Although W/W\textsuperscript{*} mutant mice are profoundly deficient in tissue mast cells, these mice do have cells with similar features of mast cells that develop from their bone marrow cells as efficiently as those from congenic +/+ mice in pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM). With cultured mast cells (CMCs), we analyzed the mechanism of mast-cell deficiency in tissues of W/W\textsuperscript{*} mice. CMCs were established from bone marrow cells of W/W\textsuperscript{*} and congenic +/+ mice with PWM-SCM, and then co-cultured with various mouse fibroblast cell lines without PWM-SCM. All the examined mouse embryo-derived fibroblast cell lines maintained CMCs derived from +/+ mice, but not CMCs from W/W\textsuperscript{*} mice, for >2 weeks. Mast cells in S phase were observed only in CMCs derived from +/+ mice under these conditions. The poor survival of W/W\textsuperscript{*} CMCs as compared with +/+ CMCs was not owing to a differential death rate but to the inability of W/W\textsuperscript{*} CMCs to continue active proliferation on fibroblasts without PWM-SCM. By synchronizing CMCs at the G\textsubscript{1} phase of the cell cycle, the defect in W/W\textsuperscript{*} CMCs was further characterized as a failure to transit G\textsubscript{1} and enter the S phase upon contact with fibroblasts. This finding indicates the indispensable function of the W gene product(s) for this response.

A DOUBLE GENE dose of mutant alleles at the W locus, located on mouse chromosome 5, produces the pleiotropic effects of macrocytic anemia, sterility, and lack of hair pigmentation.\textsuperscript{1,2} In addition to these abnormalities, Kitamura and colleagues found a depletion of mast cells in W/W\textsuperscript{*} mice.\textsuperscript{3} The skin of adult W/W\textsuperscript{*} mice contains <1% of the number of mast cells present in the skin of congenic normal (+/+ ) mice, and no mast cells have been detected in other organs.\textsuperscript{3,4} Because transfer of bone marrow cells from histocompatible +/+ donors resulted in development of donor-type mast cells in tissues of W/W\textsuperscript{*} mice, the depletion of mast cells in W/W\textsuperscript{*} mice was attributed to an abnormality of bone marrow-derived mast cell precursors, rather than to a defect in the environment necessary for the development of mast cells.\textsuperscript{3,4} However, we know neither the precise mechanism by which W mutation produces mast cell deficiency nor the gene product(s) encoded at the W locus.

In 1980 and 1981, several laboratories succeeded in growing mast cells containing chondroitin sulfate E proteoglycan, but not heparin proteoglycan, from mouse hematopoietic progenitors.\textsuperscript{5} These mast cells developed in media conditioned either by mitogen-stimulated spleen cells or a mouse leukemic cell line (WEHI-3), which are rich sources of interleukin-3 (IL-3).\textsuperscript{6} Despite the apparent depletion of mast cells in W/W\textsuperscript{*} mice, cultured mast cells (CMCs) with the same cytologic and biochemical features as those from +/+ mice can be generated efficiently from their spleen or bone marrow in the presence of IL-3.\textsuperscript{6,7} The defect of CMCs derived from W/W\textsuperscript{*} mice (W/W\textsuperscript{*} CMCs) was revealed only after transfer into the peritoneal cavity of W/W\textsuperscript{*} mice; CMCs derived from +/+ mice (+/+ CMCs), but not W/W\textsuperscript{*} CMCs, survived and synthesized heparin proteoglycan under these conditions.\textsuperscript{8,9}

Long before development of the presently used mast-cell culture systems supplemented with IL-3, Ginsburg and Sachs\textsuperscript{10} successfully cultured murine mast cells on feeder layers of embryo-derived fibroblasts. Recently, Levi-Schaffer et al\textsuperscript{11} demonstrated induction of phenotypic change in bone marrow-derived CMCs by a mouse skin-derived 3T3 fibroblast cell line. Although their systems appeared to contain mast-cell growth factors, we co-cultured CMCs with a mouse embryo-derived fibroblast cell line, NIH/3T3, in a medium not supplemented with IL-3 or IL-4\textsuperscript{12} and demonstrated that NIH/3T3 cells were able to maintain +/+ CMCs by means of direct cell-cell interactions.\textsuperscript{13} We also demonstrated that W/W\textsuperscript{*} CMCs were not supported by NIH/3T3 cells and suggested that this might be a suitable system for analysis of the function of the W gene product(s).\textsuperscript{14} In the present study, we assessed whether the defect of W/W\textsuperscript{*} CMCs could also be revealed in vitro by cocultivation with fibroblasts other than NIH/3T3 cells, and investigated the mechanism by which survival of W/W\textsuperscript{*} CMCs on NIH/3T3 cells was decreased as compared with +/+ CMCs. All examined mouse embryo-derived fibroblast cell lines supported +/+ CMCs, but not W/W\textsuperscript{*} CMCs, and the difference was owing to the decreased proliferation rate of W/W\textsuperscript{*} CMCs as compared with +/+ CMCs. Use of synchronized CMCs further characterized the defect in W/W\textsuperscript{*} CMCs as a failure to transit the G\textsubscript{1} phase of the cell cycle and enter the S phase in response to contact with NIH/3T3 cells.

MATERIALS AND METHODS

**Mice.** Mast cell-deficient (WB × C57BL/6)F\textsubscript{1}-W/W\textsuperscript{*} (W/W\textsuperscript{*}) mice, their normal littermates (+/+ ), and C57BL/6-bg'/bg' (bg'/bg') mice were raised in our laboratory. The giant granules of bg'/bg' mutant mice were used as a marker to distinguish the origin of the mast cells.\textsuperscript{15} The original stocks were obtained from the Jackson Laboratory, Bar Harbor, ME, but W\textsuperscript{*} and bg' mutant genes

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were introduced into our own C57BL/6 inbred colony by repeated backcrosses.17

**Mouse cell lines.** The National Institutes of Health (NIH)/3T3 fibroblast cell line (derived from Swiss mouse embryo cultures)18 was provided by Dr S.A. Aaronson, National Cancer Institute (NCI), Bethesda, MD. Other embryo-derived fibroblast cell lines, ie, Swiss 3T3,21 Swiss 3T6,21 BALB/3T3,20 and C3H 10T1/2-Clone 8,21 and NCTC Clone 929 (a clone of strain L derived from connective tissues) were obtained from the Japanese Cancer Research Resources Bank (JCRB). The BMA1 cell line (derived from mouse bone marrow stromal cells transfected with adenovirus DNA),22 the JPT26-3T3 cell line (a Ha-ras oncogene-transformed NIH/3T3 cell line)23 and the NWS1 fibroblast cell line (derived from adult mouse spleen) were established in our laboratory. They were all adapted to grow in α-minimal essential medium (MEM) (Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA), 100 U/mL penicillin and 100 μg/mL streptomycin.

**Establishment of CMCs and co-culture with fibroblasts.** Homogeneous populations of CMCs were obtained by culturing bone marrow cells for 4 weeks with pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) containing IL-3 and IL-4.24 Co-culture of CMCs with NIH/3T3 cells or other fibroblasts was done as described previously.13 CMCs suspended in 2 mL α-MEM containing 5% FCS and antibiotics but not PWM-SCM were added to a confluent culture of fibroblasts in a 35-mm dish. They were cultured at 37°C in a humidified atmosphere containing 5% CO2. The culture medium was aspirated and replaced with 2 mL fresh culture medium without PWM-SCM every 2 days. Mast cells were identified by staining a cytocentrifuge preparation of trypsinized cell cultures. Results were expressed as the mean values of 4 dishes.

**Clonal cell culture.** Methylocellulose culture was carried out according to the method described by Nakahata and associates.13,23 At various times after the initiation of co-culture, NIH/3T3 cells with CMCs were trypsinized. One milliliter of a culture mixture in a-minimal essential medium (MEM) (Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA), 100 U/mL penicillin and 100 μg/mL streptomycin.

**Detection of mast cells in S phase.** The proportion of cells that incorporated bromodeoxyuridine (BrdUrd) was used as an index of cell proliferation as described previously.15 Cells were incubated with 3 μg/mL BrdUrd at 37°C for 30 minutes. Cytocentrifuge preparations of tryptinated cultures were first stained with alcian blue. The cells that incorporated BrdUrd were then stained using mouse anti-BrdUrd monoclonal antibody (Becton Dickinson, Mountain View, CA) and avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Mast cells in S phase were identified by the presence of alcin blue-positive granules in the cytoplasm and dark brown granules in the nucleus.

**RESULTS**

**Maintenance of mast cells by various fibroblast cell lines.** CMCs of either +/+ or W/W* mouse origin were co-cultured with various mouse fibroblast cell lines in the absence of PWM-SCM. The proportion of mast cells in S phase was determined 48 hours after initiation of co-culture, and the numbers of mast cells remaining were counted on day 14. As shown in Table 1, all fibroblast cell lines derived from mouse embryos supported +/+ CMCs but not W/W* CMCs. The NIH/3T3 cells showed supportive activity even after transformation by the Ha-ras oncogene (ie, JPT26-3T3). In contrast with the embryo-derived cell lines, three fibroblast cell lines originating from adult mice did not support +/+ CMCs. Because NIH/3T3 was not an exceptional fibroblast cell line supporting +/+ CMCs but not W/W* CMCs, we used this cell line thereafter to analyze further the interactions between fibroblasts and CMCs in vitro.

**Cause of disappearance of W/W* CMCs on NIH/3T3 cells.** The number of surviving cells is influenced by both the proliferation and death of the cultured cells. The finding that W/W* CMCs were not in S phase 48 hours after initiation of co-culture with NIH/3T3 cells (Table 1) suggested that the decrease in number of W/W* CMCs resulted principally from arrest of proliferation. To confirm this, we determined the proportion of mast cells in S phase at various times after the initiation of co-culture with NIH/3T3 cells. We found that the proportion of mast cells in S phase was significantly lower in W/W* CMCs than in +/+ CMCs, indicating that the arrest of proliferation was a major cause of the disappearance of W/W* CMCs.
times after the initiation of co-culture. As shown in Table 2, the proportion of \(W/W^v\) CMCs in S phase decreased to <0.1% after 48 hours. This low level continued throughout the 4-week observation period, whereas 4% to 10% of \(+/+\) CMCs were shown to be in S phase during the same period (data not shown). We also determined the numbers of mast cells or CMCs may be producing a small amount of unknown factor(s) that stimulates proliferation of mast cells, either NIH/3T3 cells or \(+/+\) CMCs, which may be produced by co-cultivation or differentiation of mast cells. These results indicated that the decreased survival of \(W/W^v\) CMCs as compared with \(+/+\) CMCs on NIH/3T3 cells was owing to the decrease in the proliferative rate rather than an increase in the death rate.

Effect of normal CMCs on proliferation of \(W/W^v\) CMCs. Although NIH-3T3 cells by themselves do not appear to produce diffusible growth factors that stimulate proliferation of mast cells,\(^{12}\) either NIH-3T3 cells or \(+/+\) CMCs may produce such factors after co-cultivation or NIH-3T3 cells or CMCs may be producing a small amount of growth factors effective only at close range. To examine these possibilities, we attempted to co-culture both \(+/+\) CMCs and \(W/W^v\) CMCs with NIH-3T3 cells simultaneously. Because \(+/+\) CMCs and \(W/W^v\) CMCs are not morphologically distinguishable, we used \(bg/bg\) CMCs instead of \(+/+\) CMCs. The giant granules of \(bg/bg\) CMCs made them easily distinguishable from \(W/W^v\) CMCs.\(^{16}\) As shown in Table 3, \(bg/bg\) CMCs were well maintained on NIH/3T3 cells without PWM-SCM. When \(W/W^v\) CMCs and \(bg/bg\) CMCs were mixed, the proportion of \(W/W^v\) CMCs in S phase after 48 hours was not increased by the presence of \(bg/bg\) CMCs. Similarly, the number of \(W/W^v\) CMCs was not increased after a 2-week co-culture period with added \(bg/bg\) CMCs. This was consistent with our inability to detect IL-3 mRNA in NIH/3T3 cells after a 48-hour co-culture with \(+/+\) CMCs (data not shown). Table 3 also shows that the presence of \(W/W^v\) CMCs did not affect either the proportion of \(bg/bg\) CMCs in S phase after 48 hours or the number of \(bg/bg\) CMCs remaining after 2 weeks.

\[\text{Table 2. Comparison Between Proportion of Mast Cells in S Phase and Number of CFU-mast Maintained on NIH/3T3 Cells} \]

<table>
<thead>
<tr>
<th>Time</th>
<th>(+/+)</th>
<th>(W/W^v)</th>
<th>(+/+)</th>
<th>(W/W^v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before co-culture</td>
<td>9.7 ± 2.5</td>
<td>9.0 ± 0.9</td>
<td>37 ± 5</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>24 h after§</td>
<td>14.8 ± 3.6</td>
<td>2.9 ± 1.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>48 h after§</td>
<td>11.9 ± 1.3</td>
<td>0.1 ± 0.1</td>
<td>43 ± 4</td>
<td>38 ± 1</td>
</tr>
</tbody>
</table>

ND, not determined. Results are mean ± SE, n = 4.
*Percentage of CMCs incorporating BrdUrd.
†Cultured with PWM-SCM.
§Co-culture with NIH/3T3 cells without PWM-SCM.
\(P<0.01\) as compared with values before initiation of co-culture.

\[\text{Table 3. Co-Culture of } bg/b^g \text{ CMC and } W/W^v \text{ CMC With NIH/3T3 Cells} \]

<table>
<thead>
<tr>
<th>No. of Mast Cells Initially Plated (x 10^7/Dish)</th>
<th>Proportion of Mast Cells in S Phase After 48 h (%)</th>
<th>No. of Mast Cells After 2 wk (x 10^5/Dish)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(bg/b^g) (W/W^v)</td>
<td>(bg/b^g) (W/W^v)</td>
<td>(bg/b^g) (W/W^v)</td>
</tr>
<tr>
<td>10</td>
<td>12.7 ± 0.1</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>11.2 ± 0.7</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>12.4 ± 2.3</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>1</td>
<td>9.3 ± 3.0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>—</td>
<td>0.1 ± 0.1</td>
</tr>
</tbody>
</table>

CMCs derived from \(bg/b^g\) mice and \(W/W^v\) mice were mixed as indicated and co-cultured with NIH/3T3 cells. The giant granules of \(bg/b^g\) CMCs were used to distinguish the two different populations of CMCs. Values are mean ± SE, n = 4.
*Percentage of CMCs incorporating BrdUrd.
examined the possibility that contact with NIH/3T3 cells might drive CMCs to transit G1 and initiate DNA synthesis. Exponentially growing +/+ CMCs and W/W' CMCs were transferred from the medium containing PWM-SCM to the medium lacking PWM-SCM. When cultures were analyzed immediately after removal of PWM-SCM, cells were >96% viable and present in all phases of the cell cycle (Fig 2, t = 0). After 24 hours, the viability of both +/+ CMCs and W/W' CMCs decreased to 50% to 70%. Less than 2% of the remaining cells incorporated BrdUrd, and they displayed a single peak at G1 in the DNA histogram (Fig 2, t = 24). As expected, addition of PWM-SCM to the medium of these quiescent CMCs caused them to resume the division cycle; incorporation of BrdUrd was detected 20 to 24 hours after restimulation, regardless of the genotype of the CMCs (Fig 3A). Quiescent +/+ CMCs were also induced to resume DNA synthesis without addition of PWM/SCM after transfer onto confluent NIH/3T3 cells (Fig 3B). The time course and percentage of +/+ CMCs incorporating BrdUrd were equivalent to those restimulated by PWM/SCM. In contrast, quiescent W/W' CMCs were not induced to transit G1 phase even at 48 hours after transfer onto NIH/3T3 cells, suggesting that the difference in the proliferative response to NIH/3T3 cells between +/+ CMCs and W/W' CMCs reflected a qualitative or quantitative difference in the response rather than a difference in the time required for CMCs to resume DNA synthesis.

**DISCUSSION**

The present study shows that at least two modes of mast cell growth exist, one dependent on T-cell-derived growth factors and the other dependent on contact with fibroblasts. This is consistent with previous in vivo findings. Infections by some intestinal parasites result in a striking increase in the number of mucosal mast cells in normal mice.27,28 In contrast, T-cell--deficient nude athymic mice show a complete failure in the intestinal mast cell response to these parasitic infections and adequate functions can be restored with a thymus graft, indicating a T-cell dependency of this response.27,28 Despite the absence of T cells, however, the number of mast cells in the skin of nude athymic mice is comparable to the number observed in normal congenic mice,27,28 suggesting the presence of a mast-cell production mechanism independent of T-cell factors.

The present experiments further demonstrate that W/W' CMCs, which show a normal proliferative response to mast-cell growth factors, are not maintained by any of the embryo-derived fibroblast cell lines. On the other hand, +/+ CMCs are maintained by those cell lines under similar conditions. These and previous findings27,28 suggest that direct interactions between mast cell precursors and fibroblasts may play an important role in development of tissue mast cells in the constitutive steady state, and that the T-cell factor-dependent mechanism may represent inducible growth of mast cells for antiparasitic reactions27,28 or other inflammatory conditions. The recent finding of Galli et al29 that a large number of mast cells appeared in W/W' mice at sites of dermatitis depending on severity of inflammation is consistent with this notion. Contact with NIH/3T3 cells induced DNA synthesis in +/+ CMCs arrested at the G1 phase of the cell cycle as rapidly and efficiently as mast-cell growth factors. Further studies are necessary to determine the relationship between these two mechanisms of mast cell growth.

Under the present co-culture conditions, the number of CFU-Mast surviving on NIH/3T3 cells always paralleled
the number of CMCs. Because CMCs consisted of a pure population of cells, ~1 in 3 to 10 CMCs was a CU-FMast at the same time regardless of the genotype. This is in accord with the previous suggestion that at least a part of CU-Mast, localized in the skin or peritoneal cavity, should be derived from differentiated mast cells. Studies using in vitro colony assay has also suggested that CU-FMast of W/W' mice, present in the hematopoietic tissues and blood, but not in the skin or peritoneal cavity, might be unable to invade, proliferate within, and/or survive in the anatomic sites ordinarily supporting final mast-cell differentiation. Although the co-culture experiment we describe did not exclude other possibilities, it demonstrated that W/W' CMCs, and therefore W/W' CU-Mast, were defective in proliferation in response to contact with fibroblasts. After 48-hour co-culture with NIH/3T3 cells, no W/W' CMCs were in S phase despite the presence of CU-Mast (Table 2).

Using a long-term bone marrow culture system, Dexter and Moore successfully reproduced in vitro the hematopoietic defect observed in W/W' mice. However, their system consisted of a mixture of various stromal cells and hematopoietic cells of different differentiation stages, including potential producers of growth factors. Furthermore, the concentrations of hematopoietic progenitors were very low. In contrast, we reproduced the defect in W/W' mice by using two pure populations of cells, CMCs and NIH/3T3 cells. Sufficient CMCs can be obtained from W/W' mice as well as +/+ mice by culturing bone marrow cells with PWM-SCM. The defect in W/W' CMCs is revealed by co-culturing them with NIH/3T3 cells. The latter do not produce diffusible mast-cell growth factors and therefore allow detailed analysis of cell-cell interactions in the absence of growth factors. Furthermore, each population of cells can be manipulated independently before co-culture is started. By synchronizing CMCs before co-culture, the defect in W/W' CMCs was characterized as an inability to transit the G1 phase and start synthesizing DNA upon contact with fibroblasts. This defect did not appear to be mediated by production of diffusible growth inhibitors. A closer molecular and cellular examination of the proliferative response of +/+ CMCs to NIH/3T3 cells should therefore yield more insights into the function of the W gene product(s).

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REFERENCES
7. Galli SJ, Dvorak AM, Dvorak HF: Basophils and mast cells: Morphologic insights into their biology, secretory pattern, and function. Prog Allergy 34:1, 1984
21. Reznikoff CA, Brankow DW, Heidelberger C: Establishment and characterization of a cloned line of C3H mouse embryo cells

22. Fujita J, Yoshida O, Miyanomae T, Mori KJ: A colony-stimulating factor-producing cell line derived from mouse bone marrow cells transfected with adenovirus DNA. Gann 74:334, 1983


tion and its reconstitution with bone marrow cells. Parasite Immunol 7:429, 1985


Failure of W/Wv mouse-derived cultured mast cells to enter S phase upon contact with NIH/3T3 fibroblasts

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