The Presence of Mast Cell Precursors in Rat Peripheral Blood

By D. Zucker-Franklin, G. Grusky, N. Hirayama, and E. Schnipper

Soft agar culture of mononuclear cell fractions prepared from rat peripheral blood yielded numerous colonies consisting of mast cells. The mast cell nature of the cells was established by ultrastructural and histochemical analyses as well as by the demonstration that the colonies contained histamine and that the cells possessed receptors for the Fc component of IgE. Stringent criteria for the distinction of mast cells from monocytes/macrophages that could have metachromatic inclusions were applied. The alcian-blue-safranin technique delineated the maturation of mast cell granules by showing the loss of alcian-blue and increase in safranin-positive organelles presumed to reflect the increase in N-sulfated polysaccharides representing heparin. The mast cells exhibited low or absent reactions for peroxidase, α-naphthyl butyrate, periodic acid Schiff, and Sudan black reacting lipid, whereas macrophages stained in parallel were positive for these substances. Since it is known that extracellular conditions may cause variations in phenotypic expression, the observations have led to the hypothesis that mast cells and macrophages may have a common precursor.

The cells were also shown to contain histamine and to have membrane receptors for IgE, properties characteristic for basophils and mast cells of all species studied to date.

MATERIALS AND METHODS

Cells

Male Sprague-Dawley rats, aged 2–3 mo and weighing approximately 250 g, were used for all experiments. Peripheral blood was collected in heparin by cardiac puncture under ether anesthesia. The whole blood was diluted 1:1 with 0.85% saline and layered on a Ficoll-Hypaque gradient to separate the mononuclear cells. A purified lymphocyte fraction was obtained using Lymphocyte Separator Reagent (LSR) (Technicon Products, Tarrytown, N.Y.) as described elsewhere. On a few occasions, marrow was obtained from femurs during ether anesthesia by removing the ephphyses and flushing the marrow cavity of the diaphyses with medium. After vigorous pipetting, the marrow suspension was placed on a Ficoll-Hypaque gradient and treated like the peripheral blood cells.

Cultures

Soft agar cultures in 35 x 10 mm Falcon dishes were prepared with slight modifications of methods described by others. When cellular feeder layers were used, these were prepared with 106 Ficoll-Hypaque separated mononuclear cells per milliliter of McCoy’s medium with 10% fetal calf serum, 5% rat serum, and 0.5% agar (Difco, Detroit, Mich.). Acellular feeder layers contained monocyte conditioned medium. This was prepared in 100 mm culture dishes (Falcon, Cockeysville, Md.) containing 2 x 107 mononuclear cells in 5 ml of McCoy’s 5A medium with 10% fetal calf serum (GIBCO, Grand Island, N.Y.), and 5% rat serum. After 24 hr at 37°C in a 10% CO2 atmosphere, the medium containing the nonadherent cells was removed and replaced with fresh medium. After 7 days, the medium presumed to contain colony-stimulating factor was centrifuged and stored at ~20°C until needed. For the preparation of feeder layers, the conditioned medium was diluted 1:1 with McCoy’s medium containing 5% rat and 10% fetal calf serum. The final concentration of agar was 0.5%. The culture layers contained either post-Ficoll-Hypaque peripheral blood mononuclear cells or in a few instances, LSR-purified lymphocytes at concentrations of 106/ml of medium consisting of 5% rat serum, 10% fetal calf serum in a final concentration of 0.3% agar. When marrow was used, the cell concentration was 2 x 107/ml. A minimum of 10 dishes were prepared for each specimen obtained from 20 different animals. Ficoll-Hypaque isolated cells prepared from bone marrow...
were grown on 7 occasions. The cultures were maintained for up to 4 wk at 37°C in a humidified atmosphere containing 10% CO2.

**Histochemistry**

Colonies were removed from the agar with an attenuated Pasteur pipette, expelled into a drop of medium on a slide, and smeared. After suitable fixation, the following staining techniques were employed: toluidine blue, periodic acid Schiff reaction, Sudan black, peroxidase, naphthol-AS-dichroacetate and α-naphthyl butyrate, and alcian blue-safranin. Routine blood, marrow, and colony smears were stained with Wright's and Giemsa stains.

**Electron Microscopy**

Following colony counts with a dissecting microscope at a magnification of 30x, selected cultures were fixed by addition to the Petri dish of 3% glutaraldehyde in 0.067 M phosphate buffer with 1% sucrose overnight. Postfixation with 2% osmium tetroxide, dehydration, and embedding in epon of the intact agar disks was accomplished by a method described in detail before. The embedded colonies were cut out of the epon “block” and mounted for thin sectioning. The sections were stained with uranyl acetate and lead citrate and viewed with a Siemen's Elmiskop I electron microscope.

**Receptors for IgE**

Colonies growing on the surface were easily dislodged by directing a gentle stream of medium onto the surface of the agar. Cells from 20 to 30 dishes were pooled and suspended at a final concentration of 3.6 x 10⁶ cells/ml. The detection of IgE receptors on these cells was accomplished by a rosette technique employing sheep erythrocytes coated with DNP-BSA as described by Bloch et al. and reacted with a murine IgE hybridoma antiserum with dinitrophenol (DNP) specificity described in detail elsewhere. The murine IgE antiserum had been shown to induce allergic histamine release of rat mast cells and thus was known to be cross-reactive. A 1% suspension of DNP-BSA coated erythrocytes was reacted with 5 μl of anti-DNP hybridoma antibody (25 μg anti-DNP IgE antibody). Mast cells were mixed with the immune complex coated sheep erythrocytes in a ratio of 1:100 and incubated at 37°C for 5 min, followed by 30 min in melting ice with intermittent gentle manual agitation. The pellets were cytocentrifuged and stained with Giemsa to assess rosette formation.

**Assay for Histamine**

Samples for histamine assays were collected on two occasions in the following manner. (1) Approximately 100 colonies consisting of >100 cells/colony were aspirated with some surrounding agar, pooled, and spun to obtain a pellet. This was designated as sample 1A. An equivalent volume of 0.3% agar in McCoy's medium with 10% fetal calf serum and 5% rat serum but without cells was treated identically and served as control. It was designated sample 1B. (2) On a second occasion, the entire culture layers of 35-mm dishes were harvested with a Pasteur pipette, pooled, and resuspended in McCoy's 5A medium to a volume of 3.5 ml. After vigorous pipetting, the suspension was spun to obtain a pellet. This sample was designated 2A. The supernatant of sample 2A was removed and designated 2B. In addition, culture layers not containing any cells were prepared at the same time and treated identically. The acellular equivalent of 2A was designated 3A and its supernatant was labeled 3B. The six samples were frozen in liquid N₂ and shipped to Dr. David Levy, Department of Biochemistry, Johns Hopkins University, who kindly performed the histamine determinations without knowledge of the content of the samples. In Dr. Levy's laboratory, the specimens were frozen and thawed three times, centrifuged at 1500 rpm for 15 min, and the pellets were sonicated for 30 min in a small “cleaning bath.” In an attempt to eliminate the agar, the specimens were also filtered through a Swinex filter containing no Millipore filter. Finally, the remaining agar was precipitated with ethanol in an approximate v/v ratio of 1:1 at 4°C overnight. The precipitates were centrifuged and the supernates placed in a 0.5 ml microcup and evaporated to dryness in a vacuum dessicator. Each sample was reconstituted with 50 μl H₂O and assayed for histamine by the double isotope radioenzymatic method.

**RESULTS**

Whereas soft agar cultures prepared of human peripheral blood or marrow usually exhibit some clusters or small colonies by day 7 of incubation, the rat cells failed to show any evidence of growth at this time. Only toward the end of the second week were a small number of colonies consisting of less than 100 cells noted (Table 1). It became immediately apparent that these colonies differed from the “tight” (eosinophil) or “loose” (neutrophil/macrophage) colonies seen in human material. The cells were larger, more angular, and seemed more granulated than those observed in cultures of human cells grown under the same conditions. As illustrated in Fig. 1, the cells were rather tightly clustered. Colony counts were performed on days 14 and 21 on cultures initiated with peripheral blood mononuclear cells obtained from 11 different animals. The remainder was used for studies detailed below. With one exception (no. 11), the number of colonies per dish was low on day 14 averaging 5.3 colonies per dish. However, when incubation was continued for 3 or 4 wk, there was a marked increase in the number and size of the colonies (Table 1). Numerous colonies consisted of 1000–2000 cells, and in many dishes adjacent colonies had become

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.6 ± 2.9</td>
<td>30.6 ± 4.9</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10.3 ± 1.8</td>
<td>26.2 ± 3.7</td>
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<tr>
<td>3</td>
<td>4.9 ± 1.3</td>
<td>84.9 ± 21.2*</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>14.5 ± 2.5</td>
<td>15.0 ± 3.0</td>
<td></td>
</tr>
<tr>
<td>5A‡</td>
<td>7.8 ± 2.2</td>
<td>15.7 ± 4.1</td>
<td></td>
</tr>
<tr>
<td>5B‡</td>
<td>14.1 ± 9.3</td>
<td>21.0 ± 13.2</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>22.5 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>0</td>
<td>23.6 ± 3.7</td>
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<tr>
<td>8</td>
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<td>25.2 ± 11.4</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>3.2 ± 2.9</td>
<td>29.8 ± 10.9</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.3§</td>
<td>2.3 ± 1.6</td>
<td>26.6 ± 16.0</td>
</tr>
<tr>
<td>11</td>
<td>54.4 ± 23.3*</td>
<td>77.7 ± 25.6*</td>
<td></td>
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*Growth extremely dense, often confluent.
‡Same rat 5A, Ficoll-Hypaque fraction; 5B, monocytes removed.
§Average in 30 dishes counted.
Colonies aspirated with a Pasteur pipette were smeared on slides and stained with Wright’s and Giemsa (Plate 1A). The majority of the cells had a round or oval eccentric nucleus with a mature chromatin pattern and an invisible or inconspicuous nucleolus. The cytoplasm was replete with deeply blue-purple staining granules that made the cells indistinguishable from tissue mast cells except for their very variable and, generally, larger size. Staining with Toluidine blue yielded similar results. The diameter of the cells ranged from 8 to 40 μm (Plate 1) within the same colony and at all times of sampling. Lipid-laden macrophages were often found within and in-between colonies.

Electron Microscopy

Despite considerable effort, the preservation of the cells’ ultrastructural detail has remained unsatisfactory to date (Figs. 3 A and B). Most of the granule content became extracted leaving only some poorly defined material within a membrane-bound space. Occasional reduplication of membranes as often seen in well fixed mast cells was present. Lipid inclusions were common (see below), as is frequently the case in cultured cells, but these never attained the number or size of those in lipid-laden macrophages.27

Histochemistry

In order to characterize the cells more definitively, use was made of the demonstration by others that mast cells pass through sequential stages of maturation during which a weakly sulfated polysaccharide or “heparin precursor substance” is replaced by a highly N-sulfated polysaccharide presumed to be heparin.18 This maturation sequence is reflected first by the appearance of large Alcian-blue positive, Safranin-negative granules that gradually decrease in size and number to be completely replaced by Safranin-positive granules. When the Alcian-blue-Safranin technique was applied to the mast cell colonies, all stages of maturation could be observed (Plate 1 B–F). The mast cell colonies were Sudan-black-negative, although a few lipid inclusions could be detected among Safranin-positive (mature) cells (Plate 1G). This was in marked contrast to the lipid-laden macrophages that were present among the colonies (Plate 1H). Negative results were also obtained with histochemical techniques designed to detect peroxidase, naphthyl butyrate esterase, naphthol-ASD-chloroacetate esterase, and the periodic acid Schiff reagent.
Plate 1. (A) Cells aspirated from a presumed mast cell colony cytospun, fixed with methanol, and stained with Wright’s and Giemsa. (B) Cells aspirated from a presumed mast cell colony, stained with Alcian-blue-Safranin, show cells at various stages of maturation. Maturation sequence is illustrated to better advantage in C–F, which show (C) only Alcian-blue-positive granules, (D) a mixture of Alcian blue and Safranin-positive granules, (E) Safranin-positive granules have become predominant, (F) fully mature cell with Safranin-positive granules. Alcian-blue staining granules have disappeared. (G) Mast cell stained with Safranin and Sudan black shows a few lipid inclusions (arrow). (H) Example of a macrophage aspirated from an area in between colonies, stained with Sudan black. The cell is replete with lipid inclusions. (I) Example of a mast cell rosette formed by reacting colony cells with sheep erythrocytes coated with immune complexes containing IgE.
Fig. 3. (A) Ultrastructure of a cell representative of those seen in mast cell colonies. The granule content is often extracted during the embedding procedure. (Mag. x 9000.) (B) Detail of a mast cell showing two characteristic granules. There are also several inclusions presumed to be lipid (L). Ribosomes and some small profiles of rough endoplasmic reticulum are seen in the cytoplasm. (Mag. x 52,000.)

Receptors for IgE

On two occasions, colonies that were growing on the surface of culture layers were pooled to attain a final concentration of $1.8 \times 10^4$ cells in 0.5 ml PBS. When these were incubated with sheep erythrocytes that had been coated with an IgE immune complex (see Materials and Methods), almost all the presumed mast cells formed rosettes (Plate II). Control sheep erythrocytes coated only with DNP-BSA did not result in rosette formation.

Histamine

"Blind" assays for histamine performed on the six specimens sent to Dr. Levy revealed that this amine was only present in samples 1A and 2A, the samples containing the presumed mast cell colonies. Sample 1A contained 0.4 ng histamine and sample 2A contained 0.3 ng of histamine per 0.05 ml of their original volumes. Although precise quantitation of the amount of histamine per cell awaits more extensive studies, on the basis of rough estimates of dilutions and losses incurred during the preparatory procedures, the amount of histamine per cell has been calculated to be about 1.6 pg.

DISCUSSION

Based on a large literature, it seems that rat mast cells proliferate in vitro with relative ease. Therefore, it is perhaps not surprising that several investigators have also observed the development of mast cell colonies in soft agar cultures initiated with cells derived from various lymphoid tissues of rats as well as of mice. Since it is common knowledge that the lymphoid organs of these species normally contain numerous mast cells as well as other hematopoietic cells, the progenitor of the colonies remained in doubt. Moreover, the colonies were identified only on the basis of their morphology, which, early on, raised the question whether this criterion alone sufficed to characterize the cells. The ensuing controversy was aggravated by the observation that macrophages grown in soft agar culture often contain large numbers of metachromatic inclusions, which some investigators believe to be phagocytosed agar. More recently, however, it has been shown that the mast-cell-like colonies derived from rat thymuses indeed possess receptors for IgE. This observation has added significant support for the morphological interpretation that the cells are mast cells. It should be mentioned at this point, that our own studies originally were not designed to analyze mast cells. Rather, they formed part of a protocol aimed at the elucidation of eosinophilia in man. Since some of the experiments precluded the use of human material, and eosinopoiesis in the rat had been thoroughly investigated by others, this species seemed appropriate for our
work. The culture conditions were identical to those used in our laboratory for the differentiation of human eosinophil colonies with the exception of the addition of 5% rat serum to the medium. As with human material, the cultures were seeded with either peripheral blood mononuclear cell fractions or with blood lymphocytes from which monocytes had been removed. Such specimens contained <0.1% granulocytes. Surprisingly, no eosinophil colonies were identified at any time despite occasional inclusion in the feeder layer of agents believed to promote eosinopoiesis, such as pokeweed-stimulated lymphocyte conditioned medium or eosinophilopoietin. A plethora of colonies which, on morphological grounds, were recognized to consist of mast cells (Plate 1A) interspersed with fat-laden macrophages were the only type of colonies observed, whether the cultures were derived from blood or bone marrow. Because of the aforementioned controversy, we decided to characterize the cells by every means available at the present time. The crucial distinction to be made was whether the cells represented true mast cells or macrophages with metachromatic inclusions. The demonstration that the cells possessed receptors for IgE, a property characteristic of basophils/mast cells of all species examined to date, seemed encouraging, especially since previous studies by others had failed to detect receptors for this immunoglobulin on rat macrophages and fibroblasts. However, there are also reports claiming that activated or immune macrophages may develop IgE receptors under some circumstances. Therefore the observation that the cells possess such receptors does not provide absolute proof that they are mast cells. On the other hand, the synthesis and storage of histamine among blood cells has so far been attributed only to mast cells/basophils, and the detection of this amine in samples that contained the colonies supports the concept that the cells belong to this class. It is also relevant to mention that macrophages maintained in soft agar culture for 3 wk or longer become strongly Sudan black positive, whereas the presumed mast cells revealed only a few Sudan-black positive inclusions (Plate 1 G and I).

Among the nonspecific esterases, α-naphthyl butyrate esterase has been found to be most characteristically positive in monocytes and macrophages. The rat mast cell colonies proved to be negative in this regard, whereas human histiocyte colonies that were tested in parallel reacted strongly. The α-naphthyl acetate esterase reaction was only faintly positive in the colony cells and usually yields a much stronger result in monocytes and macrophages.

Taken together, these observations seem to indicate that rat blood is rich in precursors which, under suitable conditions, may differentiate into cells with all the properties of mast cells.

The implications of these observations are not without interest, as they may be relevant to the human mast cell/basophil system. In the first place, they suggest that, in addition to the cells constituting the reticuloendothelial system, which includes cells with such diverse properties as Kupffer cells, alveolar macrophages, and osteoclasts, maintenance of yet another connective tissue cell reservoir—the mast cell system—may also be dependent on normal bone marrow function. The long lifespan of tissue mast cells probably accounts for the absence of diseases that could be attributable to mastocytopenia as a consequence of bone marrow failure. No such disease has ever been described. On the other hand, one would anticipate that accelerated myelopoiesis would result in an increase of tissue mast cells. That this is indeed the case is exemplified by the striking increase in mast cells found in the lymphoid organs of patients with myeloproliferative syndromes and in the nodes of patients with Hodgkin's disease who also have a "reactive" marrow. Such increases in mast cells would be more difficult to explain if the cells originated from connective tissues.

Another question that deserves consideration in light of the data communicated here concerns the degree of "committedness" of precursor cells circulating in peripheral blood. Would the cells that began to form mast cell colonies after 2–3 wk in culture have developed into neutrophil/macrophage colonies earlier, i.e., within the first 10 days, if the appropriate stimulants or nutrients had been provided? Would mast cell colonies develop in the human soft agar system from the same cells that give rise to macrophage colonies under currently employed soft agar conditions, if the medium would be suboptimal for the development of macrophage/neutrophil colonies? The concept that phenotypically diverse cells may have a common origin is no longer novel. It is becoming increasingly apparent that minor changes in the microenvironment of precursor cells lead to drastically altered morphology and function. This is probably also an explanation for the large size the cells attain in culture, especially in view of knowledge that mast cell size increases with the age of the animal. Furthermore, it is known that only part of the content of the mast cell granule is synthesized endogenously. The cell takes up histidine from the environment, which it decarboxylates to form histamine. The cells are also able to take up polysaccharides, which are then gradually sulfated to form a heterogeneous group of mole-
...cules referred to as heparin. Therefore, it seems within the realm of possibility that substances in the microenvironment may dictate whether a cell “matures” into a mast cell or whether it will look and function like a macrophage. If so, the mast cell would represent yet another member of the reticuloendothelial system, in yet another guise.

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