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In vitro duplication and *in vivo* cure of mast-cell deficiency of *Sl/Sl*^{d} mutant mice by cloned 3T3 fibroblasts

(cell differentiation/cell-to-cell interaction/*W/W*^{v} mouse/interleukin 3/interleukin 4)

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ABSTRACT ***Sl/Sl*^{d} mutant mice are profoundly deficient in tissue mast cells as a result of a defect in the microenvironment promoting the development of these cells. To facilitate the analysis of the *Sl* mutation, we attempted to establish an *in vitro* system in which the *in vivo* defect of *Sl/Sl*^{d} mice could be reproduced. 3T3 cell lines were established from 17-day-old embryos of *Sl/Sl*^{d} and congenic +/+ genotypes and were cocultured with mast cells obtained *in vitro* from the bone marrow of +/+ mice. All eight 3T3 cell lines derived from +/+ embryos supported mast cells for >2 wk in the absence of T-cell-derived growth factors. By contrast, none of eight 3T3 cell lines from *Sl/Sl*^{d} embryos supported mast cells under similar conditions. The defect in *Sl/Sl*^{d} 3T3 cells was further characterized as a failure to induce the G_1-to-S transition in synchronized mast cells upon contact, suggesting that the *Sl* gene product is indispensable for this activity. When 3T3 cells of +/+ genotype, grown on pieces of cellulose acetate membrane, were transplanted into the peritoneal cavity of *Sl/Sl*^{d} mice, mast cells appeared locally in the transplanted 3T3 cell layers. These results suggested an essential role of fibroblasts *in vivo* as the tissue microenvironment promoting the development of mast cells and that they are defective in *Sl/Sl*^{d} mice. The present coculture system duplicated mast-cell deficiency of *Sl/Sl*^{d} mice *in vitro* and should prove useful for analysis of the *Sl* gene product.**

A double gene dose of mutant alleles at either the *W* locus (chromosome 5) or the *Sl* locus (chromosome 10) produces the pleiotropic effects of sterility, hypoplastic anemia, and depletion of melanocytes and mast cells (for reviews, see refs. 1 and 2). Although there is extensive similarity in the phenotypic expression of *W/W*^{v} and *Sl/Sl*^{d} mice, the underlying mechanisms are different. Bone marrow transplantation from congenic +/+ mice cures both the mast-cell deficiency and the anemia of *W/W*^{v} mice but not those of *Sl/Sl*^{d} mice (3–5). When skin pieces of either *W/W*^{v} or *Sl/Sl*^{d} mice are grafted onto the back of congenic +/+ mice, mast cells appear in the grafted skin of *W/W*^{v} mice but not in the skin of *Sl/Sl*^{d} mice (5, 6). These findings indicate that the mast-cell deficiency of *W/W*^{v} mice is due to an abnormality in bone-marrow-derived mast-cell precursors, whereas that of *Sl/Sl*^{d} mice reflects an abnormality in the microenvironment promoting mast cell differentiation *in situ*. However, at present, we do not know either the precise mechanism by which the *W* or *Sl* mutation produces mast-cell deficiency or the gene products encoded at the *W* or *Sl* locus.

A homogenous population of mast cells has been generated *in vitro* by cultivating progenitors from bone marrow in the presence of a T-cell-derived growth factor, interleukin (IL) 3 (for reviews, see refs. 7 and 8). The action of IL-3 has been shown to be enhanced by another T-cell-derived growth factor, IL-4 (9, 10). Recently we described another mode of mast-cell growth, which depends on contact with fibroblasts (11). When mast cells obtained *in vitro* from bone-marrow cells were cocultured with the NIH 3T3 fibroblast cell line, the mast cells were maintained in the absence of an exogenous source of IL-3 and IL-4. We further demonstrated that cultured mast cells derived from *W/W*^{v} mice were defective in fibroblast-dependent growth, although they responded to T-cell-derived growth factors as efficiently as those from +/+ mice (12).

In the present study we examined whether the mast-cell deficiency in *Sl/Sl*^{d} mice could be reproduced *in vitro* by a similar coculture system. No abnormality in proliferative response to either T-cell-derived growth factors or NIH 3T3 cells was detected in cultured mast cells derived from bone marrow of *Sl/Sl*^{d} mice. By contrast, a striking defect was observed in the mast-cell-supporting activity of 3T3 fibroblast cell lines established from *Sl/Sl*^{d} mouse embryos. Furthermore, the deficiency of mast cells in *Sl/Sl*^{d} mice was cured after transplantation of cloned 3T3 cells of +/+ embryo origin. Our results suggest that fibroblasts play an essential role in promoting the development of mast cells *in vivo* and that they are defective in *Sl/Sl*^{d} mice.

MATERIALS AND METHODS

Mice. C57BL/6 *Sl*^{d}/+ and WC *Sl*/+ mice as well as their normal littermates (+/+) were raised in our laboratory. The original stocks of mutant mice were derived from The Jackson Laboratory. Mast-cell-deficient adult (WB × C57BL/6)F_1 *W/W*^{v} (*W/W*^{v}) mice and (WC × C57BL/6)F_1 *Sl/Sl*^{d} (*Sl/Sl*^{d}) mice were purchased from the Shizuoka Animal Center (Hamamatsu, Japan) and The Jackson Laboratory, respectively.

Cells and Cultures. Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared as described by Nakahata *et al.* (13). Homogenous populations of mast cells were obtained by culturing bone-marrow cells at 10^6 cells per ml in α-minimal essential medium supplemented with 10^{-4} M 2-mercaptoethanol/20% (vol/vol) horse serum/10% (vol/vol) PWM-SCM. Half of the medium was replaced every 7 days. Four weeks after initiation of the culture, >98% of the cultured cells were identified as mast cells, and ≈13% of them were found to be in S phase as described (11). Therefore, those cultured for >4 wk were used in these experiments. The NIH 3T3 fibroblast cell line derived from a Swiss mouse embryo (14) or other fibroblast cell lines were cocultured with mast cells in the absence of PWM-SCM as described (11, 12). Briefly, 10^5 cultured mast cells suspended in 2 ml of medium containing 5% fetal calf serum but not PWM-SCM were added to a confluent culture

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Abbreviations: IL, interleukin; PWM-SCM, pokeweed mitogen-stimulated spleen cell-conditioned medium; BrdUrd, 5-bromodeoxyuridine.

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of fibroblasts in a 35-mm dish and incubated at 37°C in a humidified atmosphere containing 5% CO_2. The culture medium was aspirated and replaced with fresh medium without PWM-SCM every 2 days. Synchronization of mast cells at the G_1 phase of the cell cycle was carried out by culturing mast cells in the absence of PWM-SCM for 24 hr (12, 15). Mast cells were identified by staining a cytocentrifuge preparation of trypsinized cultures with Alcian blue. The proportion of mast cells in S phase was determined by identifying cells that incorporated 5-bromodeoxyuridine (BrdUrd) (11). The results described are averages of four or more dishes.

Establishment of 3T3 Cell Lines. 3T3 fibroblast cell lines were established from 17-day-old mouse embryos under the 3T3 conditions described by Todaro and others (14, 16, 17). Briefly, primary embryo cultures were plated at 3×10^5 cells per 60-mm dish in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. The cells were trypsinized every 3 days, counted, and then transferred to fresh dishes at 3×10^5 cells per dish. The +/+ embryos were obtained by mating WC +/+ and C57BL/6 +/+ mice. Because mice of the *Sl/Sl*^{d} genotype are sterile, WC *Sl*/+ and C57BL/6 *Sl*^{d}/+ mice were mated to obtain *Sl/Sl*^{d} embryos. To exclude embryos of other three genotypes (i.e., *Sl*/+, *Sl*^{d}/+, and +/+), grossly anemic embryos were first selected, and cultures were initiated from the whole embryo except the head. The head skins were transplanted under the renal capsules of recipient (WC × C57BL/6)F_1 +/+ mice, and only those whose transplanted skin developed white hairs after 10 days were considered as *Sl/Sl*^{d} embryos. Clonal sublines were obtained by isolating colonies by cloning cylinders.

Transplantation of 3T3 Cells to *Sl/Sl*^{d} Mice. 3T3 cells were grown to confluency on a piece of cellulose acetate membrane (Sartorius; 10 × 15 mm). The membranes were then inserted into the peritoneal cavity of mast-cell-deficient *Sl/Sl*^{d} or *W/W*^{v} mice under Nembutal anesthesia. Five weeks after the operation, they were killed by overinhalation of ether, and the membranes were removed and fixed in Carnoy's solution. Cell layers on both sides of the membrane were gently stripped from the membrane, mounted on glass slides, stained with Alcian blue, and examined under the microscope.

RESULTS

Maintenance of *Sl/Sl*^{d} Mast Cells on NIH 3T3 Cells. Bone-marrow cells of +/+, *Sl/Sl*^{d}, and *W/W*^{v} mice were cultured in the presence of 10% PWM-SCM. As expected, mast cells developed in all cultures. Like the normal +/+ counterparts, cultured mast cells of *Sl/Sl*^{d} mouse origin stained with Alcian blue but not with safranin or berberine sulfate. When *Sl/Sl*^{d} mast cells thus obtained were cocultured with NIH 3T3 cells without PWM-SCM, they synthesized DNA and were maintained for >2 weeks as efficiently as +/+ mast cells, whereas *W/W*^{v} mast cells were not maintained under similar conditions (Table 1).

Because the mast-cell deficiency of *Sl/Sl*^{d} mice reflects an abnormality in the microenvironment necessary for the development of these cells (2, 5), we compared the mast-cell-supporting activities of PWM-SCM derived from +/+ and *Sl/Sl*^{d} mice. However, although various concentrations of PWM-SCM were tested, no significant difference was seen in their ability to stimulate growth of +/+ mast cells (data not shown).

Establishment of *Sl/Sl*^{d} 3T3 Cell Lines and Their Failure to Support Mast Cells *in Vitro*. In the previous study, all examined mouse embryo-derived 3T3 fibroblast cell lines maintained mast cells *in vitro* (12). Therefore, we examined whether cell lines established from *Sl/Sl*^{d} mouse embryos under the 3T3 conditions could support these cells.

Table 1. Maintenance of *Sl/Sl*^{d} mast cells on NIH 3T3 fibroblasts

Origin of mast cells	Proportion of mast cells in S phase after 48 hr, %*	Mast cells after 2 weeks, no. × 10^{-4}/dish
+/+	11.1 ± 1.8	8.8 ± 1.5
Sl/Sl^d	13.1 ± 1.9	8.6 ± 1.3
W/W^v	0.2 ± 0.1	0.2 ± 0.1

Mast cells obtained *in vitro* from bone marrows of +/+, *Sl/Sl*^{d}, or *W/W*^{v} mice (1×10^5 cells per dish) were cocultured with NIH 3T3 cells without PWM-SCM. Values are mean ± SEM (n = 4).
*% of mast cells that incorporated BrdUrd.

Nine independent cultures were initiated from nine *Sl/Sl*^{d} embryos, of which eight were passaged more than 50 times and therefore considered to be established (WCB6F_1 *Sl/Sl*^{d} 3T3-1 to -8). As a control, established cell lines (WCB6F_1 +/+ 3T3-1 to -8) were initiated from eight +/+ embryos. All established cell lines displayed contact-inhibition and had a flat, polygonal, and fibroblast-like morphology similar to NIH 3T3 cells.

When these 3T3 cell lines were cocultured with +/+ mast cells and without PWM-SCM, all eight cell lines derived from +/+ embryos supported mast cells (Table 2). In contrast, none of the eight cell lines derived from *Sl/Sl*^{d} embryos supported +/+ mast cells, thus duplicating the mast-cell deficiency of *Sl/Sl*^{d} mice *in vitro*.

Because IL-3 has been shown to stimulate quiescent mast cells to transit G_1 and initiate DNA synthesis (15), we examined the possibility that +/+ 3T3 cell lines might induce this response upon contact with mast cells. Fig. 1 shows that transfer of +/+ mast cells arrested at the G_1 phase onto confluent WCB6F_1 +/+ 3T3-1 cells induced them to resume the cell cycle and start synthesizing DNA without PWM-SCM. In contrast, the G_1-arrested +/+ mast cells were not induced to synthesize DNA even at 48 hr after transfer onto WCB6F_1 *Sl/Sl*^{d} 3T3-1 cells.

Local Development of Mast Cells in Transplanted +/+ 3T3 Cell Layers in the Peritoneal Cavity of *Sl/Sl*^{d} Mice. If lack of mast cells in *Sl/Sl*^{d} mice is caused by a defect in fibroblasts as suggested *in vitro*, this lack should be cured by transplan-

Table 2. Coculture of +/+ mast cells with 3T3 cell lines established from +/+ or *Sl/Sl*^{d} mouse embryos

Origin and name of cell line	Proportion of mast cells in S phase after 48 hr, %*	Mast cells after 2 weeks, no. × 10^{-4}/dish
WCB6F_1 +/+ embryos		
WCB6F_1 +/+ 3T3-1	6.9 ± 1.4	4.6 ± 0.3
WCB6F_1 +/+ 3T3-2	7.2 ± 0.1	7.5 ± 0.4
WCB6F_1 +/+ 3T3-3	8.3 ± 1.9	3.9 ± 0.5
WCB6F_1 +/+ 3T3-4	14.3 ± 0.9	3.4 ± 0.5
WCB6F_1 +/+ 3T3-5	15.8 ± 1.5	10.2 ± 1.5
WCB6F_1 +/+ 3T3-6	3.3 ± 0.3	4.0 ± 0.2
WCB6F_1 +/+ 3T3-7	3.2 ± 0.6	1.3 ± 0.1
WCB6F_1 +/+ 3T3-8	11.6 ± 2.6	9.3 ± 2.0
WCB6 *Sl/Sl*^{d} embryos		
WCB6F_1 *Sl/Sl*^{d} 3T3-1	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-2	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-3	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-4	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-5	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-6	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-7	0	0
WCB6F_1 *Sl/Sl*^{d} 3T3-8	0	0

Cultured mast cells from +/+ mice were cocultured with each 3T3 cell line without PWM-SCM. Values are mean ± SEM (n = 4).
*% of mast cells that incorporated BrdUrd.

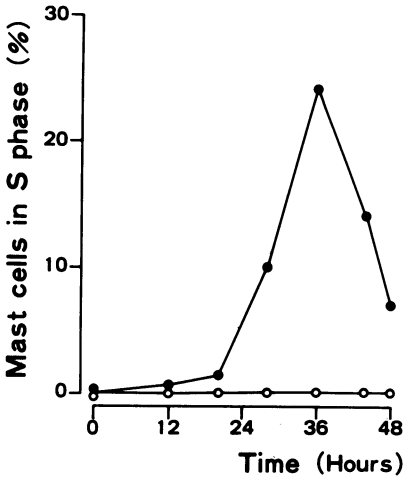

FIG. 1. G_1 transit induced in synchronized mast cells. Cultured mast cells from +/+ mice were synchronized at G_1 by culturing in suspension for 24 hr without PWM-SCM (12). Then they were cocultured with either $WCB6F_1$ +/+ 3T3-1 cells (●) or $WCB6F_1$ Sl/Sl^d 3T3-1 cells (○) established from embryos of +/+ or Sl/Sl^d genotype, respectively. At the indicated time after initiation of the coculture, percentages of mast cells incorporating BrdUrd were determined; each point represents the mean of four samples.

tation of +/+ fibroblasts. Therefore, we transplanted $WCB6F_1$ +/+ 3T3-5 cells grown on a piece of cellulose acetate membrane into the peritoneal cavity of mast-cell-deficient Sl/Sl^d mice. After 5 weeks, mast cells were identified on the transplanted membrane in all eight recipient Sl/Sl^d mice (Table 3). It should be noted that mast cells were found on the side of the membrane with $WCB6F_1$ +/+ 3T3-5 cells but not in the reverse side, which was covered only with the peritoneal resident cells of the recipient (Fig. 2). No mast cells were detected after transplantation of either $WCB6F_1$ +/+ 3T3-5 cells to W/W^v mice or $WCB6F_1$ Sl/Sl^d 3T3-1 cells to Sl/Sl^d mice (Table 3).

Support of Mast Cells by Cloned 3T3 Cells. Because the $WCB6F_1$ +/+ 3T3-5 cell line was not a clonal line, it was not certain whether appearance of mast cells in Sl/Sl^d mice was induced by transplantation of one or more types of cells. Therefore we made clonal sublines from the $WCB6F_1$ +/+ 3T3-5 cell line and examined their mast-cell-supporting activities. As shown in Table 4, all clonal 3T3 sublines supported the growth of mast cells *in vitro* and induced the appearance of mast cells *in vivo*.

DISCUSSION

We previously demonstrated that there are at least two modes of mast-cell growth, one dependent on T-cell-derived growth factors and the other dependent on contact with

Table 3. Appearance of mast cells in the peritoneal cavity of Sl/Sl^d mice after transplantation of 3T3 cells

Cell line transplanted	Genotype of recipients	Proportion of mice in which mast cells appeared
$WCB6F_1$ +/+ 3T3-5	Sl/Sl^d	8/8
$WCB6F_1$ +/+ 3T3-5	W/W^v	0/7
$WCB6F_1$ Sl/Sl^d 3T3-1	Sl/Sl^d	0/5
None*	Sl/Sl^d	0/5

Pieces of cellulose acetate membrane covered with $WCB6F_1$ +/+ 3T3-5 or $WCB6F_1$ Sl/Sl^d 3T3-1 cells were inserted into the peritoneal cavity of recipient mice. Five weeks after the transfer, the membranes were recovered and assessed for mast cells; when present, mast cells were always plentiful.

*Only pieces of cellulose acetate membrane were inserted. They were soon covered by peritoneal cells of the recipient.

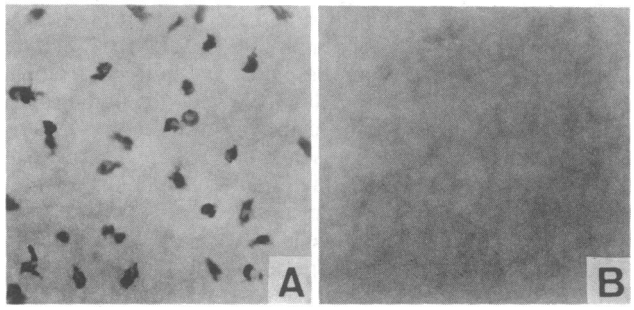

FIG. 2. Appearance of mast cells in the peritoneal cavity of Sl/Sl^d mice. Five weeks after i.p. insertion of a piece of cellulose acetate membrane covered with $WCB6F_1$ +/+ 3T3-5 cells, mast cells were identified on the side of the membrane with the $WCB6F_1$ +/+ 3T3-5 cells (*A*) but not on the other side without $WCB6F_1$ +/+ 3T3-5 cells (*B*). Stained with Alcian blue. (×100.)

fibroblasts (11). The mast cells developed *in vitro* in the presence of PWM-SCM were well maintained on the NIH 3T3 fibroblast cell line without PWM-SCM. Further screening of various cell lines showed, however, that not all fibroblast cell lines could support mast cells (12). In the present study we have described a method to reproducibly establish a cell line that has mast-cell-supporting activity. Cells from 17-day-old whole embryos of +/+ genotype were cultured under the strict 3T3 conditions as originally described by Todaro and Green (16). Out of eight trials, eight cell lines were established, all of which maintained mast cells for >2 wk in the absence of an exogenous source of mast-cell growth factors. Although all established cell lines were of flat and polygonal morphology indistinguishable from the NIH 3T3 cell line or the BALB 3T3 cell line (14, 17), the identities and natures of cell lines obtained under the 3T3-culture conditions remain to be determined.

In spite of an intrinsic defect in mast-cell precursors, mast cells are efficiently produced from the spleen and bone marrow of W/W^v mice in the presence of PWM-SCM (18, 19). The defective growth potential of W/W^v mast cells was revealed *in vitro* only when they were cocultured with NIH 3T3 or certain fibroblast cell lines without PWM-SCM (11, 12). In this study the defect in Sl/Sl^d mice was reproduced *in vitro* by a similar coculture system. Consistent with the notion that the defect resides in the tissue environment necessary for the development of mast cells rather than in their precursor cells (5), cultured mast cells derived from the bone marrow of Sl/Sl^d mice were well supported by NIH 3T3 cells. Similar to the finding with granulocyte/macrophage colony-stimulating factor (20), mast-cell growth factors were produced by the spleen cells of Sl/Sl^d mice as efficiently as those of +/+ mice. In contrast to the 3T3 cell lines derived from +/+ embryos, none of the eight 3T3 cell lines established from Sl/Sl^d embryos supported +/+ mast cells. The defect was further characterized as an inability to drive mast cells to transit the G_1 phase. This fact and the previous finding that W/W^v mast cells have a defect in the G_1-to-S transition induced by contact with fibroblasts (12) suggest that both the *W* and *Sl* gene products are indispensable for the interactions between mast cells and fibroblasts.

When cloned +/+ 3T3 cells grown on pieces of cellulose acetate membrane were transplanted into the peritoneal cavity of mast-cell-deficient Sl/Sl^d mice, mast cells appeared in the transplanted 3T3 cell layer after 5 weeks. The failure to detect mast cells after transfer of Sl/Sl^d 3T3 cells to Sl/Sl^d mice or +/+ 3T3 cells to W/W^v mice argues against the involvement of inflammation with possible production of IL-3 and/or IL-4 as the underlying mechanism for mast-cell appearance (21). Furthermore, direct contact between mast cells and/or precursors and 3T3 cells seems mandatory for

Table 4. Support of mast cells *in vitro* and *in vivo* by cloned 3T3 cells

$WCB6F_1$ +/+ 3T3-5 cell line	Coculture with mast cells		Proportion of mice in which mast cells appeared‡
	Proportion of mast cells in S phase, %*	Mast cells, no.† × 10^{-4}/dish	
Uncloned	10.9 ± 1.0	7.1 ± 0.2	7/7
Clone 1	12.2 ± 1.2	7.0 ± 1.0	4/4
Clone 2	10.2 ± 2.6	4.8 ± 0.9	4/4
Clone 3	15.6 ± 2.6	2.9 ± 0.3	4/4
Clone 4	16.7 ± 1.3	5.9 ± 0.9	4/4

*Proportion of mast cells which incorporated BrdUrd 48 hr after initiation of the coculture with +/+ mast cells.
†Number of mast cells after 2-week coculture.
‡Pieces of cellulose acetate membrane covered by uncloned or cloned $WCB6F_1$ +/+ 3T3-5 cells were inserted into the peritoneal cavity of Sl/Sl^d mice. Five weeks after the transfer the appearance of mast cells on the membrane was assessed.

the development of mast cells as observed *in vitro*, because mast cells were not detected on the side of the implanted membrane where 3T3 cells had not been cultured (Fig. 2). These findings suggest that the essential component of the *in vivo* microenvironment necessary for the development of mast cells might be 3T3-like fibroblasts.

The present coculture system reproduces, at least partly, the *in vivo* development of mast cells. The defects in W/W^v mice and Sl/Sl^d mice were revealed and clearly distinguished from each other by this system; the defect in the former appeared in mast cells and the latter in 3T3 fibroblasts. By using a long-term bone-marrow culture system, Dexter and Moore (22) successfully reproduced *in vitro* the hematopoietic defects observed in W/W^v mice and Sl/Sl^d mice. However, the defects reproduced by their system were not so marked as those reported here. Although the mutations were expressed in the hemopoietic stem cells, the hemopoietic activity was stable at approximately one-fourth of the control levels under optimal culture conditions (23). This probably reflects the *in vivo* effects of the mutations, because both the Sl/Sl^d and W/W^v mutations only decrease, but do not completely block, hemopoietic differentiation in these animals (1). Furthermore, their system consisted of mixed populations of stromal and hemopoietic cells, including potential producers of growth factors. Thus, the level of hemopoiesis, complexity of component cells, and the high variability observed in replicate flasks (23) do not lend the long-term bone-marrow culture system to further analysis of these mutations. By contrast, the present coculture system uses only two pure populations of cells, and the mutation at either the *W* or *Sl* locus markedly affects the survival of mast cells. Further studies using this system should, therefore, yield more insights into the molecular mechanisms of mast-cell growth as well as into the functions of the *W* and *Sl* gene products.

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