Hydrocortisone Differentially Affects the Ability of Murine Stromal Cells and Human Marrow-Derived Adherent Cells to Promote the Differentiation of CD34++/CD38- Long-Term Culture-Initiating Cells

By Laure Croisille, Isabelle Auffray, André Katz, Brigitte Izac, William Vainchenker, and Laure Coulombel

Very primitive human hematopoietic progenitor cells are identified indirectly by their ability to give rise to clonogenic progenitors in the presence of either human or murine stromal cells. These long-term culture-initiating cell (LTC-IC) assays are usually performed in the presence of hydrocortisone based on the observation that hydrocortisone was required for prolonged hematopoiesis in standard long-term bone marrow cultures. In this report, we investigated the role of hydrocortisone in LTC-IC assays initiated with CD34++/CD38- cells seeded onto either human bone marrow LTC-derived adherent cells or a murine marrow-derived stromal cell line, MS-5. It was found that weekly addition of hydrocortisone to the cultures reduced the frequency of LTC-IC (from 1/5 to 1/20) calculated from limiting dilution experiments and also reduced fivefold to 10-fold the number of their progeny clonogenic cells detected after 4 to 5 weeks. In contrast, the frequency and differentiative potential of CD34++/CD38- grown in the presence of MS-5 cells served as a reservoir for growth factors, has also been reported with hydrocortisone.

Expression of the functional properties of early human stem cells requires the presence of stromal cells; in the absence of an exogenous supply of human growth factors. The most primitive human progenitor cell characterized so far in vitro is indirectly identified by its ability to produce clonogenic progenitors and mature precursors of the erythroid and granulocytic lineages when cocultured with stromal cells for 4 to 8 weeks and has been referred to as long-term culture-initiating cell (LTC-IC).1,3 Successful amplification and differentiation of LTC-IC-derived myeloid progenitors in standard LTC initiated with unfractionated marrow cells requires a mixture of fetal calf serum and horse serum, maintenance of the cultures at 33°C, and the supplementation of the cultures with 10^{-6} mol/L hydrocortisone. In contrast, conditions permissive for B-lymphoid differentiation require that neither horse serum nor hydrocortisone be present. It has been inferred from these initial studies that similar conditions will be required in LTC-IC assays in which cell suspensions enriched in progenitors are cocultured with preestablished stromal cells of either human or murine origin.1,2,3,4 It is generally admitted that target cells for hydrocortisone action are the stromal components, but the exact mechanisms of action of glucocorticoids remain mysterious and somewhat contradictory. Thus, on one hand, hydrocortisone favors the development of adipocytes thought to be important for active hematopoiesis despite the lack of unambiguous proof and prevents excessive accumulation of macrophages through inhibition of response to colony-stimulating factor-1 (CSF-1).5,14 On the other hand, this hormone downregulates cytokine production by macrophages and fibroblasts,15,16 two essential components of the marrow environment. Modulation of extracellular matrix proteins such as fibronectin or proteoglycans,17 which serve as a reservoir for growth factors, has also been reported with hydrocortisone.

We recently reported that a murine marrow-derived stromal cell line MS-5 supports the differentiation of CD34+/CD38- human bone marrow (BM) cells towards myeloid lineages in LTC-IC assays.1 In an attempt to investigate whether MS-5 cells will also have a lymphoid promoting effect, we initiated CD34+/CD38- LTC-IC assays in the absence of hydrocortisone, which inhibits B-lymphoid differentiation. Unexpectedly, we observed that hydrocortisone used at 10^{-6} mol/L drastically reduced the generation of myeloid progenitors by CD34+/CD38- cocultured with MS-5 cells, even in the presence of exogenously added human cytokines. This finding contrasted with the lack of effect of hydrocortisone on the behavior of LTC-IC cultured on irradiated human marrow adherent cells derived from long-term BM cultures (LTBMC). Hydrocortisone also decreased granulocytic colony formation by CD34+/CD38- cells in methylcellulose assays performed in the presence of MS-5 and various combinations of cytokines. These studies strongly suggest that hydrocortisone might downregulate an MS-5-derived activity, promoting differentiation of...
CD34+/CD38- LTC-IC. We also believe that the systematic use of hydrocortisone in LTC-IC assays should be reevaluated and might not be necessary for prolonged active myeloid use of hydrocortisone in LTC-IC assays should carefully resuspended in fresh QMEM with 100 μg/mL DNAse labeling medium and proceeded for labeling.

MATERIALS AND METHODS

**Marrow cell preparation.** Bone fragments were obtained after obtaining informed consent from patients undergoing hip surgery and marrow cells were collected by vigorous shaking of bone fragments in a minimal essential medium (αMEM) supplemented with 100 μg/mL of deoxyribonuclease (DNase type I; Sigma Chemical Co, St Louis, MO). Cells were centrifuged once, counted, and separated on Ficoll-Hypaque. Light-density (<1.077 g/mL) cells were suspended at a concentration of 1 × 10^6 cells/mL in αMEM supplemented with 30% fetal calf serum (FCS; JBio Laboratory, Les Ulis, France) and kept at 4°C overnight. The next day, the cells were carefully resuspended in fresh αMEM with 5% FCS and 100 μg/mL DNase (labeling medium) and proceeded for labeling.

**Cell separation.** Cells (2 × 10^5 cells/mL) were incubated 45 minutes at 4°C with a 1/5 dilution of the phycoerythrin (PE)-labeled anti-CD34 monoclonal antibody (MoAb) HPCA2 and the fluorescein isothiocyanate (FITC)-labeled anti-CD38 MoAb (both purchased from Becton Dickinson, San Jose, CA). Cells were resuspended once and suspended in labeling medium at a concentration of 4 to 5 × 10^6 cells/mL for separation by cell sorting.

Cells were analyzed and sorted on an ODAM ATC 3000 cell sorter (ODAM/Bruker, Wissembourg, France) equipped with an INNOVA 70-4 Argon ion laser (Coherent Radiation, Palo Alto, CA) tunneled at 488 nm and operating at 500 mW. The wide angle light scatter (WALS) was first extracted with a 510 DCLP filter and the two fluorescence signals were then separated with a 550 DCSP filter and finally isolated using a 530 DF 30 filter for FITC and a 575 DF 26 filter for PE (all filters from Omega Optical Inc, Brattleboro, VT). Compensation for double-stained samples was set up with single-stained samples. Data were analyzed on the ATC 3000 computer (ODAM/Bruker) and all parameters were displayed on a linear scale. Cells "negative" with respect to CD38 and expressing high levels of CD34 (referred to as CD34+/CD38- cells) were sorted. To increase the purity of the sorting, the morphologic two-parameter histogram (WALS v electric measurement of the cell volume) was acquired for the cells falling in the CD38 and an additional gate was applied to retain a well-defined homogeneous population and to reject highly diffusive cells (which may be autofluorescent) and/or too large objects (which may correspond to cell doublets). The four-parameter sorting was run at 3,000 to 3,500 cells per second and the sorted cells were collected in 3.5 mL of αMEM with 10% FCS. For limiting dilution experiments, cells were directly sorted in the 96-well plate by the automatic autoclone apparatus.

**Establishment of LTC of CD34+/CD38- cells with human LTBMCDerived adherent cells and a murine stromal cell line MS-5.** Human and murine stromal cells were selected as previously described in details. Briefly, human marrow adherent cells were derived from 4- to 6-week-old human LTBMCDerived established as described previously. Adherent cells were trypsinized and replated in 96- or 24-well plates and irrigated at 10 Gy once confluency was reached. The MS-5 stromal cell line was kindly provided by K. Mori and maintained in αMEM supplemented with 10% FCS. Unirradiated MS-5 feeders were preestablished in 24-well (4 × 10^4 cells/well) or in 96-well plates (6 × 10^5 cells/well) in 100 μL. To avoid early detachment of the stromal layer, plastic wells were precoated with 1% gelatin immediately before seeding of the stromal cells.

Cocultures were established by incubating CD34+/CD38- cells, either in 24-well (2,000 to 5,000 cells/well) or in 96-well (2 to 100 cells/well) plates precoated with human subcultured adherent marrow cells or cells from the murine stromal line MS-5. Cultures were initiated in LTC medium (αMEM supplemented with 12.5% FCS, 12.5% horse serum [Hyclone Laboratories, Logan, UT], 10^-4 mol/L 2-mercaptoethanol). Hydrocortisone (10^-4 mol/L; hydrocortisone 21-hemisuccinate; Sigma) was added where indicated. In some experiments, reversibility of the effect of hydrocortisone was tested by first initiating cultures with or without hydrocortisone during an initial 2-week period and then switching to LTC medium without or with hydrocortisone, respectively, for the next 2 to 4 weeks. All cultures were fed every week by half-medium change. The progenitor content of each well was assessed by sacrificing wells after a total of 4 to 6 weeks in culture and plating cells in methylcellulose colony assays (see below) supplemented by 100 U/mL recombinant human interleukin-3 (rhuIL-3), 50 ng/mL recombinant human Steel factor (rhuSF), 10 ng/mL recombinant human granulocyte colony-stimulating factor (rhu-G-CSF) (both kindly provided by Amgen, Thousand Oaks, CA), and 2 U/mL recombinant human erythropoietin (rhuEpo). For cocultures initiated in 24-well plates, nonadherent and adherent fractions (the latter detached by a short trypsinization and plated at 3 to 4 × 10^4 cells/mL) were separately assessed. For limiting dilutions, the entire content of each well (96-well plate) was harvested and plated in 1 mL of methylcellulose medium after trypsin treatment and at least 10 different wells were terminated per each time point and each culture condition. Ten thousand MS-5 cells were added to colony assays of cells generated from LTC-IC maintained on subcultured human marrow adherent cells to ensure stable conditions in the colony assays.

**Regular Dexter-type LTBMCD.** Primary LTBMCD were established from unseparated marrow according to procedures previously described. Briefly, 20 to 25 × 10^6 unfractionated marrow cells were suspended in 8 mL of LTC medium supplemented or not with 10^-8 mol/L final hydrocortisone and placed in 60-mm Falcon tissue culture dishes. Cultures were incubated for the first 3 to 4 days at 37°C and subsequently at 33°C in a fully humidified atmosphere and 5% CO₂. At weekly intervals, half of the nonadherent cells in half of the medium was removed and 4 mL of fresh medium was added back to the dish. In two experiments, some dishes initiated with or without hydrocortisone were switched at week 2 or 3 without or with hydrocortisone, respectively. At the end of 4 to 6 weeks in culture, nonadherent cells were collected and adherent cells were detached by trypsinization. Both cell fractions were plated in methylcellulose assays at a concentration of 5 × 10^6 cells/mL.

**Assessment of hematopoietic clonogenic progenitors.** Erythroid (colony-forming unit-erythroid [CFU-E], mature burst-forming unit-erythroid [mBFU-E], and immature BFU-E [ibBFU-E]); granulocytic (CFU-granulocyte-macrophage [CFU-GM]); and mixed (CFU-granulocyte, erythroid, monocyte, megakaryocyte [CFU-GEMM]) progenitors were quantified in the sorted CD34+/CD38- and in 24- or 96-well long-term cocultures using previously described methylcellulose assays.

**CD34+/CD38- cells were plated at a concentration of 0.5 to 1 × 10^5 cells/mL of complete methylcellulose medium (0.8% methylcellulose in Iscove’s medium, 30% FCS, 1% deionized bovine serum albumin [BSA], 10^-4 mol/L β-mercaptoethanol; purchased from the Terry Fox Laboratory, Vancouver, Canada). Development of erythroid and pluripotent progenitors was best stimulated by rhuSF (50 ng/mL), rhuIL-3 (100 U/mL), and rhuEpo (2 U/mL) and 10,000 MS-5 cells were added to the plates. CFU-GM were quantified separately in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 10 ng/mL; kindly provided by Immunex), rhuSF, and rhu-G-CSF. Plates were incubated at 37°C in an air atmosphere supplemented with 5% CO₂ and saturated with humidity. Progenitors were scored at days 10 (CFU-E and mBFU-E), 15 or 16 (primitive ibBFU-E and CFU-GM), and 22 to 24 (CFU-GEMM and blast progenitors) using previously detailed criteria.

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RESULTS

Frequency and proliferative potential of CD34+/CD38- LTC-ICs cultured on either LTBMC-derived adherent human cells or murine MS-5 stromal cells. To measure absolute numbers of LTC-IC in the different culture conditions, we determined LTC-IC frequency in LTC established at limiting dilution on human marrow adherent feeders or MS-5 cells. Frequency of LTC-IC was appreciated by Poisson statistical analysis of the proportion of negative wells (a negative well contained no progenitor in clonogenic assay performed at week 4 or 5) in experiments initiated with 2 to 50 CD34+/CD38- cells. Frequency of LTC-IC in CD34+/CD38- fraction cocultured on MS-5 cells in the presence of regular LTC-medium with hydrocortisone was 1/23; in cocultures established on human marrow adherent cells, the frequency was 1/12 (Fig 1). This corresponds on average to 1 LTC-IC per 50,000 initial unfractionated marrow cells. The clonogenic output per LTC-IC, which reflects the proliferative potential of these cells, and the distribution of the number of progenitors per positive well, which reflects the heterogeneity of LTC-IC potential, were also determined and results from one representative experiment are shown on Fig 2. In further experiments, the proportion of positive (or negative wells) was used as an estimate of the frequency of LTC-ICs (see below).

Effect of hydrocortisone on the frequency and clonogenic cell progeny of LTC-ICs cultured on the murine stromal cell line MS-5. Seventeen separate experiments were performed in which different numbers of CD34+/CD38- cells ranging from 2 to 100 were cultured on MS-5 cells in LTC medium with or without the addition of 10^-6 mol/L hydrocortisone. In five experiments, cocultures were established simultaneously on human marrow LTBMC-derived adherent cells. Cultures were maintained as described in the Materials and Methods. A dramatic effect of hydrocortisone was observed in cocultures established on MS-5 stromal cells and the results of six selected experiments are shown on Table 1 and Fig 2. A significant increase in both the proportion of positive wells and the clonogenic output per well was observed in all experiments. Thus, in 268 wells initiated with 2 CD34+/CD38- cells (n = 3) and maintained without hydrocortisone, the proportion of positive wells reached 38%, whereas only 15% of 240 wells (n = 2) initiated with 2 cells in the presence of hydrocortisone were positive after 4 weeks

![Graph](http://www.bloodjournal.org)
in culture. Thirty-two percent of wells initiated with 5 cells (n = 56), 90% of those initiated with 20 cells (n = 59), and 100% of those initiated with 30 cells (n = 45) and grown without hydrocortisone produced at least one progenitor after 4 weeks, whereas, in the presence of hydrocortisone, 80 to 100 cells were required to obtain 100% positive wells (Table 1). In two experiments in which at least three cell concentrations were used in sufficient numbers of wells, the frequency of LTC-IC calculated using Poisson statistics was found to be 1/2 to 1/5 without hydrocortisone, whereas it was 1/16 to 1/25 with hydrocortisone (Fig 1). Differences in the proportion of positive wells in assays performed with and without hydrocortisone were statistically significant as analyzed by the *t*-test in paired experiments initiated with 2, 5, and 10 CD34+/CD38− cells.

Withdrawing hydrocortisone from the medium not only increased the frequency of LTC-IC but also increased 15-± 2.6-fold (n = 15, *P* < .01) the number of progenitors produced per positive well after 4 weeks in culture as compared with cultures established with hydrocortisone. Differences in the numbers of clonogenic progenitors generated in LTC-IC assays performed with or without hydrocortisone were highly significant (*t*-test in paired experiments, *P* < .001) in all experiments independently of the numbers of wells used to initiate the cultures. Thus, most of the wells initiated with 2 to 10 cells contained less than 3 progenitors on average, whereas parallel wells maintained without hydrocortisone contained 10 to 30 progenitors (Table 1 and Fig 2). This increase in progenitors output was almost entirely accounted for by CFU-GM, which represented 86% of the progenitors detected. Examination of the phenotype of these colonies indicated that progenitors generated from LTC-IC grown with hydrocortisone were more primitive than those generated from LTC-IC grown without hydrocortisone. Thus, a high proportion of granulocytic progenitors generated in cultures established without hydrocortisone generated colonies of 200 to 1,000 differentiated cells at day 13 and pure macrophagic colonies were also observed. In contrast, all CFU-GM detected in LTC-IC assays supplemented with hydrocortisone generated huge colonies whose optimal size was reached only at days 20 to 25 and there were no pure macrophagic colonies. Consistent with this hypothesis that removal of hydrocortisone led to amplification of clonogenic progenitors derived from LTC-IC, the proportion of CFU-GEMM (calculated from the total number of progenitors per experiment, i.e., per 10 to 150 wells) decreased from 16% ± 3% (n = 15) in cultures with hydrocortisone to 4% (n = 19) without hydrocortisone. Consequently, high amounts of nucleated cells were produced in wells maintained without hydrocortisone, whereas cell proliferation was barely detectable in wells containing hydrocortisone (data not shown).

**Effect of hydrocortisone on the frequency and clonogenic cell progeny of LTC-ICs cultured on human marrow LTBMC-derived adherent cells.** In 11 experiments, a series of wells was initiated with 5 to 100 cells on irradiated LTBMC-derived human stroma with and without hydrocortisone; results from 4 experiments are shown in Table 1. In contrast to the dramatic effect of hydrocortisone on cultures established on MS-5, withdrawing glucocorticoids from cultures established on subcultured human marrow adherent cells had very little effect on either the frequency of positive wells or the number of progenitors produced per well at week 4 or 5 (Table 1). Thus, in 2 experiments, the frequency of LTC-IC was 1/10 in the presence of hydrocortisone and 1/10 and 1/5 in the absence of hydrocortisone (Fig 1). Furthermore, there was no significant increase (1.5- ± 0.2-fold) in the number of progenitors produced per positive well with or without hydrocortisone (Table 1) and the phenotype of the colonies was unchanged and characteristic of very immature progenitor cells, as shown by the size of the colonies and their late appearance (days 20 to 25). Consequently, in the absence of hydrocortisone, the mean clonogenic output at week 4 or 5 was much lower in individual wells initiated on human marrow feeders than in those initiated on MS-5 cells. Thus, on human adherent cells, an average of 2.5 and 15 progenitors were produced in wells initiated with 5 and 100 CD34+/CD38− cells, respectively, whereas in parallel experiments performed on MS-5, 15 and 120 progenitors were

<table>
<thead>
<tr>
<th>Cells/Well</th>
<th>No. of Wells</th>
<th>% Positive Wells</th>
<th>No. of Progenitors per Positive Well</th>
<th>No. of Wells</th>
<th>% Positive Wells</th>
<th>No. of Progenitors per Positive Well</th>
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<td>Cocultures on MS-5 cells</td>
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<tr>
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<tr>
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<td>100</td>
<td>14</td>
<td>12</td>
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</table>

Limiting dilution cocultures were established on MS-5 or human stromal cells in culture medium containing 12.5% horse serum and 12.5% FCS with or without 10−8 mol/L hydrocortisone. After 5 weeks in culture, the content in clonogenic progenitors was analyzed by plating the whole content of each well in methylcellulose.
produced. However, as pointed out earlier, the maturation stage of clonogenic progenitors generated by LTC-IC grown on MS-5 and human marrow adherent cells was not identical.

Similar differences were observed in cocultures initiated on human marrow feeders or MS-5 cells in 24-wells plates with 4,000 to 10,000 CD34+/CD38- cells (data not shown).

Hydrocortisone counteracts the effect of cytokines on the proliferation of CD34+/CD38- in methylcellulose colony assays or in LTC-IC assays on MS-5. In cocultures established on MS-5 in the absence of exogenously added human growth factors and hydrocortisone, the number of progenitors progressively increased and peaked at week 4 (1,414 progenitor cells produced per 10 wells initiated each with 100 CD34+/CD38-; Fig 3). When IL-3, IL-6, and SF were added weekly to the cocultures in the absence of hydrocortisone, the maximum number of progenitors obtained was 1,577, which is not significantly different from the 1,414 progenitors produced in the absence of cytokines. However, kinetics of growth were different and, in the presence of cytokines, high numbers of progenitors were already present at week 2 (Fig 3). Similar kinetics were observed when IL-3, IL-6, SF, G-CSF, and GM-CSF were added, although in this condition, 2,500 progenitors were produced, which is twofold higher than with the three-cytokine combination.

The addition of hydrocortisone completely abrogated the stimulatory effect of either cytokine combination in cocultures on MS-5 cells (Fig 3).

In a second series of experiments, we compared at week 2 the numbers of clonogenic progenitor cells found in cultures initiated with 50 CD34+/CD38- cells on subcultured adherent marrow cells versus murine stromal cells with and without hydrocortisone and cytokines. The addition of IL-3, IL-6, and SF to LTC established on human marrow feeders without hydrocortisone resulted in a twofold to 2.5-fold increase in progenitor cells numbers at week 2 (from 8 ± 1 to 21 ± 4 per 50 input CD34+/CD38- cells in experiment no. 1 from Fig 4) and on MS-5 to a 10- to 20-fold increase (from 15.4 ± 1.2 to 129 ± 21 per 50 input CD34+/CD38- in experiment no. 1 from Fig 3). This increase was abolished by addition of hydrocortisone to the cultures (Fig 4).

Cultures performed in 24-well plates gave similar results (data not shown). Thus, in the absence of hydrocortisone, MS-5 cells synergized with cytokines to amplify progenitor cells production to a much higher extent than did human marrow adherent cells; this synergistic effect was abolished by hydrocortisone. We have previously reported that MS-5 cells added to methylcellulose colony assays of sorted CD34+/CD38- selectively promote the development of primitive BFU-E, CFU-GEMM, and CFU-GM in synergy with SF, IL-3, Epo, and G-CSF. In a series of 5 experiments, we tested the effect of weekly addition of hydrocortisone on colony formation by CD34+/CD38- cells in the presence of MS-5 and various combinations of cytokines (Table 2). A variable but significant decrease in the number of CFU-GM was observed (mean ± SEM; 40% ± 2.1%, P < .01, t-test in 5 paired experiments) and the size of granulocytic colonies was reproducibly much lower when hydrocortisone was present. In contrast, the growth of iBFU-E and CFU-GEMM was unaffected. Hydrocortisone had no effect on CD34+/CD38- progenitor growth in the absence of MS-5 cells (Table 2).

In 2 experiments, 20× concentrated medium conditioned by MS-5 cells grown in the presence or in the absence of hydrocortisone was added to colony assays supplemented with optimal or suboptimal concentrations of cytokines. MS-5 supernatant had neither a stimulatory nor an inhibitory effect on the growth of CD34+/CD38- cells and did not substitute for MS-5 cells (data not shown).

The effect of hydrocortisone on LTC is reversible. In one experiment, cocultures were established with 5 CD34+/CD38- per well (40 to 60 wells per condition) with or without hydrocortisone. After 2 weeks in culture, hydrocortisone was either added or withdrawn depending of the initial condition and the cultures were continued for 4 weeks. Progenitor cell content was compared with that of cultures grown continuously with or without hydrocortisone. As shown in Table 3, the frequency of positive wells at week 4 was 17% if hydrocortisone was present during the entire culture period. This proportion increased to 29% in cultures in which hydrocortisone had been removed during the last 2 weeks. The corresponding progenitor cell content per positive well was 1.7 and 6, respectively. The frequency of positive wells was 83% and the mean progenitor cell content per well 17 at week 4 in the absence of hydrocortisone during the entire culture. The frequency of positive wells and mean progenitor content were 67% and 5, respectively, when hydrocortisone was added to the wells during the last 2 weeks. Similar results were observed at week 6 (Table 3).

Hematopoiesis is not sustained in Dexter-type LTC initiated with unseparated marrow cells in the absence of hydrocortisone. To ensure that, in our hands, the addition of
EFFECT OF HYDROCORTISONE ON STROMAL CELLS

Fig 4. Effect of hydrocortisone and cytokines on the production of clonogenic cells in LTC-IC assays initiated on human stromas and MS-5. Limiting dilutions experiments (11 wells per condition) were initiated by plating 50 (exp 1) or 30 (exp 2) CD34+/CD38- cells on either MS-5 cells or human stromal cells. Culture medium was supplemented once a week with rhulL-3 (100 U/mL), rhulL-6 (100 U/mL), rhSF (50 ng/mL), and hydrocortisone (10^-8 mol/L) as indicated. After 2 weeks in culture, wells were sacrificed and their progenitor cell content was individually quantitated in methylcellulose clonogenic assays. Each histogram represents the mean (±SEM) number of progenitor cells present in 11 wells.

hydrocortisone was necessary for prolonged hematopoiesis in standard human LTC, we established a series of 4 different LTBMCS with unfractionated marrow cells according to standard procedures with and without 10^-6 mol/L hydrocortisone and measured after 4 to 6 weeks their content in progenitors. As shown in Table 4, in agreement with the initial observation by Greenberger, both the number of nucleated and progenitor cells was much lower in the absence of hydrocortisone. In the absence of hydrocortisone, no confluent adherent layer formed and macrophages were the predominant cell type. It is noteworthy that, in experiment no. 6, in which an adherent layer developed even in the absence of hydrocortisone, progenitors were virtually absent, demonstrating that the stroma was not competent to maintain active hematopoiesis. Reversibility of the effect of hydrocortisone was tested in 2 experiments following the same strategy as described above for limiting dilution experiments on MS-5. The retrieval of hydrocortisone after 2 weeks in standard conditions did not significantly modify the progenitor content, which remained high. In contrast, delayed addition of hydrocortisone did not permit us to increase the progenitor output (Table 4).

DISCUSSION

Supplementation of human and murine standard LTBMC by glucocorticoids has been shown to be crucial for prolonged granulopoietic differentiation. On the basis of this finding, 10^-8 mol/L hydrocortisone has been added regularly to the recently developed LTC-IC assay, which derives from the standard LTC procedure except that purified cells with a primitive phenotype (CD34+/CD38- or CD34+/HLADp^low) are grown on subcultured marrow LTC-derived adherent cells or murine stromal cell lines. Results from this study show that hydrocortisone dramatically altered both the frequency and the proliferative potential of CD34+/CD38- LTC-IC grown on murine, but not on human, stromal cells. If we consider an LTC-IC as a cell that generates at least one clonogenic progenitor after 4 to 5 weeks of culture, then 1 in 5 (and, in some experiments, 1 in 2) CD34+/CD38- cells cocultured on MS-5 in the absence of hydrocortisone was an LTC-IC. In the presence of hydrocortisone, this proportion decreased to 5%, or 1 in 20 cells. Regardless of the mechanisms involved, this finding indicates that hydrocortisone prevents most LTC-IC from generating clonogenic progenitor cells, thus impeding their indirect identification.

Our data strongly suggest that hydrocortisone interferes with the amplification of LTC-IC-derived clonogenic progenitor cells rather than on LTC-IC recruitment. First, each LTC-IC cultured on MS-5 generated on average 10 clono-
That such mechanisms might also be operative in vivo is reminiscent of the action of macrophage and TGF-β. Activity of prim-
cycling towards the granulocytic pathway. This difference suggests that hydrocortisone blocks cellulinose.

At week 2 in experiments no. 4 through 6, hydrocortisone was either deleted without HC or added to the cultures initiated with HC. At the end of the culture, the progenitor content of each well was assessed in methylcellulose colony assays.

Table 4. Effect of Hydrocortisone on Progenitor Cell Production in Standard LTC Initiated With Unfractionated Marrow Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Week</th>
<th>No. of Positive Wells (%)</th>
<th>No. of Progenitors per Positive Well</th>
<th>No. of Positive Wells With BFU-E + CFU-GM</th>
<th>No. of Positive Wells With CFU-GEMM (%)</th>
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</thead>
<tbody>
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<td>1.75</td>
<td>1 (25)</td>
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</tr>
<tr>
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<td>6</td>
<td>1/12 (8)</td>
<td>1</td>
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<tr>
<td>+HC switch</td>
<td>4</td>
<td>7/24 (29)</td>
<td>6</td>
<td>3 (43)</td>
<td>2 (28)</td>
</tr>
<tr>
<td>+HC at week 2</td>
<td>6</td>
<td>9/24 (37)</td>
<td>1.6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>-HC</td>
<td>4</td>
<td>21/24 (87)</td>
<td>17</td>
<td>14 (70)</td>
<td>7 (33)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9/12 (75)</td>
<td>14</td>
<td>1 (7)</td>
<td>0</td>
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<tr>
<td>-HC switch</td>
<td>4</td>
<td>18/24 (67)</td>
<td>5</td>
<td>8 (50)</td>
<td>6 (37)</td>
</tr>
<tr>
<td>+HC at week 2</td>
<td>6</td>
<td>9/24 (37)</td>
<td>1.3</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Cocultures were established at limiting dilutions by incubating 5 CD34+/CD38- cells on MS-5 cells in LTC medium with or without hydrocortisone (HC; 10⁻⁶ mol/L) for an initial 2-week period. Then, for the next 2 to 4 weeks (total culture length 4 to 6 weeks) HC was either removed or added (switch - HC) or added (switch + HC). In cultures initiated with or without HC, respectively, and cultures were subsequently kept in the new medium until they were ended. The content of each culture in progenitor cells was assessed at the indicated time by plating nonadherent and trypsinized adherent cells in methyl-

Table 4. Effect of Hydrocortisone on Progenitor Cell Production in Standard LTC Initiated With Unfractionated Marrow Cells

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Week</th>
<th>No. of Progenitors per Culture (% Adherent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>1,471 (70)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,272 (70)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>7,740 (42)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1,863 (70)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>3,450 (66)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2,986 (67)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>3,698 (60)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2,967 (67)</td>
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<tr>
<td>5</td>
<td>4</td>
<td>1,257 (70)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2,062 (67)</td>
</tr>
</tbody>
</table>

1LTBMC were established with 25 x 10⁶ cells per dish in LTC medium with or without 10⁻⁶ mol/L hydrocortisone and maintained at 33°C for the time indicated. At week 2 in experiments no. 4 through 6, hydrocortisone was either deleted (switch - HC) or added (switch + HC). In cultures initiated with or without HC, respectively, and cultures were subsequently kept in the new medium until they were ended. The content of each culture in progenitor cells was assessed at the indicated time by plating nonadherent and trypsinized adherent cells in methyl-

1α may play a role in the regulation of LTC-IC grown on MS-5 cells and human marrow feeders, none of these is likely to mediate hydrocortisone effect in LTC on MS-5. (1) The different effect of hydrocortisone on human marrow-derived adherent cells and MS-5 observed in this study strongly argues against this hypothesis. (2) The MIP-1α gene is not transcribed in MS-5 cells grown without hydrocortisone (Kobari, submitted). (3) The medium conditioned by MS-5 cells grown in the presence of hydrocortisone did not inhibit colony formation by CD34+/CD38- (not shown) or CD34+/CD38- committed progenitor cells, and did not contain increased TGF-β bioactivity tested in the CCL64 assay (data not shown). Even though high mRNA and bioactive levels of TGF-β have been detected in human adherent layers and murine stromal cells, these are not affected by various compounds, including hydrocortisone. In contrast to the effect of hydrocortisone, the inhibitory effect of TGF-β can be counteracted by high concentrations of cytokines.

Another alternate mechanism that fits with our experimental data is the downregulation by hydrocortisone of a synergistic factor produced by MS-5. Our previous studies have clearly shown that MS-5 synergizes with cytokines to promote the development of human primitive CD34+/CD38- progenitors in colony assays and, more recently, we showed that this murine stromal cell line can substitute for GM-CSF, IL-3, or Epo in supporting the long-term growth of the factor-dependent UT-7 cell line.

The present study provides evidence that a synergistic factor produced by MS-5 cells operates also in LTC and promotes the amplification of CD34+/CD38-derived clonogenic progeny. Thus, in cocultures supplemented or not with IL-3, SF, and IL-6, and in the absence of hydrocortisone, the number of progenitor cells produced after 2 to 4 weeks was 8 to 10 times higher in wells maintained on MS-5 as compared with human marrow-derived adherent cells. The addition of hydrocortisone drastically counteracts this synergistic effect of MS-5 and lowers the production of progenitor cells to the level of or even below that observed on human marrow feeders. These observations are consistent with the hypothesis that hydrocortisone down-regulates this synergistic activity, which is undetectable in.
human marrow feeders. This hypothesis is further supported by the selective decrease in CFU-GM number and/or size induced by weekly addition of hydrocortisone to colony assays of CD34+/CD38− cells stimulated by optimum concentrations of SF, IL-3, and G-CSF in the presence of MS-5. In contrast, hydrocortisone had no effects on any progenitor type in the absence of MS-5 cells.

Suppression by hydrocortisone of a synergistic factor produced by MS-5 is in agreement with the known downregulation by glucocorticoids of most of the cytokines produced by fibroblasts, lymphocytes, and macrophages, including stimulatory as well as inhibitory molecules such as IL-1, IL-3, IL-4, IL-6, GM-CSF, and TNFα. Except for SF, none of the known cytokines thought to regulate early steps of human hematopoietic differentiation (IL-3, IL-1, GM-CSF, or IL-6) is involved here either because they are not transcribed by MS-5 grown in the absence of hydrocortisone (IL-3, IL-1, and GM-CSF; Kobari, submitted) or because they do not cross species barriers. Furthermore, our observation that hydrocortisone counteracts the stimulatory effect of SF, IL-3, and IL-6 exogenously added at optimum concentrations on CD34+/CD38− LTC-IC indicates that additional molecules are required, as also suggested by others. Incidentally, the lack of effect of hydrocortisone in cocultures performed on marrow-derived adherent cells also indicate that stromal-derived cytokines such as IL-6, G-CSF, GM-CSF, and IL-1, which are likely to be downregulated by corticosteroids, may not be of crucial importance for CD34+/CD38− LTC-IC development. This finding again emphasizes the role of yet unknown growth regulators on the proliferation of primitive hematopoietic cells.

It is important to point out that our observation that hydrocortisone retrieval does not affect hematopoiesis in assays established on preformed human marrow feeders does not contradict previous reports on the role of this hormone in LTC-IC. Thus, hydrocortisone was absolutely necessary for the development of a competent adherent layer and consequently for prolonged active hematopoiesis in regular Dexter-type LTC established with unfractionated human marrow cells. However, once a competent stroma is established, removing hydrocortisone may not be detrimental for LTC-IC development. Therefore, the need to supplement LTC-IC assays that are established with purified stromal cells and hematopoietic progenitors should probably be reevaluated. In addition, this approach, which allows us to manipulate the properties of a stromal cell line to sustain hematopoiesis, may be exploited to characterize molecules involved in the regulation of the stem cell compartment.

ACKNOWLEDGMENT

We thank K. Mori for providing the MS-5 cell line; Amgen, Immunex, Genetics Institute for their gifts of recombinant cytokines; and F. Sainteny and L. Kobari for helpful discussion and advice on the use of the MS-5 cell line. We also thank Drs Brunet, Missenard, and Lapresle (Clinique Arago, Paris, France); Dr Koechlin (Hospital Croiz-Rouge, Paris, France); and Dr Judet (Clinique Jouvenet, Paris, France) for providing marrow samples.

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Hydrocortisone differentially affects the ability of murine stromal cells and human marrow-derived adherent cells to promote the differentiation of CD34++/CD38- long-term culture-initiating cells

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