Proliferative Potential of Degranulated Murine Peritoneal Mast Cells

By Akira Kuriu, Sanae Sonoda, Yuzuru Kanakura, Kiyoshi Jozaki, Atsushi Yamatodani, and Yukihiko Kitamura

The fate of mast cells after degranulation was investigated. Purified peritoneal mast cells of WBB6F_, +/+ mice were sensitized with monoclonal anti-dinitrophenol (DNP) IgE antibodies and stimulated with DNP conjugated with human serum albumin. Mast cells were vitally stained with neutral red, and highly degranulated mast cells were identified under a phase-contrast microscope and individually picked up with the micromanipulator. When these highly degranulated mast cells were individually plated in methylcellulose, their potential to produce a cluster or a colony was comparable to that of morphologically intact mast cells. Moreover, when highly degranulated mast cells were injected into the skin of genetically mast cell-deficient WBB6F_, -/- mice, the proportion of injection sites at which mast cell clusters appeared was comparable to the value observed when morphologically intact mast cells were injected. The present result indicates that proliferative potential of mast cells is not reduced by their degranulation.

MAST CELLS HAVE high-affinity IgE receptors on their surface, and the immunologic activity of mast cells is mediated through these IgE receptors. Binding of antigens to IgE molecules results in formation of linkages between IgE receptors and then release of the basophilic granules. This process constitutes an important step in the immediate hypersensitivity reaction that occurs in allergic diseases such as urticaria, bronchial asthma, and allergic rhinitis. In addition to having a role in allergic diseases, mast cells have a physiologic role as an effector of host defense mechanisms in intestinal helminth infection and dermal tick infestation.

The mechanisms responsible for initiating the degranulation have been studied intensively. However, the events which follow degranulation have received comparatively little attention. Regranulation of mast cells has been described by several investigators, but they dealt with suspended mast cells as a mass. When morphology of individual mast cells was described, the cells appeared to have been selected rather arbitrarily. Since the response of each mast cell to antigens may not be homogenous, we consider that investigation of the fate of a single degranulated mast cell is indispensable. In the present study, we examined the proliferative potential of a single degranulated mast cell either in methylcellulose or in the skin of genetically mast cell-deficient WBB6F_, -/- mice.

MATERIALS AND METHODS

Mice and cell suspensions. WBB6F_, (+/+, W/W_) mice were raised in the Shizuoka Laboratory Animal Center and were used at age 8 to 10 weeks. Mice were anesthetized by ether and were killed by decapitation; 3 mL Tyrode's buffer containing 0.1% gelatin (Sigma Chemical, St. Louis) was injected into the peritoneal cavity; the abdomen was gently massaged for 30 seconds. The peritoneal cavity was opened, and the fluid containing peritoneal cells was aspirated and placed with a pasteur pipette. The peritoneal cells were sedimented at 150 g for 10 minutes at room temperature and resuspended in Tyrode's buffer. Spleen and bone marrow cells were suspended in α-medium (Flow Laboratories, Rockville, MD) as described previously.

Purification of peritoneal mast cells. Mast cells were separated from major components of mouse peritoneal cells, macrophages, and small lymphocytes according to the method described by Yurt et al.. Cells from ten mice (6 to 10 x 10^7) in 1 mL Tyrode's buffer were layered on 2 mL 22.5% wt/vol metrizamide (density, 1.120 g/mL, Nyegaard, Oslo) and centrifuged at room temperature for 15 minutes at 400 g. The cells remaining at the buffer–metrizamide interface were aspirated and discarded; the cells in pellet were washed and resuspended in 1.0 mL Tyrode's buffer. Mast cells represented 70% to 80% of nucleated cells in this preparation. To obtain mast cell suspensions of ≥99% purity, the procedure just described was repeated using the 70% to 80% pure mast cells.

Cell counts. Cells were counted with a standard hemocytometer. Mast cells were identified either by staining with neutral red (0.2% in 0.9% NaCl) or by phase-contrast microscope. These methods gave similar results.

Degranulation of mast cells. Purified peritoneal mast cells were passively sensitized with monoclonal mouse anti-DNP IgE antibodies, a gift from Dr Teruko Ishizaka, Johns Hopkins University School of Medicine. The mast cells were suspended in α-medium supplemented with 10% fetal bovine serum (FBS 3 to 5 x 10^5/mL, HyClone, Logan, UT) and were incubated with anti-DNP IgE (30 μg/mL) for 40 hours at 37°C in a humidified atmosphere flushed with 5% CO2. The mast cells were washed three times and then incubated with DNP conjugated with human serum albumin (DNP-HSA, 0.01 μg/mL) for 30 minutes at 37°C. After the treatment with antibodies, mast cells were washed three times with appropriate buffer.

Separation of highly degranulated mast cells. Discontinuous density gradients were made by sequentially overlaying 2 mL each of 22.5% and 18.5% metrizamide (density 1.120 and 1.100 g/mL, respectively). IgE-sensitized and DNP-HSA-stimulated mast cells were washed, resuspended in 1 mL Tyrode's buffer, overlaid on the gradients, and centrifuged at room temperature for 15 minutes at 400 g. The supernatant (light fraction), interface (middle fraction), and pellet (dense fraction) were collected, washed three times, and resuspended with appropriate buffer. Cells from each fraction were spun in a cytospin (Cytospin, Shandon Southern, Elliot, IL) at 600 rpm for five minutes and stained with May-Grünwald-Giemsa solution.

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Clonal culture and suspension culture. The method of clonal culture in methylcellulose was described previously. Mast cell clusters (four to 63 cells) and colonies (≥64 cells) were counted on day 16. The method of obtaining suspension-cultured mast cells from the bone marrow was described previously. Suspension-cultured mast cells were used 4 weeks after the initiation of the culture. At that time, ≥95% of cells in the suspension culture were identified as mast cells.

Single-cell culture. The light fraction of stimulated mast cells were resuspended with calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS) and stained with neutral red. Individual cells that did not contain neutral red-positive granules were aspirated with a fine microtube attached to a micromanipulator (Narishige, Tokyo). Each cell was transferred to a well of 24-well microtiter plates (Corning, NY) containing 0.2 mL methylcellulose culture medium. The culture plates were incubated for 16 days, and development of mast-cell clusters and colonies was examined.

Staining of cultured cells. Individual colonies were lifted as described. The samples were washed, spun in a cytocentrifuge, and fixed with Carnoy's fluid. The cytocentrifuge preparations were stained with a fluorescent dye, berberine sulfate, as described by Enerbäck. The specimens were examined with an Olympus epifluorescent microscope. Enerbäck demonstrated by cytofluorometry that berberine sulfate specifically stains heparin-containing granules of mast cells. We confirmed this by showing that the fluorescence disappeared after heparinase digestion.

Direct injection of cells into the skin. Highly degranulated mast cells which did not contain neutral red-positive granules were individually aspirated with the micromanipulator. Five cells were transferred to an Eppendorf tube, mixed with India ink, and directly injected into the skin of WBB6F₁-W/W' mice that genetically lack mast cells. The WBB6F₁-W/W' mice were killed 5 weeks after the injection, and the appearance of mast cell clusters was examined as described. Serial sections (25 μm thick) of injection sites which could be identified as blotted black spots, were prepared and stained with acidified toluidine blue. A group of >100 mast cells at the largest cross-section was defined as a mast cell cluster. In some cases, sections were stained with berberine sulfate to identify heparin-containing mast cells.

Histamine content. Histamine concentration was assayed by high-performance liquid chromatography (HPLC) coupled with fluorometry as described by Yamatodani et al and the content of histamine per 10⁶ mast cells was calculated.

RESULTS

Purified peritoneal mast cells were passively sensitized with monoclonal anti-DNP IgE antibodies. Although the

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Fractionation</th>
<th>Fraction*</th>
<th>Proportion to Mast Cells Belonging to Each Group‡†</th>
<th>Histamine Content‡ (ng/10⁶ Cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>No</td>
<td>—</td>
<td>98 ± 1 2 ± 1 0</td>
<td>14,400 ± 1,170</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>—</td>
<td>16 ± 35 70 ± 55 14 ± 35</td>
<td>6,300 ± 2,010§</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Dense</td>
<td>55 ± 75 45 ± 75 0</td>
<td>10,400 ± 1,380</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Middle</td>
<td>3 ± 15 90 ± 15 7 ± 15</td>
<td>4,500 ± 1,230§</td>
</tr>
<tr>
<td>Yes</td>
<td>Yes</td>
<td>Light</td>
<td>1 ± 15 52 ± 55 47 ± 55</td>
<td>740 ± 340§</td>
</tr>
</tbody>
</table>

* Sensitized mast cells were cultured with DNP-HSA and fractionated by discontinuous density gradient centrifugation: dense fraction > 1.120 g/mL, middle fraction 1.100 to 1.120 g/mL, light fraction < 1.100 g/mL.
† Group 1, apparently intact mast cells; group 2, partially degranulated mast cells which contained significantly fewer granules than intact mast cells; group 3, highly degranulated mast cells containing less than five granules.
‡ Mean ± SE of four to eight independent results.
§ P < .01 as compared with the value of IgE-sensitized but DNP-HSA–nonstimulated mast cells by t test.
number of viable cells slightly decreased during incubation, the sensitized mast cells were morphologically indistinguishable from freshly isolated peritoneal mast cells. When DNP-HSA was added to the suspension of IgE-sensitized mast cells, 63.9% ± 10.4% of total associated histamine was released in 30 minutes (mean ± SE of eight samples). The histamine release from the control mast cells incubated without DNP-HSA was 5.9% ± 0.9% (mean ± SE of eight samples).

Morphology of mast cells sensitized with anti-DNP IgE and subsequently stimulated with DNP-HSA was heterogeneous. We classified them into the following three groups; group 1, apparently intact mast cells; group 2, partially degranulated mast cells in which granules were significantly less than in intact mast cells; and group 3, highly degranulated mast cells which contained less than five granules (Fig 1).

To enrich highly degranulated mast cells belonging to group 3, IgE-sensitized and antigen-stimulated mast cells were fractionated with discontinuous density-gradient centrifugation. Highly degranulated mast cells were enriched in the light fraction, as shown in Table 1.

Table 3. Proliferative Potential of Individually Identified and Individually Plated Mast Cells in Methylcellulose

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Morphology*</th>
<th>Neutral Red Stain</th>
<th>No. of Plated Cells</th>
<th>No. of Clusters</th>
<th>No. of Colonies</th>
<th>Proportion of Proliferation (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE-sensitized but antigen-nonstimulated</td>
<td>Intact</td>
<td>No</td>
<td>96</td>
<td>23</td>
<td>31</td>
<td>56</td>
</tr>
<tr>
<td>IgE-sensitized but antigen-nonstimulated</td>
<td>Intact</td>
<td>Yes</td>
<td>96</td>
<td>20</td>
<td>24</td>
<td>48</td>
</tr>
<tr>
<td>IgE-sensitized and antigen-stimulated</td>
<td>Highly degranulated‡</td>
<td>Yes</td>
<td>191</td>
<td>42</td>
<td>38</td>
<td>42</td>
</tr>
</tbody>
</table>

*Each mast cell was identified under phase-contrast microscope.
†P > .1 when proportion of mast cells which made either a cluster or a colony was compared among experimental groups by chi-square test.
‡Mast cells without any neutral red-positive granules were selected.

Table 2. Development of Mast Cell Clusters and Colonies After Plating of 10⁶ Mast Cells That Received Various Treatments

<table>
<thead>
<tr>
<th>Plated Mast Cells</th>
<th>No. of Experiments</th>
<th>No. per 10⁶ Plated Cells (Mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE-sensitized but antigen-nonstimulated</td>
<td>7</td>
<td>379 ± 29</td>
</tr>
<tr>
<td>IgE-sensitized and antigen-stimulated</td>
<td>7</td>
<td>436 ± 50*</td>
</tr>
<tr>
<td>Light fraction of IgE-sensitized and antigen-stimulated mast cells</td>
<td>5</td>
<td>458 ± 55*</td>
</tr>
</tbody>
</table>

*P > .1 as compared with the number of clusters or colonies derived from IgE-sensitized but antigen-nonstimulated mast cells by t test.

Table 4. Proportion of Berberine Sulfate-Positive Mast Cells in Various Mast Cell Populations

<table>
<thead>
<tr>
<th>Mast Cells</th>
<th>Proportion of Berberine Sulfate-Positive Mast Cells (Mean ± SE)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact peritoneal mast cells†</td>
<td>98 ± 5 (5)</td>
</tr>
<tr>
<td>Bone marrow-derived suspension-cultured mast cells</td>
<td>0 (7)</td>
</tr>
<tr>
<td>Pooled mast cells from colonies produced by intact peritoneal mast cells‡</td>
<td>23 ± 3 (8)</td>
</tr>
<tr>
<td>Pooled mast cells from colonies produced by highly degranulated mast cells§</td>
<td>1 ± 1 (8)</td>
</tr>
</tbody>
</table>

*Number of samples is shown in parentheses.
†IgE-sensitized but antigen-nonstimulated mast cells.
‡Colonies were produced by IgE-sensitized but antigen-nonstimulated mast cells.
§Colonies were produced by IgE-sensitized and antigen-stimulated mast cells. Only highly degranulated mast cells were picked up with a micromanipulator and individually plated in methylcellulose.
cells were used as a negative control. The proportion of mast cells with berberine sulfate-positive granules was significantly lower in mast cells derived from colonies of either origin than in the positive control (Table 4). Even when colony-derived mast cells contained berberine sulfate-positive granules, the ratio of berberine sulfate-positive granules to the total granules was low (data not shown). In contrast, all granules of IgE-sensitized but antigen-nonstimulated mast cells were actually berberine sulfate positive. When the proportion of mast cells with berberine sulfate-positive granules was compared between colonies derived from IgE-sensitized but antigen-nonstimulated mast cells and colonies from highly degranulated mast cells, the proportion was significantly higher in the former colonies (Table 4).

Peritoneal mast cells can proliferate not only in methylcellulose but also in the skin of WBB6F1/W/W' mice. In the next experiment, we examined whether highly degranulated mast cells produced mast cell clusters in the skin of WBB6F1/W/W' mice as well. Mast cells without neutral red-positive granules were picked up by the micromanipulator. Five cells were pooled and injected into a single injection site. Proportions of injection sites at which a mast cell cluster appeared were comparable between apparently intact mast cells and highly degranulated mast cells (Table 5).

In some cases, mast cell clusters in the skin were stained with berberine sulfate. Granules of all mast cells were stained with this fluorescent dye. There was no significant difference in the strength of the fluorescence between mast cell clusters which developed at the injection sites of apparently intact mast cells and those that developed at the injection sites of highly degranulated mast cells.

**DISCUSSION**

When mast cells sensitized with IgE antibodies underwent degranulation by exposure to antigens, they changed in morphologic appearance, density, and histamine content. Mast cells that released nearly all granules resembled lymphocytes in morphology and density. The present study clearly indicates that such highly degranulated mast cells can proliferate and restore the morphology of mast cells. When degranulated mast cells made clusters or colonies in methylcellulose, the proportion of mast cells with berberine sulfate-positive granules was low; furthermore, the ratio of berberine sulfate-positive granules to the total granules was low even in berberine sulfate-positive mast cells. However, when highly degranulated peritoneal mast cells were injected into the skin of WBB6F1/W/W' mice, all granules of mast cells which developed at the injection sites were berberine sulfate positive. This shows that highly degranulated connective tissue-type mast cells can restore the original morphology in appropriate environments.

Dvorak et al. observed the recovery of degranulated human mast cells with an electron microscope and proposed the morphologic cycles between intact granulated mast cells and highly degranulated mast cells resembling lymphocytes. A similar change of rat peritoneal mast cells was also observed by Slutsky et al. with a phase-contrast microscope. Ginsburg et al. described recovery of degranulated mouse mast cells that had been cultured on a mouse fibroblast monolayer. These results and our present result are not mutually exclusive. Probably two types of mast cell cycles are present: the cycle without proliferation and the cycle with proliferation.

The proportion of mast cells which made a cluster or a colony was comparable between highly degranulated mast cells without any detectable granules and apparently intact mast cells. Therefore, mast cells do not appear to proliferate at a more rapid rate after degranulation. This is consistent with clinical observations that allergic patients experiencing repeated episodes of mast cell degranulation in target tissues exhibit either no increase in mast cells or only two- to threefold increase. The latter possibly is explained by the prolonged inflammatory reaction.

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