unit	$\log_{10}$ Total cells† per VC unit
Control	10	11·9±0·2	—	11·9±0·2	
Control L-DOPA treated	5	11·7±0·4	—	11·7±0·4	
Day 8	6	1·2±0·8	—	1·2±0·8	
Day 10	6	2·1±0·7	0·1±0·1	2·2±0·7	0·1983±0·16
Day 11	5	6·8±1·9	0·4±0·3	7·3±2·2	0·7847±0·13
Day 12	9	11·7±2·5	5·0±2·2	16·7±4·4	1·1262±10·10
Day 14	8	51·3±4·7	48·6±5·1	99·9±8·3	1·9874±0·04
Day 16	7	50·8±4·6	41·2±7·0	92·0±10·3	1·9475±0·05
Day 19	7	53·2±2·3	30·1±5·0	83·3±7·1	1·9116±0·04
Day 35	4	26·8±0·7	12·5±0·4	39·4±0·4	1·5952±0·007

VC unit = villus crypt unit—see text

* Mast cells plus GL cells (mean±SE).

† Mast cells plus GL cells ($\log_{10}$ of the geometric mean±SE).

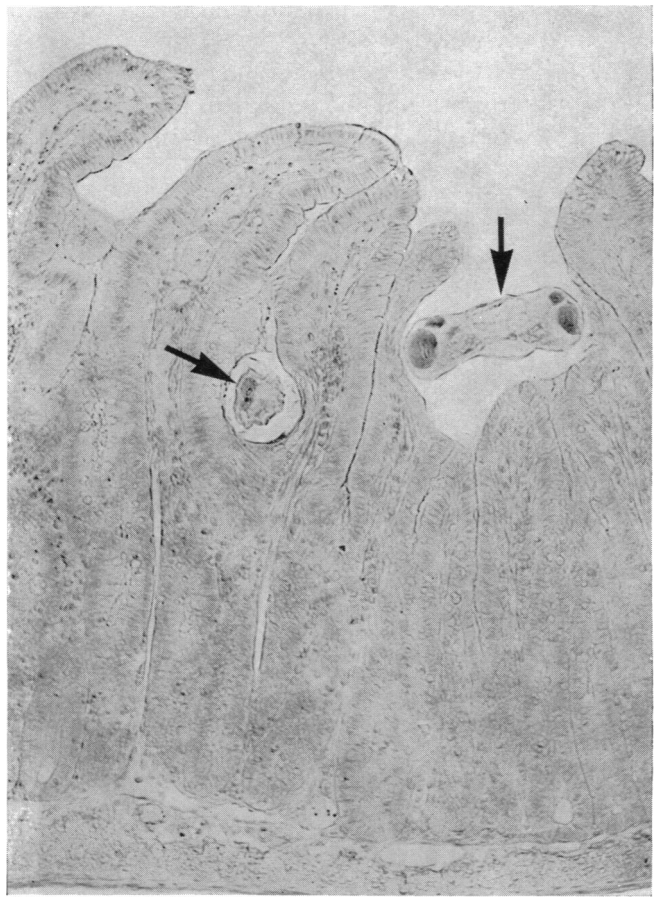

FIG. 2. Jejunal mucosa of a rat 8 days after infection with *N. brasiliensis*. Mast cells are absent from the lamina propria and the villus outline has been altered in the vicinity of the parasites (arrows). Astra blue/safranin. × 150.

TABLE 2

SIZE OF VILLUS-CRYPT UNIT

Day of infection	No. of rats	Villus area (mm^2)*
Control	5	0·049 ± 0·002
Day 8†	—	—
Day 10	5	0·074 ± 0·003
Day 11	5	0·071 ± 0·003
Day 12	5	0·070 ± 0·003
Day 14	5	0·070 ± 0·004
Day 16	5	0·074 ± 0·003
Day 19	5	0·074 ± 0·003
Day 35	4	0·069 ± 0·002

* Mean ± SE.

† Because of severe villus distortion no measurements were taken.

Control compared with all groups except on day 14 $P < 0·001$.
Control compared with group on day 14 $P < 0·01$.
Difference between days 10, 11, 12 and 14 N.S. (*t*-test).

against time show that the rise was exponential in character with a slope, $b = 0{\cdot}44 \pm 0{\cdot}03$ (Fig. 4).

On the 11th and 12th days the mast cells and GL cells were morphologically similar to those on the 10th day after infection, but tended to have larger numbers of granules. GL cells were found in greatest concentrations where mast cells were most abundant,

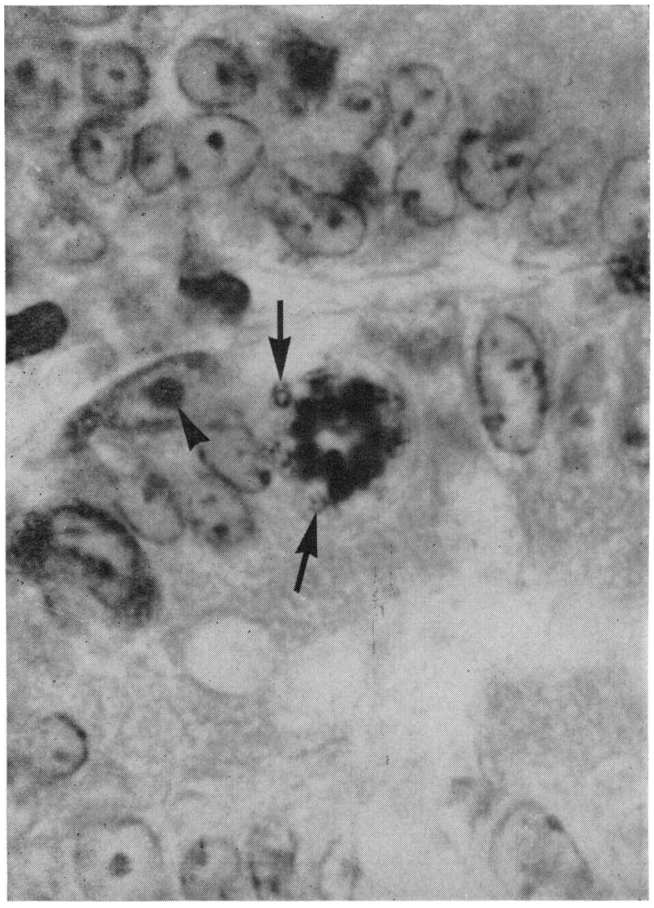

FIG. 3. A globule leucocyte in mitosis in the jejunal epithelium of a rat 12 days after infection with *N. brasiliensis*. Granules (arrows) can be distinguished around the mitotic figures and also in a nearby cell which has a prominent nucleolus (arrowhead). Astra blue/safranin. ×2000.

although the distribution of both in the mucosa varied. Mitotic figures were observed in both cell types (Fig. 3).

The maximum number of GL cells was observed on the fourteenth day after infection (Table 1). Some seemed to be fully granulated and were indistinguishable from mast cells seen in normal rats; however, most of the GL cells apparently contained fewer granules than their counterparts in the lamina propria. Mast cells in some areas were fragmented and were, for this reason, difficult to count. Mitoses were found readily in both GL cells and mast cells on this day.

The greater part of the GL cell population was located in the upper regions of the gland crypt epithelium. Very few were present in the epithelium overlying the villus and those that were, usually contained very few granules. GL cells contributed an increasing proportion of the population from the 10th to the 14th day of infection by which time they formed approximately half of the total (Table 1). On the 16th day, the distribution and morphology of the granulated cells were similar to those seen on the 14th day. Mitoses were, however, rarely observed, although nucleoli could still be distinguished in some of the nuclei. The numbers of mast cells remained at approximately the same level between the 14th and 19th days, but the proportions of GL cells decreased steadily and by the 35th day, GL cells formed one-third of the total (Table 1).

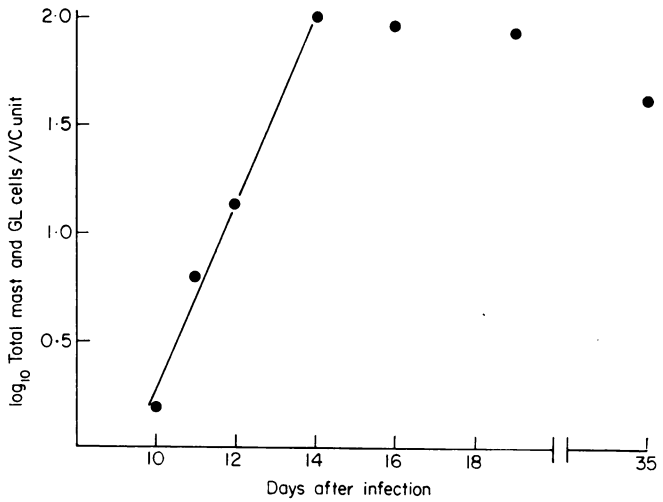

FIG. 4. The kinetics of the combined mast and GL cell populations during the immunological expulsion of *N. brasiliensis*.

On the 19th and 35th day of infection, the mast cells were fully granulated and compact (Fig. 5) and very few appeared to be fragmented. GL cells were morphologically similar to and distributed in the same way as those seen on day 14.

DISCUSSION

Examination of the changes in the intestinal mast cells of the rat during the expulsion of *Nippostrongylus brasiliensis* reveals several important points. In the first place, there was an exponential increase in the total number of cells between the 10th and 14th days of infection. These cells underwent mitosis at all stages of the phase of population expansion and there was evidence of mast cell differentiation and maturation. Secondly, when cell numbers were at a maximum on day 14, approximately half of them were globule leucocytes. Previous studies have shown that the latter are mast cells which have migrated intraepithelially (Murray *et al.*, 1968) and they were, for this reason, included in the total counts. Amine has, however, been lost from GL cell granules and there has been a change in the relationship between the acid mucopolysaccharide and basic protein in the granule matrices (Murray *et al.*, 1968). In effect, the globule leucocytes are partially discharged cells and the proportion of them present gives an indication of the extent of mast

cell discharge. Finally, there is a striking temporal relationship between the expulsion of the parasites and the rise in mast cell numbers. Self-cure occurred between the 11th and 19th days of infection, thus following the pattern described by Jarrett *et al.* (1968a, b, 1969). Mast cells reappeared in the mucosa by the 10th day and, allowing for mucosal expansion, were very numerous during self-cure. As will be discussed below, there is evi-

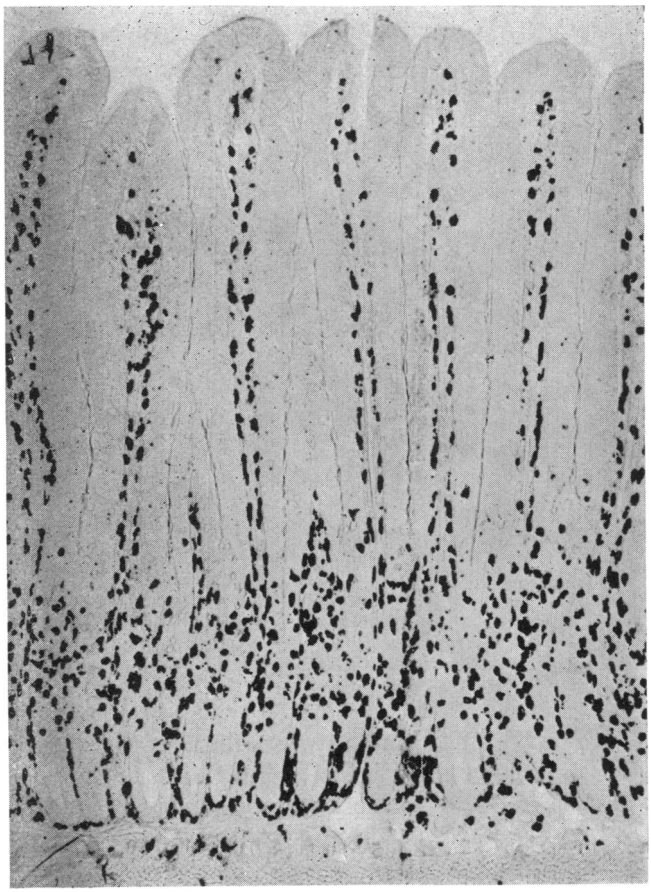

FIG. 5. Mast cells and globule leucocytes are abundant in the jejunal mucosa 19 days after infection. Astra blue/safranin. × 150.

dence, apart from the coincidence in the timing of these events, that the mast cells have an important role in worm expulsion.

Evaluation of quantitative methods

Several factors had to be considered in selecting a method for counting the cells. These included ease of identification, the distribution of the cells in the mucosa, alterations in tissue volume, and the facility with which the two cell populations could be compared.

The copper phthalocyanine dyes, Astra blue and Alcian blue, when used at pH 0·3 are highly specific for the sulphated acid mucopolysaccharides of mast cell granules (Bloom

and Kelly, 1960; Enerbäck, 1966a). In the intestinal mucosa, only the granules of the latter (Enerbäck, 1966a; Murray *et al.*, 1968) and of the globule leucocyte (Murray *et al.*, 1968) are stained. Thus, the method provides a simple and reliable means of detecting the cells. Safranin O, when used as a counterstain, gives a weak background coloration which outlines the villus structure and at the same time provides strong staining of mitotic figures and nucleoli.

Mast cells were evenly dispersed in the mucosa in normal rats. However, during *Nippostrongylus* infections a larger proportion of the cells were concentrated in the crypt region, and the villi were expanded so that the gaps between them were narrowed. There were, therefore, several advantages in the use of the villus-crypt (VC) unit as compared with fixed-field methods. The subjective selection of fields, which is difficult to avoid in the latter technique, became less of a problem. The VC unit facilitated differential counts of mast and GL cell numbers and was flexible in the event of volume changes so that two of the three dimensional alterations were already included within it. The villi must, however, be sectioned longitudinally to avoid artefacts of cell distribution which could arise from oblique sections of the crypt region.

Mast cell changes

Very few mast cells were found in the intestinal mucosa on the 8th day of infection. This has been confirmed by ultrastructural studies which show that mast cells are damaged as soon as the parasites reach the intestinal lumen, that they are progressively destroyed, and that their remnants are eventually phagocytosed by macrophages (Miller, 1970). Preliminary experiments indicate that metabolites, secreted by the parasites, are responsible for mast cell damage (Miller, 1970) and it has been suggested that *N. brasiliensis*, like *Ascaris* (Uvnäs and Wold, 1967) secretes a mast cell degranulating factor and this may be responsible for producing a subepithelial vascular leak (Murray *et al.*, 1968). The resultant villus oedema could benefit the larvae either by facilitating their feeding or possibly by allowing them a more secure hold when intertwined between the villi.

The immature mast cells, which appeared in the mucosa 10 days after infection, differentiated from cells with large nuclei and prominent nucleoli. This is in agreement with the findings of Taliaferro and Sarles (1939).

Electron microscopy shows these precursor cells to be of the blast type with an abundant cytoplasmic content of ribosomes and they would appear to be of lymphoid origin (Miller, 1971a). The exponential character of the population expansion, the presence of mitotic figures, and the morphology of the cells point to an increase by cell division and differentiation, and this agrees well with the ultrastructural findings (Miller, 1971a). The early stages of growth and maturation between the 10th and 12th days of infection are reflected by a wide range of individual counts and by the irregular distribution of poorly granulated cells within the intestinal lamina propria of each rat. By the 14th day the range had narrowed and mast cells were more mature. This was apparent from the greater numbers of granules contained within individual cells, although, even at this stage, both mast and GL cells were often seen to be in mitosis.

The stimuli required to bring about increased numbers of mast cells are not known. They have, however, been observed to develop from lymphoid cells of the thymus (Ginsburg, 1963), from thoractic duct cells (Ginsburg and Lagunoff, 1967) from cultures of lymph node cells (Ginsburg and Lagunoff, 1967) and to multiply within lymph nodes (Miller and Cole, 1968). The proliferation of mast cells in cultures of thoracic duct cells

implies that they were derived by differentiation of precursor cells rather than by multiplication of an existing population of mast cells; Miller and Cole (1968) postulated that both mechanisms might be functional in the lymph nodes. Proliferation of mast cells was most marked after antigenic stimulation (Ginsburg and Lagunoff, 1967; Miller and Cole, 1968) and it is possible that *N. brasiliensis* also provides a stimulating factor responsible for mast cell differentiation and proliferation, although other influences, such as degranulation of the original mast cell population, might also have an effect.

A decline in the total number of granulated cells occurred after the fourteenth day of infection and three factors may have contributed to this.

1. Increasing proportions of the cells migrate intraepithelially to become GL cells during the period of population expansion. The eventual fate of the GL cells has not yet been clarified, but the majority are partially discharged as they reach their location within the epithelium (Murray *et al.*, 1968; Miller, 1970) and it is probable that, as they are carried towards the villus tip between migrating epithelial cells, they continue to discharge their granule contents. Eventually the complete loss of stainable acid mucopolysaccharide from the granules may render the cells less easily detected by conventional histological methods. In any event, GL cells could be seen in the crypt region but were present only in small numbers higher up the villus. It is possible that they are able to migrate back into the lamina propria, but none was seen to migrate into the lumen. Their demise would, however, contribute to the overall decline in the number of recognizable mast cells.

2. Histologically, many mast cells were fragmented, and ultrastructural (Miller, 1971b) as well as histochemical studies (Miller, 1971c) suggest that a large proportion of them were partially discharged or were even more severely damaged on the 14th and 16th days of infection. The severity of these changes was striking (Miller, 1971b) and may be a major factor in the cessation of the phase of population increase.

3. After the 14th day, the rate of cell division and differentiation was probably declining. It was beyond the scope of the present work to estimate the mitotic activity of mast cells during *Nippostrongylus* infection, but many were seen in mitosis on the 14th day, whereas mitotic figures were rare on the 16th day and none was observed on later days of infection. Population growth by cell division had probably almost ceased by the 19th day of infection.

Mast cells and self-cure

The experiments of Barth *et al.* (1966) showed that a localized anaphylactic reaction played a part in worm rejection, probably by increasing the permeability of the mucosa to anti-worm antibody. Natural infections by *N. brasiliensis* are associated with the production of reagin-like antibodies (Ogilvie, 1964, 1967; Wilson and Bloch, 1968), and these react with an allergen extracted either from the parasites or from their metabolites (Jones and Ogilvie, 1967; Wilson, 1967). The present quantitative findings, backed up by histochemical and ultrastructural studies (Miller, 1971b, c) show the intestinal mucosa to be packed with mast cells, many of which are discharging their amines. These results support the hypothesis of Jarrett *et al.* (1968a, 1969) that mast cell disruption is effected by an allergen–reaginic antibody mediated system. This hypothesis is given added weight by the fact that mast cells in the peritoneum are sensitized to release histamine as early as 10 days after infection (Wilson and Bloch, 1968). However, other discharge mechanisms, such as the action of a specific mast cell degranulator produced by the parasite, cannot at present be excluded.

When antihistaminics or reserpine, a depletor of 5-hydroxytryptamine (5-HT), are administered to rats undergoing self-cure, the expulsion of the parasites is inhibited (Urquhart *et al.*, 1965; Sharp and Jarrett, 1968). Such results suggest a role for histamine and 5-HT in the reaction.

During self-cure there are extensive mucosal alterations characterized by a breakdown of the epithelium in the gland crypts and by disruption of the vascular endothelium (Miller, 1970). Horseradish peroxidase when given intravenously escapes readily through the epithelium on the 14th day of infection whereas in normal rats it does not penetrate beyond the junction between intact epithelial cells (Murray, Jarrett, Jennings and Miller, 1970). Quantitative experiments using ^{131}I-labelled polyvinylpyrrolidone have shown a marked increase in the leakage of this tracer into the intestinal lumen during worm expulsion, a peak being evident on the 14th day of infection in male rats (Murray, Jarrett and Jennings, 1971).

These findings demonstrate a striking relationship between the mast cell changes, the alteration of mucosal permeability and the expulsion of the parasites. We have suggested (Jarrett *et al.*, 1969) that biogenic substances such as 5-HT, histamine and proteases, released from the mast cells, are responsible for the mucosal changes. The increase in permeability would facilitate the rapid passage of antibody, whether of systemic or of local origin, into the intestinal lumen. Clearly such a mechanism would have an effect on the expulsion of the parasites. Whether it merely augments the passage of antibody, thus increasing the rate of expulsion, or whether it is an essential part of the self-cure phenomenon, has not been clarified.

ACKNOWLEDGMENT

We are grateful to Mr A. Finney for the preparation of the figures.

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