Infection of Nippostrongylus brasiliensis Induces Normal Increase of Basophils in Mast Cell-Deficient Ws/Ws Rats With a Small Deletion at the Kinase Domain of c-kit

By Tsutomu Kasugai, Manabu Okada, Masahiro Morimoto, Naoki Arizono, Kazutaka Maeyama, Minoru Yamada, Hideki Tei, Kayoko Doimae, Hitoshi Onoue, George F.J. Newlands, Takehiko Watanabe, Yoshitake Nishimune, Hugh R.P. Miller, and Yukihiko Kitamura

All basophils, mucosal-type mast cells (MMC) and connective tissue-type mast cells (CTMC) are derived from the multipotent hematopoietic stem cell. Mutations at the c-kit locus result in deficiency of MMC and CTMC in both mice and rats. To investigate the role of the c-kit receptor tyrosine kinase for production of basophils, we used white spotting/white spotting (Ws/Ws) mutant rats that have a small deletion at the tyrosine kinase domain of the c-kit gene. When Ws/Ws, nude athymic, and normal (+/+ ) rats were infected with Nippostrongylus brasiliensis (NB), the number of basophils increased greater than 50-fold in the peripheral blood of Ws/Ws and +/+ rats but did not increase in that of nude rats. Blood histamine concentration increased significantly in Ws/Ws and +/+ rats but did not increase in nude rats. Immature basophils increased greater than 10-fold in the bone marrow of Ws/Ws and +/+ rats but did not increase in that of nude rats. Mature and immature basophils that developed after the NB infection were identified by electron microscopy. The present result confirms that T-cell-derived cytokines are indispensable for the augmented production of basophils and suggests that stimulation via the c-kit receptor may not be necessary for the augmented production.

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BOTH BASOPHILS and mast cells have prominent basophilic granules containing proteoglycan and histamine, and both of them have high-affinity IgE receptors on their surface.1-4 Both basophils and mast cells are involved in immediate hypersensitivity reaction through IgE molecules bound to the receptors.5-7 Basophils and mast cells are derived from the multipotent hematopoietic stem cell.6,11 However, the differentiation process of mast cells and that of basophils are quite different. Precursors of mast cells leave the bone marrow (BM), migrate in peripheral blood (PB), invade into connective or mucosal tissue and then differentiate into morphologically identifiable mast cells.7,10,12 Mast cells retain an appreciable proliferative potential after the morphological differentiation,12,14 and even degranulated mast cells can proliferate and regranulate.15,16 On the other hand, basophils complete differentiation within the BM.6,10,11 Mature basophils leave the BM, migrate in PB and may invade into connective or mucosal tissue, especially during some allergic inflammations.6,10,11-12 However, the proliferation of mature basophils within tissues is not considered to occur.23 Differentiation of mast cells of mice and rats is regulated by T-cell-dependent and fibroblast-dependent mechanisms.12 The T-cell-dependent mechanism is mediated by interleukin-3 (IL-3), IL-4, IL-9 and IL-10,12,24-27 whereas the fibroblast-dependent mechanism is mediated by the growth factor encoded by the mouse SI locus (hereafter called SI factor).28-31 The receptor of the SI factor is encoded by the W locus of mice; the W locus is identical with the c-kit proto-oncogene that encodes a receptor tyrosine kinase (hereafter called c-kit receptor).32,33 Homozygous or double heterozygous mutant mice at either W or SI locus are deficient in mast cells,8,12,34 indicating the essential role of the SI factor and the c-kit receptor for differentiation of mast cells.

Although mice of W/W” genotype are deficient in mast cells,8 the number of basophils in the PB of W/W” mice has been reported to be comparable with that of normal control (+/+ ) mice.35 Basophils of W/W” mice may infiltrate into the skin infested with Dermacentor variabilis ticks.22 However, mice do not appear to be suitable for quantitative examination of blood basophils because of their small blood volume and low proportion of basophils.35 We recently found a rat mutant with a 12-base deletion in the tyrosine kinase domain of the c-kit gene.36,37 Because the heterozygous rat has deformed areas on the forehead and abdomen, the mutant allele was designated as Ws (white spotting). Homozygous Ws/Ws rats are black-eyed, white-coated, and deficient in mast cells.36 Because sequential sampling of PB is easier in rats than in mice, we examined whether the c-kit receptor played an important role for development of basophils by using Ws/Ws rats. A greater than 50-fold increase of basophils was observed in the PB of Ws/Ws rats infected with a helminth, Nippostrongylus brasiliensis (NB), and the magnitude of the increase in Ws/Ws rats was comparable with that of +/+ rats.

MATERIALS AND METHODS

Rats and helminth infection. The origin of, and the breeding procedure for Ws/Ws rats have been described.36 Although the original heterozygous Ws/+ rat with coat color dilution and white spots was found in the Brown Norway (BN) inbred strain, homozygous Ws/Ws rats with white hair and black eyes could not be obtained in the

From the Department of Pathology, Medical School, and the Research Institute for Microbial Diseases, Osaka University; the Department of Medical Zoology, Kyoto Prefectural University of Medicine; the Department of Pharmacology, Tohoku University, School of Medicine, Japan; the Moredun Research Institute, Edinburgh; and the Department of Veterinary Clinical Studies, University of Edinburgh, Edinburgh, UK.

Submitted September 4, 1992; accepted December 16, 1992.

Supported by grants from the Ministry of Education, Science and Culture and the Ministry of Health and Welfare, the Cosmetology Research Foundation, and the Hokuriku Seiyaku Allergy Award.

Address reprint requests to Yukihiko Kitamura, MD, Department of Pathology, Osaka University Medical School, Yamada-oka 2-2,Suita, Osaka, 565 Japan.

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Blood, Vol 81, No 10 (May 15), 1993: pp 2521-2529 2521
athymic rats were maintained in our laboratory as a closed colony. Bars show SE. Each point represents the mean of 8 to 14 rats; numbers of basophils and eosinophils directly under the microscope. For basophil counts, three solutions were prepared according to Harriet and Leonard with a slight modification: solution A, 0.1% EDTA-Na2 in saline; solution B, deionized water containing 0.076% cetylpyridinium chloride, 0.7% lanthanum chloride - 6H2O, 0.9% NaCl, 0.21% Tween 20 (Sigma Chemical Co, St Louis, MO), and 0.143% alcian blue 8 GX (Eastman Kodak, Rochester, NY), filtered through a piece of filter paper; and solution C, 1N HCl. Blood (0.1 mL) was diluted with 0.4 mL solution A, and 0.45 mL solution B was added to the diluted blood. After 1 minute of gentle agitation, 0.05 mL solution C was added, and the mixture was gently agitated again. Counts were performed with the Bürker-Türk hemocytometer. The number of basophils counted in ten chambers was multiplied by 1.11 × 105 to give the total number of basophils per liter of blood. For eosinophil counts, Hinkelmans solution was used. Hinkellman's solution consists of 0.5% yellow eosin, 0.5% phenol, and 0.5% formalin. Blood (0.1 mL) was diluted with 0.9 mL Hinkellmans solution. After 30 seconds of gentle agitation, counts were performed with the Bürker-Türk hemocytometer. The number of eosinophils per liter of blood was calculated as described above.

**Histamine contents.** To avoid the contamination of tissue fluid, blood sample was collected through the tail artery. Histamine was measured with a high performance liquid chromatography (HPLC) coupled with fluorometry as described by Yamamoto et al. Briefly, 100 μL of whole blood was deproteinized by addition of 200 μL of 4% perchloric acid containing 5 mmol/L EDTA-Na2. The mixture was centrifuged at 10,000g for 20 minutes at 4°C, and 50 μL of the supernatant was applied to the HPLC-fluorometry. Histamine contents were expressed as nmol/L.

**Purification of basophils.** The method described by Dvorak et al was used with a slight modification. Rats were anesthetized with ether, and blood was collected through the inferior vena cava using a syringe containing sufficient heparin to provide a final concentration of 10 U/mL. Carbonyl iron particles (Nacalai Tesque, Kyoto, Japan), 10 mg/mL, were added to the blood, and phagocytes were allowed to ingest these particles during an incubation of 30 minutes at 37°C. Erythrocytes were removed by a sedimentation step, in which 2 parts of freshly prepared 3% gelatin solution were added to 1 part of blood; the bulk of erythrocytes sedimented over the course of 45 minutes at room temperature. The leukocyte-rich supernatant was centrifuged at 150g for 10 minutes at room temperature and resuspended in 20 mL calcium, magnesium-free Hanks' balanced salt solution (CMF-HBSS). At this and subsequent steps it was essential to add fresh rat serum to the CMF-HBSS at a final concentration of 10%; in the absence of fresh rat serum, basophil granules stained poorly or not

**Counts of leukocytes.** Blood samples were obtained from the retroorbital sinus. Numbers of leukocytes were counted with a Bürker-Türk hemocytometer, and differentials were performed using blood smears stained with May-Grünewald-Giemsa. Numbers of neutrophils and lymphocytes were calculated from the leukocyte counts and differentials, but numbers of basophils and eosinophils, especially before the NB infection, were too low to obtain reliable values from the leukocyte counts and differentials.
Table 2. Histamine Content of Blood Before and After the NBI in +/+ , Ws/Ws, and Nude Rats

<table>
<thead>
<tr>
<th>Time of Examination</th>
<th>Rats</th>
<th>No. of Rats</th>
<th>Histamine Content* (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before NBI</td>
<td>+/+</td>
<td>10</td>
<td>288 ± 29</td>
</tr>
<tr>
<td></td>
<td>Ws/Ws</td>
<td>7</td>
<td>155 ± 14†</td>
</tr>
<tr>
<td></td>
<td>nude</td>
<td>8</td>
<td>219 ± 22†</td>
</tr>
<tr>
<td>2 weeks after</td>
<td>+/+</td>
<td>7</td>
<td>1,326 ± 220†</td>
</tr>
<tr>
<td>NBI</td>
<td>Ws/Ws</td>
<td>6</td>
<td>1,162 ± 190†</td>
</tr>
<tr>
<td></td>
<td>nude</td>
<td>7</td>
<td>166 ± 19†</td>
</tr>
</tbody>
</table>

* Mean ± SE.
† P < .01, when compared with the value of +/+ rats at the same day of infection by t-test.
‡ P < .01, when compared with the value observed in rats of the same genotype before the NBI by t-test.

at all, probably because of degranulation. Phagocytic cells containing carbonyl iron and remaining free iron particles were removed by the passage through a magnetic field. A stock solution of Percoll (Pharmacia, Piscataway, NJ) was prepared by mixing 90 mL Percoll with 8.96 mL CMF-HBSS (10×), 1 mL HEPES buffer, and 455 mL HCl (1 N). To obtain the desired density, this stock solution of Percoll was diluted with CMF-HBSS (1×) supplemented with 1% HEPES buffer and 10% fresh rat serum. The final density of Percoll was measured by PAAR Digital Density Meters (Anton Paar, Graz, Austria). The cell suspension (20 mL) was carefully layered over 30 mL Percoll (1.084 g/mL) in a 50-mL conical-bottomed plastic tube. The tube was centrifuged using a swing-out rotor. Centrifugation at 400g for 45 minutes at 20°C led to a clear band of mononuclear cells at the interface between plasma and Percoll, and a “buffy coat” of basophils and the other leukocytes immediately above the erythrocytes at the bottom. The cells in the “buffy coat” and the pellet were collected and washed twice with CMF-HBSS containing 10% fresh rat serum. Residual erythrocytes were lysed by exposure to 16 vols of 0.17 mol/L Tris-0.75% NH₄Cl solution, pH 7.2. Nearly all erythrocytes were lysed within 5 minutes and the resulting suspension was used for the next step. The basophil-enriched fraction was harvested by underlaying the suspension with 0.5 mL fresh rat serum using a 23-gauge needle attached to 1.0 mL tuberculin syringe; after centrifugation at 150g for 10 minutes at 4°C, basophils and contaminating leukocytes were pelleted, whereas the erythrocyte stroma remained at the serum interface as a fluffy white band. Basophil recovery was assessed by the direct count as described above.

Proportion of immature basophils in the BM. The BM of femurs was removed with forceps and fixed in Carnoy’s fluid. The paraffin sections (4 μm thick) were cut and stained with alcian blue (pH 0.5) and nuclear-fast red. By counting about 1,000 nucleated cells of the BM, the ratios of immature basophils and mast cells to the total nucleated cells were calculated.

Paired immunofluorescence study of rat mast cell proteases (RMCP) I and II. Paired immunofluorescence was performed according to Huntley et al. In brief, the BM was fixed in 4% paraformaldehyde for personal use only. on November 16, 2017. For personal use only.

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**Fig 2.** Ultrastructural features of a basophil (A), eosinophil (B), or neutrophil (C) leukocyte in the PB of a Ws/Ws rat. The basophil has a segmented nucleus with condensed chromatin. The cytoplasm of the basophil contains large and round or oval granules with homogeneous matrices. The surface architecture of the basophil is comparable with that of the eosinophil and the neutrophil. Original magnification × 9,000.
Fig 3. BM sections of +/+ (A, B) and Ws/Ws (C, D) rats 1 week after the NB infection. Sections were stained with alcian blue and nuclear-fast red (A, C, D). The cytoplasm of mast cells were stained diffusely (A) and that of immature basophils contains distinct granules (A, C, D). The cytoplasm of immature basophils stained faintly (arrowheads) and that of relatively mature basophils stained densely (arrows) (A, C, D). A, B, C: X 425. B is a section adjacent to A, and binding of RMCP I is shown by immunofluorescent staining. D is a higher magnification (X 1,000) of basophils that are shown by an arrow and arrowheads in C.

Electron microscopy. The basophil-enriched fraction was centrifuged and the cell pellet was fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde, 0.2% CaCl₂, and 0.1 mol/L cacodylate buffer, pH 7.4 for 2 hours at 4°C. After washing with 0.1 mol/L cacodylate buffer, the cell pellet was quickly mixed with warmed 2% agar, centrifuged, and placed in a 4°C ice bath for 30 minutes. The agar pellet was postfixed for 1 hour in 1% osmium tetroxide, and was immersed in 1% tannic acid for 30 minutes at 4°C. The agar pellet was washed three times in 1% Na₂SO₄ and 1% CH₃COONa at 4°C to remove phosphate, stained en bloc at 4°C overnight with 0.5% uranyl acetate, and embedded in Epon 812 (Nacalai Tesque).

For demonstration of endogenous peroxidase, the BM was fixed in a mixture of 2.5% glutaraldehyde, 2% paraformaldehyde, 0.2% CaCl₂, and 0.1 mol/L cacodylate buffer, pH 7.4 for 1 hour at 4°C.
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After washing with 0.05 mol/L Tris HCl buffer, pH 7.6, the sample was incubated for 1 hour at room temperature in Graham and Karnovsky's medium (0.05% 3-3'diaminobenzidine tetrachloride and 0.01% hydrogen peroxide in 0.05 mol/L Tris HCl buffer, pH 7.6).44 The sample was then washed three times in 1% osmium tetroxide for 1 hour, and embedded in Epon 812. Ultrathin sections were cut, stained lightly with lead citrate, and observed with the JEM-100CX II electron microscope (JEOL Ltd, Kyoto, Japan).

RESULTS

Increase of basophils in PB of Ws/Ws rats. Ws/Ws and control +/+ rats were infected with NB, and blood samples were obtained from the retroorbital sinus at various days after the infection. Numbers of neutrophils and lymphocytes were calculated from the leukocyte counts and differentials, but only <0.03% basophils and <2.7% eosinophils were present in blood smears of noninfected rats. Therefore, we counted basophils and eosinophils directly under the microscope after specific staining. Numbers of basophils, eosinophils, neutrophils, and lymphocytes increased significantly after infection in both Ws/Ws and +/+ rats, and no significant differences were detectable between Ws/Ws and +/+ rats (Fig 1). The magnitude of the increase was the largest in basophils. Because the most remarkable increase in basophils and eosinophils was observed 2 weeks after infection, we counted the number of basophils, eosinophils, neutrophils, and lymphocytes in blood of nude rats before and 2 weeks after the NB infection.

In contrast to Ws/Ws and +/+ rats, no significant increase of basophils, eosinophils, neutrophils, and lymphocytes was observed in nude rats after infection (Table 1).

The histamine contents in blood of Ws/Ws, +/+, and nude rats were determined before and 2 weeks after infection. Before infection, the concentration of histamine in whole blood was significantly higher in +/+ rats than in Ws/Ws rats, implicating the presence of mast cell-derived histamine in blood of +/+ and nude rats. However, two weeks after infection blood histamine significantly increased to comparable levels in both Ws/Ws and +/+ rats. No such increase was observed in the blood of nude rats (Table 2).

Electron microscopical features of blood basophils. Electron microscopy is the most reliable method for identification of basophils.2,10,11,45,46 Because ultrastructural examination of basophils in blood of noninfected rats is difficult because of the low proportion of such cells, we obtained blood from Ws/Ws rats 2 weeks after the NB infection. Moreover, we augmented the proportion of basophils by removing the cells that phagocytized carbonyl iron and by the subsequent centrifugation in Percoll gradients. Samples of nucleated cells containing 1.9% to 3.0% basophils were obtained. In these specimens, basophils were detectable with ease and distinguishable from other granulocytes (Fig 2). Basophils had homogeneously electron-dense cytoplasmic granules; the granules had no crystalloid structures as observed in eosinophil granules. Their granules were larger and more uniform in shape than those of neutrophils; more round than those of eosinophils. Other features of basophils were shared to some extent with neutrophils and eosinophils. The plasma membrane had irregular, short, and thick processes. The nucleus was segmented into multiple lobes, with chromatin heavily condensed beneath the nuclear membrane. There was a small Golgi region, occasional free ribosomes, and strands of rough endoplasmic reticulum (RER). These ultrastructural features were consistent with those of mouse, guinea pig, and human basophils described by Dvorak et al.10,11,45,46

Although mucosal-type mast cells (MMC) did develop in the jejunum of Ws/Ws rats after the NB infection,41 cells with electron microscopical features of MMC were not detectable in blood. MMC were characterized by the presence of RMCP II, and connective tissue-type mast cells (CTMC) by RMCP I. Binding of anti-RMCP I and anti-RMCP II antibodies was examined using cytocentrifuge preparations of the basophil-enriched fraction obtained from the NB-infected Ws/Ws rats, but neither RMCP-I+ nor RMCP-II+ cells were detectable (data not shown).

Immature basophils in the BM. The BM sections of Ws/ Ws, +/+, and nude rats were stained with alician blue. Two types of basophilic cells were observed in +/+ and nude rats. The cytoplasm was stained diffusely in the first type and was distinctly granulated in the second (Fig 3A). Only the second type was observed in the BM of Ws/Ws rats (Fig 3, C and D). The diffusely stained cells in the BM of +/+ rats were RMCP-I-RMCP-II (Fig 3B). On the other hand, neither RMCP-I+ nor RMCP-II+ cells were detectable in the BM of Ws/Ws rats (data not shown). Therefore, we considered that cells of the first type were CTMC and that the distinctly granulated basophilic cells were immature basophils. The proportion of immature basophils to total nucleated cells was comparable among Ws/Ws, +/+ , and nude rats before infection (~1% of nucleated cells). After infection with NB, the proportion of immature basophils rapidly increased about 10-fold and reached a peak 1 week after infection in the BM.

![Graph showing proportion of immature basophils and mast cells in BM of Ws/Ws and +/+ rats at various days after NB infection](image-url)
of Ws/Ws and +/+ rats (Fig 4). In contrast, such an increase was not detectable in the BM of nude rats (data not shown). The proportion of CTMC to total nucleated cells did not increase after infection in the bone marrow of +/+ rats (Fig 4). The ultrastructural features of immature basophils were studied in the BM of Ws/Ws rats. One week after infection, most cells that contained basophil-specific large granules had a round or oval nucleus. The size of these immature granules was larger than that of mature basophils, and their cytoplasm was filled with moderately dilated strands of RER; we considered them to be basophilic myelocytes (Fig 5A). Two weeks after infection, the majority of immature basophils appeared to be more differentiated. The dispersed chromat in pattern of the nucleus became condensed and the nucleus was lobulated; we considered them to be basophilic metamyelocytes (Fig 5C). Although diluted RER cisternae were detectable, these structures appeared to decrease in size.

Because mast cells of rats are reported to be peroxidase- and basophilic myelocytes to be peroxidase-, we examined the peroxidase activity of immature basophils in the BM of Ws/Ws rats and that of CTMC in the BM of +/+ rats. In basophilic myelocytes, peroxidase was shown in the RER, perinuclear cisterna, and all cisternae of the Golgi complex (Fig 5B). All cytoplasmic granules showed a positive peroxidase reaction as well. Peroxidase synthesis apparently ceased later in maturation, and no reaction product was present in the RER or Golgi complex of basophilic metamyelocytes (Fig 5D). The peroxidase activity of granules varied from cell to cell, and among granules of the same cell. In most basophilic metamyelocytes, some granules were peroxidase- and others peroxidase- or very weakly peroxidase-. All CTMC examined in the BM of +/+ rats were peroxidase- (Figs 5E and 5F).

DISCUSSION

The number of basophils in PB increased greater than 50-fold in Ws/Ws and +/+ rats infected with NB, but no significant increase of basophils was observed in the PB of infected nude rats. Infection also induced a greater than 10-fold increase in the number of immature basophils in the BM of Ws/Ws rats and +/+ rats but not in the BM of nude rats. This increase in +/+ rats but not in nude rats is consistent with the previous result reported by Ogilvie et al. The cytokines produced by T cells such as IL-3 and IL-4 may play an essential role in the production of basophils in rats. On the other hand, the stimulus mediated by the SF factor and the c-kit receptor did not appear to be necessary for the augmented production of basophils. The potential importance of T-cell-derived cytokines for the production of basophils is consistent with the result of Mayer et al. that administration of human recombinant IL-3 induced dose-dependent and time-dependent increase of basophils in rhesus monkeys. IL-3 is also the principal factor that induces in vitro development of basophils from human BM.

The observation of Steeves and Allen that basophils infiltrate into the skin of W/W mice infected with D variabilis ticks is also consistent with the present result. The truncated c-kit protein produced by the W mutant allele does not participate in the formation of c-kit receptors because of the lack of the extracellular domain. Therefore, c-kit receptors composed of W c-kit proteins have weak but detectable tyrosine kinase activity, there is a possibility that the residual c-kit kinase activity may be necessary for the production of basophils in W/W mice. We have not yet determined whether low level of c-kit kinase activity is retained in Ws/Ws rats. If it is the case, such a low c-kit activity may play a role for the augmented production of basophils in Ws/Ws rats. We are now attempting to assess the activity of c-kit kinase in cultured mast cells derived from the BM of Ws/Ws rats. On the other hand, Ws/Ws rats appeared to be more suitable than W/W mice for the quantitative evaluation of basophil production, because repeated sampling of PB was much easier in Ws/Ws rats than in W/W mice.

Despite remarkable increase of immature basophils in the BM of the NB-infected Ws/Ws rats, neither RMCP-I+ CTMC nor RMCP-II+ MMC developed in their BM. The number of CTMC did not increase and MMC did not develop in the BM of +/+ rats after infection, either. On the other hand, MMC did develop in the jejunum of the NB-infected Ws/Ws rats, although the number of MMC in the Ws/Ws rats was about 10% that of the control +/+ rats. The concentration of T-cell-derived cytokines may be sufficient for differentiation of MMC in the jejunum but not in the BM. Such a low concentration of T-cell-derived cytokines appeared to be enough for augmented production of basophils in the BM.

The histamine concentration in the PB of +/+ rats was about twice that of Ws/Ws rats. Because practically no mast cells are detectable in noninfected Ws/Ws rats, the low histamine concentration in noninfected Ws/Ws rats was attributable to their lack of mast cells. The origin of histamine detected in the PB of noninfected Ws/Ws rats remains unclear. After infection with NB, the histamine concentration increased 7.5-fold in Ws/Ws rats and 4.5-fold in +/+ rats. In contrast, no such increase occurred in nude rats. Changes in histamine concentration of +/+ Ws/Ws and nude rats was consistent with the changes in number of basophils. His-
tamine content per 10⁶ basophils (HC) was calculated from HC and number of basophils (No. of Baso) before and after the NB infection (NBI) using the following formula:

\[
HC = \frac{HC \text{ After NBI} - HC \text{ Before NBI}}{\text{No. of Baso After NBI} - \text{No. of Baso Before NBI}}
\]

HC in Ws/Ws rats was 2.92 nmol and was 4.44 nmol in +/- rats when calculated by the above formula. The estimated histamine content of rat basophils is 100-fold greater than that of mouse basophils, but is comparable with that of human basophils. The histamine content of rat basophils is 100-fold smaller than that of rat peritoneal mast cells. Electron microscopy is the most reliable method for identification of basophils. The ultrastructural features of basophils in the PB of Ws/Ws and +/- rats were consistent with those of guinea pigs, humans, and mice described by Dvorak et al. The ultrastructural features of blood basophils were also consistent with those of basophils invading into the lamina propria of the small intestine. Basophilic cells that increased in the BMs of Ws/Ws and +/- rats after the NBI were identified as immature basophils both ultrastructurally and by peroxidase enzyme histochemistry. Immunohistochemically, no RMCP-I⁺ and/or -II⁺ cells were detectable in the BM of Ws/Ws rats infected with NB. Because confusion with mast cells can now be avoided, we believe that the NB-infected Ws/Ws rats are a suitable animal model for quantitative studies of basophil differentiation and recruitment.

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