Macrophage Inflammatory Protein 1α, Interleukin 3 and Diffusible Marrow Stromal Factors Maintain Human Hematopoietic Stem Cells for at least Eight Weeks In Vitro

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Summary

Factors that induce proliferation of the human hematopoietic stem cell are ill-defined. Primitive hematopoietic progenitors can be maintained and differentiate in stroma-dependent, long-term bone marrow cultures (LTBMC), originally described by Dexter et al. (Dexter, T. M., L. H. Coutinho, E. Spooncer, C. M. Heyworth, C. P. Daniel, R. Schiro, J. Chang, and T. D. Allen. 1990. Molecular Control of Haemopoiesis). However, 70–80% of primitive progenitors capable of reinitiating secondary stromal cultures (LTBMC-initiating cells [IC]) are lost over a period of 5 wk in such cultures. We have recently described a novel "stroma-noncontact" culture system, in which hematopoietic progenitors are separated from the stromal layer by a 0.4-μm microporous filter membrane. Primitive progenitors in such cultures can not only differentiate into committed progenitors, but are also maintained to a greater extent than in "Dexter" cultures. However, still only 50% of the originally seeded LTBMC-IC are recovered at week 5. Since maintenance of primitive progenitors may depend not only on growth-promoting factors but also on factors that inhibit differentiation and/or proliferation, we evaluated the effect of macrophage inflammatory protein 1 α (MIP-1α) or "stem cell inhibitor"' in combination with the growth-inducing factor interleukin 3 (IL-3) on the recovery of LTBMC-IC from stroma-noncontact cultures. We demonstrate that addition of MIP-1α alone to stroma-noncontact cultures does not change the number of LTBMC-IC present after 8 wk, indicating that this factor may not directly inhibit or stimulate proliferation of primitive progenitors. Addition of the growth stimulatory cytokine, IL-3, alone results in exhaustion of LTBMC-IC after 8 wk of culture, possibly as a result of their terminal differentiation. However, LTBMC-IC can be maintained for at least 8 wk when grown in stroma-noncontact cultures supplemented with both MIP-1α plus IL-3. This effect depends on soluble (ill-defined) stromal factors, and results from a direct interaction of these cytokines with the progenitor population or its progeny, but not the stroma.

Mechanisms that govern induction of a quiescent state, proliferation, and differentiation of primitive human hematopoietic progenitors, are not well understood. In vivo, hematopoiesis takes place in close proximity with the bone marrow microenvironment where hematopoietic stem cells reside, usually in Go, and occasionally proliferate and differentiate into more mature precursors. In vitro, this process is mimicked relatively closely in stroma-dependent, long-term bone marrow cultures (LTBMC)[1], originally described by Dexter et al. (1). It is thought that proliferation of primitive progenitors in these cultures is the result of the relative availability of both growth-promoting cytokines, which can be induced by a variety of stimuli, and the negative influence of growth inhibitory cytokines such as TGF-β (2). However, human stroma-dependent LTBMC have a limited life span, and we and others (3, 4) have demonstrated that 70–80% of primitive progenitors capable of reinitiating secondary stromal cultures (LTBMC-initiating cell [IC]) are lost over a period of 5 wk of culture.

We have recently developed an alternative long-term culture system in which hematopoietic progenitors are grown at a distance of the stromal layer in transwell inserts ("stroma-noncontact" culture) (4, 5). Cells in the transwell cannot physically interact with stromal cells present in the bottom well underneath the transwell, but diffusible factors can freely pass the 0.4-μm filter membrane. We have demonstrated that primitive $CD34^+/DR^-$ cells grown in such cultures can not only

[1] *Abbreviations used in this paper:* BM, bone marrow; CFC, colony-forming cell; IC, initiating cell; LDA, limiting dilution analysis; LIF, leukemia inhibitory factor; LTMBC, long-term bone marrow culture; MIP-1α, macrophage inflammatory protein 1α; rHu, recombinant human; SCF, stem cell factor.

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differentiate into committed progenitors and mature myeloid elements, but that significantly more LTBMC-IC can be recovered from these cultures than from stromal cultures in which the hematopoietic cells are cultured in direct contact with the stromal layers. However, the recovery of LTBMC-IC at week 5 of culture is still not >50% of the originally seeded LTBMC-IC. To improve the recovery of LTBMC-IC in vitro, we have previously examined the effect of various, known growth-promoting cytokines, including IL-1, IL-3, IL-6, G-CSF, GM-CSF, leukemia inhibitory factor (LIF), and stem cell factor (SCF), on the recovery of LTBMC-IC from stroma-noncontact cultures (5). Although several of these cytokines improve the total cell expansion and production of committed progenitors in these cultures, none of the tested cytokines, either alone or in combination, were able to increase the number of LTBMC-IC present after 5 wk.

Macrophage inflammatory protein 1α (MIP-1α), also termed stem cell inhibitor (6, 7), is an 8-kD chemokine produced by macrophages, T lymphocytes, and bone marrow (BM) stromal cells (7). MIP-1α reversibly inhibits the proliferation of murine day 12-CFU-S and CFU-A both in vitro and in vivo (6, 8). In humans, MIP-1α reversibly inhibits the proliferation of CFU-granulocyte, erythrocyte, macrophage, megakaryocyte (GEMM), burst forming unit-erythroid (BFU-E), and CFU-GM, whereas more mature CFU-E and single lineage CFU-G and CFU-M progenitors (9, 10) are stimulated. These studies suggest that MIP-1α may have an important role in maintaining immature hematopoietic progenitors in a quiescent state. Since loss of primitive progenitors from ex vivo long-term cultures may be the result of excessive stimulation of the usually quiescent primitive hematopoietic compartment resulting in their irreversible terminal differentiation, we elected to evaluate the effect of MIP-1α on the recovery of immature LTBMC-IC from long-term stroma-noncontact cultures.

Materials and Methods

Cell Separations

BM was aspirated from the posterior iliac crest in heparin from healthy young volunteers after informed consent. Primitive CD34$^+$/HLA-DR$^-$ cells (DR$^-$ cells) were purified by sequential Ficoll-Hypaque centrifugation (s.g. 1077) (Sigma Chemical Co., St. Louis, MO), counterflow centrifugation elutriation (11, 12), depletion of T lymphocytes and NK cells by sheep erythrocyte rosetting (13), and depletion of committed lymphoid and myeloid/monocytic cells using immunomagnetic beads and anti-CD11b, anti-CD19 (Becton Dickinson & Co., Mountain View, CA), anti-MY8 (Coulter Cytometry, Hialeah, FL) and anti-glycophorin-A antibodies (AMAC, Inc., Westbrook, ME) (14). The resultant lineage-negative cells were selected on a FACStar Plus® laser flow cytometry system (Becton Dickinson & Co.) equipped with a Consort 32 computer for low vertical and low horizontal light scatter properties and expression of CD34, but absence of HLA-DR antigens using mouse IgG1-PE and mouse IgG2a-FITC antibodies as controls (14).

Long-term Culture Systems

Complete LTBMC media consisted of IMDM with 12.5% FCS (Hyclone Laboratories, Logan, UT), 12.5% horse serum (Terry Fox Laboratories, Vancouver, Canada), 2 mM L-glutamine (GIBCO BRL, Gaithersburg, MD), 1,000 U/ml penicillin, 100 U/ml streptomycin (GIBCO BRL), and 10^{-6} M hydrocortisone (14).

Stroma-free Cultures. DR$^-$ cells were suspended in transwell inserts (14 × 10^3/5 ml for 6-well plates) placed in stroma-free wells. Culture media consisted of complete LTBMC media with or without cytokines.

Stroma-noncontact Cultures. Transwell cultures consisted of allogeneic irradiated stromal cells subcultured in the bottom well of 6- (1.75 × 10^6 cells suspended in 3 ml) or 24- (0.35 × 10^6 cells suspended in 0.8 ml) well plates. A collagen-treated transwell insert (0.4-μm microporous filter) (Costar Corp., Cambridge, MA) was then placed above the stromal layer, and sorted DR$^-$ cells plates in the upper wells (2 × 10^3 cells/well of 24-well plates, or 4.5–14 × 10^3 DR$^-$ cells/5 ml of 6-well plates).

Conditioned Media Cultures. DR$^-$ cells (14,000 cells/well of 6-well plates) were plated in transwell inserts of stroma-free wells. Culture media for these cultures consisted of stroma-conditioned media obtained from T-150 flasks containing irradiated allogeneic stromal layers. Media was transferred from the flask to the hematopoietic cell containing wells 2, 4, and 7 d after feeding the stroma-containing culture vessel. Upon transfer of the media from the T-150 flask, cytokines were added to obtain the final desired concentration.

Maintenance of Cultures. All cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. At weekly intervals stroma-noncontact and stroma-free cultures were fed by removing half the cell-free media from the bottom wells only and replacing with fresh complete media. Conditioned media cultures received media conditioned by allogeneic irradiated stromal layers for 2, 4, or 7 d after feeding.

Enumeration of Cell Number. Cells recovered from stroma-free, stroma-noncontact, or conditioned media cultures were recovered at week 1, 2, 3, 5, or 8 from the transwell inserts after vigorous washing. Cells were resuspended in IMDM plus 20% FCS, and an aliquot obtained for enumeration under hemocytometer.

Enumeration of Colony-forming Cell (CFC) by Methylcellulose Culture. Cells harvested at week 1, 2, 3, or 5 from transwell inserts of stroma-noncontact cultures were plated in methylcellulose progenitor assay supplemented with 3 IU erythropoietin (Epoietin; Amgen Biologicals, Thousand Oaks, CA) and 5–10% 5637 bladder carcinoma conditioned media as described (14). The cultures were assessed at day 18 of culture for the presence of secondary CFC.

Enumeration of the Absolute Number of LTBMC-IC by Limiting Dilution Assays (LDA). At day 0, DR$^-$ cells (22 replicates per concentration; 400, 150, 50, and 15 DR$^-$ cells/well) were plated onto 3 × 10^4 irradiated allogeneic stromal cells or irradiated M2-10B4 murine stromal cells subcultured in 96-well plates. Likewise, cells recovered after 5 or 8 wk from stroma-noncontact cultures, stroma-free cultures, and conditioned media cultures initiated at day 0 with 13,530 DR$^-$ cells were plated in LDA (cell number is the equivalent of 400, 150, 50, and 15 DR$^-$ cells at day 0) as described (4). In each experiment, day 0 LDAs and LDAs performed at weeks 5 and 8 with cultured cells, were always performed either on marrow stromal feeders or on M2-10B4 feeders. Cultures were maintained at 37°C and 5% CO_2 in a humidified atmosphere for an additional 5 wk. At weekly intervals, 75 μl media was removed and replaced by 75 ml fresh complete media. At week 5, all media was removed and the stromal layers with hematopoietic cells overlaid with 200 μl methylcellulose media supplemented with 30% FCS, 5–10% supernatant of the bladder cancer cell line 5637 and 3 IU erythropoietin. 2 wk later, wells were scored for the presence or absence of CFC as described (5). The absolute number of LTBMC-IC present in the different cell populations was calculated

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as the reciprocal of the concentration of test cells that gives 37% negative cultures using the Poisson statistics (15) and the weighted mean method (16).

Cytokines

Recombinant human (rHu) IL-3 (Genetics Institute, Cambridge, MA) was added to cultures three times per week to obtain a final concentration of 5 ng/ml (as indicated in the Results section). rHu stem cell factor (SCF) (Amgen) was added to stroma-free cultures three times per week to obtain a final concentration of 20 ng/ml (as indicated in the Results section). rHuMIP-1α (R&D Systems, Inc., Minneapolis, MN) was added to cultures three times per week to obtain a final concentration of 10, 50, or 100 ng/ml as indicated (concentrations were based on studies demonstrating a growth inhibitory effect of 100 ng/ml MIP-1α on committed human hematopoietic progenitors [9, 10]). TGF-β1 (R&D Systems, Inc.) was added once weekly 4 d after the regular feeding. Since cyclical proliferation of immature CFC in LTBMC is thought to be the result of increased production of growth-promoting factors early after feeding followed by the proliferation inhibitory effects of TGF-β in the second part of the week after feeding, we elected to add TGF-β 4 d after feeding only in association with IL-3 three times per week to mimic cytokine fluxes thought to induce cyclical proliferation of primitive CFC in LTBMC (2).

Stromal Layers

BM Stromal Layers. BM stromal layers were generated as previously described (15). In short, BM mononuclear cells from normal individuals were suspended in complete LTBMC media and incubated for 4 d at 37°C in a humidified atmosphere with 5% CO_2, and afterwards at 33°C. Cultures were demipopulated weekly and media replaced with fresh complete media. Once confluent (3–5 wk after initiation of the culture) stromal layers were irradiated with 1,250 rad using a Mark-1 cesium irradiator (Shepard and Associates, Glendale, CA). Stromal layers were then subcultured in wells of 6-, 24-, or 96-well plates before use.

M2-10B4. M2-10B4, a murine BM stroma cell line, was maintained in RPMI-1640 supplemented with 10% FCS. M2-10B4 was subcultured in the wells of a 96-well plate (Costar Corp.). When confluent, plates were irradiated with 6,000 rad using a cesium irradiator, and all media replaced by complete LTBMC media. Irradiated plates were used 2–7 d later. Several studies have demonstrated that maintenance of LTBMC-IC and their differentiation into CFC is similar when DR$^-$ cells are cultured either on BM stromal layers or on murine M2-10B4 feeders (3, 17).

Statistics

Results of experimental points obtained from multiple experiments were reported as the mean ± 1 SEM. Significance levels were determined by two-sided Student's t test analysis.

Results and Discussion

To determine if addition of MIP-1α alone or in conjunction with factors with known growth-promoting activity to stroma-noncontact cultures could maintain a constant level of primitive human hematopoietic progenitors in in vitro cultures, DR$^-$ cells, which contain the LTBMC-IC (14, 18), and transplantable hematopoietic stem cells (19), were cultured in stroma-noncontact cultures supplemented with or without MIP-1α, IL-3, or MIP-1α plus IL-3. Cell expansion was significantly greater in stroma-noncontact cultures supplemented with IL-3 alone or MIP-1α+IL-3 than in cytokine-free cultures (Fig. 1) or cultures with MIP-1α alone. Significantly more CFC were present in stroma-noncontact cultures supplemented with IL-3 or MIP-1α+IL-3, 3 and 5 wk after initiation of the culture (Fig. 2) than in cytokine-free or MIP-1α supplemented cultures. DR$^-$ derived progeny, recovered 5 wk after initiation of the stroma-noncontact

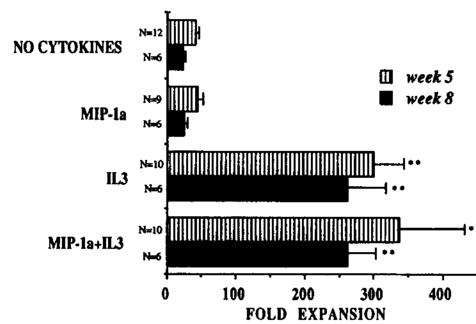

Figure 1. 1–2.5 × 10^3 FACS® sorted DR$^-$ cells/ml were cultured in stroma-noncontact cultures supplemented without cytokines, MIP-1α (100 ng/ml, three times per week), IL-3 (5 ng/ml, three times per week), or 5 ng/ml IL-3 and 100 ng/ml MIP-1α three times per week for 5 and 8 wk. Cells were collected from the transwell insert and enumerated under hemocytometer. Fold expansion was calculated as the number of cells present in transwell inserts at the different time points divided by the number of cells used to initiate the cultures. Differences between cytokine-free or MIP-1α–supplemented cultures and either IL-3 or MIP-1α+IL-3–supplemented cultures were significant at the <0.001 level (**). Differences between cytokine-free and MIP-1α–supplemented were not significant. Differences between IL-3 and MIP-1α+IL-3–supplemented cultures were not significant.

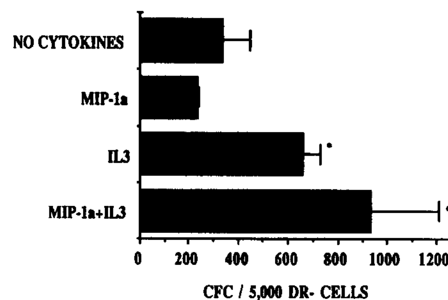

Figure 2. FACS® sorted DR$^-$ cells (n = 3) were cultured in stroma-noncontact cultures supplemented without cytokines, MIP-1α (100 ng/ml three times per week), IL-3 (5 ng/ml, three times per week), or IL-3 and MIP-1α (5 and 100 ng/ml, three times per week) for 5 wk. Cells were collected from the transwell insert and replated in methylcellulose progenitor culture (*). The cultures were assessed at day 18 of culture for the presence of secondary CFC as previously described (*). Comparison between cytokine-free and IL-3–supplemented cultures: (*) $p \leq 0.01$. Comparison between cytokine-free or MIP-1α–supplemented cultures and MIP-1α+IL-3–supplemented cultures: (¶) $p < 0.05$. Differences between cytokine-free and MIP-1α–supplemented cultures were not significant. Differences between IL-3 and MIP-1α+IL-3–supplemented cultures were not significant.

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cultures, was also replated in LDA onto secondary stromal feeders to enumerate the absolute number of LTBMC-IC present in the transwell inserts. Approximately 50% of LTBMC-IC present in originally sorted DR$^-$ cells were still present at week 5 in cytokine-free stroma-noncontact cultures (Fig. 3). Addition of MIP-1α or IL3 alone to stroma-noncontact cultures did not significantly change the number of LTBMC-IC present at week 5 compared to cytokine-free cultures. However, addition of the combination of MIP-1α+IL3 to stroma-noncontact cultures resulted in the recovery of 95–200% of the initially seeded LTBMC-IC after 5 wk of culture.

Whereas addition of early acting, growth-promoting cytokines, such as IL-3, LIF, G-CSF, or SCF to stroma-noncontact cultures does not increase the recovery of LTBMC-IC, combined addition of supraphysiological amounts of these cytokines results in a significantly greater loss of primitive progenitors over time (5). This suggests that multiple cytokines may overwhelm the (ill-defined) antidifferentiation effect of stroma-derived factors and may result in irreversible differentiation of primitive progenitors. To determine if continued presence of high concentrations of MIP-1α+IL-3 in stroma-noncontact cultures would result in a similar exhaustion of the primitive LTBMC-IC pool, stroma-noncontact cultures with or without these cytokines were maintained for 8 wk before replating the DR$^-$ progeny in LDA onto secondary stromal feeders (Fig. 3). Culture of DR$^-$ cells in cytokine-free stroma-noncontact cultures or cultures supplemented with MIP-1α alone for 8 rather than 5 wk resulted in an ~60% loss of LTBMC-IC, which was not significantly different than that observed at week 5. Interestingly, culture of DR$^-$ cells in IL-3 supplemented stroma-noncontact cultures for 8 wk resulted in a significant depletion of LTBMC-IC (<5% of the originally sorted DR$^-$ population). However, combined addition of MIP-1α+IL-3 resulted in an insignificant decrease in LTBMC-IC number compared with week 5 and continued recovery of 80–150% of day 0 LTBMC-IC. We demonstrate that addition of 10, 50, or 100 ng/ml MIP-1α with IL-3 to stroma-noncontact cultures resulted in a similar recovery of LTBMC-IC (data not shown). These results are consistent with those described by Mantel et al. (20) who recently demonstrated that addition of MIP-1α at concentrations ranging from 10 to 500 ng/ml to methylcellulose cultures had equivalent effects on committed murine hematopoietic progenitor growth.

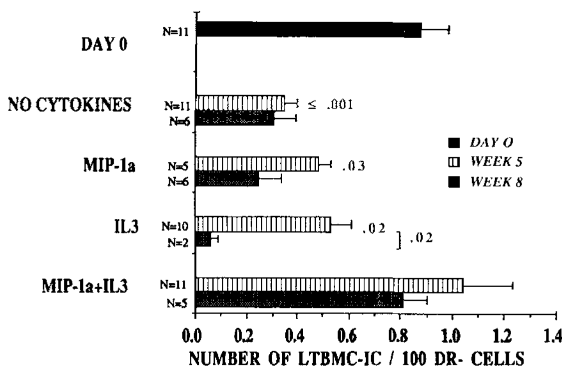

Figure 3. Freshly sorted DR$^-$ cells were plated in limiting dilution on bone marrow stromal layers or M210-B4 feeders (22 replicates of 400, 150, 50, and 15 cells) for 5 wk (*Day 0*). 14,000 DR$^-$ cells were plated in stroma-noncontact cultures supplemented without cytokines, with MIP-1α alone (100 ng/ml, three times per week), IL-3 (5 ng/ml, three times per week), or IL-3 and MIP-1α (5 and 100 ng/ml, three times per week) for 5 and 8 wk. Cells recovered from the transwell inserts were then replated in LDA on either bone marrow stromal layers or M210-B4 feeders for an additional 5 wk (cell number = the equivalent of 400, 150, 50, and 15 DR$^-$ cells at day 0). After 5 wk, stromal layers were overlayed with methylcellulose-containing media supplemented with erythropoietin and supernatant from the bladder CA cell line 5637, and wells were scored 2 wk later for the presence or absence of CFC and the absolute number of LTBMC-IC present in the different cell populations calculated (*). Comparison between day 0 and week 5 is noted above the week 5 bars. Comparison between weeks 5 and 8 is only significant for cultures supplemented with IL-3.

The mechanism(s) through which MIP-1α+IL-3 in stroma-noncontact cultures maintain primitive progenitors capable of reinitiating long-term cultures are unclear. Although the receptor for MIP-1α has recently been identified as a seven *trans*-membrane spanning receptor belonging to the superfamily of G protein–linked receptors (21), the molecular mechanism(s) underlying the growth-modulating effects of MIP-1α are unknown. We hypothesize that the increased recovery of LTBMC-IC from cultures supplemented with MIP-1α+IL-3 may be the result of an increased proliferation without terminal differentiation of LTBMC-IC. MIP-1α was originally described as "stem cell inhibitor" since it inhibits in vitro and in vivo proliferation of immature CFU-A, CFU-S, and CFU-GEMM progenitors (6–10). Our observations may, however, not be the result of growth inhibition. In contrast to the effect of MIP-1α+IL-3 on LTBMC-IC recovery, combined addition of TGF-β+IL-3 to stroma-noncontact cultures resulted in a decreased recovery of cells (data not shown), CFC (data not shown), and LTBMC-IC after 5 wk of culture compared with cultures to which IL-3 alone was added (Fig. 4). It is well known that TGF-β inhibits cell proliferation by preventing their transition through the cell cycle (22, 23). A direct antiproliferative effect of TGF-β may therefore

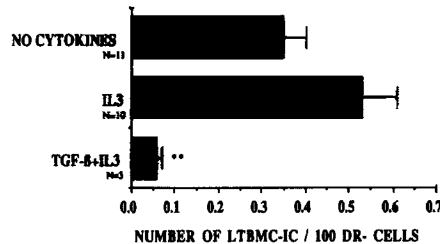

Figure 4. DR$^-$ cells were cultured for 5 wk in stroma-noncontact cultures supplemented without cytokines or with either IL-3 alone (5 ng/ml three times per week) or TGF-β+IL-3 (5 ng/ml TGF-β once per week, 4 d after the regular feeding and 5 ng/ml IL-3 three times per week). LTBMC-IC content was determined as described in Materials and Methods. Comparison between cultures supplemented with IL-3 alone or cytokine-free cultures and cultures supplemented with TGF-β+IL-3, (**) $p \leq 0.001$.

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underlie the decreased recovery of LTBMC-IC from TGF-β plus IL-3–supplemented cultures, whereas this mechanism may not be operative in cultures supplemented with MIP-1α + IL-3. Recent in vivo studies have demonstrated that MIP-1α may not maintain the mouse long-term repopulating stem cell, which is more primitive than the CFU-S and CFU-A progenitor, in a quiescent state when triggered into cycle by 5-fluorouracil treatment (24). These studies, together with data presented here indicating that addition of known proliferation inhibitory factors such as TGF-β results in significantly decreased recovery of LTBMC-IC from stroma-noncontact cultures, indicate that MIP-1α + IL-3 may not inhibit the proliferation of LTBMC-IC. Increased recovery of LTBMC-IC from MIP-1α + IL-3 supplemented cultures could result from a proliferation-inducing effect of MIP-1α. However, since addition of MIP-1α without the growth-promoting cytokine IL-3 failed to increase the recovery of LTBMC-IC, MIP-1α may not have a direct proliferation inducing effect on primitive human hematopoietic progenitors. Prolonged stimulation of DR$^-$ cells in stroma-noncontact cultures with IL-3 alone results in eventual depletion of primitive LTBMC-IC (Fig. 3), likely as a result of their terminal differentiation. We believe therefore that MIP-1α may prevent the terminal differentiation of immature progenitors induced to proliferate by relatively high levels of growth-promoting factors, such as IL-3, and thus result in expansion and/or accumulation of primitive progenitors.

Recent studies employing a stroma-free culture system have demonstrated that culture of unfractionated murine marrow cells or Sca$^+$Lin$^-$ cells in the presence of SCF, IL-11, and MIP-1α results in a significantly greater expansion of CFU-A compared with cultures supplemented with SCF and IL-11 alone (25). To examine if stroma is required to maintain a constant number of LTBMC-IC for 5–8 wk in the presence of MIP-1α + IL-3, DR$^-$ cells were also cultured in "stroma-free" cultures, in which DR$^-$ cells were suspended in transwell inserts placed in empty wells. These cultures were fed weekly with fresh complete media, not conditioned by stromal layers, and supplemented three times per week with MIP-1α + IL-3 + SCF. Only 5% of LTBMC-IC present at day 0 in freshly sorted DR$^-$ cells were recovered from stroma-free cultures supplemented with MIP-1α + IL-3 + SCF (Fig. 5), indicating that additional stroma-derived soluble factors are synergyzing with MIP-1α + IL-3 to maintain a constant level of human LTBMC-IC in in vitro cultures.

MIP-1α receptors are present on monocytes/macrophages (7) and MIP-1α induces chemotaxis and oxidative burst in monocytes (26). Observations described here could therefore be the result of the induction of secondary differentiation-inhibitory or proliferation-inducing factors by stromal macrophages. Addition of MIP-1α + IL-3 did, however, not significantly change the level of cytokines, including GM-CSF and IL-1β, present in stromal supernatants, as determined by ELISA (data not shown). To examine if the observed effects of MIP-1α + IL-3 are the result of their direct interaction with hematopoietic progenitors or of an indirect effect on stromal cells, we cultured DR$^-$ cells in LTBMC

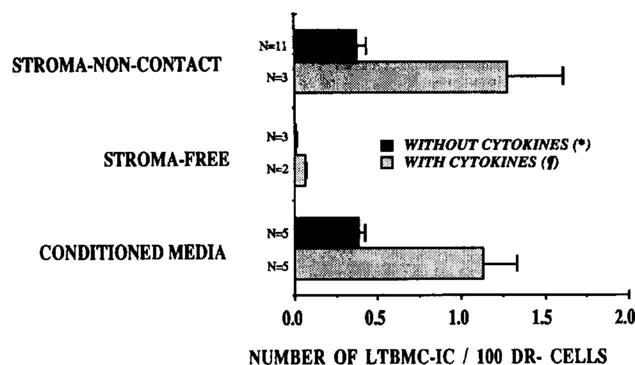

Figure 5. 14,000 DR$^-$ cells were plated in stroma-noncontact cultures supplemented with (*) or without MIP-1α+IL-3 (¶). Alternatively, 14,000 DR$^-$ cells were plated in stroma-free cultures supplemented with SCF (20 ng/ml three times per week) and IL-3 (5 ng/ml three times per week) and with (¶) or without (*) MIP-1α (100 ng/ml three times per week) for 5 wk. 14,000 DR$^-$ cells were also plated in conditioned media cultures. Cytokines were added to the conditioned media upon transfer from the stroma-containing T-150 flask to the DR$^-$ cell–containing culture vessel to obtain the final desired concentration in those cultures with cytokines (¶) (5 ng/ml IL-3 three times per week and 50 ng/ml MIP-1α three times per week). Cells recovered from the transwell inserts of all three culture systems were replated in LDA on either bone marrow stromal layers or M210-B4 feeders for an additional 5 wk (cell number = the equivalent of 400, 150, 50, and 15 DR$^-$ cells at day 0) and the absolute number of LTBMC-IC present in the different cell populations was calculated as described.

media conditioned by allogeneic stromal layers present in a separate culture vessel. MIP-1α + IL-3 was added to the conditioned media when transferred to the hematopoietic cell–containing well and was therefore never in contact with the stromal layer. Culture of DR$^-$ cells in conditioned media without cytokines results in maintenance of an equivalent number of LTBMC-IC as when cultured in stroma-noncontact cultures (Fig. 5). When MIP-1α + IL-3 were added to the conditioned media, a similar number of LTBMC-IC was present after 5 wk of culture compared with that in MIP-1α + IL-3 containing stroma-noncontact cultures (Fig. 5). This suggests that MIP-1α + IL-3 exert their effect through direct interaction with DR$^-$ cells or their progeny rather than through altering the bone marrow microenvironment milieu.

We demonstrate for the first time that at least 100% of LTBMC-IC can be maintained in vitro for a minimum of 2 mo when cultured in the presence of MIP-1α + IL-3 in stroma-noncontact cultures. MIP-1α + IL-3 require additional undefined diffusible factors from stroma to maintain a constant number of LTBMC-IC for 5–8 wk in culture. The effect of MIP-1α + IL-3 is, moreover, the result of the direct interaction of MIP-1α + IL-3 with the DR$^-$ cell population or its progeny, but not with the stromal layer. It remains to be seen if the addition of other early-acting, growth-promoting cytokines, such as IL-6 (11), IL-11 (27), LIF (28), SCF (29), or G-CSF (30), to the combination of MIP-1α, IL-3, and stroma-derived factors will further expand the human primitive progenitor compartment ex vivo, but not trigger the

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primitive cell compartment into irreversible terminal differentiation. The observation that a constant level of very primitive LTBMC-IC can be maintained for at least 8 wk in ex vivo cultures when grown in the presence of MIP-1α, IL-3, and stroma-derived soluble factors suggests a possible clinical application for such culture systems for in vitro stem cell expansion and retroviral gene transfection protocols.

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