Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6485–6488, September 1986
Cell Biology

Coculture of interleukin 3-dependent mouse mast cells with fibroblasts results in a phenotypic change of the mast cells

(chondroitin sulfate E proteoglycan/heparin proteoglycan)

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Contributed by K. Frank Austen, May 27, 1986

ABSTRACT **The heparin-containing mast cells that reside in the connective tissue of the mouse, but not the chondroitin sulfate-containing mast cells in the gastrointestinal mucosa, stain with safranin when exposed to alcian blue/safranin. Mouse bone marrow-derived mast cells (BMMC), the probable *in vitro* counterparts of *in vivo* mucosal mast cells, were cultured for 14 days with mouse skin-derived 3T3 fibroblasts in RPMI 1640 medium containing 10% fetal calf serum and 50% WEHI-3 conditioned medium. Although the BMMC adhered to the fibroblast monolayer, they continued to divide, probably due to the presence of interleukin 3 in the conditioned medium. The mast cells remained viable throughout the period of coculture, since they failed to release lactate dehydrogenase and because they increased their histamine content ≈15-fold. After 12–14 days of coculture, >50% of the BMMC changed histochemically to become safranin$^+$; 30–40% of the ^{35}S-labeled glycosaminoglycans on the proteoglycans synthesized by these cocultured mast cells were heparin, whereas heparin was not detected in the initial BMMC. In the absence of WEHI-3 conditioned medium, BMMC adhered to the fibroblast monolayer, and after 8 days of coculture, the number of mast cells did not change and their histamine content remained the same. However, these mast cells also became safranin$^+$ and synthesized 40% heparin glycosaminoglycans. Thus, coculture of BMMC with fibroblasts induces a phenotypic change so that the resulting mast cells stain safranin$^+$ and synthesize heparin proteoglycans, whereas the presence of WEHI-3 conditioned medium stimulates proliferation and an increase in histamine content.**

The T-cell-dependent mast cells that reside in the mucosa of the mouse are distinct from the mast cells that reside in the serosal cavity (1). Bone marrow-derived mast cells (BMMC) that are analogous ultrastructurally and histochemically to mucosal mast cells are derived by culture of progenitors (2–4) with the T-cell-derived lymphokine interleukin 3 (IL-3) (5). These BMMC differ from serosal mast cells in that their granules stain alcian blue$^+$/safranin$^-$, contain lower levels of histamine, and have chondroitin sulfate E proteoglycans (6) rather than heparin proteoglycans. The serosal mast cells of the rat can be maintained *ex vivo* without changing phenotypically on mouse 3T3 fibroblasts (7). In this study, we demonstrate that when BMMC are cocultured for 14 days with 3T3 fibroblasts in the presence of IL-3, they become adherent, continue to divide, increase their histamine content ≈15-fold, change histochemically to resemble the serosal mast cell by becoming alcian blue$^+$/safranin$^+$, and synthesize heparin proteoglycans. These results indicate that fibroblasts induce a change in the phenotype of BMMC toward that of a serosal mast cell.

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METHODS

Establishment of Mast Cell Fibroblast Cocultures. Mouse BMMC were obtained as described (2, 5) by culturing BALB/c bone marrow cells for 3 weeks in plastic culture flasks with 50% enriched medium [RPMI 1640 medium, 10% heat-inactivated fetal calf serum (56°C, 30 min), penicillin (100 units/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 0.1 M nonessential amino acids, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] and 50% WEHI-3 conditioned medium. The culture medium was changed weekly and the BMMC not adhering to the plastic were used for further culture. The contact-inhibited Swiss albino mouse-skin-derived 3T3 fibroblast cell line (American Type Culture Collection) was seeded in 35-mm culture dishes at a density of 1×10^4 cells per 2 ml of enriched medium. These culture dishes, which were normally used 7 days later, contained $0.5\text{–}1 \times 10^6$ fibroblasts. Three-week-old cultures of BMMC were washed twice with enriched medium by centrifugation at $120 \times g$ for 5 min. Each cell pellet was resuspended in 50% enriched medium and 50% WEHI-3 conditioned medium at a density of 2.5×10^5 BMMC per ml, and 2 ml were seeded into the culture dishes containing the confluent monolayer of fibroblasts. Cocultures were maintained in a humidified atmosphere of 6% CO_2. The culture medium was aspirated every 48 hr, and the monolayers were washed once with 2 ml of enriched medium to remove the nonadherent mast cells. In each experiment, eight mast cell fibroblast cocultures were established, and the number of mast cells present for one culture at each time point was determined by quantitating the number of metachromatically staining cells that were nonadherent and floating in the medium and the number that were adherent and dispersed by Pronase treatment of the monolayer.

Determination of Histamine Content. The histamine content (mean ± SD) per 10^6 adherent and 10^6 nonadherent cocultured mast cells was determined for four separate experiments by using a radioenzymatic assay (8). The adherent mast cells were scraped from the culture dishes with a Teflon policeman. All preparations were resuspended in 1 ml of WEHI-3 conditioned medium and sonicated (Branson Sonifier, 20 pulses, 50% pulse cycle, setting 4) before being analyzed. Counts for adherent and nonadherent mast cells were obtained from a replicate culture to permit calculation of histamine content per 10^6 mast cells.

Safranin-Positive Staining of Adherent Mast Cells. Suspensions of BMMC and nonadherent and dispersed adherent mast cells obtained from coculture as well as non-dispersed cocultured mast cells were air-dried and incubated for 5 min with a solution of 0.5% alcian blue/0.3% acetic acid (9, 10).

Abbreviations: BMMC, bone marrow-derived mast cells; IL-3, interleukin 3.

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The alcian blue-treated cultures were washed with distilled water and were incubated for 5 min with a solution of 0.1% safranin/0.1% acetic acid, washed, air-dried, and examined microscopically.

Analysis of ^{35}S-Labeled Proteoglycans. Starting BMMC and mast cells cultured for 14 days on fibroblasts were incubated at 37°C with 100 μCi of [^{35}S]sulfate (≈4000 Ci/mmol; 1 Ci = 37 GBq) for 24 hr. After radiolabeling the cocultures, BMMC were processed as usual (6). The nonadherent ^{35}S-labeled mast cells were removed with the culture medium, centrifuged at 120 × *g*, and washed three times with fresh enriched medium. To obtain an enriched population of adherent ^{35}S-labeled mast cells, the cocultures were washed three times with RPMI 1640 medium and treated for 20 min at 37°C with calcium- and magnesium-free Hanks' balanced salt solution (HBSS$^-$) containing 10% fetal calf serum and Pronase (1 mg/ml). After the Pronase-dispersed cells were centrifuged at 275 × *g* for 15 min through 13% metrizamide in HBSS$^-$, 30.6% ± 12.9% (mean ± SD; *n* = 9) of the previously adherent mast cells in the coculture were recovered at the bottom of the metrizamide gradient at a purity of 79.1% ± 8.2% (mean ± SD; *n* = 11) as assessed by light microscopic analysis of cells stained with toluidine blue. The cell pellets from the [^{35}S]sulfate-labeled BMMC, nonadherent mast cells and adherent mast cells were each extracted with 1% (wt/vol) Zwittergent 3-12 detergent and 4 M guanidine·HCl (6), and solid CsCl was added to the detergent and guanidine·HCl extracts to give final densities of 1.4 g/ml. After centrifugation at 95,000 × *g* for 48 hr (6, 11), the bottom 50% of each gradient was dialyzed, lyophilized, and then dissolved in 0.5 ml of water. The extent of incorporation of [^{35}S]sulfate into proteoglycans was determined by filtration of 10% of each cell extract on Sephadex G25/PD-10 prepacked columns under dissociative conditions (6). The presence of ^{35}S-labeled heparin and chondroitin ^{35}S-labeled sulfate glycosaminoglycans was assessed by determining the susceptibility of the proteoglycans to nitrous acid degradation (12) and chondroitinase ABC digestion (13). Digests and hydrolysates were chromatographed on the Sephadex G25/PD-10 columns, with degradation being assessed by determining the shift in ^{35}S radioactivity from the void volume (V_o) to the included volume.

RESULTS

Mast Cell Proliferation. When 3-week-old cultures of 5 × 10^5 mouse BMMC were seeded onto a confluent monolayer of mouse-skin-derived 3T3 fibroblasts in the presence of 50% WEHI-3 conditioned medium as a source of IL-3, 41.4% ± 21.6% (mean ± SD; *n* = 7) of the BMMC adhered to the monolayer within the first 48 hr of the coculture as assessed by counting the adherent mast cells. The culture medium was changed every 24–48 hr, and the monolayers were washed at each medium change to remove the nonadherent mast cells. Under these culture conditions, the total number of adherent mast cells in the culture increased progressively from day 2 to day 10 and remained relatively constant thereafter (Fig. 1), whereas the number of nonadherent cells removed with each medium change remained almost the same throughout the experiment. By the 10th day of the coculture, the fibroblast monolayers were covered with 1.3–1.6 × 10^6 mast cells (*n* = 4). Only 7% of the total cytosolic enzyme, lactate dehydrogenase (14), was released into the culture medium during the 2 weeks of the standard coculture, demonstrating further the viability of the proliferating mast cells.

Histamine Content. The amount of histamine per 10^6 cells increased significantly in both the adherent and nonadherent mast cells during the coculture (Fig. 2). This increase was evident after only 2 days and continued incrementally in both the adherent and nonadherent populations of cells throughout the coculture period. After 2 weeks, the histamine content increased from 0.18 ± 0.09 μg per 10^6 cells (mean ± SD; *n* = 4) in the starting BMMC to 2.77 ± 0.91 μg per 10^6 cells (mean ± SD; *n* = 4) in the adherent cells and 3.00 ± 1.61 μg per 10^6 cells (mean ± SD; *n* = 4) in the nonadherent cells.

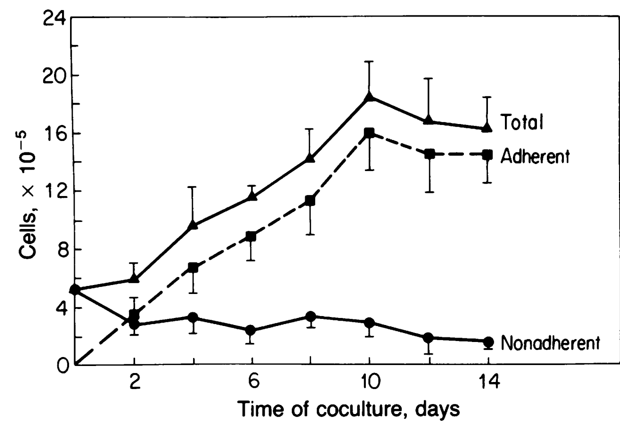

FIG. 1. Variation in numbers of total, adherent, and nonadherent mast cells with days of coculture. The data for total (▲), adherent (■), and nonadherent (●) mast cells are the mean ± SD of four separate experiments.

Safranin-Positive Staining. Adherent and nonadherent mast cells were examined histochemically throughout the coculture period with a staining technique that had been used to distinguish alcian blue$^+$/safranin$^+$ mouse serosal mast cells from alcian blue$^+$/safranin$^-$ mouse mucosal mast cells (9, 10). All of the granules of the starting population of BMMC were alcian blue$^+$/safranin$^-$ (Fig. 3*a*). After 12–14 days of coculture with the fibroblasts, in three experiments almost all of the mast cells in the coculture possessed granules in which ≈50% were safranin$^-$ and ≈50% were safranin$^+$ (Fig. 3*b*); in two other experiments, >95% of the granules in all of the mast cells were safranin$^+$ (Fig. 3*c*). The cytoplasm of the fibroblasts (either cultured alone or in the presence of BMMC) stained poorly with both cationic dyes.

Appearance of Heparin Glycosaminoglycans. The rate of [^{35}S]sulfate incorporation into macromolecules for dispersed adherent and nonadherent ^{35}S-labeled mast cells was 15,800 ± 7400 cpm·hr^{-1} per 10^6 cells (mean ± SD; *n* = 7) and 6100

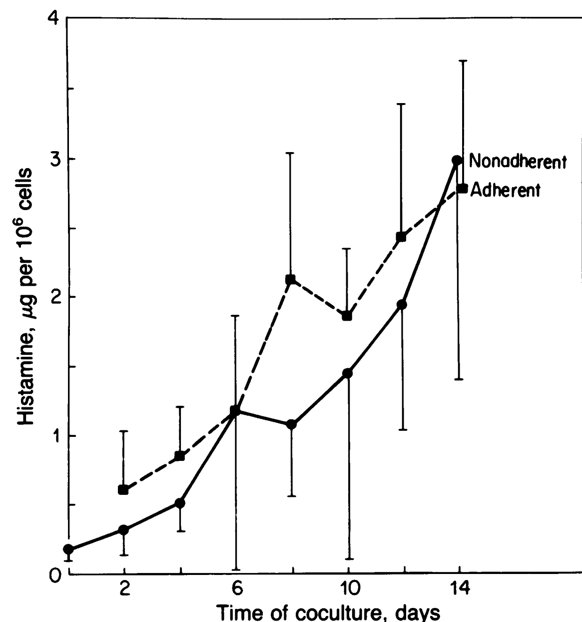

FIG. 2. Increase in histamine content (±SD) of adherent (■) and nonadherent (●) mast cells with days of coculture.

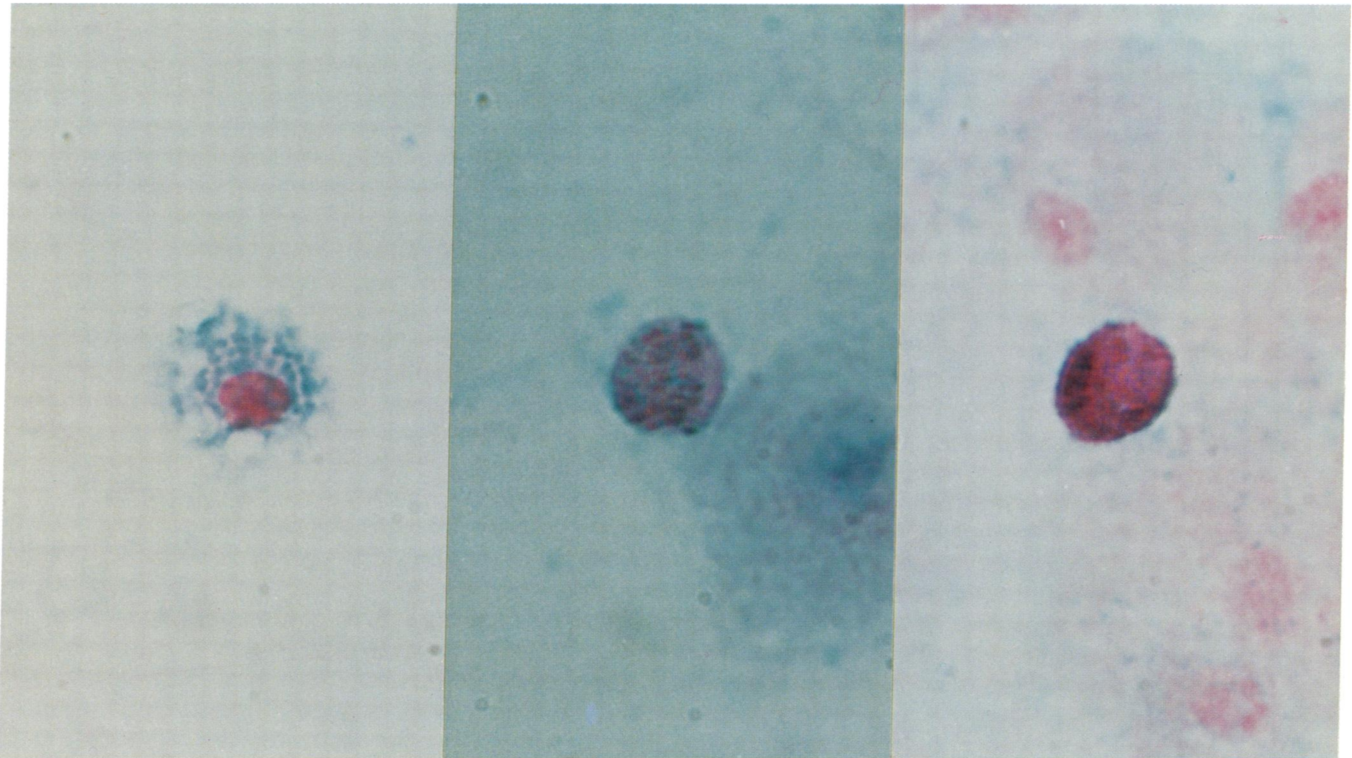

FIG. 3. Acquisition of safranin-positive staining of adherent mast cells with coculture: (*a*) Starting BMMC; (*b*) a 14-day coculture in which ≈50% of the granules in the mast cells are safranin$^+$; (*c*) a 14-day coculture in which >95% of the granules in all of the mast cells are safranin$^+$.

± 2200 cpm·hr^{-1} per 10^6 cells (mean ± SD; n = 4), respectively. Upon density-gradient centrifugation, 91% ± 4% (mean ± SD; n = 8) of the total ^{35}S-labeled macromolecules from the dispersed adherent mast cells and 73% ± 14% (mean ± SD; n = 4) from the nonadherent mast cells cultured for 1 or 2 weeks with fibroblasts were recovered in the high buoyant-density fractions, consistent with the preferential incorporation of [^{35}S]sulfate into proteoglycans rather than sulfated glycolipids or glycoproteins. When the mast cell ^{35}S-labeled proteoglycans were tested for their susceptibility to chondroitinase ABC, 45% ± 12% (mean ± SD; n = 8) of the ^{35}S-labeled macromolecules from the dispersed adherent mast cells (Fig. 4*F*) and 51% ± 9% (mean ± SD; n = 3) of the ^{35}S-labeled macromolecules from the nonadherent mast cells were digested, as compared to >88% (n = 3) for the starting BMMC (Fig. 4*C*). Upon nitrous acid treatment, 39% ± 7% (mean ± SD; n = 8) of the ^{35}S-labeled macromolecules from the dispersed adherent mast cells (Fig. 4*E*) and 28% ± 3% (mean ± SD; n = 3) of the ^{35}S-labeled macromolecules from the nonadherent mast cells were degraded, as compared to <5% for the starting population of BMMC (Fig. 4*B*). Two-dimensional cellulose acetate electrophoresis of glycosaminoglycans (6, 15), obtained by β elimination (6, 16) of proteoglycans from dispersed adherent mast cells cocultured for 14 days, revealed the presence of a second glycosaminoglycan that possessed the electrophoretic mobility of standard preparations of heparin (data not shown); this glycosaminoglycan was not detected in the initial BMMC. Thus, coculture of mouse BMMC with mouse fibroblasts resulted in the appearance of proteoglycans in the mast cells that contained heparin glycosaminoglycans in addition to chondroitin sulfate glycosaminoglycans.

Coculture of BMMC with fibroblasts in the absence of WEHI-3 conditioned medium resulted in the adherence of 10% (mean; n = 4) of the starting population of mast cells to the monolayer after 2 days of coculture. Continued coculture for 8 days in the absence of the WEHI-3 conditioned medium did not result in a significant change in the number of mast cells [57,300 ± 27,300 versus 76,300 ± 35,400 (mean ± SD; n = 4)] or in a significant increase in the histamine content per cell [0.3 ± 0.14 μg per 10^6 cells versus 0.6 ± 0.5 μg per 10^6 cells (mean ± SD; n = 3)] but was associated with a change in histochemical staining from alcian blue$^+$/safranin$^-$ to alcian blue$^+$/safranin$^+$. Furthermore, when radiolabeled with [^{35}S]sulfate, 40% (mean; n = 2) of the total ^{35}S-labeled macromolecules in the mast cells maintained on 3T3 fibroblasts without proliferation and then obtained by Pronase digestion and density gradient isolation were ^{35}S-labeled heparin glycosaminoglycans. In separate experiments, BMMC cocultured in the presence of WEHI-3 conditioned medium for 10 days and then for 8 additional days in medium lacking WEHI-3 conditioned medium remained safranin$^+$ and continued to synthesize heparin. Thus, coculture of BMMC with fibroblasts induces a phenotypic change so that the resulting mast cells stain safranin$^+$ and synthesize heparin proteoglycans, whereas the presence of WEHI-3 conditioned medium stimulates proliferation and an increase in histamine content.

DISCUSSION

In vivo attempts to induce further differentiation of the mouse BMMC have led to conflicting results. Crapper *et al.* (17) transferred mouse C57BL/6J-bg^J/bg^J BMMC into the skin of mast cell-deficient F_1 hybrid (B10pd/W$^{f/+}$ × DBA/W$^{f/+}$) W^f/W^f mice and found that the injected BMMC did not survive unless the IL-3-producing WEHI-3 tumor was also injected into the animal. Because the transferred mast cells did not change histochemically, these investigators conclud-

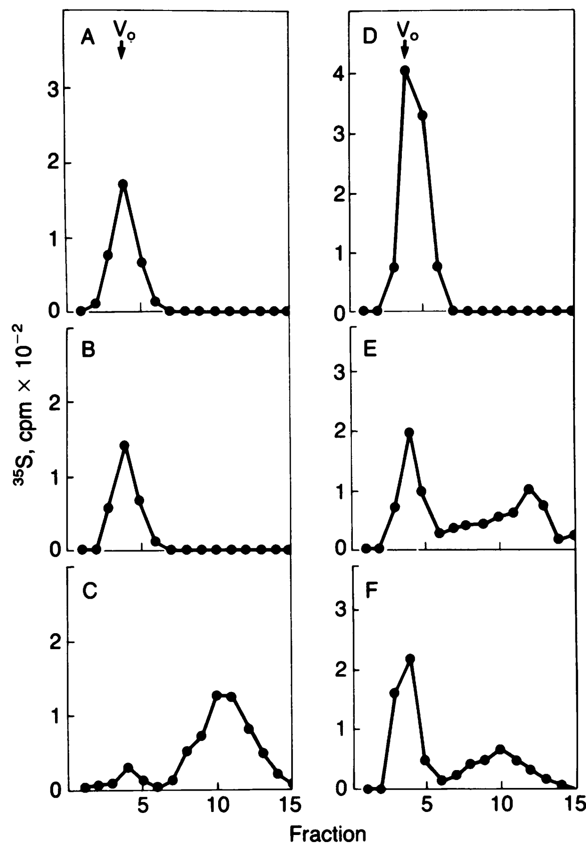

FIG. 4. Appearance of heparin glycosaminoglycans in adherent mast cells with coculture. The presence of ^{35}S-labeled heparin and ^{35}S-labeled chondroitin sulfate glycosaminoglycans in the BMMC before (*A–C*) and after (*D–F*) culture for 14 days on fibroblasts was assessed by determining the susceptibility of the extracted proteoglycans to nitrous acid degradation (*B* and *E*) and chondroitinase ABC digestion (*C* and *F*), respectively, as assessed by Sephadex G-25/PD-10 gel filtration under dissociative conditions.

ed that mouse BMMC cannot differentiate into serosal-like mast cells. Nakano *et al.* (18) carried out similar *in vivo* reconstitution experiments in mice but used the more severely mast cell-deficient WBB6F$_1$-W/W^v strain as the recipient for the culture-derived alcian blue$^+$/safranin$^-$ BMMC. They concluded that the mouse BMMC is a precursor of both the mucosal and serosal subclasses of mast cells because alcian blue$^+$/safranin$^+$ mast cells were detected in the serosal cavity, and alcian blue$^+$/safranin$^-$ mast cells appeared in the mucosa of the glandular stomach of the reconstituted animal. In previous attempts to induce further differentiation of BMMC *in vitro*, treatment with either sodium butyrate (19, 20) or normal rat serum (21) resulted in a marked inhibition of cellular proliferation and an increase in the amount of histamine per cell but did not induce the cells to synthesize heparin proteoglycans.

Ginsburg and co-workers observed that culture of antigen-stimulated lymph node cells on a monolayer of embryonic cells resulted in a preferential increase in mast cells of two morphologic types (22), one of which contained heparin proteoglycans (23). Using cloned fibroblasts rather than embryonic cells and IL-3 differentiated BMMC rather than lymph node cells to delineate the interacting cell types, we demonstrate that *in vitro* differentiated mouse BMMC cocultured with fibroblasts in the presence of WEHI-3 conditioned medium continue to divide, increase their histamine content, and synthesize heparin proteoglycans. Although all of the granules of the starting population of BMMC were alcian blue$^+$/safranin$^-$, most of the mast cells cocultured for 2 weeks became safranin$^+$. The detection in three experiments of both types of stainable granules in most of the individual mast cells suggests that the fibroblasts are inducing a change in the phenotype of most of the starting BMMC. The additional finding that the safranin$^-$ BMMC stop dividing and become heparin-containing safranin$^+$ mast cells when cocultured with fibroblasts in the absence of WEHI-3 conditioned medium confirms that these phenotypic changes do not depend on continual proliferation. We conclude that IL-3 is required for the differentiation of mouse bone marrow precursors to an intermediate mast cell that resembles the mucosal subclass (5, 24) and that fibroblasts induce their further differentiation toward cells of the serosal subclass.

This work was supported by Grants AI-22531, AI-23483, and HL-36110 from the National Institutes of Health. F.L.-S. is the recipient of a Chaim Weizmann Fellowship from the Weizmann Institute of Science, Rehovot, Israel. R.L.S. is an American Heart Association Established Investigator.

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