



Title	FATE OF BONE MARROW-DERIVED CULTURED MAST CELLS AFTER INTRACUT ANEOUS, INTRAPERITONEAL, AND INTRAVENOUS TRANSFER INTO GENETICALLY MAST CELL-DEFICIENT W/W ^v MICE Evidence that Cultured Mast Cells Can Give Rise to Both Connective Tissue Type and Mucosal Mast Cells
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FATE OF BONE MARROW-DERIVED CULTURED MAST
CELLS AFTER INTRACUTANEOUS, INTRAPERITONEAL,
AND INTRAVENOUS TRANSFER INTO GENETICALLY
MAST CELL-DEFICIENT W/W^m MICE

Evidence that Cultured Mast Cells Can Give Rise to Both Connective
Tissue Type and Mucosal Mast Cells

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Cells with many of the features of mast cells develop in cultures of normal mouse hematopoietic cells containing mast cell growth factor (1–8), an activity similar or identical to interleukin 3 (IL-3)¹ (9–11), histamine-producing cell-stimulating factor (9), or P cell-stimulating factor (12–14). Large numbers of these cells (>10⁸) can be generated as virtually homogeneous populations or as clones, thereby facilitating a wide variety of biochemical and functional studies (reviewed in 15). Yet, despite their popularity as an experimental model, the precise relationship of these cells to mast cell populations observed *in vivo* is controversial.

It has been clear from the beginning that the cultured cells differ in certain important respects from the best-characterized cell type available for detailed comparative studies: mature peritoneal mast cells (reviewed in 15). Cultured mast cells appear immature by ultrastructure (1, 2, 15), contain low levels of histamine (1, 2, 5, 12, 15–18), and express fewer surface receptors for IgE than do mature peritoneal mast cells (2). In addition, cultured mast cells incorporate Na₂³⁵SO₄ into granule-associated chondroitin sulfates (2, 10, 17, 19), including

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¹ *Abbreviations used in this paper:* CFU-S, spleen colony-forming unit; CTMC, connective tissue type mast cell(s); IL-3, interleukin 3; MMC, mucosal mast cell(s); PWM-SCM, pokeweed mitogen-stimulated spleen cell-conditioned medium.

an oversulfated chondroitin that is designated chondroitin sulfate E (10, 19). By contrast, normal mouse peritoneal mast cells synthesize heparin (2, 19).

However, peritoneal mast cells are representative of only one of two phenotypically distinct mast cell populations that have been identified in murine rodents. Maximow was probably the first to recognize that certain mast cells in the rat intestinal mucosa were atypical, in that their staining characteristics differed from those of mast cells observed in other anatomical sites (20). Enerbäck greatly extended these observations and defined conditions of fixation and histochemical staining that discriminated between such atypical or mucosal mast cells (MMC) and the connective tissue type mast cells (CTMC) of the skin, peritoneal cavity, and other sites (21).

We (1, 2, 15) and several other investigators (10, 14, 17) have noted that cultured murine mast cells express certain characteristics similar to those of MMC. MMC contain low levels of histamine (21, 22), and microspectrophotometric evidence suggests that their granules contain a poorly sulfated glycosaminoglycan similar to chondroitin sulfate (23). Cultured mast cells and MMC also exhibit similar patterns of responsiveness to certain secretagogues (17) and, in the rat, contain a similar or identical granule-associated protease (24).

Furthermore, the growth of both cultured mast cells and MMC may be regulated by similar signals. Mucosal mast cells are exquisitely sensitive to T cell regulation. Striking proliferation of MMC occurs during T cell-dependent responses to certain intestinal parasites in normal mice and rats (for reviews, see 15, 21, 25–27). By contrast, athymic nude mice lack such a response (28). The nude mouse intestinal mucosa contains MMC precursors (27), however, and MMC proliferation does occur if nude mice are reconstituted with T cells before intestinal infection (28). Cultured mast cells proliferate in response to mast cell growth factor (IL-3), which can be elaborated by cloned $Ly-1^{+}2^{-}$ inducer T cells (1–4). In addition, Crapper et al. (29) reported that the survival of cultured mast cells injected into the skin of (B10pd \times DBA/2) F_1 - W^J/W^J (B10D2 F_1 - W^J/W^J) mast cell-deficient mice required that the mice also contain a subcutaneous inoculum of the WEHI-3B tumor as a source of mast cell growth factor.

Although some authors have suggested that cultured mast cells may be committed to express the MMC phenotype, we (1, 2, 15) and Yung and Moore (8) entertained an alternative hypothesis: that many of the properties of cultured mast cells might reflect their immaturity. However, attempts to induce further maturation of cultured mast cells in vitro met with only limited success. The inducing agent sodium butyrate caused a marked inhibition of cultured mast cell proliferation, resulted in increased storage of histamine and chondroitin sulfate, and favored partial maturation of cytoplasmic granules (2, 30). But the cells did not appear fully mature by ultrastructure nor did they synthesize detectable amounts of heparin.

In the present study, we adopted a different strategy: we injected cultured mast cells directly into mast cell-deficient (WB \times C57BL/6) F_1 - W/W^v (WBB6 F_1 - W/W^v) mice and examined the cells' histochemical characteristics and ultrastructure at different intervals up to 10 wk later. We performed similar experiments using partially purified peritoneal mast cells from WBB6 F_1 -+/+ mice. When injected in vivo, cultured or peritoneal mast cells gave rise to mast cells in several

different anatomical sites. In the peritoneal cavity, skin, spleen, and glandular stomach muscularis propria, these mast cells exhibited features of CTMC. By contrast, adoptively transferred mast cells identified in the mucosa of the glandular stomach resembled MMC. These results suggest that mast cell phenotype can be strikingly influenced by local microenvironmental factors.

Materials and Methods

Mice. The tissues of adult WBB6F₁-W/W^v mice contain <1% the number of CTMC as do their normal (+/+) littermates (31, 32). WBB6F₁-W/W^v mice also lack MMC (33). Both of these populations can be restored by transplantation of bone marrow cells derived from WBB6F₁-+/+ mice (31–33). WBB6F₁-(W/W^v, +/+) and C57BL/6-*bg^J/bg^J* mice were raised at the Osaka University Medical School or Shizuoka Laboratory Animal Center. Cells derived from C57BL/6-*bg^J/bg^J* mice were used in some experiments because their giant granules represent a morphologic marker that can unequivocally identify the cells as of donor origin (34, 35). The original stocks of mutant mice were obtained from The Jackson Laboratory, Bar Harbor, ME, but W^v and *bg^J* mutant genes have been maintained in C57BL/6 mice of our own inbred colony (more than 29 and 16 backcrosses, respectively, at the time of the present experiments) (36, 37). Mice were used at 2–6 mo of age.

Cell Suspensions. Mice were killed by decapitation. Bone marrow cells were recovered and suspended in Eagle's medium as described previously (38). To harvest peritoneal cells, 2 ml of Eagle's medium containing 10 IU/ml heparin and 0.1% bovine serum albumin (BSA) was injected into the peritoneal cavity, and the abdomen was massaged gently for 30 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated with a pasteur pipette. When peritoneal cells were used for microscopical observations, heparin was omitted from the Eagle's medium, which was injected into the peritoneal cavity.

Cell Counts. Cells were counted in a standard hemocytometer. Peritoneal and cultured mast cells were identified either by staining with neutral red (0.02% in 0.9% NaCl) or by using phase contrast microscopy. These methods gave similar results.

Conditioned Medium. Pokeweed mitogen (Gibco Laboratories, Grand Island, NY)-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described in Nakahata et al. (39). Spleen cells were incubated for 5 d at 2×10^6 cells/ml in α medium (Flow Laboratories, Irvine, Scotland) containing a 1:300 dilution of PWM, 10% fetal bovine serum (Irvine Scientific, Santa Ana, CA), 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co., St. Louis, MO). The conditioned medium was centrifuged, filtered through a 0.22 μ m filter (Millipore Corp., Bedford, MA), and stored at -80°C .

Culture of Mast Cells. Bone marrow cells were cultured at 10^6 /ml in α medium supplemented with 10^{-4} M 2-mercaptoethanol, 20% horse serum (Sterile Systems, Inc., Logan, UT) and 10% PWM-SCM. Culture flasks (Nunc, Roskilde, Denmark) were incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Half of the medium was replaced every 7 d. The cells were washed and resuspended in Eagle's medium for injection in vivo.

Separation of Peritoneal Mast Cells. The method has been described in detail (40). Briefly, most mouse peritoneal cells are macrophages and small lymphocytes. Macrophages were removed with a magnet after being permitted to ingest carbonyl iron (GAF Co., New York). Small lymphocytes and mast cells then were separated by Percoll density gradient centrifugation. After centrifugation, mast cells (30–50% pure) were washed and resuspended in Eagle's medium for injection in vivo.

Cell Injection. Cultured or peritoneal mast cells were injected into the skin, peritoneal cavity, or tail vein of WBB6F₁-W/W^v mice. To facilitate direct injections of cells into the skin, an area of dorsal skin was shaved with hair clippers. Cells (in 0.05 ml Eagle's medium per site) were injected with a tuberculin syringe. Each mouse received six injections, which were marked by mixing India ink with the suspension medium. Intraperitoneal and intravenous injections were done with injection volumes of 0.5 and 0.2 ml, respectively.

Limiting Dilution Analysis of Mast Cell Precursors. The principle of the method has

been described in detail (41). WBB6F₁-W/W^v mice were killed 5 wk after the direct injection of cells into the skin. The dorsal skin was reflected; each injection site that could be identified by the presence of ink was removed in a 1.5 × 1 cm piece of skin, which then was attached to a piece of thick filter paper to keep it flat, and fixed in Carnoy's fluid (42). Skin pieces were embedded in paraffin; 25-μm-thick serial sections were cut parallel to the skin surface from the subcutaneous tissue to the epithelium, and the sections were stained with alcian blue-safranin (42). All of the serial sections were examined under the microscope to detect mast cell clusters, defined as a group of mast cells numbering >100 in its largest cross sections. When at least one mast cell cluster was identified, we assumed that the injected cell suspension had contained mast cell precursor(s). The concentration of mast cell precursors was calculated from the proportion of injection sites at which no clusters appeared (defined as the "proportion of nonappearance") for various cell doses by limiting dilution analysis as described by Finney (43), Porter and Berry (44), and Breivik (45).

Assay of Spleen Colony-forming Units (CFU-S). The method of Till and McCulloch was used to determine the number of CFU-S (46). Cultured or peritoneal cells of WBB6F₁-+/+ mouse origin were injected intravenously into syngeneic mice irradiated (850 rad) as previously described (40). The mice were killed 8 d after cell injection; spleens were removed and fixed in Bouin's solution, and colonies were counted at a magnification of 7× with a dissecting microscope. Each experiment contained at least 10 mice.

Evaluation of Mast Cell Development, Morphology, and Histochemistry. Mice were killed at various intervals after intraperitoneal or intravenous injection of cells. Peritoneal cells were harvested as described above, and were spun in the cytocentrifuge. A piece of dorsal skin, and the spleen and the stomach were removed. The cytocentrifuge preparations and the tissues were fixed in Carnoy's fluid. Paraffin sections (10 μm) of tissues were made by routine methods and were stained with alcian blue-safranin. Mast cells in the skin, spleen, and glandular stomach were counted as described previously to determine whether successful transfer and development of mast cells had occurred (32, 47). ~10⁵ cells were examined in cytocentrifuge preparations of peritoneal cells after staining with alcian blue-safranin. We judged that successful transfer of mast cells to the peritoneal cavity had occurred if mast cells represented >0.1% of the peritoneal cells.

Mast Cell Staining with Berberine Sulfate. In most experiments, representative cytocentrifuge preparations and tissue specimens were stained with the fluorescent dye berberine sulfate (Sigma Chemical Co.), as described by Enerbäck (48), and were examined in an epifluorescence microscope (Olympus Corp., Tokyo). Under these conditions of fixation and staining, the cationic dye selectively binds to phosphate- and/or sulfate-containing polyanions, including DNA and heparin (48). However, staining of DNA and heparin are distinguishable because heparin-bound berberine sulfate exhibits rapid fading upon continuous illumination whereas DNA-bound dye slowly increases in fluorescence intensity (48). Furthermore, when heparin-containing mast cells are examined, the cytoplasmic granules exhibit bright yellow fluorescence whereas the nucleus appears a dull green (48). Dimlich et al. (49) demonstrated that berberine sulfate staining of tissue sections of rats or mice at pH 4.0 identified connective tissue type mast cells, such as those in the skin. By contrast, berberine sulfate did not stain mast cells in the gastric mucosa (49).

Even though bright cytoplasmic staining with berberine sulfate at pH 4.0 appears to be highly selective for heparin-containing mast cells, we provided further evidence that the dye was identifying heparin in our preparations, by treating some specimens either with heparinase (heparin lyase from *Flavobacterium heparinum*; Seikagaku Chemical Industry Co. Ltd., Tokyo) or with chondroitinase ABC (chondroitin ABC lyase from *Proteus vulgaris*; Seikagaku Chemical Industry) before staining. For treatment with enzymes, specimens were fixed in chilled calcium acetate formalin at 4°C for 18 h according to Yamada (50) and overlaid with Tris-HCl buffer (0.1 M) containing either heparinase (0.5 IU/ml, pH 7.0) or chondroitinase ABC (5.0 IU/ml, pH 8.0). After washing away the enzyme solutions with Tris-HCl buffer of the same pH, specimens were stained with berberine sulfate. In control experiments, treatment with heparinase for 30 min abolished the berberine sulfate-dependent fluorescence of granules of freshly isolated peritoneal

mast cells, whereas treatment with chondroitinase ABC for 4 h did not. Treatment of specimens with either of the buffer solutions (pH 7.0 or 8.0) alone had no effect on the berberine sulfate staining.

In some cases, mast cells were counted after staining with berberine sulfate, and then the same specimens were stained with alcian blue; mast cells in the same optical field were counted again, and the ratio of berberine sulfate-positive mast cells to alcian blue-positive mast cells was calculated.

Electron Microscopy. Cultured cells or peritoneal cells were centrifuged at 500 g for 10 min. The cell pellets were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 45 min, and were washed with 0.1 M cacodylate buffer containing 4.5% sucrose. The cells were postfixed with 1% osmium tetroxide in veronal acetate buffer (pH 7.4) for 1 h at 4°C, dehydrated in a graded series of ethanol followed by acetone, and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD), stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope.

Histamine Content. Prior to assay, small aliquots of the cultured or peritoneal cells were taken to determine the number of mast cells present; the remaining cells were stored at -80°C. Histamine concentration was assayed by high performance liquid chromatography coupled with fluorometry as described by Yamatodani et al. (51), and the content of histamine per 10⁶ mast cells was calculated.

Results

Development of Mast Cells and Mast Cell Precursors in Suspension Culture

Bone marrow cells of WBB6F₁-+/+ mice were cultured in the presence of 10% PWM-SCM. Aliquots of the cultured cells were harvested when half of the medium was changed and the concentrations of CFU-S, mast cell precursors, and morphologically identifiable mast cells were determined. The number of CFU-S decreased exponentially, and none were detectable 3 wk after initiation of the culture (Fig. 1). By contrast, the numbers of mast cell precursors and morphologically identifiable mast cells increased rapidly. 4 wk after initiation of the culture, ~2% of the cultured cells had the ability to form mast cell clusters in the skin of WBB6F₁-W/W^v mice, and >95% of the cultured cells were identified as immature mast cells (Fig. 1). The immature mast cells stained with alcian blue, but with neither safranin or with berberine sulfate (Fig. 2, Table I).

To determine the concentrations of mast cell precursors reported in Fig. 1, WBB6F₁-W/W^v recipient mice were killed 5 wk after the direct cutaneous injection of cells harvested 4 wk after the initiation of culture. In one experiment, WBB6F₁-W/W^v mice were killed 10 wk after the injection of cultured mast cells. Mast cell clusters persisted at the skin injection sites in these mice, and most of the mast cells in the clusters stained with berberine sulfate. Some of these mast cells also stained with safranin.

Intraperitoneal Injection of Cultured Mast Cells

Characteristics of mast cells recovered from the peritoneal cavity. Cultured mast cells of WBB6F₁-+/+ mouse origin were harvested 4 wk after the initiation of the culture and 10⁶ cells were injected into the peritoneal cavity of each WBB6F₁-W/W^v mouse. Peritoneal cells of the recipient mice were recovered at various times for preparation of cytocentrifuge specimens or for electron microscopy. Mast cells that stained with berberine sulfate were observed 3 wk after intraperitoneal injection and, by 10 wk, accounted for ~90% of the recovered cells

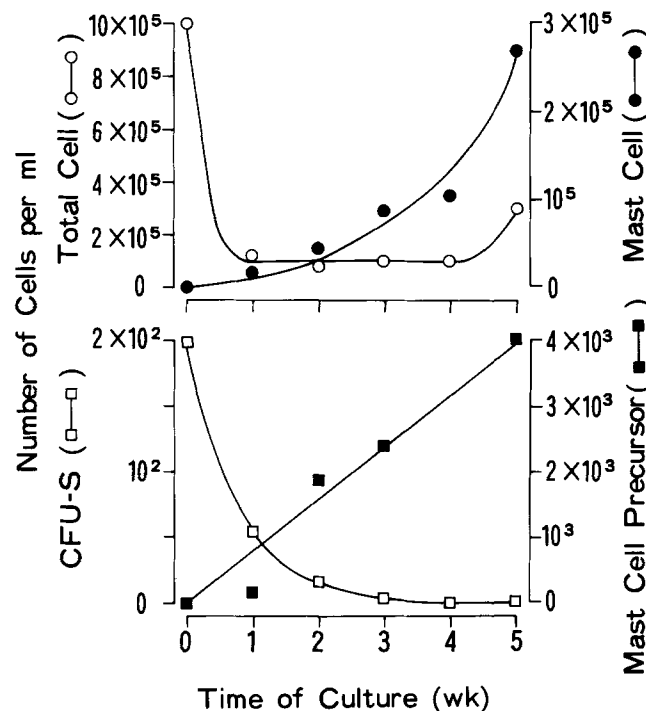


FIGURE 1. Concentrations of total cells, morphologically identifiable mast cells, CFU-S, and mast cell precursors, at various times after the initiation of suspension culture of WBB6F₁-/+ mouse bone marrow cells (10^6 /ml) in 10% PWM-SCM. Cells were fed by replacement of half of the medium every week; cell concentrations were determined at that time.

(Table I, Fig. 2). Berberine sulfate staining of the recovered mast cells was prevented by first incubating the cells with 0.5 IU/ml of heparinase for 30 min (Fig. 2). By contrast, incubation of the cells with the heparinase buffer solution, or with 5 IU/ml of chondroitinase ABC for 4 h, did not significantly affect the intensity of berberine sulfate staining (Fig. 2).

In addition to acquiring the ability to stain with berberine sulfate, the recovered cells exhibited other alterations in phenotype when compared with cultured mast cells studied before adoptive transfer. Thus, mast cells studied 10 wk after intraperitoneal injection stained with safranin and exhibited ultrastructural features similar to those of mature peritoneal mast cells recovered from normal WBB6F₁-/+ mice (Fig. 2). Moreover, mast cell histamine content progressively increased after intraperitoneal injection (Table I).

Two experiments were done to exclude the possibility that intraperitoneal injection of cultured mast cells might induce the development of mast cells of WBB6F₁-W/W^v (recipient) origin. First, we injected cultured mast cells (10^6) of C57BL/6-*gb^J/gb^J* mouse origin into the peritoneal cavity of WBB6F₁-W/W^v mice. 10 wk after injection, most of the mast cells stained with berberine sulfate and many of them stained with safranin. All of the recovered mast cells exhibited the giant cytoplasmic granules characteristic of the *gb^J/gb^J* mutation.

For the second experiment, bone marrow cells of either WBB6F₁-W/W^v or

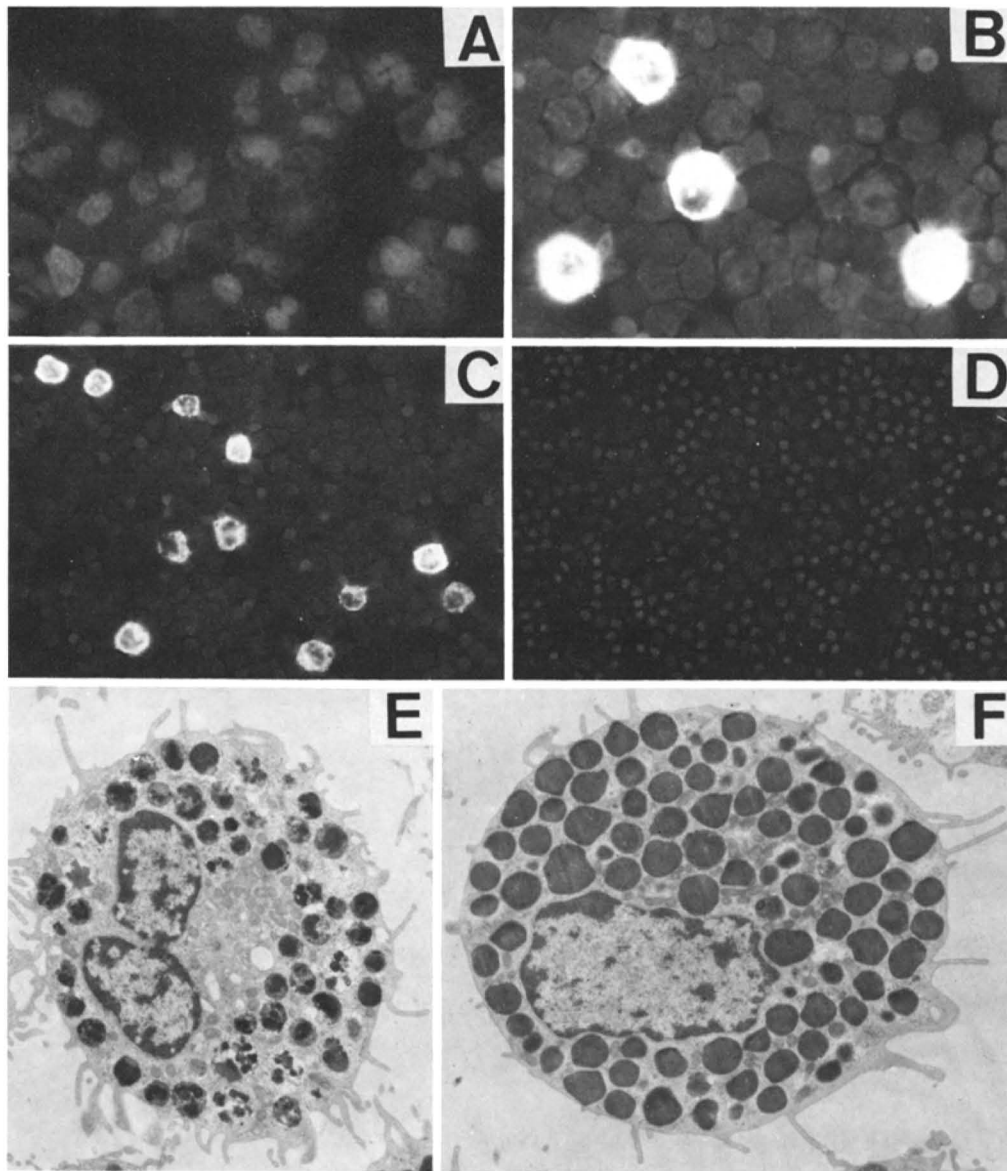


FIGURE 2. (A–D) Cytocentrifuge preparations stained with berberine sulfate. (A) Cultured mast cells 4 wk after initiation of culture; only nuclei showed faint fluorescence. $\times 400$. (B) Peritoneal cells recovered from a WBB6F₁-W/W^v mouse 10 wk after the intraperitoneal injection of 10^6 cultured mast cells of WBB6F₁-+/+ mouse origin. The cytoplasm of the mast cells exhibited intense fluorescence. $\times 400$. (C) A specimen made from the same cell suspension shown in B, but treated with chondroitinase ABC before staining. Bright cytoplasmic fluorescence of mast cells was retained. $\times 200$. (D) A specimen made from the same cell suspension shown in B and C, but treated with heparinase before staining. Heparinase treatment abolished staining of mast cells by berberine sulfate. $\times 200$. (E) The ultrastructure of a cultured mast cell 4 wk after initiation of the culture. The cytoplasmic granules exhibited heterogeneous content. $\times 5,800$. (F) Ultrastructure of a mast cell recovered from the peritoneal cavity of a WBB6F₁-W/W^v mouse 10 wk after intraperitoneal injection of cultured mast cells. The cytoplasmic granules appear uniformly electron dense. $\times 5,800$.

TABLE I
Proportion of Berberine Sulfate-positive Cells and Histamine Content of Mast Cells Recovered from the Peritoneal Cavity of WBB6F₁-W/W^v Mice After Intraperitoneal Injection of 10⁶ Cultured Mast Cells of WBB6F₁-+/+ Mouse Origin

Type of cells	Berberine sulfate-positive cells as a percent of alcian blue-positive cells*	Histamine content of peritoneal cells* (ng/10 ⁶ mast cells)
Cultured mast cells		
Before i.p. injection	<0.1 [‡] (7)	120 ± 9 (4)
1 wk after	<0.1 [‡] (7)	430 ± 100 (4)
3 wk after	5 ± 1 (6) [§]	470 ± 130 (7)
5 wk after	60 ± 11 (5) [§]	1,540 ± 310 (6) [§]
10 wk after	93 ± 3 (10) [§]	2,730 ± 140 (8) [§]
Peritoneal mast cells of WBB6F ₁ -+/+ mice	98 ± 5 (5)	13,800 ± 2,100 (7) [§]

* Mean ± SE; number of mice is shown in parenthesis.

[‡] No berberine sulfate-positive cells seen in >1,000 alcian blue-positive cells.

[§] $P < 0.01$, when compared with the value listed above by t test.

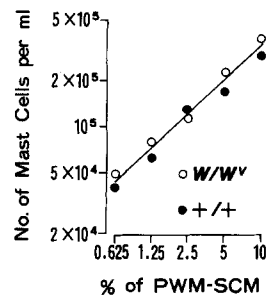


FIGURE 3. Comparison of in vitro growth of cultured mast cells of WBB6F₁-W/W^v or WBB6F₁-+/+ mouse origin. Cultured mast cells were harvested 4 wk after initiation of the original cultures, which had been maintained in medium supplemented with 10% PWM-SCM. The W/W^v or +/+ cells were then incubated (3.0×10^4 cells/ml) with various concentrations of PWM-SCM for 1 wk, after which the number of mast cells was determined.

WBB6F₁-+/+ mice were cultured in the presence of PWM-SCM. As was reported by Yung and Moore (7), mast cells developed in both cultures. Like WBB6F₁-+/+ cells, cultured mast cells of WBB6F₁-W/W^v origin stained with alcian blue but with neither safranin or berberine sulfate. 4 wk after the initiation of culture, we compared the growth response of the two populations by placing the cells in various concentrations of PWM-SCM. No significant differences between the responses of WBB6F₁-W/W^v and -+/+ cells were observed (Fig. 3). Although the experiment shown in Fig. 3 used PWM-SCM prepared with WBB6F₁-+/+ cells, an experiment using PWM-SCM prepared with WBB6F₁-W/W^v cells gave similar results (data not shown).

In striking contrast to the results obtained in vitro, marked differences in the behavior of WBB6F₁-W/W^v and -+/+ cells were observed when the cultured mast cells were injected in vivo (Table II). Indeed, no mast cells of WBB6F₁-W/

TABLE II
*Disappearance of Cultured Mast Cells of WBB6F₁-W/W^v Mouse
 Origin After Transplantation into the Peritoneal Cavity of
 WBB6F₁-W/W^v Mice*

Weeks after injection	Genotype of cultured cells	No. of recipients	No. of mast cells per 10 ⁵ peritoneal nucleated cells*
1	+/+	4	23.2 ± 6.9
	W/W ^v	6	2.5 ± 0.6 [‡]
3	+/+	6	26.3 ± 3.5
	W/W ^v	7	0.6 ± 0.3 [‡]
5	+/+	6	22.3 ± 2.7
	W/W ^v	7	<0.1 [‡]
10	+/+	4	27.5 ± 2.8
	W/W ^v	7	<0.1 [‡]

Cultured mast cells were injected intraperitoneally into WBB6F₁-W/W^v recipients (10⁶ cells per mouse) 4 wk after the initiation of the cell cultures.

* The results are shown as the mean ± SE.

[‡] $P < 0.01$, when compared with the value for +/+ mice by *t* test.

W^v origin were detectable 3 wk after intraperitoneal injection, and no mast cells that stained with safranin or berberine sulfate were detected at any intervals.

Tissue Distribution and Characteristics of Mast Cells Recovered after Intraperitoneal Injection of Cultured or Peritoneal Mast Cells

To determine whether mast cells injected intraperitoneally might migrate to other anatomical sites, we injected cultured mast cells of WBB6F₁-+/+ mouse origin into the peritoneal cavity of WBB6F₁-W/W^v mice, and searched for mast cells in various tissues of these mice 10 wk later. Partially purified (30–50% pure) peritoneal mast cells, which, like our preparations of cultured mast cells, contained no detectable CFU-S (data not shown), were injected for comparison. Injection of either cultured or peritoneal mast cells resulted in the development of mast cells in the peritoneal cavity, spleen, skin, and glandular stomach (Table III). Mast cells were detected more frequently in the muscularis propria; most of them were situated in the outer layer of the muscle.

Most mast cells in the peritoneal cavity, spleen, skin, and muscularis propria of the stomach were stained with berberine sulfate, and some of them stained with safranin. By contrast, when mast cells were observed in the stomach mucosa, they stained only with alcian blue. In occasional sections, mast cells were observed in both the muscularis propria and the mucosa. In these stomachs, berberine sulfate- or safranin-positive mast cells were observed only in the muscularis.

Tissue Distribution and Characteristics of Mast Cells Observed After Intravenous Injection of Cultured or Peritoneal Mast Cells

10 wk after intravenous injection of cultured or peritoneal mast cells of WBB6F₁-+/+ origin, mast cells were observed in the peritoneal cavity, spleen, skin, and glandular stomach of recipient WBB6F₁-W/W^v mice (Table IV). As with intraperitoneal injections of cells, most of the mast cells observed in

TABLE III
Appearance of Mast Cells in WBB6F₁-W/W^v Mice 10 Wk After Intraperitoneal Injection of Cultured or Peritoneal Mast Cells of WBB6F₁-+/+ Mouse Origin

Type of mast cells	No. of cells	Proportion of cases in which mast cells appeared				
		Peritoneal cavity	Spleen	Skin	Glandular stomach	
					Muscle	Mucosa
Cultured*	10 ²	0/5	0/5	0/5	0/5	0/5
	10 ³	2/5	0/5	0/5	3/5	0/5
	10 ⁴	5/6	0/6	0/6	5/6	2/6
	10 ⁵	6/6	1/6	1/6	5/6	4/6
Peritoneal†	10 ³	2/10	0/10	0/10	0/10	0/10
	10 ⁴	6/9	0/9	3/9	4/9	1/9
	10 ⁵	10/10	10/10	3/10	9/10	5/10

* Cultured mast cells were harvested 4 wk after the initiation of the culture.

† Peritoneal mast cells were partially purified (30–50%); the number shown in the table refers to the number of mast cells injected.

TABLE IV
Appearance of Mast Cells in WBB6F₁-W/W^v Mice 10 Wk After Intravenous Injection of Cultured or Peritoneal Mast Cells of WBB6F₁-+/+ Mouse Origin

Type of mast cells	No. of cells	Proportion of cases in which mast cells appeared				
		Peritoneal cavity	Spleen	Skin	Glandular stomach	
					Muscle	Mucosa
Cultured*	10 ³	0/6	1/6	0/6	0/6	0/6
	10 ⁴	0/5	1/5	0/5	0/5	0/5
	10 ⁵	0/6	5/6	0/6	1/6	1/6
	10 ⁶	1/6	6/6	2/6	5/6	5/6
Peritoneal†	10 ³	0/8	3/8	0/8	0/8	0/8
	10 ⁴	0/8	6/8	0/8	1/8	1/8
	10 ⁵	6/6	6/6	0/6	5/6	5/6

* Cultured mast cells were harvested 4 wk after the initiation of the culture.

† Peritoneal mast cells were partially purified (30–50%); the number shown in the table refers to the number of mast cells injected.

connective tissue sites stained with berberine sulfate and some of them stained with safranin. But in the glandular stomach, berberin sulfate- or safranin-positive mast cells were observed only in the muscularis propria; all mast cells in the mucosa stained only with alcian blue (Fig. 4). Intravenous injection of cells resulted in the more frequent development of mast cells in the spleen than in the peritoneal cavity or stomach (Table IV). The opposite was true when cells were injected intraperitoneally (Table III). Intravenous injection of cultured or peritoneal mast cells had no measurable effect on the macrocytic anemia of the recipient WBB6F₁-W/W^v mice (data not shown).

Discussion

The mast cells that developed in suspension cultures of mouse bone marrow cells supplemented with PWM-SCM resembled the growth factor-dependent

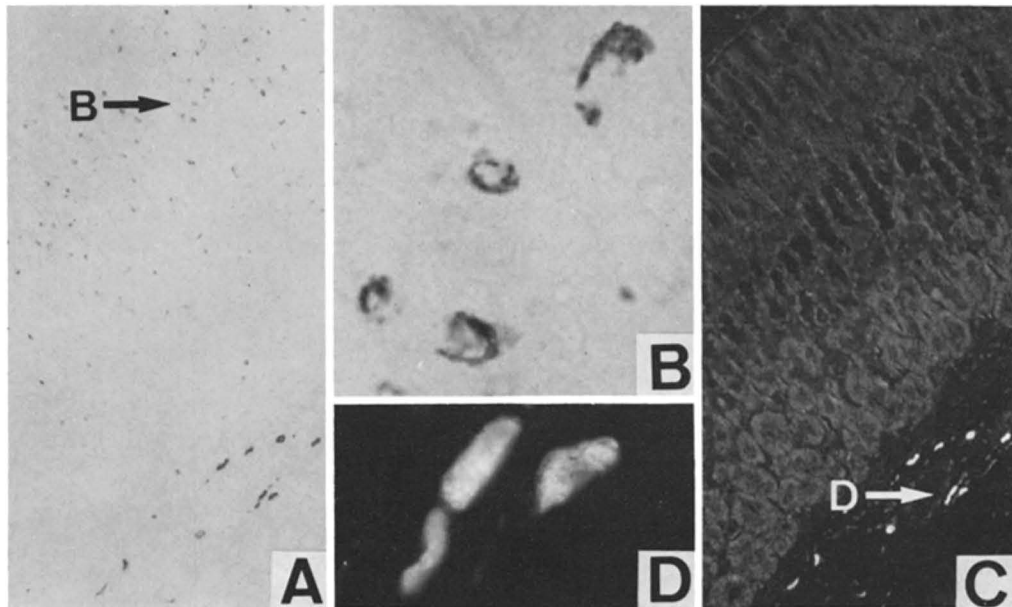


FIGURE 4. Glandular stomach of a WBB6F₁-W/W^v mouse fixed 10 wk after the intravenous injection of 10⁶ cultured mast cells of WBB6F₁-/+ mouse origin. The cultured mast cells had been harvested for injection 4 wk after initiation of the culture. (A) Stained with alcian blue-safranin. Many mast cells stained with alcian blue were observed in the mucosa. The area indicated by the arrow is shown in higher magnification in B. (C) The same section stained with berberine sulfate; only mast cells in the muscle layer showed bright cytoplasmic fluorescence. The area indicated by the arrow is shown in higher magnification D. (A and C) $\times 100$. (B and D) $\times 1,000$.

mast cells characterized in the laboratory of S. J. Galli (1, 2, 15) and by several other investigators (5–7, 10, 12–14, 16–18, 39, 52). The cells had the ultrastructural features of immature mast cells, contained low levels of histamine, and synthesized cytoplasmic granules that stained with alcian blue but with neither safranin or with berberine sulfate. We found that, as the number of morphologically identifiable mast cells in the cultures increased, the number of cells capable of giving rise to mast cell clusters when injected into the skin of WBB6F₁-W/W^v mice increased roughly in parallel. Such mast cell precursors represented $\sim 1\%$ of the cells present 4–5 wk after the initiation of culture. By contrast, the number of CFU-S decreased exponentially, and none were detectable after 3 wk of culture.

Mast cell clusters were identifiable in the skin of WBB6F₁-W/W^v mice examined even 10 wk after intracutaneous injection of the cultured cells. Some of these mast cells stained with safranin and most of them stained with berberine sulfate, a pattern mimicking that of normal cutaneous mast cells of the connective tissue-type (21, 48, 49). By contrast, Crapper et al. (29) found that cultured mast cells derived from B10.D2 or C57BL/6J-*bg^J/bg^J* mice did not survive >2 wk after injection into the skin of B10D2F₁-W^f/W^f mice. If the recipient mice bore a subcutaneous inoculum of the IL-3-producing WEHI-3B tumor, mast cells sur-

vived in the skin for 4 wk after injection (29). But these mast cells stained only with alcian blue; no safranin-positive mast cells were observed.

The discrepancy between our results and those of Crapper et al. (29) may be attributable to differences in the host animals. The mast cell deficiency of B10D2F₁-W^f/W^f mice is not as severe as that of WBB6F₁-W/W^v mice (29). We found that, when mast cell precursors of C57BL/6-*bg*^J/*bg*^J mouse origin were injected into the skin of different mice doubly heterozygous for mutations at the *W* locus (e.g., WBB6F₁-Wⁿ/W^f, -Wⁿ/W^h, and -W/W^v), the frequency of development and size of mast cell clusters were inversely proportional to the concentration of mast cells in the skin of the host mice (unpublished data). Whatever the explanation for the differences between our findings and those of Crapper et al. (29), our data indicate that exogenous sources of IL-3 or other growth factors are not required for the survival of cultured WBB6F₁-+/+ mast cells in WBB6F₁-W/W^v mice. In addition, our histochemical findings indicate that cultured mast cells injected directly into the skin of WBB6F₁-W/W^v mice acquire at least some of the characteristics of mature CTMC.

An analogous result was obtained when cultured mast cells were injected into the peritoneal cavity of WBB6F₁-W/W^v mice. Many of the mast cells recovered 5 wk after intraperitoneal injection of cultured cells were safranin and berberine positive; by 10 wk, ~90% of the mast cells stained with berberine sulfate. In addition, the histamine content of the recovered mast cells was substantially increased compared with that of cells taken directly from culture, and the recovered cells closely resembled normal mature peritoneal mast cells by ultrastructure (53).

Two lines of evidence indicate that the mast cells recovered from the peritoneal cavity of recipient WBB6F₁-W/W^v mice were derived from the injected cultured cells. First, when the experiment was performed using cultured mast cells of C57BL/6-*bg*^J/*bg*^J origin, the mast cells recovered 10 wk later contained the giant cytoplasmic granules characteristic of the beige mutation. Second, when cultured mast cells derived from WBB6F₁-W/W^v mice were injected, identifiable mast cells were no longer present 5 wk after intraperitoneal injection. The latter finding argues against the notion that the mast cell recovered from the peritoneal cavity of WBB6F₁-W/W^v mice injected with cultured mast cells of WBB6F₁-+/+ origin, were of host origin.

The differences in the behavior of WBB6F₁-W/W^v and -+/+ mast cells after injection into WBB6F₁-W/W^v mice are of interest. Even though cultured mast cells derived from WBB6F₁-W/W^v or, -+/+ bone marrow cells gave similar responses to growth factors present in PWM-SCM when tested 4 wk after the initiation of culture, the WBB6F₁-W/W^v mast cells could not survive as well as WBB6F₁-+/+ cells after injection in vivo. This result may reflect a defect in the ability of cultured mast cells of WBB6F₁-W/W^v origin to respond to growth or differentiation factors present in vivo but not in vitro. On the other hand, Yung and Moore (7) reported that cultured mast cells of WBB6F₁-W/W^v origin survive in culture less well than control cells of WBB6F₁-W/+ origin, with differences between these populations first evident at about 5 wk of culture. This result raises the possibility that the disappearance of WBB6F₁-W/W^v cells in vivo may

have occurred as a result of an intrinsic cellular defect that can also be expressed *in vitro*.

The phenotype of mast cells developing in the tissues of recipient mice after intravenous or intraperitoneal transfer of cultured cells varied predictably according to anatomical site. Many mast cells in the skin, spleen, or peritoneal cavity of recipient WBB6F₁-W/W^v mice stained with berberine sulfate, and some of them stained with safranin. These cells thus resembled by histochemistry the resident populations of CTMC observed at these sites in normal +/+ mice. The same was true when the histochemistry of mast cells developing in the stomachs of recipient WBB6F₁-W/W^v mice was considered. Like the mast cells in the mucosa of the glandular stomach of +/+ mice, the mast cells that developed in the gastric mucosa of recipient WBB6F₁-W/W^v mice stained only with alcian blue; none of the cells stained with either safranin or berberine sulfate. By contrast, many of the mast cells developing in the glandular stomach muscularis propria of recipient WBB6F₁-W/W^v mice stained with safranin and with berberine sulfate.

Enerbäck (48) and Dimlich et al. (49) demonstrated that berberine sulfate staining is highly specific for heparin-containing mast cells of the connective tissue type. We confirmed the specificity of the reaction in our material by demonstrating that the staining was prevented by prior treatment of the cells with heparinase but not chondroitinase ABC. These findings strongly suggest that cultured mast cells or their progeny acquire the ability to synthesize and store heparin when they are transplanted to appropriate anatomical locations *in vivo*. This represents an important observation, since under usual conditions of suspension culture (2, 10, 17, 19), or even when exposed to the inducing agent sodium butyrate (2), mouse mast cells synthesize chondroitin sulfates rather than heparin. It should be acknowledged, however, that histochemistry represents an indirect approach to the identification of cellular constituents. It therefore will be important to use direct biochemical methods to confirm the presence of heparin in the mast cells. Evaluations of other aspects of the phenotype of the recovered cells, such as expression of membrane structures characteristic of cultured as opposed to normal peritoneal mast cells (10, 54) will also be of interest.

Taken together, our histochemical and ultrastructural evidence indicates that, after injection into WBB6F₁-W/W^v recipients, cultured mast cells and/or their progeny are able to acquire characteristics of either CTMC or MMC. Whether such phenotypic plasticity is a property of all cultured mast cells, or only a subpopulation, remains to be determined. Whatever the answer to this question may be, our observations argue against the notion that cultured mouse mast cell populations maintained under ordinary *in vitro* conditions are irrevocably committed to the MMC phenotype.

Several mechanisms might account for the expression of the two murine mast cell phenotypic patterns recognized in our experiments. One possibility is that the cultured cells (and immature mast cells *in vivo*) contain or can generate two distinct committed lineages, one of which gives rise to cells with the phenotype of MMC, the other of which gives rise to CTMC. Certain complex culture systems containing embryonic fibroblasts permit the expression of two mast cell

phenotypic patterns *in vitro* (55). However, if commitment to two separate mast cell lineages occurred in our experiments, it probably happened *in vivo*. First, cultured mast cell populations, even when not cloned, for the most part exhibit remarkably uniform characteristics (reviewed in 15). Expression of type II major histocompatibility complex (MHC) products may vary, but probably in response to differences in levels of soluble factors such as γ -interferon (2, 5, 15, 56). Second, in a recent collaborative study with Dr. T. Nakahata and his colleagues at Shinshu University, Matsumoto, Japan, we injected cloned mast cells derived from individual methylcellulose colonies into both the skin and the gastric mucosa of WBB6F₁-W/W^v mice (manuscript in preparation). The mast cells that developed in the skin expressed the histochemical characteristics of CTMC, whereas the mast cells developing in the gastric mucosa stained only with alcian blue. This result indicated that each clone contained cells capable of generating both mast cell phenotypes.

However, the occurrence of phenotypically distinct mast cell populations does not necessarily require the existence of distinct mast cell lineages. For example, a single lineage might give rise to populations whose phenotype varied according to such factors as stage of differentiation/maturation, anatomical location, or state of functional activity (15, 57). Our investigation of the development of mast cells after intraperitoneal or intravenous injection of representative, freshly isolated CTMC suggests that the phenotypic pattern ordinarily expressed by an individual mast cell population *in vivo* can be altered if those cells are transferred to a different anatomical location.

After intravenous or intraperitoneal injection of partially purified (30–50% pure) peritoneal mast cells, we observed mast cells in both the connective tissues and gastric mucosa of WBB6F₁-W/W^v recipients. Remarkably, the mast cells in the gastric mucosa stained only with alcian blue. The CFU-S represents a precursor of the mast cell lineage (32, 47), but normal mouse peritoneal mast cells can exhibit extensive proliferative potential when injected intracutaneously into WBB6F₁-W/W^v mice (40). Taken together, these observations suggest that the alcian blue–positive mast cells in the gastric mucosa were actually derived from the injected peritoneal mast cells, and raises the possibility that, under certain circumstances, CTMC give rise to cells with at least some of the features of MMC. The extent of alteration of peritoneal mast cell phenotype associated with location of the cells in the gastric mucosa, and the mechanisms regulating such changes, are currently under investigation.

In addition to their relevance to the controversy about mast cell subsets, our observations suggest a new approach to the analysis of mast cell function. Mast cell–deficient mice represent a useful model for the analysis of mast cell biology *in vivo*. However, mast cell–deficient mice and their normal +/+ littermates differ in characteristics other than the number of mast cells: mast cell–deficient mice of W/W^v genotype are anemic, sterile, and lack cutaneous melanocytes (31). Adoptive transfer of WBB6F₁-+/+ bone marrow into WBB6F₁-W/W^v mice produces an animal (+/+ → W/W^v) whose mast cell defect is largely corrected, but whose anemia is cured as well (31, 32). Under certain circumstances, it may be difficult to determine whether an alteration of a particular biological response in +/+ → W/W^v mice reflects the reconstitution of mast cells, normalization of

the hematocrit, or some other effect of transplantation of unfractionated bone marrow cells. However, cultured mast cell populations exhibit much more stringent lineage commitment than do normal bone marrow cells. When tested after several weeks in vitro they contained undetectable levels of CFU-S (present study) or erythroid burst-forming units (BFU-E) (H. Steinberg and S. J. Galli, unpublished data). Furthermore, injection of cultured mast cells into WBB6F₁-W/W^v mice partially reconstituted mast cell populations without having any measurable effect on the recipient animals' macrocytic anemia. Transplantation of cultured mast cells into WBB6F₁-W/W^v mice thus may represent an attractive strategy for selectively reconstituting the mast cell compartment of these animals.

Summary

Both connective tissue mast cells and mast cells grown in vitro are derived from multipotential hematopoietic stem cells, but these two mast cell populations exhibit many differences in morphology, biochemistry, and function. We investigated whether the phenotype of cultured mast cells or their progeny was altered when the cells were transferred into different locations in vivo. Cultured mast cells were immature by ultrastructure, and stained with alcian blue but with neither safranin or berberine sulfate, a fluorescent dye that binds to the heparin of connective tissue mast cell granules. By contrast, mast cells recovered from the peritoneal cavity of congenitally mast cell-deficient (WB × C57BL/6)F₁-W/W^v (WBB6F₁-W/W^v) mice 10 wk after intraperitoneal injection of cultured WBB6F₁-+/+ or C57BL/6-*bg^J/bg^J* mast cells stained with both safranin and berberine sulfate. Staining with berberine sulfate was prevented by treatment of the cells with heparinase but not chondroitinase ABC, suggesting that the adoptively transferred mast cell population had acquired the ability to synthesize and store heparin. Furthermore, the recovered mast cells were indistinguishable by ultrastructure from the normal mature peritoneal mast cells of WBB6F₁-+/+ mice, and contained substantially more histamine than mast cells studied directly from culture.

Intravenous injection of cultured mast cells resulted in the development of safranin- and berberine sulfate-positive mast cells in the peritoneal cavity, spleen, skin, and glandular stomach muscularis propria. Mast cells also developed on the glandular stomach mucosa, but these cells stained with alcian blue rather than safranin, and did not stain with berberine sulfate. This result suggests that cultured mast cells can give rise to mast cells of either the connective tissue type or mucosal phenotype, depending on anatomical location. Furthermore, transplantation of cultured mast cells into WBB6F₁-W/W^v mice had no measurable effect on the anemia of the recipient mice, suggesting a possible strategy for repairing the mast cell deficiency of WBB6F₁-W/W^v mice without affecting other bone marrow-derived populations such as erythrocytes.

Intravenous injection of representative connective tissue type mast cells (30–50% pure peritoneal mast cells derived from WBB6F₁-+/+ mice) gave results similar to those obtained with cultured mast cells: mast cells developing in the peritoneal cavity, skin, spleen, and glandular stomach muscularis propria of WBB6F₁-W/W^v recipients stained with safranin and berberine sulfate, whereas mast cells developing in the mucosa of the glandular stomach stained only with

alcian blue. This result offers further support to the notion that mast cell phenotype may be regulated, at least in part, by the tissue microenvironment.

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