Infection of *Nippostrongylus brasiliensis* Induces Development of Mucosal-Type But Not Connective Tissue-Type Mast Cells in Genetically Mast Cell-Deficient *Ws/Ws* Rats

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*Ws/Ws* rats have a small deletion at the tyrosine kinase domain of the *c-kit* gene and are deficient in both mucosal mast cells (MMC) and connective tissue-type mast cells (CTMC). The role of the *c-kit* receptor in the development of MMC and CTMC was investigated by infecting *Ws/Ws* and control +/+ rats with *Nippostrongylus brasiliensis* (*NB*), which induces T-cell-dependent mast cell proliferation. Although mast cells did not develop in the skin of *Ws/Ws* rats, a significant number of mast cells developed in the jejunum after NB infection. These mast cells had the MMC protease phenotype (rat mast cell protease [RMCP] 1+/1+1/2 and lacked heparin because they were not stained with berberine sulfate. Globule leukocytes were also detected in the mucosal epithelium of these rats. However, the number of MMC and the serum concentration of RMCP II in NB-infected *Ws/Ws* rats were only 13% and 7% of those of *NB*-infected +/+ rats, respectively. A small number of mast cells also developed in the lung, liver, and mesenteric lymph nodes of *Ws/Ws* rats after NB infection. Although mast cells in these tissues had the MMC phenotype throughout the observation period, the increased mast cells in the lung and liver of +/+ rats acquired a CTMC-like phenotype and were RMCP 1+/1+1/2, berberine sulfate 1+, and formalin resistant. These results indicate that the need for the stimulus through the *c-kit* receptor appears to be greater in the development of CTMC in the skin as well as for CTMC-like mast cells in the lung and liver than for the development of MMC.

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There are two induction mechanisms for the differentiation/proliferation of mast cells. One is dependent on T-cell-derived cytokines and the other is dependent on a fibroblast-derived growth factor. The T-cell-derived cytokines, interleukin-3 (IL-3), IL-4, IL-10, and probably IL-9, are produced at the time when intestinal helminth infection induces a remarkable increase of mast cells in the intestinal mucosa of rats 1 and mice. 1.6. This type of mast cell reaction does not occur in nude athymic rats 7,8 and mice, 9 but does occur in nude athymic mice injected with IL-3. 10 Despite the absence of an intestinal mastocytosis, nude athymic mice and rats have normal numbers of mast cells in the skin and a normal number of mast cells in the intestinal mucosa. 11,12 Thus, the T-cell–dependent mechanism appears to be essential for the inductive, but not the constitutive, differentiation/proliferation of mast cells. 13

The fibroblast-dependent mast cell differentiation/proliferation is mediated by a growth factor encoded by the *SI* locus of the mouse, 15,16 hereafter referred to as the *SI* factor. The receptor for the *SI* factor is encoded by the *W* locus of the mouse; the *W* locus is identical to the *c-kit* proto-oncogene and encodes a receptor tyrosine kinase. 17,20 Hereafter, we shall call it the *c-kit* receptor. A double gene dose of mutant alleles at either the *W* (*c-kit*) or *SI* locus results in the deletion of both connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC). 21 Although intact *W/Wv* mice lack CTMC and MMC, a small number of MMC did develop in the small intestine of *W/Wv* mice after infection with *Trichinella spiralis*. 22 CTMC also developed in the skin of *W/Wv* mice in association with a chronic idiopathic dermatitis or in response to repeated epicutaneous application of phorbol 12-myristate-13-acetate (PMA). 23,24 Moreover, perfusion with recombinant IL-3 resulted in the development of both CTMC and MMC in *W/Wv* mice. 25 These results indicate that signal transduction through the *c-kit* receptor may not be indispensable for development of mast cells.

Recently, we found a rat mutant with a 12-base deletion in the tyrosine kinase domain of the *c-kit* cDNA. 26,27 The mutant allele was designated as *W* (white spotting); the homozygous *Ws/Ws* rats are deficient in both CTMC and MMC. CTMC and MMC are more clearly characterized in rats than in mice. Rat CTMC are stained with safranine and berberine sulfate, contain mast cell protease (RMCP) I, and the granules are preserved by formalin fixation. On the other hand, rat MMC are not stained with safranine or berberine sulfate, contain RMCP II, and the granules are not shown after formalin fixation. 28,31

When compared with the topical application of PMA or the perfusion of recombinant IL-3, infection by helminths appears to represent a more physiologic condition that induces differentiation/proliferation of mast cells. Because the infection of rats with *Nippostrongylus brasiliensis* (*NB*) induces the most prominent intestinal mast-cell proliferation among helminth infections of rats and mice, we examined the response of *Ws/Ws* rats to *NB* infection to clarify the role of the *c-kit* receptor in the development of MMC and CTMC.
MATERIALS AND METHODS

Animals and NB infection. Ws/Ws and +/+ rats were produced by mating male and female Ws/+ rats, which show a diluted coat color and a large white spot on the abdomen.8 Ws/Ws and +/+ rats of 10 to 12 weeks of age received a subcutaneous injection of 2,000 infective-stage larvae of NB. After the infection, the number of NB eggs per gram of feces was counted daily for 2 weeks.

Tissue preparation. Rats were killed by overinhalation of ether 1, 15, 22, 30, and 56 days after the infection. Tissues were removed and fixed in Carnoy’s fluid. To examine formalin sensitivity, some tissues were also fixed in buffered 3.7% formalin (pH 7.3). Paraffin sections were cut and stained with alcin blue, pH 0.5, or with berberine sulfate at pH 4.0. Specimens for electron microscopy were fixed and processed as described previously.25

Mast cell counting. Mast cell counts were performed on 5-μm, alcin blue-stained sections. The number of mast cells in the villosus crypt unit (VCU) of jejunal mucosa was determined according to the method described by Miller and Jarrett.3 The number of mast cells per square millimeter in the liver and lung was estimated as described previously.28

Labeling of mast cells in S phase. Rats received an intraperitoneal (IP) injection of 5-bromo-2’-deoxyuridine (BrdU; Sigma Chemical Co, St Louis, MO) at a dose of 100 mg/kg 1 hour before being killed. Tissues were processed as described above and 5-μm sections were cut. Labeled nuclei of mast cells were stained using anti-BrdU monoclonal antibody (MoAb) as described previously.3 The proportion of labeled mast cells was estimated by counting more than 1,000 mast cells.

Measurements and staining of RMCP. Blood samples were obtained from the retro-orbital sinus and centrifuged. Sera were then stored at −80°C. The level of RMCP II in the serum was measured by enzyme-linked immunosorbent assay (ELISA) using an anti-RMCP II mouse MoAb and horseradish peroxidase-conjugated sheep F(ab’)2 anti-RMCP II as described by Huntley et al.31

A paired immunofluorescence study of RMCP I and II in mast cells was performed as described by Huntley et al.4 In brief, tissues were fixed with 4% paraformaldehyde for 6 hours and then with 70% alcohol for 24 hours. Paraffin sections were cut and incubated with 3-amino-9-ethyl-carbazole and then with 10% albumin in phosphate-buffered saline (PBS). Sections were then successively incubated with rabbit F(ab’)2 anti-RMCP I, sheep antirabbit IgG-tetramethylrhodamine isothiocyanate, anti-RMCP II mouse MoAb, and sheep antimouse IgG fluorescein isothiocyanate. After washing, slides were mounted in Citifluor AFI mount (Citifluor Ltd, London, UK) and viewed under an epifluorescent microscope (Olympus, Tokyo, Japan) sequentially excited with blue and green light.

Statistics. The Mann Whitney U-test (two-tailed) was used to evaluate the significance of differences.

RESULTS

Ws/Ws and control +/+ rats were infected with NB and the number of NB eggs in feces were counted. The numbers of eggs reached a peak on day 7 or 8 in both groups of rats. In +/+ rats, the egg output on day 8 was 133,300/g of feces, which was 3.5-fold greater than the value (38,100) found in Ws/Ws rats (P < .01). In both Ws/Ws and +/+ rats, the number of eggs declined rapidly thereafter, and reached a low level on day 11, with no significant differences between both groups of rats. Some rats were killed on day 15, and only a few worms were found in the small intestines of both Ws/Ws and +/+ rats.

At various periods after the NB infection, Ws/Ws and +/+ rats were killed and the numbers of mast cells in the lamina propria and the epithelium of the jejunum were counted. For this purpose, the jejunum was fixed with Carnoy’s fluid and stained with alcin blue. The number of mast cells in the lamina propria of +/+ rats declined on day 7 after NB infection, but markedly increased on day 15 (Fig 1A). The number reached a peak on day 22 and then gradually decreased. Although no mast cells were detected in the lamina propria of uninfected Ws/Ws rats, mast cells did develop on day 15 after infection with NB. The number of mast cells also reached a peak on day 22 in Ws/Ws rats, but the value was 13% that of +/+ rats (Fig 1A). After day 22, the number of mast cells gradually decreased in Ws/Ws rats, but small numbers of mast cells were detectable even 56 days after infection.

The proportion of mast cells in S phase of the cell cycle was evaluated by the incorporation of BrdU. Peak values in the proportion of mast cells in S phase were observed on day 15 after NB infection in both Ws/Ws and +/+ rats; no significant difference was observed between the two groups (Table 1). Thereafter, the proportion declined to the preinfection level.
Table 1. Rate of BrdU Labeling of Jejunal Mucosal Mast Cells in Ws/Ws and +/+ Rats After Infection With N brasiliensis

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<th>Days After Infection</th>
<th>BrdU Labeling of Mast Cells (%)</th>
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<tr>
<td></td>
<td>Ws/Ws</td>
</tr>
<tr>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>7</td>
<td>ND</td>
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<tr>
<td>15</td>
<td>6.6*</td>
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<tr>
<td>22</td>
<td>0.7</td>
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<td>30</td>
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Rats received an IP injection of BrdU 1 hour before being killed. Labeled nuclei of mast cells were immunohistochemically stained using anti-BrdU MoAb. Data shown are mean of 4 to 7 rats.

Abbreviation: ND, not determined because of the paucity of mast cells.

* Significantly different from the value for day 0 of +/+ rats (P < .05).

levels on day 22, when the number of mast cells reached the peak value, indicating the limited duration of mast cell proliferation.

Mast cells that invade into the epithelial layer of the intestine are designated globule leukocytes. These were not detectable in either +/+ or Ws/Ws rats before NB infection, but the number reached a peak on day 22 in both groups of rats (Fig 1B). The number of globule leukocytes rapidly decreased after day 22, and none was observed 30 days after infection.

Because Woodbury et al have shown that the amount of RMCP II in serum represents the levels of mast cell activation induced by the NB infection, we assayed RMCP II in Ws/Ws and +/+ rats on various days after infection using an ELISA. Although RMCP II was not detectable in the serum of noninfected Ws/Ws rats, a significant amount was present on day 15 (Table 2). However, the value observed in Ws/Ws rats was only 7% that of +/+ rats. In both rats, serum RMCP II was maintained at rather high levels even 56 days after the infection.

The fixation and staining properties of mast cells that developed in the lamina propria of the jejunum of Ws/Ws rats were investigated. These mast cells were stained with alcian blue but not with safranine or berberine sulfate (Fig 2), and were sensitive to formalin fixation. When binding of anti-RMCP I and II antibodies to these cells was examined, practically all mast cells were RMCP I/II+ (Fig 2). No significant differences between Ws/Ws and +/+ rats were observed in either the staining properties or formalin sensitivity of the intestinal mast cells, and these parameters were consistent with those reported for MMC. The ultrastructural features of the mast cells that developed in the jejunum of Ws/Ws rats were also consistent with those of MMC.

The numbers of mast cells in tissues other than the jejunum were examined in Ws/Ws and +/+ rats after NB infection. As already reported, practically no mast cells were detectable in any of the examined tissues of noninfected Ws/Ws rats, but 15 days after infection, small numbers of mast cells were detected in the mucosa of the stomach and colon, an area of mesentery adjacent to the intestine, the sinus of mesenteric...
lymph nodes, the portal area of the liver, and the peribronchiolar area of the lung. Mast cells did not develop in the skin of Ws/Ws rats throughout the observation period. The numbers of mast cells in the liver and lung are shown in Fig 3. The numbers were much smaller in Ws/Ws than in +/+ rats. Moreover, the fixation and staining properties of liver and lung mast cells differed between Ws/Ws and +/+ rats. Mast cells were not shown in the liver and lung of Ws/Ws rats after formalin fixation, and were not stained with berberine sulfate after Carnoy's fixation throughout the observation period. Mast cells containing RMCP I also did not develop in the liver and lung of Ws/Ws rats. Although most mast cells in the liver and lung of +/+ rats were not shown after formalin fixation at day 15 of NB infection, a significant number of formalin-resistant mast cells developed in the liver and lung of +/+ rats 8 weeks after NB infection (Fig 3). On day 15 of infection, most of the increased hepatic and pulmonary mast cells in +/+ rats were RMCP I+/II+ and berberine-. However, 8 weeks after infection, some were RMCP I+/II+, and others were RMCP I+/II+ (Fig 4). The proportion of RMCP I+/II+ mast cells to the total was comparable with the proportion of berberine sulfate-positive mast cells.

**DISCUSSION**

Mast cells and globule leukocytes developed in the jejunum of Ws/Ws rats after the NB infection. These mast cells were sensitive to formalin fixation, did not stain with berberine sulfate, and were RMCP I+/II+. These histochemical features are consistent with those of MMC.28-30 Because mast cell numbers did not increase in the jejunum of nude athymic rats after NB infection,7,8 our present results suggest that the mast cell proliferation in Ws/Ws rats is T-cell dependent. However, the number of mast cells in the jejunum of Ws/Ws rats was only 13% that of control +/+ rats. The concentration of RMCP II in the serum of Ws/Ws rats was 7% that of the control +/+ rats. The poor MMC response in Ws/Ws rats may be attributed to a defective function of the mutant c-kit receptor with a deletion of four amino acids. In fact, tyrosine kinase activity of the mutant c-kit protein was apparently defective (Tei et al, manuscript in preparation). Thus, the present results suggest a synergistic effect of the SI factor and T-cell-derived cytokines for the induction of mucosal mastocytosis. On the other hand, because stimulated mast cells reportedly produce IL-3 and IL-4,26,38 the lack of mast cells in noninfected Ws/Ws rats may also result in the low local concentration of these cytokines after NB infection. Thus, it is possible that both signals through the c-kit and IL-3/IL-4 receptors are reduced in NB-infected Ws/Ws rats.

Even though the absolute number of MMC developed in the jejunum of NB-infected Ws/Ws rats was significantly lower than that in the jejunum of NB-infected +/+ rats, a comparable proportion of mast cells in S phase was found in the jejunum of both Ws/Ws and +/+ rats. This suggests that MMC were as efficient to proliferate at least in response to T-cell-derived cytokines in Ws/Ws rats as in the +/+ counterpart. It has been reported that recombinant human stem cell factor (SCF; a ligand for c-kit receptor) in combination with other growth factors, such as IL-3, granulocyte-macrophage colony-stimulating factor, or granulocyte colony-stimulating factor, caused a highly immature population of CD34+lin- precursor cells to form an increased number of colony-forming cells in vitro, but only a few colonies were formed in the presence of either SCF alone or each of the growth factors.39 Furthermore, administration of SCF in baboons or mice induced an increased number of colony-forming cells both in the marrow and blood.40,41 Taken together,
Our results may implicate the importance of the SI factor and c-kit receptor as a signal favoring mainly the production of mast cell precursors rather than morphologically identifiable mast cells. We are now attempting to measure the concentration of mast cell colony-forming cells in the bone marrow, peripheral blood, and various other tissues in both Ws/Ws and control +/+ rats with or without the NB infection.

MMC have been reported to have a role in the rejection of certain parasites. When W/W<sup>+</sup> mice were infected with *Trichinella spiralis*<sup>42</sup> or *Strongyloides ratti*,<sup>43</sup> the worm rejection was prolonged compared with the infection in +/+ mice, but the prior reconstitution of W/W<sup>+</sup> mice by transplantation of +/+ bone marrow cells normalized the timing of rejection. In NB-infected W/W<sup>+</sup> mice, the rejection was prolonged as well, but the bone marrow transplantation did not normalize the rejection.<sup>44</sup> The present data of fecal egg count suggest not only that NB was rejected rapidly in Ws/Ws rats, but that the number of worms infested in Ws/Ws rats might have been fewer than the +/+ counterpart. This suggests that Ws/Ws rats may be more efficiently protected than the +/+ rats. Although MMC and globule leukocytes developed in Ws/Ws rats, the numbers were much smaller than in +/+ rats, and the concentration of RMCP II in the serum were much lower in Ws/Ws rats than in +/+ rats. Thus, our results indicate that MMC are not the major effector cells, at least in the rejection of NB. Several factors such as NK-cell function<sup>45</sup> and hypersecretion from goblet cells<sup>46</sup> were also reported to be involved in worm rejection. At present, we cannot point out the mechanism with which Ws/Ws rats rejected NB more efficiently than +/+ rats did.

The number of mast cells did not increase in the skin of either Ws/Ws or +/+ rats even after the NB infection. On the other hand, normal numbers of mast cells are present in the skin of T-cell–deficient nude athymic rats.<sup>12</sup> Therefore, the SI factor, but not T-cell–derived cytokines, appears to play a physiologically important role for the differentiation of skin mast cells, which display the typical CTMC phenotype. However, this does not imply that the SI factor alone can induce the development of CTMC. We have already shown that nerve growth factor (NGF) can induce development of CTMC-like cells from cultured mast cells, of which survival is supported by IL-3.<sup>47</sup> Ody et al<sup>43</sup> induced development of CTMC in the skin of W/W<sup>+</sup> mice by perfusion of recombinant IL-3. An explanation of the result of Ody et al is as follows. Invasion of mast-cell precursors and their differentiation to immature MMC-like cells is supported by a non-physiologically large amount of IL-3, and the acquisition of CTMC phenotype is induced by NGF produced by skin fibroblasts. On the other hand, production of IL-3 by spleen cells of nude athymic mice has been reported by Kimoto et al.<sup>48</sup> However, because the number of MMC did not significantly increase in the jejunum of NB-infected nude rats,<sup>7,8</sup> a small amount of IL-3 produced in nude rats does not seem to play an important role in the production of normal numbers of mast cells in the skin.

After NB infection, a small number of mast cells developed in various tissues of Ws/Ws rats, such as liver, lung, and mesenteric lymph nodes. These mast cells showed MMC phenotype and did not acquire CTMC-like features throughout the observation period. In the control +/+ rats, the numbers of mast cells in the lung, liver, and mesenteric lymph nodes increased after NB infection. Most of the newly recruited mast cells in the lung and liver were of the MMC phenotype on day 15, but some acquired a CTMC-like phenotype 8 weeks after the NB infection. The CTMC-like mast cells in +/+ rats were resistant to formalin fixation, and were berberine sulfate<sup>+</sup> and RMCP I<sup>+</sup>/II<sup>+</sup>. Because most skin mast cells are RMCP I<sup>+</sup>/II<sup>+</sup>, the phenotype of those that increased in the lung and liver of +/+ rats is not exactly the same as that of skin mast cells. These results suggest a phenotypic change from RMCP I<sup>+</sup>/II<sup>+</sup> to RMCP I<sup>+</sup>/II<sup>+</sup> mast cells. Because this change was observed only in +/+ rats, stimulation through the c-kit receptor appears to be necessary for the phenotypic change. In this respect, our results are consistent with those of Tsai et al.,<sup>49</sup> who reported that the injection of recombinant SI factor induces the development of CTMC-like mast cells.

Taken together, the present results suggest that the need for the stimulus through the c-kit receptor is greater in the development of CTMC in the skin as well as for CTMC-like cells in the lung and liver than for the development of MMC.
**MAST CELL-DEFICIENT Wb/Ws RATS**

*Wb/Ws* rats infected with *NB* may be a useful tool for studying the differentiation mechanisms of CTMC and MMC.

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Infection of *Nippostrongylus brasiliensis* induces development of mucosal-type but not connective tissue-type mast cells in genetically mast cell-deficient Ws/Ws rats

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