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Effect of a *Trichinellia spiralis* infection on the distribution of mast cell precursors in tissues of thymus-bearing and non-thymus-bearing (nude) mice determined by an *in vitro* assay

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SUMMARY

The frequency of precursor cells capable of giving rise to cells with characteristics of mucosal mast cells in tissues from thymus-bearing and non-thymus-bearing (nude) mice orally infected with *Trichinella spiralis* was determined with an *in vitro* assay. Analysis of the frequency of mast cell precursors in bone marrow, blood, spleen and small intestinal tissue revealed similar frequencies of mast cell precursors in bone marrow from both thymus-bearing and athymic mice. These frequencies in bone marrow were not affected by infection. However, in blood and spleen from thymus-bearing mice at Day 7 post-infection (p.i.), and in the gut at Day 14 p.i., significant increases of mast cell precursor frequencies were detected. In contrast, no significant increase was observed in the tissues of infected nude mice. These data are in accordance with *in vivo* findings, indicating that a mucosal mast cell response in the gut is both thymus and antigen dependent. It was concluded (i) that a mucosal mast cell response to infection with *T. spiralis* is probably due to local proliferation and maturation of residing mast cell precursors, (ii) that this response might be amplified by an influx of precursor cells from the blood into the gut, and (iii) that both phenomena are T-cell dependent.

INTRODUCTION

Intestinal helminth infections like *Trichinella spiralis* or *Nippostrongylus brasiliensis* in rodents are characterized by increased numbers of mucosal mast cells (MMC) in the gut and associated lymphoid tissues. Intestinal mastocytopoiesis is a T-cell dependent phenomenon (Ruitenberg & Elgersma, 1976; Mayrhofer, 1979). MMC responses can be accelerated by the adoptive transfer of immune T cells in infected thymus-bearing recipients (Nawa & Miller, 1979; Alizadeh & Wakelin 1981). However, in infected congenitally nude mice the adoptive transfer of immune T cells had no effect (Parmentier *et al.*, 1982). Thus, other factors in the host may also be involved in restricting MMC responses. These restricting factors might include a lack of MMC precursor cells. In addition, T-cell regulation of MMC proliferation and maturation might be deficient in nude mice, even after the transfer of immune lymphocytes.

It has been shown that mast cells, including MMC, originate from the bone marrow (Kitamura *et al.*, 1979; Crowle, 1983). Whether intestinal mastocytosis is due to the recruitment of precursor cells from the bone marrow that migrate via the blood to the intestine and merely differentiate at the site, or reflects local proliferation and maturation, as has been proposed earlier (Nawa & Miller, 1979; Guy-Grand *et al.*, 1984), was the subject of this study.

In vitro cultures of mast cells may provide a tool to analyse further the kinetics of MMC responses *in vivo*. Several studies report the successful culture of mast cells *in vitro*. These cells have many of the features of MMC, i.e. a low histamine content (Schrader *et al.*, 1981; Sredni *et al.*, 1983), the absence of heparin and presence of a unique proteoglycan (Haig *et al.*, 1982; Razin *et al.*, 1982), and ultrastructural, biochemical and functional similarities (Sredni *et al.*, 1983; Nabarra & Dy, 1984). Therefore, several authors tentatively identify these cultured mast cells as MMC (Crapper & Schrader, 1983; Sredni *et al.*, 1983). Mast cell colony growth and differentiation *in vitro* require the administration of mast cell growth factors, currently designated interleukin-3 (IL-3) (Ihle *et al.*, 1983), derived from concanavalin A (Con A) (Razin, Gordon-Coude & Good, 1981), or antigen-stimulated lymphocytes (Ginsburg *et al.*, 1981; Haig *et al.*, 1982, Haig, Jarett & Tas, 1984).

In vivo, increasing numbers of IL-3-producing cells have been detected in the spleen of *N. brasiliensis*-infected mice, coinciding with intestinal mastocytosis (Filho *et al.*, 1983). This suggests that, *in vivo*, intestinal MMC responses are also IL-3 dependent.

We have cultured bone marrow, blood and spleen cells and

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gut leucocytes from *T. spiralis*-infected mice in the presence of Con A-stimulated spleen cell supernatant in an *in vitro* assay. Thymus-bearing and congenitally athymic (nude) mice were used to study the effect of a *T. spiralis* infection and T-cell regulation on the distribution of cells capable of forming colonies of cells with MMC characteristics in the putative tissue of origin, during transport and in the specific target tissue. The results indicate that, besides a local proliferation and maturation of precursor mast cells, MMC responses to infection with *T. spiralis* are mediated by the recruitment of precursor mast cells to the gut. Both processes are T-cell dependent.

MATERIALS AND METHODS

Animals

Female B10LP +/*nu* (thymus-bearing) and congenitally nude (athymic) *nu*/*nu* mice were used. Animals 6 weeks of age were obtained from the Central Laboratory for the Breeding of Laboratory Animals (TNO, Zeist, The Netherlands) where the mice were maintained by conventional back-crossing between B10LP and nude B10LP *nu*/*nu* animals. The animals were maintained for 2 weeks in a laminar flow cabinet prior to use.

Parasite and parasitological methods

The *Trichinella spiralis* strain was originally isolated from an infected pig in Poland and maintained in the National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands, from 1960 onwards in Laboratory Wistar rats and Swiss mice. Passages were performed every 6 weeks. Larvae used for oral infection were obtained from infected Swiss mice after conventional digestion with HCl and pepsin (Köhler & Ruitenberg, 1974). Digestion was not prolonged beyond 2 hr in order to prevent possible interference with the infectivity of the larvae.

Source of MMC-like cell precursors

The frequency of mucosal mast cell-like cell precursors in bone marrow, blood, spleen and jejunal mucosa in *T. spiralis*-infected +/*nu* and *nu*/*nu* mice at Days 7 and 14 p.i. was determined by an *in vitro* assay. Groups of five mice each were orally infected with 300 *T. spiralis* larvae at Day 0. At Days 7 and 14 post-infection (p.i.) the animals were autopsied and tissues were prepared for *in vitro* culture. Bone marrow cells were prepared by flushing the femur and tibia with Ca^{2+}- and Mg^{2+}-free Hanks' buffered saline (pH 7·2), washed, pelleted and resuspended in culture medium. Cell suspensions from spleens were obtained by gently pressing the organs through nylon gauze filters, with continuous addition of Hanks' buffer, pelleted and resuspended in culture medium. Blood was collected in Hanks' buffer containing 10 IE heparin/ml. After pelleting, cells were resuspended for 60 seconds in 0·155 M NH_4Cl, 10 mM $KHCO_3$, 0·1 mM EDTA, pH 7·2, washed in Hanks' buffer, and resuspended in culture medium. Intestinal tissue was sliced with a pair of scissors longitudinally. Two-cm parts were washed in Hanks' buffer and incubated for 10 min in 1 mM dithiotreitol (Merck, Darmstadt, FRG) in RPMI-1640 (Gibco Paisley, Renfrewshire, U.K.) with 10 IE heparin/ml. After washing, the intestinal pieces were gently scraped with forceps and the resultant suspension was passed through a 1-cm nylon wool column, washed and resuspended in culture medium. Intestinal leucocytes as well as cells from the other organs were counted in a haemocytometer with trypan blue exclusion. Cells were suspended in 96-well flat-bottomed Costar plates in numbers ranging from 1×10^7 to 50 cells per well in duplicate per tissue per animal. At various days cultures were analysed for colony growth in the wells and cells were fixed for microscopical examination. Wells with the lowest number of suspended cells at Day 0 in which mast cell colonies were determined were used to calculate the precursor frequency. This calculation was performed according to the weighted mean estimation described by Taswell (1981). Thus, the weighed mean frequency estimate f for separate tissues per group was calculated as:

$$f = \frac{\sum\left(\frac{-\ln p_i}{x_i}\right)\left(\frac{x_i r_i}{1-p_i}\right)}{\sum\left(\frac{x_2^i r_i}{1-p_i}\right)}.$$

Briefly, x_i represents the number of cells tested in each replicate culture of each dose i; r_i the number of negatively responding cultures of each dose; and $p_i = r_i/n_i$, the fraction of negatively responding cultures of each dose; n_i is the number of replicate cultures of each dose.

Culture of MMC-like cells

Mast cell cultures were performed with RPMI-1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and penicillin-streptomycin (each 100 units/ml) (growth medium) and conditioned medium (1 : 1 v/v), incubated at 37°, 5% CO_2 in humidified atmosphere. Every 7 days cell cultures were provided with new medium. At day 28 cells were washed and cytocentrifuge slides were prepared.

Conditioned medium (CM)

Pooled spleen cells from 10 B10LP +/*nu* mice (1×10^6 cells/ml) were cultured for 48 hr in RPMI-1640, 2 mM L-glutamine, 10% FCS and antibiotics and 10 μg/ml Con A in 150-cm^2 tissue culture flasks (Costar, Cambridge, MA), incubated at 37°, 5% CO_2, humidified air. Supernatant was harvested after centrifuging cells at 1000 *g* for 10 min.

During the experiments mentioned above this single batch of CM was used for all cultures.

Identification of cultured cells

Cultured cells were examined for their capacity to incorporate serotonin. Cells (5×10^5/ml) were washed with PBS and incubated for 30 min at 37° with 1 miCu/ml ^{3}H-serotonin (Amersham International, Amersham, Bucks, U.K.). After washing, cells were transferred into scintillation vials containing 1 ml of a mixture of Triton X-100 (1%) and Insta Fluor (Packard-Becker BV, Groningen, The Netherlands (1 : 1 v/v). Counts per minute were determined with an ISO cap/300 liquid scintillation counter (Nuclear Chicago, Des Plaines, IL). A similar procedure for peritoneal mast cells obtained by peritoneal washings was performed. Furthermore, morphology (metachromatically staining granules), ultrastructure, histamine content and sulphation of glycosaminoglycans were determined.

Cytocentrifuge slides fixed in methanol were stained with Toluidin Blue O or May-Grünwald Giemsa.

For transmission electron microscopy, cells were washed in PBS (pH 7·2) and fixed in 2·5% (v/v) glutaraldehyde in 0·1 M Sörensen phosphate buffer with 0·15 M sucrose (pH 7·4) for 150

min at 4°. After washing with Sörensen's buffer with 5% (w/v) sucrose, cells were post-fixed in 2% (w/v) OsO_4 with 1·5% (w/v) K4Fe $(CN)_6$ in Sörensen's buffer for 90 min. After dehydration in ethanol, cells were embedded in an epon-equivalent epoxy-resin, glycid ether 100 (Merck). Ultrathin sections were double-stained with uranyl acetate and lead citrate. Sections were observed with a Philips 201 transmission electron microscope. For scanning electron microscopy, cells fixed as described above were mounted on poly-L-lysine-coated cover slips, and dried with CO_2 in a Polaron E 3000 critical point drying apparatus (4 hr, four flushes of 2 min each). The cover slips with cells were mounted on aluminium stubs and coated with a thin layer of gold in a Polaron E 5000 sputter coater. Samples were observed using a Philips 501 scanning electron microscope at 15 kV.

Determination of histamine content of cultured mast cells was kindly performed by Dr Wolthers and Mr Beukelman, Academic Hospital, University of Groningen, The Netherlands, using a gas chromatographic method with nitrogen phosphorus detection (Keyzer *et al.*, 1982).

Determination of sulphation of glycosaminoglycans in cultured mast cells was performed according to methods described previously (Tas & Geenen, 1975; Tas & Berndsen, 1977). Briefly, cytospin slides fixed in Böhm's solution (10% formalin, 5% acetic acid in methanol) were stained for 2 min with 0·01% Toluidin Blue dissolved in 20 mM citrate-phosphate buffer (pH 5·0), washed in distilled water and mounted in water-free glycerol. Metachromatic spectra from cultured and peritoneal mast cells were recorded microspectrophotometrically at different times after mounting. Overall mean of peak wavelengths as well as the overall standard deviation per 10 cells were determined.

Statistical analysis

The probability of significant differences between groups of mice was calculated by Student's *t*-test.

RESULTS

From *in vitro* analysis it appeared that in the presence of Con A-stimulated spleen cell supernatant, cells with characteristics of MMC were cultured from bone marrow, spleen, blood and intestinal tissue dissected from *T. spiralis*-infected and non-infected thymus-bearing B10LP +/*nu* and athymic *nu*/*nu* mice. Colony growth was observed after 7 days and culture was performed for 28 days, during which period colonies steadily grew. The cultured cells were able to incorporate tritiated serotonin up to 25% of the level of isolated connective tissue mast cells from the peritoneum (Fig. 1). The cells were characterized by metachromatically staining granules (Fig. 2) and a histamine content of 0·1 pg/cell, whereas 13–14 pg per cell was estimated for peritoneal mast cells. The overall mean peak wavelength measured in equilibrium phase was around 550 nm for Toluidin Blue O stained cultured mast cells, which indicated the presence of low sulphated proteoglycans, i.e. with fewer than two sulphate groups per disaccharide unit. For peritoneal mast cells, a mean equilibrium peak wavelength was estimated at 519 nm, demonstrating the presence of a highly sulphated proteoglycan, i.e. heparin (Table 1).

Scanning electron microscopy revealed that cultured mast cells from all tissues examined had similar characteristics, i.e. numerous villous projections, filopodia and/or microvilli

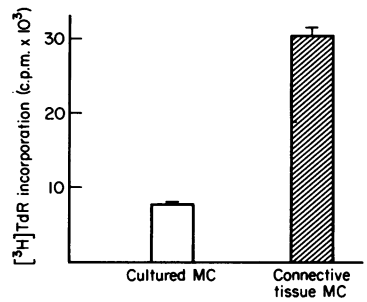

Figure 1. Uptake of ^{3}H-serotonin by 2×10^4 cultured and 2×10^4 connective tissue mast cells (MC) of B10LP +/*nu* mice (five animals per group, means ± SE).

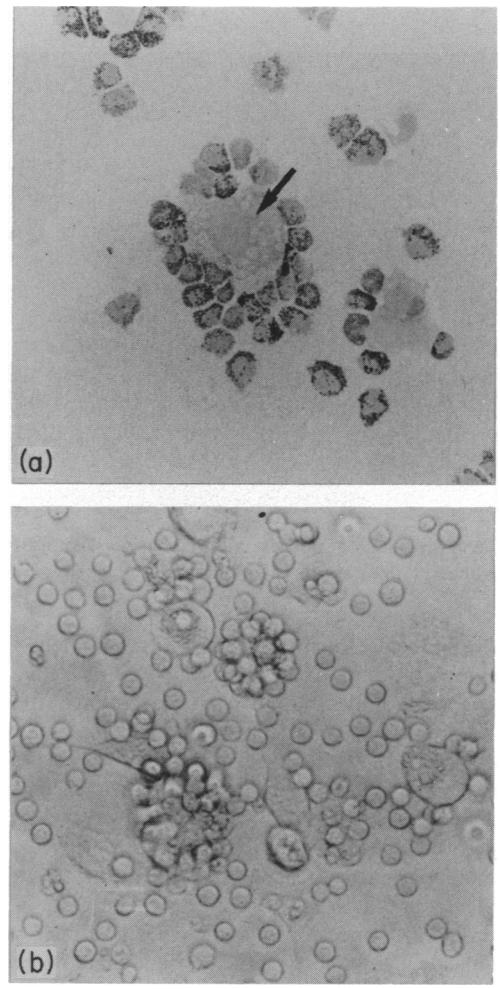

Figure 2. Bone marrow cells from B10LP +/*nu* mice after 14 days of culture in the presence of Con A-stimulated spleen supernatant. Metachromatically staining cells. Note the large blastic cell (arrow). (a) Methanol, toluidin blue, magnification ×400. (b) Phase contrast, magnification ×320.

(Fig. 3). Transmission electron microscopy (Fig. 4) showed excentrically or peripherally located pleomorphic nuclei containing a nucleolus and marginally condensed heterochromatin. The cytoplasm showed small mitochondria, free ribosomes, rough endoplasmic reticulum in short cisternae and inconsis-

Table 1. Histamine content and mean metachromatic peak wavelength in equilibrium phase during microspectrophotometric determination of sulphatation of proteoglycans in cultured mast cells from B10LP +/*nu* mice

	Histamine (pg/cell)*	Mean metachromatic peak wavelength (nm ± SD)†
Bone marrow	0·09–0·1	551 ± 4
Spleen	0·08–0·09	550 ± 4
Gut	0·05–0·5	549 ± 3
Blood	0·01–0·2	550 ± 4
Peritoneum	13–14	519 ± 3

* Determined in 10^5–10^6 cells.
† $n = 10$.

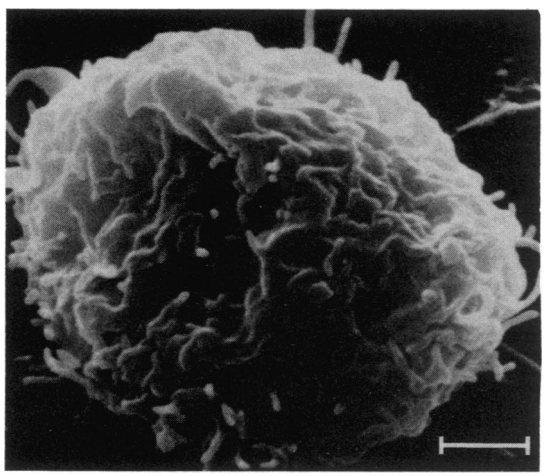

Figure 3. Scanning micrograph of a cultured mast cell from B10 LP +/*nu* mouse characterized by ridges and filopodia. Bar represents 1 μm.

tently a prominent Golgi complex, in a moderately electron-dense cytoplasmic matrix. Numerous peripherally located heterogeneous granules bound by a perigranular unit membrane were found containing fine or coarse aggregated granular material and small 50–75 nm membraneous vesicles (Fig. 4b). Sometimes larger membrane-bound vesicles with electron-dense material were observed. Cells that were cultured from B10LP *nu/nu* mice were similar to those cultured from +/*nu* littermates.

From the presence of mast cell colonies in the titrated wells, it was calculated that in the bone marrow from both +/*nu* and *nu/nu* non-infected mice approximately 5 per 10^4 cells were able to form mast cell colonies *in vitro*. In bone marrow from both +/*nu* and *nu/nu* mice this frequency remained at the same level during infection *in vivo* (Fig. 5a and b). The frequency of mast cell precursors in the blood from non-infected animals was 1 per 10^5 cells in +/*nu* and 3 per 10^6 cells in *nu/nu* mice. However, whereas a significant 15-fold increase was found at Day 7 p.i. in the blood of +/*nu* mice, no such increase was observed in the blood of infected nude mice. At Day 14 p.i. precursor levels in +/*nu* blood had returned to control levels.

Similar results weres obtained with spleen cultures. The frequency of mast cell precursors at Day 0 in the spleens of +/*nu* mice, 3 per 10^5 cells, increased to 2 per 10^4 cells at Day 7 p.i., and resulted at Day 14 p.i. in levels comparable with Day 0. Again, in

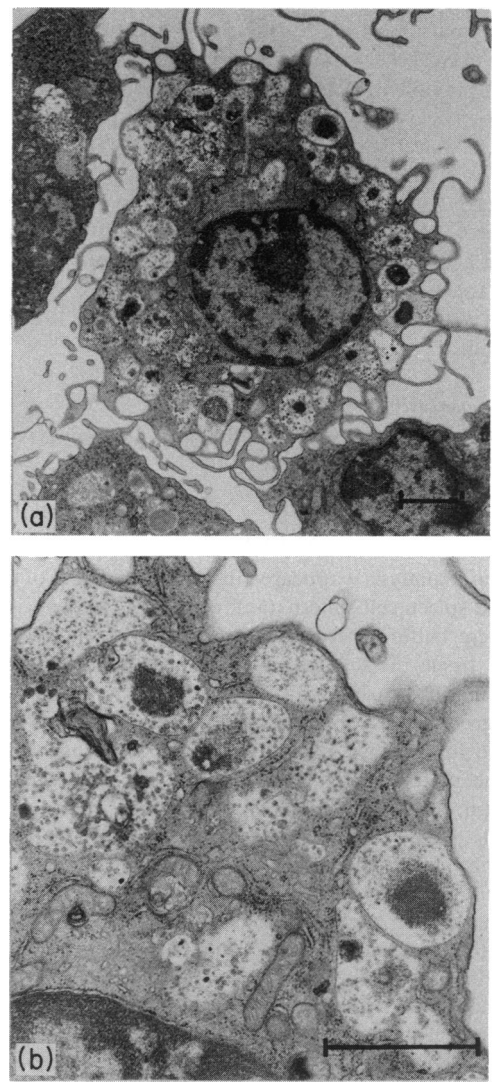

Figure 4. Electron micrograph of a cultured mast cell from a B10LP +/*nu* mouse (a) and detail of the same cell (b). Bar represents 1 μm.

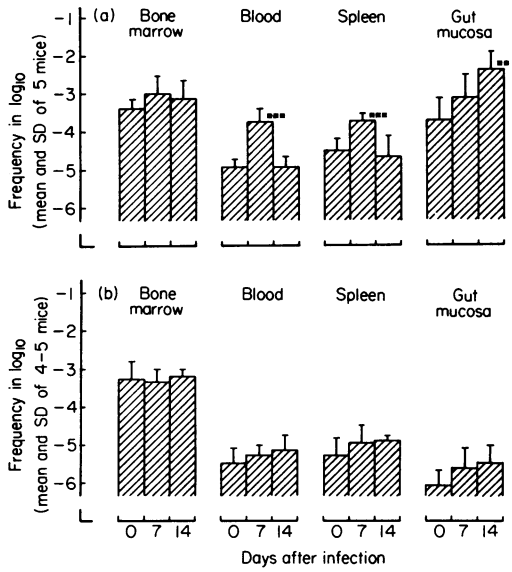

Figure 5. Frequency of mucosal mast cell-like precursor cells in tissues from B10LP+/*nu* (a) and *nu/nu* (b) mice after oral infection with 300 *T. spiralis* larvae each, assayed *in vitro*. Student's *t*-test: ** $P<0{\cdot}01$, *** $P<0{\cdot}001$.

the spleens of infected nude mice no changes in precursor frequency were found.

Cells cultured from jejunum from infected +/*nu* animals showed steadily increasing precursor frequencies, from 2 per 10^4 cells to 5 per 10^3 cells, i.e. a significant 25-fold increase. As was true for the other tissues, precursor frequencies remained unchanged in the gut of nude mice.

Phase-contrast microscopy revealed a resemblance between bone marrow- and spleen-derived mast cell colonies. In these cultures regularly large blast-like cells (Fig. 2a) were noticed, sometimes connected with mast cells. These large cells were not observed in blood nor in cultures of gut cells.

DISCUSSION

The proliferation of mucosal mast cells (MMC) in laboratory rodents during intestinal infections with *Trichinella spiralis* and other helminths is thymus dependent (Ruitenberg & Elgersma, 1976; Mayrhofer, 1979) and is mediated by recirculating T cells (Nawa & Miller, 1979; Alizadeh & Wakelin, 1981). Since *in vivo* no intestinal MMC response occurs in nude mice, and transfer of immune lymphocytes fails to induce mastocytosis in these mice (Parmentier *et al.*, 1982), we have investigated the distribution of MMC-like precursor cells in tissues from normal and athymic mice during a *T. spiralis* infection. Cells with many features of MMC, including a low histamine content, absence of heparin, a relatively lower uptake of serotonin, and ultrastructural similarities with IL-3-dependent mast cells (Tertian *et al.*, 1982; Sredni *et al.*, 1983; Nabarra & Dy, 1984) and murine intestinal lamina propria mast cells (J. S. Teppema, unpublished results) were cultured from bone marrow, spleen, blood and intestinal tissue from normal and nude mice, when Con A-stimulated spleen cell supernatant [i.e. T-cell derived IL-3 (Ihle *et al.*, 1983)] was administered. This suggests that MMC precursors are present in mice regardless of the presence of the thymus. The results of the present study show that the distribution of MMC precursors in tissues of euthymic mice during infection corresponds with intestinal mastocytosis *in vivo*, whereas in nude mice no changes in distribution during an infection were observed.

Bone marrow and intestinal tissue in non-infected +/*nu* mice contain approximately the same level of MMC precursors, which exceeds the corresponding frequencies in blood and spleen, respectively. Recently, it has been suggested that intestinal mast cell responses in *T. spiralis*-infected mice are due solely to local MMC proliferation regulated by T cells, since no change of MMC precursor frequencies was observed in bone marrow (Dillon & MacDonald, 1986). However, these authors have only examined bone marrow at Day 4 p.i. Our data, obtained by studying other tissues and other time-points after infection, confirm that there are no changes in precursor frequencies in bone marrow, but indicate that in blood, spleen and intestines these frequencies do change. A *T. spiralis* infection results in increasing MMC precursor frequencies in the intestine of +/*nu* mice, preceded by increased frequencies in blood and spleen. These observations are consistent with those obtained by others (Crapper & Schrader, 1983; Guy-Grand *et al.*, 1984) and suggest that MMC precursors arise in the bone marrow and are seeded out via the blood. In nude mice these changes in precursor frequencies do not occur, indicating that T cells or T-cell products are involved in the regulation of these phenomena. It has been suggested that bone marrow-derived MMC precursors might also be attracted from the blood to the gut by local factors other than antigen or T-cell factors such as IL-3, since nude and germ-free mice were shown to contain levels of MMC precursors in the gut comparable to those of normal mice (Guy-Grand *et al.*, 1984). In the latter study different murine strains were compared and MMC precursors in infected nude mice were not determined. Mast cell deficient W/W^v mice, which do not show MMC responses to parasites (Alizadeh & Murrell 1984), contain normal frequencies of MMC-like precursors in bone marrow and spleen, but not in the gut (Crapper & Schrader, 1983). It was speculated that these mice have a defect of homing of MMC precursors to the gut, although a defect in the antigen-dependent amplification of their numbers in the intestine cannot be excluded.

Taken together, we conclude that these data indicate that a MMC response to *T. spiralis* is due to local proliferation and maturation of residing mast cell precursors, that this response might be amplified by an influx of precursor cells from the blood into the gut, and that both phenomena are T-cell dependent.

Amplification of the MMC response by recruitment of precursor mast cells, as indicated by preceeding increasing frequencies in the blood, is supported by our earlier studies, in which we have demonstrated that the intestinal increase of MMC precursors is regulated by the release of serotonin, probably facilitating local vasopermeability (Parmentier *et al.*, 1987). Since our data show that MMC-like precursor cells are present in nude mice, but MMC responses could not be induced in these animals after transfer of 14-day immune lymphocytes (Parmentier *et al.*, 1982), we speculated that nude mice may lack mechanisms that direct IL-3-producing T cells to the gut (Parmentier *et al.*, 1987). It has been shown in earlier studies that serotonin release, causing local vasopermeability which facilitates entry of inflammatory cells in extra vascular tissues, requires regulation by T cells other than lymphokine-producing

cells, which are induced early after sensitization (van Loveren & Askenase, 1984). These latter T cells are not present in nude mice and are apparently not in the transferred immune lymphocytes harvested 14 days after infection either. *In vitro* analysis of MMC precursor frequencies in tissues from antigen-sensitized mice treated with serotonin antagonists may resolve whether this mechanism also accounts for the local increase at the site, or in associated lymph nodes, reported in other models, in which parasitic (Guy-Grand *et al.*, 1984) or other antigenic stimuli (Crapper & Schrader, 1983; Ahlstedt *et al.*, 1986) have been studied.

According to Guy-Grand *et al.* (1984), MMC precursors upon activation by IL-3-producing T cells undergo a cyclic traffic via the thoracic duct seeding the whole length of the gut, and proliferate and mature after re-entering intestinal tissues regulated by simultaneously recycling IL-3-producing T cells. This opinion is in agreement with our results, and it may account for the observation that during *T. spiralis* infections in mice, increasing MMC numbers have also been found in mesenteric lymph nodes and coecum (Parmentier *et al.*, 1982). Furthermore, cannulation or extirpation of the thoracic duct results in decreased MMC responses in the jejunum of *T. spiralis*-infected rats (J. Buys, unpublished results).

In vivo, IL-3-producing T cells have been demonstrated in the spleen from *N. brasiliensis*-infected mice. Splenic IL-3 production coincided with intestinal mastocytopoiesis (Filho *et al.*, 1983). This might be explained by circulating antigen-stimulated IL-3-producing T cells; however, serum levels of IL-3 remained very low. Our results show a strong correlation between MMC precursor frequencies in blood and spleen. Whether precursor levels in spleen simply reflect levels in the blood or suggest a contribution from the spleen to a rise of mast cell precursors in the blood cannot be decided. Like bone marrow cultures, splenic cultures from both +/*nu* and *nu*/*nu* mice are characterized by large blastic cells, which have not been observed in the gut and blood cultures. The nature and function of these cells are obscure, but they might be related to both mucosal mast cell and megakaryocyte stem cell lineages (Schrader, 1983). These blast cells were absent in the intestinal tissues and blood. Peripheral MMC-like precursors were unable to form colonies in spleen after adoptive transfer. These data may suggest that MMC precursors in both bone marrow and spleen are less committed to becoming MMC during a *T. spiralis* infection, as compared to peripheral precursor cells. Both compartments may therefore act as the source of MMC precursors. However, since during a helminth infection the level in bone marrow remained unchanged, in contrast to the level in the spleen, these data may also suggest that during a helminth infection the spleen serves as the source to meet the demand for the surplus of MMC precursors, and that hence T-cell dependent recruitment of MMC precursors from the spleen via the circulation occurs. This compartment, in contrast to bone marrow, reacts to T-cell signals (IL-3) during an infection. Similar to the spleen, other lymphoid organs, i.e. (mesenteric) lymph nodes, may also be the source of MMC precursors during an infection, although blast-like nurse cells have not been reported in these organs (Crapper & Schrader, 1983; Ginsburg *et al.*, 1978; Denburg, Befus & Bienenstock, 1980; Davidson *et al.*, 1983).

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