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Effect of coculture of rodent mast cells with murine chronic graft-versus-host disease (cGVHD)–derived fibroblasts

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We investigated whether murine chronic graft-versus-host disease (cGVHD)–derived skin fibroblasts maintain the viability and functional activity of rat peritoneal connective tissue–mast cells (CTMCs) and whether they affect the change in phenotype of mouse bone marrow–derived mast cells (BMMCs). Skin fibroblasts were isolated before the development of fibrosis (day 5), during overt fibrosis (day 28), and after recovery from fibrosis (day 120). cGVHD fibroblasts of days 5 and 28 exhibited enhanced proliferation, a property that was maintained through several subcultures. CTMCs adhered to the same extent, did not divide, and maintained their viability in all the different cultures. When CTMCs were activated with compound 48/80, they released ~80% of their histamine content, indicating that coculture with cGVHD fibroblasts did not adversely affect CTMC function. The amount of histamine found in the medium of 8 days CTMC/cGVHD fibroblast coculture was similar to that found in control culture. These findings suggest that the degranulation of dermal MCs, characteristic of cGVHD, is not due to a direct activating effect of the cGVHD fibroblasts on the MCs. BMMCs seeded on cGVHD fibroblasts acquired the capacity for safranin staining and increased their histamine content, indicative of a change to CTMCs. Thus, cGVHD fibroblasts are able to provide a microenvironment adequate for maintaining viability and activity of CTMCs and for promoting maturation and change in phenotype of BMMCs. (J ALLERGY CLIN IMMUNOL 1992;89:501-9.)

Key words: Rat peritoneal mast cells, mouse bone marrow–derived mast cells, chronic graft-versus-host disease, fibroblasts, coculture

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cGVHD results from the reactions of immunocompetent grafted cells against the host. cGVHD is a syndrome observed in both man and animals and induces a syndrome of inflammatory and fibrotic changes that resemble, in some respects, those observed in scleroderma.^{1, 2} The fibroblast is a major participant or target cell in cGVHD. It has been demonstrated that the dermal fibroblasts produce increased levels of collagen.³ Fibroblasts may also have a role in the inflammation observed in cGVHD. Fibroblasts have been ascribed immunoregulatory and immunomodulatory roles in various inflammatory processes, primarily because of their ability to produce cytokines^{4, 5} and products of arachidonic acid metabolism.⁶ We have recently demonstrated that the ability of dermal fibroblasts to secrete IL-1 in response to various stimuli is significantly impaired in animals with cGVHD.⁷

cGVHD can be induced experimentally in BALB/c mice by sublethal irradiation and subsequent administration of B10.D2 spleen cells intravenously.^{8, 9} Toluidine blue staining of the skin of these mice with cGVHD reveals a striking decrease in stainable MCs beginning on day 12. MCs are virtually undetectable in the dermis of animals with cGVHD during a period of 2 to 3 months.¹⁰ Cutaneous MCs reappear about 120 days after cGVHD induction.¹¹ Recently, we have demonstrated that peritoneal MCs undergo changes similar to skin MCs.¹² Tongue and kidney capsule MCs also disappear at the onset of fibrosis.¹³ It has been proposed that the disappearance of MCs, which appears to involve all the CT locations, is due to a gradual state of chronic MC activation and degranulation.^{10, 14} However, it is possible that the altered microenvironment resulting from changes in fibroblast function during cGVHD also plays a contributing role in the decrease in MC number. This notion has not yet been evaluated.

We have previously demonstrated that rat peritoneal MCs (CTMC), cocultured with 3T3 skin-derived fibroblasts, maintain their viability, phenotype, and functional activity for more than 1 month.¹⁵ In the present study, we examined the effects of coculturing peritoneal MCs with cGVHD fibroblasts. In a second series of experiments, we also performed studies with mouse BMMCs, a putative *in vitro* counterpart to the mucosal-type MC. Such cells mature and change phenotype to the CT type when they are cocultured with 3T3 skin-derived fibroblasts for more than 7 days.¹⁶⁻¹⁸ We examined whether a similar change in phenotype occurs when such cells are cocultured with cGVHD fibroblasts.

Abbreviations used

cGVHD:	Chronic graft-versus-host disease
B→B:	BALB/c →BALB/c
B:	BALB/c
MC:	Mast cell
CTMC:	Connective tissue-type mast cell
BMMC:	Bone marrow-derived mast cell
DMEM+:	Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (50 µg/ml), and glutamine (2 mmol/L)
EM:	RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, glutamine (2 mmol/L), penicillin (100 U/ml), streptomycin (100 µg/ml), and nonessential amino acids (0.1 mmol/L)
TG:	Tyrode's buffer with gelatin
IL:	Interleukin
HR:	Histamine release

MATERIAL AND METHODS

cGVHD model

We used a model of cGVHD across minor histocompatibility barriers.⁸ Donors were B10.D2 mice (H-2^d, mls b) or BALB/c mice (also H-2^d, mls b), and recipients were BALB/c mice. To induce cGVHD, recipients received 600 rad of ⁶⁰cobalt whole-body irradiation just before intravenous cell transfer with 5×10^7 spleen cells. Three groups of mice were used: group A, irradiated BALB/c mice that received B10.D2 spleen cells (cGVHD); group B, irradiated BALB/c mice that received BALB/c spleen cells (B→B); group C, healthy control BALB/c mice (B). Mice were purchased from Harlan Olac, Ltd. (Bicester Oxon, U.K.). All animals were cared for according to the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research.

Fibroblast cultures

Biopsy specimens were obtained from shaved abdominal mouse skins. Fibroblast cultures were established from explants through subculture of the outgrowing cells, which were grown to confluence in DMEM+ (Kibbut, Bet Haemek, Israel). Cultures were maintained at 37° C in a CO₂ incubator (5% CO₂ and 95% humidity) until the tenth passage. Swiss albino mouse skin-derived 3T3 fibroblast cell line was obtained from American Type Culture Collection (Rockville, Md.). For the coculture experiments, fibroblasts from the third through the tenth passages inclusive were seeded in 35 mm culture dishes (Falcon; Becton Dickinson, Cockeysville, Md.) at a density of $4 \times 10^3/2$ ml of DMEM+. Cultures were used 7 days later at fibroblast confluency.

Fibroblast-proliferation assay

Fibroblasts, 10^4 in 100 μ l DMEM + , were incubated for 48 hours at 37° C in a humidified atmosphere of 5% CO₂ in 96-well flat-bottom microtiter plates (Nunc GMB, Roskilde, Denmark). One microcurie of [³H]thymidine (New England Nuclear, North Billerica, Mass.) was added to each well for the last 18 hours of incubation. Supernatants were harvested onto fiberglass filters with a semiautomated cell harvester. Pellets were harvested onto the same filter after trypsinization (5 minutes at 37° C) with vigorous washing. The incorporation of [³H]thymidine was determined by scintillation counting (LKB Wallac, Turku, Finland). Assays were performed in sextuplicate.

Coculture of rat peritoneal MCs and cGVHD fibroblasts

Rat peritoneal MCs were obtained from Sabra male rats (an outbred strain of the Hebrew University) weighing 250 to 300 gm. MCs were cocultured with 3T3 or cGVHD fibroblasts with the protocol previously described for 3T3 fibroblasts.¹⁹ Briefly, MCs were isolated and purified from the peritoneal cavity with a sterile procedure. They were then cultured at a density of $0.8 \times 10^5/2$ ml in enriched medium, EM. Cocultures were maintained in a CO₂ incubator (5% CO₂ at 95% humidity at 37° C) and fed by aspirating the culture medium and replacing it with fresh medium every 2 to 3 days.

Coculture of mouse BMMCs and cGVHD fibroblasts

Mouse BMMCs were obtained by culturing BALB/c bone marrow cells for 3 weeks in plastic culture flasks (Falcon; Becton Dickinson) with 50% EM-50% WEHI-3 (myelomonocytic cell)-conditioned medium, as previously described.¹⁶ The culture medium was changed every 7 days. After 3 weeks, >98% of the cells in the suspension cultures were MCs, as assessed by metachromatic staining with toluidine blue. Three-week-old BMMCs were washed with EM by centrifugation (5 minutes at 120 g). Cell pellets were resuspended in EM-WEHI at a density of 2 to 3×10^5 BMMCs per 2 ml and seeded onto the various fibroblast monolayers. In such cultures, only some of the BMMCs adhere to the fibroblasts; therefore, there are two BMMC populations present in the cultures, one adherent and the other nonadherent. Cocultures were maintained in a CO₂ incubator (37° C in 5% CO₂ and 95% humidity). Culture medium was replaced every 2 to 3 days. Feeding was performed by aspirating the culture medium together with the nonadherent BMMCs and then plates were washed with 1 ml of EM to remove all the nonadherent BMMCs before adding the fresh medium.

MC number and viability

The viability and number of rat MCs and mouse BMMCs were assessed after each feeding. Viability was determined by the trypan blue exclusion assay in which the cocultures

were incubated with 0.4% trypan blue (Sigma Chemical Co., St. Louis, Mo.) for 5 minutes at room temperature. Rat MCs and BMMCs that adhered to the fibroblast monolayers were stained with toluidine blue (0.07%, pH 3.5, for 40 seconds) and counted under an inverted microscope equipped with a graduated grid at original magnification $\times 100$.^{15, 19} A minimum of 14 fields of the culture dish were routinely examined. Nonadherent BMMCs were pelleted by centrifuging the culture medium (for 5 minutes at 120 g). The pellet was resuspended in 0.1 ml of EM-WEHI, and BMMCs were counted with an hemocytometer after staining with an equal volume of acidic toluidine blue.

MC morphology

Rat MC and mouse BMMC fibroblast cocultures, as well as the nonadherent BMMCs from the cultures, were stained with alcian blue for 5 minutes, followed by safranin for 5 minutes.²⁰ The staining characteristics and morphology of the MCs were evaluated under a light microscope at original magnification $\times 1000$.

Histamine assay

Histamine was assessed in rat MCs and mouse BMMCs, and the medium supernatants by a radioenzymatic assay with ¹⁴C-labeled methyl-S-adenosyl-L-methionine (New England Nuclear) and a crude preparation of rat kidney methyltransferase, as described by Shaff and Beaven.²¹ Every 2 days, when the rat MC fibroblast cocultures were fed, culture media were saved to determine their histamine content. On the last day of the experiment, MC-associated histamine was measured by scraping the different rat MC/fibroblast monolayers from the plate with a Teflon (Costar Corp., Cambridge, Mass.) policeman into 1 ml of TG. The cell suspensions were disrupted by sonication (output 5, 50% duty cycle; W380, Heat Systems Ultrasonics, Inc., Farmingdale, N.Y.). These data from these experiments are presented as percentage of HR, calculated by the following formula: micrograms of histamine in culture medium (days 2 + 4 + 6 + 8) $\times 100$ μ g of histamine in culture medium (days 2 + 4 + 6 + 8) + micrograms of histamine in cells (day 8).

Histamine content of BMMCs was evaluated in both the nonadherent and adherent populations. Every 2 to 3 days, the nonadherent BMMCs were collected by sedimentation (as above) and resuspended in 0.2 ml of TG. The cells were then counted, sonicated, and assayed for histamine content. BMMCs that adhered to the fibroblast monolayers were counted, scraped from the plates in 1 ml of TG, sonicated, and assayed for histamine content. These data from these experiments are expressed as micrograms of histamine per 10^6 BMMCs.

Rat MC activation with compound 48/80

Rat MC cocultured with cGVHD or control fibroblasts were tested for their responsiveness to compound 48/80, a known MC activator. For these experiments, media were

TABLE I. Proliferation of cGVHD and control fibroblasts

	[³ H]Thymidine incorporation (cpm ± SD)		
	cGVHD	B→B	<i>p</i> Value
Day 5	25,963 ± 926	12,740 ± 417	<0.001
Day 28	19,224 ± 504	12,180 ± 590	<0.002
Day 120	16,759 ± 905	17,708 ± 1215	NS

NS, Not significant.

cGVHD and B → B fibroblasts (third subculture) were incubated with [³H]thymidine to assess cell proliferation as described in MATERIAL AND METHODS.

Data are the mean ± SD of three experiments performed in sextuplicate.

withdrawn from the cultures, and monolayers were washed once with 1 ml of TG⁺⁺ (TG containing 1.8 mmol/L of CaCl₂ and 0.9 mmol/L of MgCl₂). Compound 48/80 (3 µg/ml) in 1 ml of TG⁺⁺ buffer or 1 ml of TG⁺⁺ alone was added to the cultures for 10 minutes at 37° C. To assess MC activation, histamine content in the supernatants and in the MCs was determined as described above. Data are expressed as the percentage of histamine released.

Statistical analysis

Data were analyzed with the Student's *t* test.

RESULTS

Viability and number of rat MCs cocultured with cGVHD fibroblasts

To confirm that cGVHD fibroblasts differ from control fibroblasts regarding proliferation and that this characteristic is maintained throughout the subculture passages, we measured [³H]thymidine incorporation by cGVHD and B → B control fibroblasts. As presented in Table I, cGVHD fibroblasts from days 5 and 28 from the third passage incorporated significantly greater amounts of this radiotracer in their DNA than control B → B fibroblasts. cGVHD and control fibroblasts obtained 120 days after transplantation did not differ in their proliferation rates. Moreover, the exact same pattern of [³H]thymidine incorporation was maintained throughout the subcultures, that is, from the third to tenth passages (data not presented).

Rat MCs were seeded on cGVHD or control fibroblasts, and the number of MCs adherent to the various fibroblast monolayers was evaluated (Fig. 1). Rat MCs adhered to the cGVHD (day 28) and control fibroblasts within a few minutes. Every 2 days for 6 days, the number of MCs attached to the monolayer and their viability was tested. That the number of rat MCs dropped slightly in all cultures because of a loss of attachment and subsequent death in 20% to 40% of the initially adherent MCs is illustrated in Fig. 1.

MC numbers significantly decreased (*p* < 0.05) between days 2 and 4 only on cGVHD fibroblasts; smaller decreases on the other monolayers were insignificant. cGVHD and control fibroblasts did not induce MCs to divide, as assessed by the stability of cell counts and by the absence of [³H]thymidine incorporation into the MCs (data not presented). At all time points, adherent MCs were fully viable, as demonstrated by trypan blue exclusion (>95%). Similar results were observed when MCs were cocultured on cGVHD fibroblasts obtained 5 and 120 days after the cGVHD induction (data not presented).

Effect of cGVHD fibroblasts on rat MC HR

To evaluate whether culturing rat MCs with cGVHD fibroblasts affects MC functional activity we assessed their response to activation with compound 48/80 on the sixth day of coculture. MCs activated with compound 48/80 released between 70% and 95% of their histamine content, regardless of the fibroblast monolayer on which they were cultured. MCs cultured on cGVHD monolayers, day 5, day 28, and day 120, released between 79.2% ± 3.9% to 87.7% ± 7.1% of their histamine content. MCs cultured on 3T3 or control monolayers released 95.7% ± 0.9% and 68.8% ± 1.8%, respectively. Spontaneous HR in all the monolayers measured during incubation of the cocultures with buffer alone ranged between 3.7% ± 0.2% and 18.1% ± 2.8% and was slightly higher in MCs cultured on cGVHD fibroblasts of day 28 and day 120 (13.8% ± 0.8% and 18.1% ± 2.8%, respectively).

We next determined whether cGVHD fibroblasts can themselves directly induce rat MC to release histamine on prolonged coculture. Histamine released in the different cultures was estimated by measuring the amount of histamine in the culture medium throughout 8 days of coculture and by measuring histamine content in the cells on day 8 (Fig. 2).

When MCs were cocultured on 3T3 fibroblasts, basal HR was 13.4% ± 2.9%. Coculture of MCs with cGVHD fibroblasts isolated 5 and 28 days after disease induction only slightly increased this basal HR (27.7% ± 10.0% and 24.0% ± 0.3%, respectively; *p* < 0.1). MCs cultured on control fibroblasts released a similar percentage of histamine (17.1% ± 5.9% and 24.4% ± 5.8% in BALB/c → BALB/c and healthy BALB/c, respectively).

Number, histamine content, and phenotype of mouse BMMCs cocultured with cGVHD fibroblasts

When mouse BMMCs are cocultured on 3T3 fibroblasts in the presence of IL-3, they continue to divide, and some adhere to the monolayer. (These are

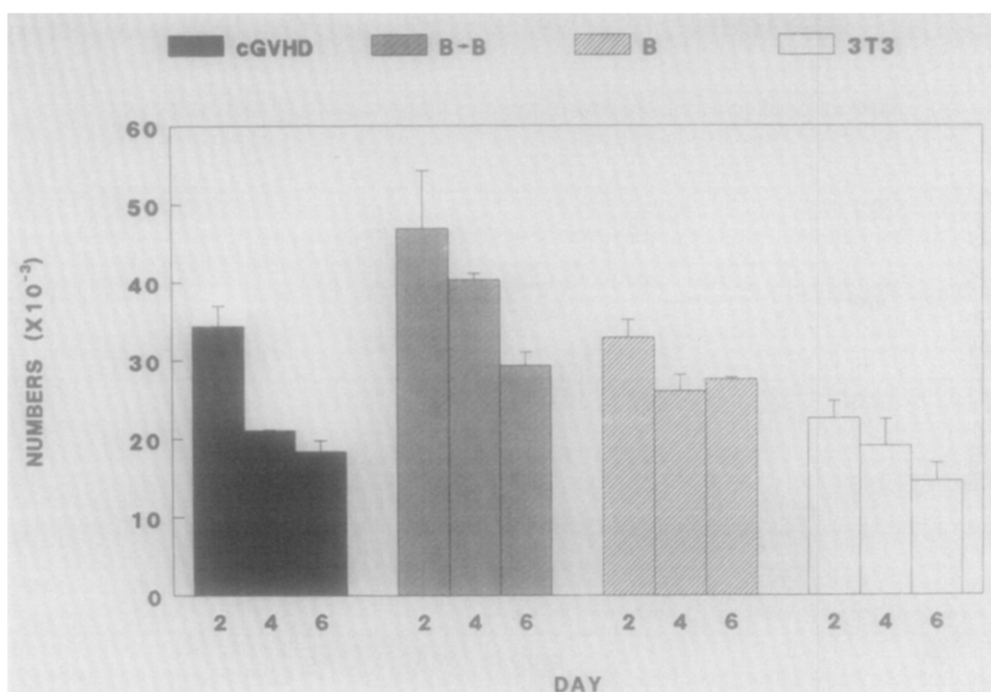


FIG. 1. Number of rat MCs adherent to cGVHD and control fibroblasts. Fibroblasts were isolated from mice 28 days after induction of disease. Rat MCs (80×10^3) were seeded on different fibroblast monolayers in EM. Every 2 days, MCs were counted during medium changing. Data are mean $\pm$ SE of two experiments performed in duplicate.

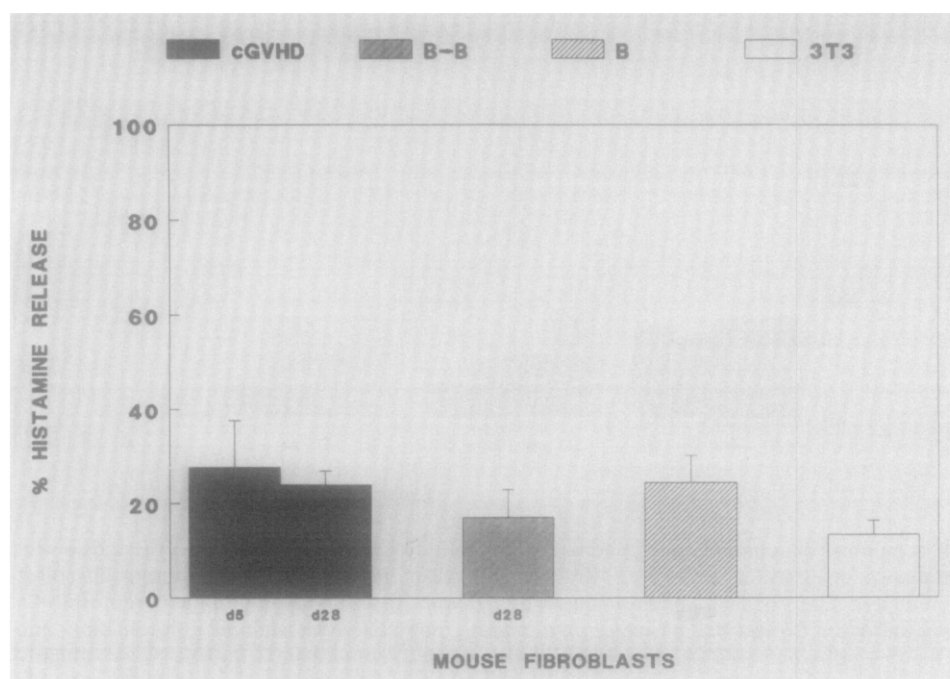


FIG. 2. Histamine released from rat MCs cocultured with cGVHD and control fibroblasts. Rat MCs were seeded on different fibroblast monolayers, and every 2 days, culture supernatants were removed and replaced with fresh media. Removed supernatant was assessed for histamine content. On day 8, experiment was terminated by sacrificing cell monolayer and assessing its histamine content. Levels of histamine released from rat MCs are listed as percentages and were calculated as described in MATERIAL AND METHODS. Data are mean $\pm$ SE of three experiments performed in duplicate.

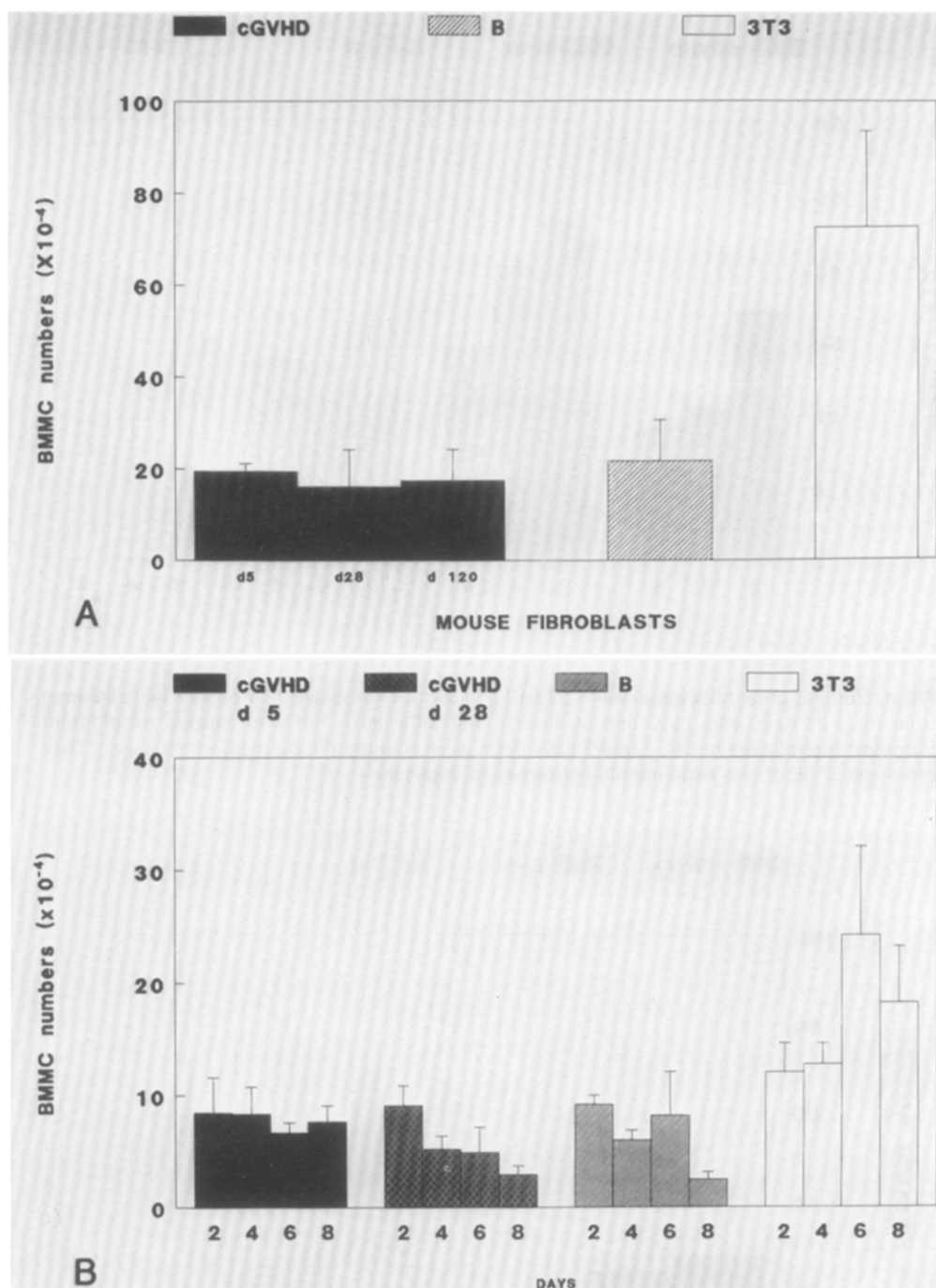


FIG. 3. Number of adherent and nonadherent mouse BMMCs cocultured with cGVHD and control fibroblasts. **A**, BMMCs, 20 to $30 \times 10^4/2$ ml EM-WELI, were cultured on different fibroblast monolayers. Every 2 days, medium was changed with fresh media, and nonadherent BMMCs were removed. On last day of experiment (day 8), monolayer was stained with toluidine blue to identify adherent BMMCs, and cells were counted. **B**, Nonadherent BMMCs were similarly counted every 2 days. Data are mean $\pm$ SE of three experiments performed in duplicate.

termed adherent BMMC.) In the experiments depicted in Fig. 3, BMMCs ($30 \times 10^4/2$ ml) were seeded on cGVHD (day 5, 28, or 120), control, or 3T3 fibroblasts, and the number of adherent BMMCs was eval-

uated on the eighth day of coculture. The 3T3 monolayer demonstrated the highest number of adherent BMMCs ($72.4 \times 10^4 \pm 29.7$), which was significantly higher than that detected on the other mono-

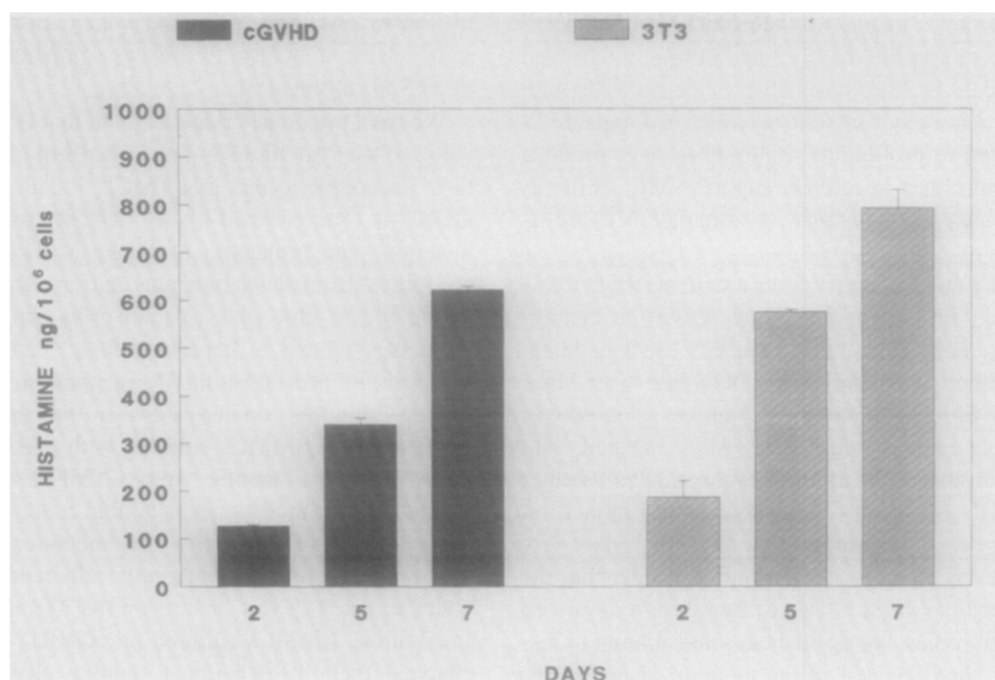


FIG. 4. Histamine content of adherent mouse BMMCs cocultured with day 28 cGVHD and 3T3 fibroblasts. Data are mean $\pm$ SE of two experiments performed in duplicate.

layers (Fig. 3, A). Similarly, when the number of nonadherent BMMCs was quantitated (every 2 days for 8 days), the highest number of cells was found in the 3T3 cocultures (Fig. 3, B). Viability of both adherent and nonadherent BMMCs was always $>95\%$.

The histamine content of adherent and nonadherent BMMCs cocultured with cGVHD fibroblasts (day 28) was evaluated as a function of the number of days of coculture. The results demonstrated that, in nonadherent BMMCs, histamine content increased approximately fourfold in 7 days (from 140.2 ± 43.0 to 598.0 ± 169.7 ng/ 10^6 cells). BMMCs seeded on cGVHD fibroblasts obtained 5 and 120 days after cGVHD induction demonstrated similar increases in histamine. In BMMCs adherent to cGVHD fibroblasts of day 28 histamine content increased to an extent similar to that of nonadherent cells (Fig. 4). BMMCs cocultured with 3T3 fibroblasts (that adhered to the monolayer) increased their histamine content in the 7 days of the experiment to a somewhat greater extent (792.5 ± 54.5 versus 621.5 ± 10.5 ; $p < 0.1$).

Adherent and nonadherent BMMCs were examined histochemically throughout the coculture period with the alcian blue/safranin-staining technique. This procedure differentiates between mouse CTMCs (alcian blue plus/safranin plus) and mouse mucosal MCs (alcian blue plus/safranin minus). After 4 to 5 days of coculture, both nonadherent and adherent BMMCs, cocultured on any of the fibroblast monolayers, dis-

played a mixed granule population in which both safranin plus and safranin minus granules were detected within the same cell. By the last day of coculture (day 9) $>95\%$ of the granules in the BMMCs were safranin plus, that is, CT phenotype.

DISCUSSION

Rat peritoneal MCs that belong to the CT subtype can be maintained in vitro on 3T3 mouse skin-derived fibroblasts.¹⁵ In contrast, mouse BMMCs maintained on such a monolayer change their mucosal phenotype toward the CT type.¹⁶⁻¹⁸ In this study we investigated the effect of culturing both subtypes of MCs on confluent monolayers of murine cGVHD skin-derived fibroblasts. Skin fibroblasts were isolated from mice with cGVHD before the development of fibrosis (day 5), during overt fibrosis (day 28), and after the recovery from cGVHD (day 120). We evaluated the degree of adherence of rat MCs seeded on cGVHD fibroblasts that were isolated on days 5, 28, and 120 after disease induction. We found that the rat MCs adhered to all three monolayers with a similar efficiency (Fig. 1). Moreover, a comparable number of MCs remained adherent to cGVHD and control fibroblasts. MCs did not proliferate in any of the cultures tested. We have previously demonstrated that rat MCs cocultured with 3T3 fibroblasts do not proliferate even in the presence of IL-2, IL-3, IL-4, or a mixture of IL-3 and IL-4. Rather, the addition of these cytokines

caused the MCs to release their histamine content.²² Therefore, it appears that 3T3 and normal mouse and cGVHD-derived fibroblasts all provide rat MCs with a "final" microenvironment in which CT-type MCs can no longer proliferate. Fibroblasts may produce factors that either selectively suppress MC proliferation or which are inadequate to stimulate MC proliferation.

Rat MCs cocultured for 6 days with cGVHD fibroblasts maintained their functional activity as demonstrated by their ability to release 80% of their histamine content when they were activated with compound 48/80. The amount of histamine released was comparable to that released by MCs cultured with control fibroblasts. In addition, we evaluated whether the cGVHD fibroblasts themselves could induce rat MCs to release histamine (Fig. 2). This possibility was excluded by experiments demonstrating that the culture medium of rat MCs cocultured for 8 days with cGVHD fibroblasts contained the same amount of histamine as that detected in the medium of control fibroblasts. This result suggests that cGVHD fibroblasts do not provide inductive stimuli for MC activation and HR. Thus, the impressive MC degranulation observed at both cGVHD onset and later during overt disease^{10, 13} cannot be attributed to MC-activating factors produced by the fibroblasts. The notion of another source of MC-activating factor(s) is supported by recent experiments performed in our laboratory demonstrating that rat and mouse MC-activating factor(s) is/are released by splenocytes of cGVHD mice.¹⁴ Thus, the results of the previous and present studies suggest that in cGVHD MCs undergo activation through a selective factor produced by cells found in various anatomic locations rather than in local CT cell populations.

In the second series of experiments, we examined the effect of culturing BMMCs with cGVHD fibroblasts. We have previously demonstrated that BMMCs seeded on a confluent monolayer of 3T3 fibroblasts in the presence of IL-3 continue to proliferate and that some of the cells adhere to the monolayer. In the present study we observed that BMMCs seeded on cGVHD and control fibroblasts adhered to the monolayers, although this was less efficient than on the 3T3 fibroblasts. It should be remembered that in these experiments nonadherent BMMCs were removed at every medium changing, and as a result of this procedure, the total number of BMMCs in cGVHD and control cultures after 8 days of coculture was smaller than in the 3T3 cultures (Fig. 3, A). It is not clear why the BMMCs adhered better to the 3T3 monolayers. However, it would appear that the molecules involved in the attachment of BMMCs to fibroblasts are

present in lower concentrations or have a lower affinity in cGVHD and control fibroblasts compared with that in 3T3 fibroblasts.

We have previously demonstrated that BMMCs cocultured for 7 to 10 days with 3T3 fibroblasts change their phenotype to that of CTMC.¹⁶⁻¹⁸ This change to CTMC is characterized by an increased synthesis of histamine and proteases, a change in histochemical properties (from alcian blue plus/safranin minus staining to alcian blue plus/safranin plus staining), the onset of synthesis of heparin proteoglycans, and the synthesis of prostaglandin D₂ in addition to leukotrienes C₄ and B₄, on activation. In the current study we found that BMMCs cultured with cGVHD fibroblasts similarly "mature" into CTMCs, as demonstrated by their increase in histamine content (Fig. 4). After 7 days of coculture, this mediator increased approximately fourfold both in the adherent and in the nonadherent BMMCs, just as on the 3T3 monolayer. In addition, BMMCs cultured on cGVHD fibroblasts changed their histochemical staining characteristics from safranin minus to safranin plus after 9 days of coculture; BMMCs cocultured on 3T3 and control fibroblasts also displayed this change in phenotype.

Several lines of evidence indicate that cGVHD fibroblasts differ from normal fibroblasts. For example, murine skin-derived cGVHD fibroblasts appear to produce more collagen per cell than do normal fibroblasts.³ In addition, we have recently demonstrated *in vitro* that cGVHD fibroblasts, at the early phase of cGVHD, synthesize significantly greater amounts of prostaglandin E₂ than control fibroblasts²³ and have a pronounced depression in IL-1 generation on stimulation with exogenous lymphokines/cytokines and liposaccharides. This finding was observed over a wide range of concentrations of lymphokines/cytokines and liposaccharides. The depressed ability of cGVHD fibroblasts to generate IL-1 was pronounced even after few passages of the cells *in vitro* and on stimulation in culture, that is, outside the suppressive milieu of the animal.⁷

The results of the present study provide corroborating evidence that cGVHD fibroblasts are inherently different from their normal counterparts. We found that cGVHD (days 5 to 28) fibroblasts have a higher proliferation rate than control B → B fibroblasts. This property too was maintained throughout several subcultures *in vitro*. Similarly, it has been demonstrated that fibroblasts derived from skin biopsy specimens of patients with scleroderma maintain their enhanced capacity to synthesize collagen and glycosaminoglycans on consecutive *in vitro* subcultures.²⁴⁻²⁶

In summary, these data obtained in the present study demonstrate that the altered biochemical characteris-

tics displayed by cGVHD fibroblasts do not adversely affect their ability to maintain the viability and functional activity of rat MCs and to induce a change in phenotype of mouse BMMCs.

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