Early increase of gut intraepithelial mast cell precursors following *Strongyloides venezuelensis* infection in mice

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SUMMARY

The time-course of differentiation/proliferation of mast cells in gut epithelium was investigated in mice infected with the nematode *Strongyloides venezuelensis*. After infection, expression of proliferating cell nuclear antigen increased in gut intraepithelial mast cells on days 7 to 11, followed by an increase in the number of intraepithelial mast cells from days 11 to 14. Mast cell precursors were defined as cells that formed mast cell colonies in methylcellulose culture. After infection, the numbers of mast cell precursors in the population of gut intraepithelial mononuclear cells (IEMNC) increased significantly on day 3 and returned to the pre-infection level by day 7. Mast cell precursors in Peyer's patches, mesenteric lymph nodes (MLN), and spleen also increased from day 7 p.i. Production of IL-3 and IL-4 in MLN and spleen were increased between 7 and 11 days p.i. These results show that murine intestinal mastocytosis is initiated by an early increase in mast cell precursor number in the gut epithelium followed by proliferation/differentiation of mast cells. Mast cell precursor numbers increased even before the production of IL-3 and IL-4 in MLN and spleen, suggesting that some local factors might be involved in this phenomenon.

Key words: nematode, *Strongyloides venezuelensis*, mast cell, mast cell precursor, interleukin-3, interleukin-4.

INTRODUCTION

Infection with intestinal nematodes such as *Nippostrongylus brasiliensis* and *Trichinella spiralis* induces mucosal mast cell hyperplasia (mastocytosis) in the gut mucosa (Miller & Jarrett, 1971). Nematode-induced mastocytosis does not occur in nude athymic rats (Mayrhofer & Fisher, 1979; Arizono *et al.* 1990) or mice (Ruitenberg & Elgersma, 1976), and appears to be dependent on the T cell-derived mast cell growth/differentiation factors interleukin (IL)-3, IL-4, IL-10, and probably IL-9, which are produced at the time when intestinal nematode infection induces marked mastocytosis in rats and mice (Kitamura *et al.* 1993).

Mast cells are derived from haematopoietic precursors (Kitamura *et al.* 1993). Mast cell precursors are distributed not only in the bone marrow but also in various other tissues. Rodent intestinal mucosa and epithelium are particularly rich in these cells which have been suggested to give rise to local mast cell hyperplasia (Guy-Grand *et al.* 1984; Dillon & MacDonald, 1986; Parmentier *et al.* 1987; Kasugai *et al.* 1995). Although previous studies have shown that numbers of mast cell precursors increased after nematode infection, the timing of these increases varied according to the nematode species and the tissues examined.

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Strongyloides venezuelensis, a rodent intestinal nematode, has a life-cycle similar to that of *N. brasiliensis*, and has also been reported to induce significant intestinal mastocytosis (Horii, Khan & Nawa, 1993). In the present study, we investigated the temporal sequence of the appearance of mast cell precursors in various tissues in comparison with the timing of proliferation/differentiation of gut intraepithelial mast cells in *S. venezuelensis*-infected mice.

MATERIALS AND METHODS

Animals and S. venezuelensis *infection*

Female BALB/c mice, 8 weeks old, were purchased from Japan SLC Inc. (Hamamatsu, Japan). The strain of *S. venezuelensis* was kindly provided by Professor Nawa at Miyazaki Medical College. Mice were injected subcutaneously in the hip region with 2000 *S. venezuelensis* infective-stage larvae in 0·2 ml saline. The numbers of *S. venezuelensis* eggs per gram of faeces were counted on days 3, 7, 11, 14, 21 and 28 p.i.

Preparation of S. venezuelensis *antigen*

After stringent washing of infective-stage *S. venezuelensis* larvae with saline, the larvae (4×10^5/ml) were suspended in PBS, homogenized with a Polytron (Kinematica, Luzern, Switzerland), and further

sonicated for 30 sec using an ultrasonic disruptor (Tomy-Seiko Ltd, Tokyo, Japan). The homogenate was centrifuged at 12000 *g* for 20 min at 4 °C, the supernatant fraction was collected, filtered through 0·45 μm filters (Millipore Corp., Bedford, USA) and stored at −80 °C until use.

Staining of intestinal mast cells and estimation of mast cell numbers

Mice were killed by an overdose of ether, and a piece of the small intestine 5 cm from the pyloric ring was removed. The intestine was opened longitudinally, flattened on a filter paper and fixed in Carnoy's fluid for 3 h at room temperature or in a mixture of methanol-formalin-acetic acid (MFAA) for 24 h at 4 °C (Mayrhofer, 1980). Paraffin sections 5 μm thick were cut and stained with 0·2 % alcian blue dissolved in 0·5 M HCl for 30 min. The numbers of mast cells in the epithelium and lamina propria mucosa were counted in 20–50 villous crypt units (VCU) according to the method described by Miller & Jarrett (1971).

Immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in tissue sections

After staining with alcian blue, sections of the small intestine were incubated in 2 % hydrogen peroxide for 30 min and then with normal rabbit serum for 1 h. The slides were further incubated with mouse monoclonal antibody against PCNA (PC10; 1:50 dilution; Oncogene Science Inc., Uniondale, NY, USA) at 4 °C overnight. After washing, the slides were incubated with rabbit anti-mouse IgG (1:100 dilution; Wako Chemical Ltd, Osaka) for 1 h, followed by incubation with mouse peroxidase-anti-peroxidase complex (1:400 dilution; Jackson Lab. Inc., West Grove, USA) for 1 h. The slides were finally incubated in 0·05 M Tris buffer (pH 7·4) containing 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (Dojin Lab., Kumamoto, Japan) and 0·03 % hydrogen peroxide. $PCNA^+$ mast cells had brown-labelled nuclei and blue cytoplasm. The $PCNA^+$ mast cell rate was determined by counting a minimum of 50 mast cells.

Cell suspensions

Bone marrow cells were suspended in α-minimal essential medium (α-MEM; Flow Laboratories Inc., Irvine, UK) as previously described (Kasugai *et al.* 1995). Peyer's patches were excised with scissors before the gut was opened. Single cells from Peyer's patches, mesenteric lymph nodes (MLN) and the spleen were prepared as described previously (Uchikawa *et al.* 1994). Peripheral blood was collected by cardiac puncture using a syringe containing sufficient heparin to provide a final concentration of 10 U/ml, and peripheral blood mononuclear cells (PBMNC) were isolated by density-gradient centrifugation using lymphocyte separating medium (Lymph Prep; Nacalai Tesque, Kyoto, Japan).

Isolation of intraepithelial mononuclear cells (IEMNC) from the small intestine

IEMNC in the gut epithelium were isolated according to the previously described method (Davies & Parrott, 1981) with minor modifications. Briefly, the small intestine was obtained from mice and the MLN, connective tissues, and Peyer's patches were carefully removed and discarded. Faecal contents were removed by purging with cold Hanks' balanced salt solution (HBSS) containing 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamycin (Gibco BRL, Gaithersburg, USA). The small intestine was then opened longitudinally and cut into short segments (~ 1 cm). The tissues were then incubated with magnetic stirring at 37 °C for 15 min in HBSS containing 2 % foetal calf serum (FCS; ICN Biological Inc., Haemek, Israel) and 5 mM EDTA-Na_2 (pH 7·2), and transferred into 50 ml vol. centrifugation tubes (Nunc Inc., Naperville, USA), shaken vigorously for 15 sec, and the isolated cells were recovered. The harvested cells were resuspended in HBSS containing 5 % FCS, and then passed through a column loosely packed with glass wool (0·2 g packed into a 10 ml syringe) to remove dead cells and tissue debris. Discontinuous density gradients were prepared using Percoll (Pharmacia LKB, Uppsala, Sweden) by layering from the bottom 80 % and then 40 % Percoll. Cell suspensions mixed with 40 % Percoll were layered onto the gradient and centrifuged at 600 *g* for 20 min at room temperature. The cells in the interface between the 80 and 40 % layers were then collected and washed in HBSS. Approximately 0·5–$1·0 \times 10^6$ cells/mouse with > 95 % cell viability were consistently obtained by this procedure. Tissue sections of the small intestine after this treatment showed that villous epithelial cells were removed while lamina propria mucosa remained intact. Further, plasma cells which are abundant in propria mucosa did not appear among the isolated cells. These observations indicated that few, if any, lamina propria cells were isolated along the IEMNC.

Spleen cell conditioned medium

Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM–SCM) was prepared as described by Nakahata *et al.* (1982) with minor modifications. Spleen cells obtained from uninfected mice were cultured (2×10^6/ml) for 5 days at 37 °C in a humidified 5 % CO_2 atmosphere in α-MEM containing PWM (1:300 dilution; Gibco BRL), 10 % FCS and 10^{-4} M 2-mercaptoethanol (Sigma

Chemical Co., St Louis, USA). The medium was centrifuged, filtered through 0·45 μm filters, and stored at −80 °C until use. The concentrations of IL-3, IL-4 and IL-10 in PWM-SCM were measured by ELISA before use.

Clonal cell culture

Methylcellulose culture was carried out according to the method described by Nakahata *et al.* (1982) with minor modifications. After washing with PBS, 5×10^4 IEMNC or bone marrow cells, or 1×10^6 cells from other tissues were mixed with 1 ml of a mixture containing α-MEM, 0·9% (w/v) methylcellulose (Nacalai Tesque), 10% (v/v) FCS, 10^{-4} M 2-mercaptoethanol, and 10% (v/v) PWM–SCM, and were plated in 35 mm dishes (Becton Dickinson Labware, Lincoln Park, USA). Dishes were incubated at 37 °C in a humidified 5% CO_2 atmosphere. On days 16–18, colonies containing > 50 cells were counted under an inverted microscope.

Staining of cultured cells

Individual colonies were lifted from the methylcellulose medium using 10 μl Eppendorf pipettes under direct microscopical visualization. The cells were spun in a cytocentrifuge (Auto-Smear; Sakura Seiki Inc., Tokyo, Japan) at 800 rpm for 5 min, and the slides were stained with May-Gründwald-Giemsa solution.

Measurement of IL-3, IL-4, IL-5 and IL-10

Spleen, MLN, and Peyer's patch cells (1×10^6/ml) were cultured in α-MEM containing 10% FCS, 10^{-4} M 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin with or without PWM (1:300) or 10 μg/ml of nematode antigen at 37 °C in a 5% CO_2 atmosphere for 72 h. Supernatants were collected by centrifugation (2000 ***g*** for 15 min) and stored at −80 °C until cytokine measurement. Levels of IL-3, IL-4, IL-5 and IL-10 in the culture supernatant were determined using ELISA kits (Endogen Inc., Cambridge, USA) following the manufacturer's instructions.

Statistical analysis

Student's *t*-test was used to determine the significance of differences. *P* values less than 0·05 were considered to be significant.

RESULTS

Increase of intraepithelial mast cell number in the small intestine after S. venezuelensis *infection*

After *S. venezuelensis* infection, the number of eggs in faeces was counted. The number of eggs reached a peak on day 7, decreased on day 11, and had totally disappeared from the faeces from day 14 (data not shown).

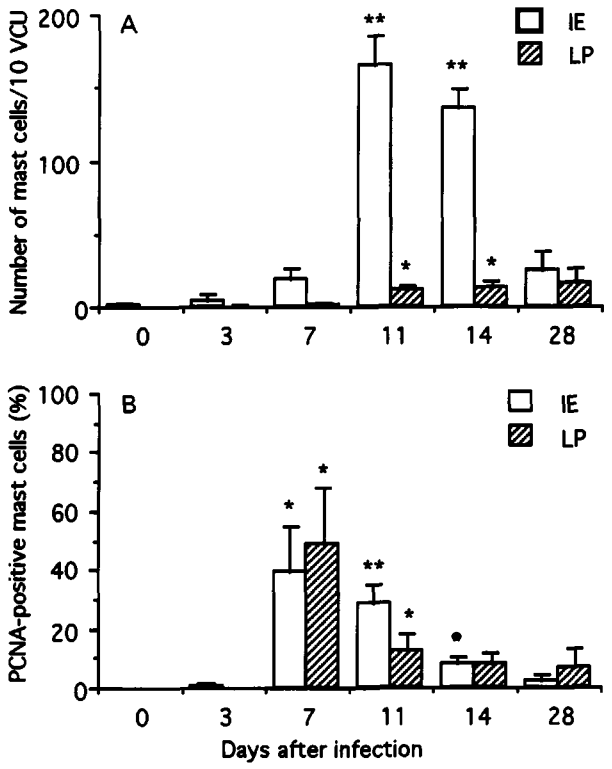

Fig. 1. Numbers of mast cells (A) and the rate of PCNA-positive mast cells (B) in the small intestine of mice after *Strongyloides venezuelensis* infection. IE: intraepithelial mast cells. LP: lamina propria mast cells. Columns and bars represent means ± S.E.M.
*, ** Significantly different from the corresponding value for day 0 (* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$).

Numbers of mast cells in the epithelium and lamina propria mucosa of the small intestine were examined before and after infection. Intraepithelial mast cell numbers began to increase on day 7 and peaked between days 11 and 14 p.i. (Fig. 1A). Mast cells were rarely found in the lamina propria mucosa of uninfected mice. Nematode infection also induced mast cells in the lamina propria mucosa, but in significantly smaller numbers than intraepithelial mast cells. By day 28, the number of intraepithelial mast cells had decreased to 1/7 of the peak level (Fig. 1A).

PCNA expression was examined in intraepithelial and lamina propria mast cells. The ratio of PCNA-positive mast cells was significantly increased on days 7 and 11 in both the epithelium and lamina propria mucosa (Fig. 1B). The peak increase of PCNA expression occurred several days earlier than that of intraepithelial mast cell numbers.

Increases of mast cell precursors in various tissues

Mast cell precursors that form mast cell colonies in methylcellulose culture containing PWM–SCM

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Table 1. Number of mast cell and other (granulocyte and/or macrophage) colonies in various tissues of uninfected mice in methylcellulose culture with PWM–SCM

(Data are means ± S.E.M. of 4 dishes)

	Number of colonies per 10^6 MNC		
Origin of cells	Mast	Others	Total
Bone marrow	180 ± 38 (40 ± 7)*	268 ± 28 (60 ± 8)*	448 ± 23 (100)*
Blood	24 ± 3 (76 ± 6)	7 ± 2 (24 ± 6)	31 ± 2 (100)
Spleen	2 ± 2 (6 ± 6)	21 ± 3 (94 ± 6)	23 ± 5 (100)
IEMNC	830 ± 43 (100)	0 (0)	830 ± 43 (100)
Payer's patch	35 ± 3 (100)	0 (0)	35 ± 3 (100)
MLN	11 ± 1 (100)	0 (0)	11 ± 1 (100)

* Percentage in total colonies.

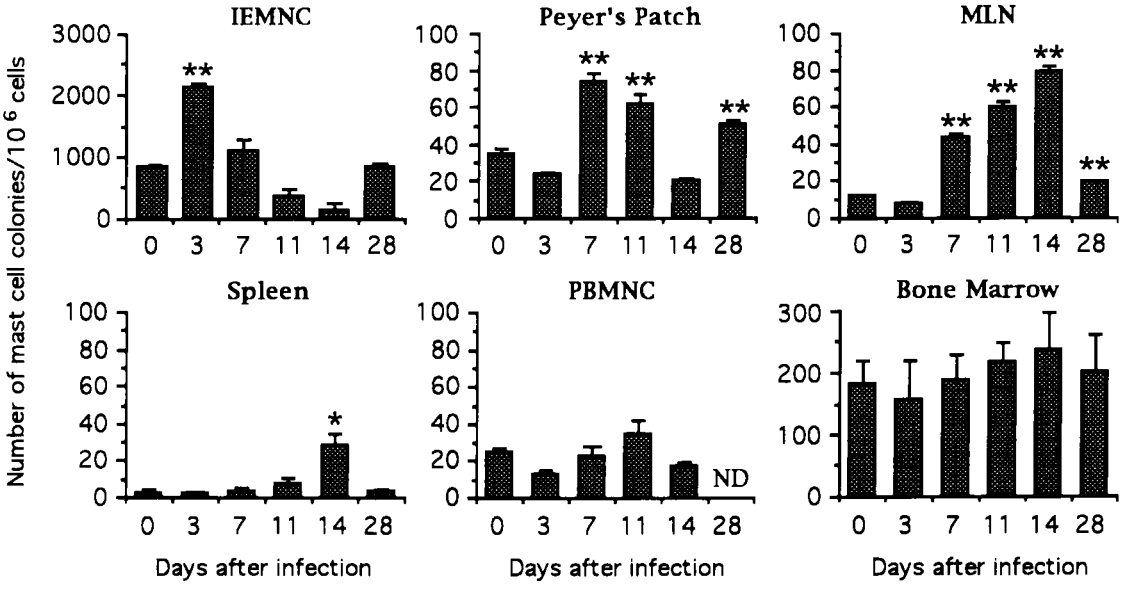

Fig. 2. Numbers of mast cell colonies derived from various tissues before and after *Strongyloides venezuelensis* infection. Isolated cells were cultured in methylcellulose containing PWM–SCM for 16–18 days. Columns and bars represent means ± S.E.M. MLN: mesenteric lymph node. ND: not determined. *, ** Significantly different from the corresponding values for day 0 (* $P < 0.05$, ** $P < 0.01$).

were examined in IEMNC, Peyer's patches, MLN, spleen, bone marrow and PBMNC. After 16–18 days of culture, colonies were picked and spun down on glass slides, and then stained with May–Grünwald–Giemsa solution. Colonies derived from bone marrow cells, PBMNC, or spleen cells included not only mast cell colonies but also granulocyte and/or macrophage colonies (Table 1). In contrast, when IEMNC, Peyer's patch cells and MLN cells were cultured, only mast cell colonies developed regardless of the time after infection.

The numbers of mast cell colonies in IEMNC increased as early as 3 days p.i. and returned to pre-infection level by day 7 (Fig. 2). The numbers of mast cell colonies also increased in Peyer's patches, MLN and spleen cells from day 7, several days after the increase of the IEMNC-derived colonies. In PBMNC and bone marrow cells, the numbers of mast cell colonies did not change significantly. In uninfected mice, a significant number of mast cell colonies developed from IEMNC; 4-fold that of bone marrow-derived mast cell colonies and > 20-fold those of MLN cells, Peyer's patch cells, spleen cells and PBMNC (Table 1).

Cytokine production by lymphoid organs

IL-3, IL-4, IL-5, and IL-10 production was examined in culture supernatants of MLN cells, spleen cells, and Peyer's patch cells stimulated with PWM or with nematode antigen for 72 h. MLN and spleen cells produced significant amounts of IL-3 and IL-4 from 7 to 11 days p.i. (Fig. 3). IL-5 production also increased in accordance with IL-3 and IL-4 production in the MLN and spleen cells (data not shown). A low level of IL-10 was detectable only in MLN cell cultures stimulated with PWM from 7 to 11 days p.i. (Fig. 3). Peyer's patch cells did not produce detectable levels of the cytokines examined (data not shown).

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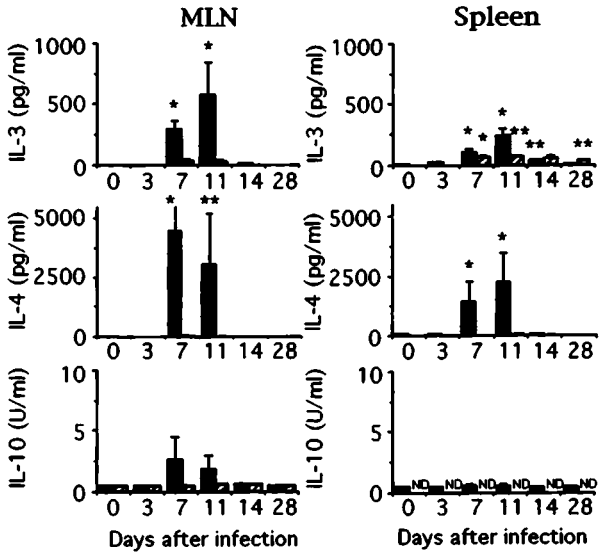

Fig. 3. Production of IL-3, IL-4 and IL-10 by mesenteric lymph node and spleen cells after *Strongyloides venezuelensis* infection. Mesenteric lymph node (MLN) and spleen cells (1×10^6 cells/ml) were cultured with PWM (■) or with *S. venezuelensis* antigen (▨) for 72 h, and the cytokine levels in the supernatant were assayed by ELISA. Columns and bars represent means ± S.E.M. ND: not determined. *, ** Significantly different from the corresponding values for day 0 (* $P < 0{\cdot}05$, ** $P < 0{\cdot}01$).

The concentrations of IL-3, IL-4 and IL-10 in PWM–SCM used for methylcellulose culture were 640 pg/ml, 130 pg/ml and 2·7 U/ml, respectively, which were significantly higher than the levels at day 0 as shown in Fig. 3, probably due to higher cell concentration and longer incubation period of spleen cell cultures to obtain large amounts of PWM–SCM.

DISCUSSION

In the normal mouse small intestine, mast cells reside mainly in the villous and cryptic epithelium, while they are only rarely found in the lamina propria mucosa. In studies using limiting dilution assays, significant numbers of mast cell precursors were found in the gut epithelium and lamina propria mucosa of mice in numbers equivalent to or more than those in the bone marrow (Crapper & Schrader, 1983; Guy-Grand *et al.* 1984; Dillon & MacDonald, 1986; Schrader, 1986). The present results obtained by *in vitro* colony-forming assay confirmed that isolated IEMNC contained significant numbers of mast cell precursors: > 4-fold higher than in the bone marrow, and > 20-fold higher than in PBMNC, spleen, MLN, and Peyer's patches. The number of mast cell precursors in the gut epithelium increased by approximately 2·5-fold as early as day 3 p.i. This result confirmed a previous finding that the frequency of intraepithelial mast cell precursors increased significantly early after *Trichinella spiralis* infection in mice (Dillon & MacDonald, 1986). The present study further showed that this increase in the number of intraepithelial mast cell precursors occurred several days earlier than the increase of the rate of PCNA expression in mast cell nuclei which peaked on day 7. PCNA is synthesized in proliferating cell nuclei mainly in S phase of the cell cycle (Bravo & Macdonald-Bravo, 1985), indicating that the mast cells were replicating rapidly during the period of high PCNA expression. All these findings indicate that gut mastocytosis begins with an early increase in mast cell precursor number in the epithelium which is followed by differentiation/proliferation of mast cells. Some studies have shown that mast cell precursor number increased in gut mucosa 9–10 days or 2 weeks p.i. (Guy-Grand *et al.* 1984; Parmentier *et al.* 1987). However, in these studies, the analysed cell population contained large numbers of lamina propria cells rather than IEMNC.

Mast cell precursors also increased in number in the MLN, Peyer's patches and spleen 7–14 days p.i., when the number of intraepithelial mast cells had increased, suggesting that the increase in mast cell precursors in those sites has no direct relationship with gut intraepithelial mastocytosis. Inconsistent results have been obtained regarding the frequency of bone marrow mast cell precursors after nematode infection. Haig, Jarrett & Tas (1984) reported that *N. brasiliensis* infection caused an increase in the frequency of mast cell precursors in rat bone marrow, while other reports and the present results showed that the frequency did not change significantly (Guy-Grand *et al.* 1984; Dillon & MacDonald, 1986; Parmentier *et al.* 1987; Kasugai *et al.* 1995). The reasons for these discrepancies are not clear, but it is possible that the response of mast cell precursors in the bone marrow might show strain-dependent variations as reported by Reed *et al.* (1988). In rats infected with the nematode *N. brasiliensis*, numbers of mast cell precursors in PBMNC were significantly decreased at the time when the number of mast cell precursors was increased in the gut mucosa, suggesting that the peripheral blood mast cell precursors might have directly invaded into the gut mucosa (Kasugai *et al.* 1995). In the present study in mice, the numbers of mast cell precursors in PBMNC were decreased slightly in accordance with the increases in intraepithelial mast cell precursors, although this relationship was not statistically significant.

It has been reported that nematode-induced mastocytosis is dependent on various T cell factors such as IL-3, IL-4, IL-9 and IL-10 (Filho *et al.* 1983; Guy-Grand *et al.* 1984; Hültner *et al.* 1990; Ashman *et al.* 1991; Thompson-Snipes *et al.* 1991), and that injection of antibodies against IL-3 and IL-4 significantly suppressed the occurrence of intestinal mastocytosis in *N. brasiliensis*-infected mice (Madden *et al.* 1991). In the present study, the levels of IL-3 and IL-4 were markedly increased in cultures of cells from MLN and the spleen on days 7–11 p.i.,

when the rate of PCNA expression in mast cells was increased. Parmentier *et al.* (1987) reported that the increase in frequency of mast cell precursors in gut lamina propria was also T cell-dependent, since the frequencies of mast cell precursors did not increase in nematode-infected nude athymic mice. However, the early increase in number of intraepithelial mast cell precursors observed in the present study occurred earlier than the production of IL-3 and IL-4 in MLN and spleen, suggesting that some local factors might be involved in triggering the increase in number of intraepithelial mast cell precursors. For instance, it is possible that some nematode-derived molecules stimulate intraepithelial lymphocytes to produce cytokines that would play a critical role in the increase in mast cell precursor number in the epithelium. On the other hand, it is also possible that T cell-independent mechanisms might be involved in the early increase of precursors. In fact, it was suggested that antigenically stimulated gut T cells are not necessarily required for the homing of mast cell precursors (Guy-Grand *et al.* 1984) and that certain nematodes induce T cell-independent increases in IL-3 gene expression very early after infection (Svetic *et al.* 1993). Further, stem cell factor (SCF) induces the development of mast cells both *in vivo* and *in vitro* (Galli, Geissler & Zsebo, 1994; Kitamura *et al.* 1995; Lantz & Huff, 1995). SCF also promotes chemotaxis of mast cells which is potentiated by costimulation with IL-3 (Meininger *et al.* 1992). Newlands *et al.* (1995) reported that injection of anti-SCF antibody into *N. brasiliensis*-infected rats resulted in marked decreases in number of jejunal mast cells, suggesting that SCF makes an important contribution to gut mastocytosis. In mast cell-deficient *Ws/Ws* rats which have a double gene dose of mutant alleles at the *c-kit* locus, *N. brasilienses* infection induced mastocytosis at a significantly lower level than in +/+ animals (Arizono *et al.* 1993). Thus, SCF may also be relevant to the increase of mast cell precursors in the epithelium.

Taken together, the present results suggest that murine intestinal mastocytosis is initiated by an increase in mast cell precursor number in the epithelium followed by differentiation/proliferation of mast cells. Factors that trigger this early increase of mast cell precursors are still to be elucidated.

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