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Proliferation of Normal Human Promyelocytes and Myelocytes After a Single Pulse Stimulation by Purified GM-CSF or G-CSF

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Enriched populations of either normal human promyelocytes and myelocytes or blast cells were obtained by fluorescence-activated cell sorting with the monoclonal antibody WEM-G11. These populations were used to study the effect of pulse stimulation by purified recombinant human granulocyte-macrophage colony–stimulating factor (GM-CSF) or cross-reacting purified murine granulocyte colony–stimulating factor (G-CSF). Maximal clone formation by promyelocytes and myelocytes was observed in 1-mL agar cultures stimulated continuously with 400 units of either CSF and in cultures of cells that were pulse stimulated by 3,200 units (or greater) of either CSF. Pulse stimulation by 800 units of GM-CSF or G-CSF generated 75% clone formation, and pulse stimulation by 200 units CSF gave 50% clone formation. The majority of clones formed by pulse-stimulated cells were only two cells in size; however, some clones were up to 15 cells in size after a single exposure to CSF. Clone formation was not observed in cultures of blast cell populations after a single pulse stimulation with GM-CSF or G-CSF.
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THE COLONY-STIMULATING FACTORS (CSFs) are a group of glycoprotein hormones controlling the production of granulocytes and macrophages in vitro and in vivo. One action of these molecules is to support the survival of progenitor cells[1] and mature cells[2] in vitro. Fewer than 5% of murine granulocyte-macrophage progenitor cells are able to survive more than 24 hours in the absence of CSF.[3,4] Transfer of colonies initiated by CSF to cultures lacking CSF leads to the cessation of colony growth and the death of colony cells.[5,6] In addition, the proliferation of hematopoietic cells in vitro to form maturing progeny is absolutely dependent on the continual presence of CSF.[6,7] CSF can influence both the entry into the cell cycle[8,9] and the duration of cell cycle time,[9,10] with few cells being able to complete a cell cycle in progress at the time of CSF withdrawal.[11] CSF-stimulated proliferation is concentration dependent,[12] and CSF-mediated survival occurs at lower concentrations than that required for CSF-stimulated proliferation.[1,2]

Recently, the fluorescence-activated cell sorter (FACS) and monoclonal antibody WEM-G11 were used to obtain an enriched population of normal human promyelocytes and myelocytes,[13] the transient proliferation of which was stimulated in vitro by granulocyte CSF (G-CSF) and granulocyte-macrophage CSF (GM-CSF).[14,15] When using these cells, however, it was evident that limited proliferation consistently occurred in the apparent absence of CSF. This was seen both in experiments where no contaminating monocytes or lymphocytes (potential sources of GM-CSF and G-CSF) were detected in the promyelocyte-myelocyte cell population[13] and also in experiments where developing promyelocyte-derived clones were transferred from CSF-stimulated to unstimulated cultures.[14] The experiments presented here were performed to explore one possible mechanism for the limited proliferation observed for these normal human cells in the apparent absence of exogenous CSF. This mechanism is based on the observation that maximally biologically effective doses of CSFs result in only a low level of receptor occupancy, thereby implying that only a few of the available receptors are used at any one time. Therefore high doses of CSFs delivered in a single pulse that occupy all available receptors may allow the cells to continue to respond with the normal slow receptor utilization rate in the absence of exogenously added CSF, at least for a few cell divisions.

MATERIALS AND METHODS

Bone marrow cells. All bone marrow samples were obtained after informed consent of patients in accordance with a protocol approved by the Ethics Committee of The Walter and Eliza Hall Institute of Medical Research. Normal bone marrow cells were obtained from individuals undergoing cardiac surgery who had no observed hematologic abnormalities.

The cells were washed with Eisen's balanced salt solution, layered over Ficoll-Hypaque (1.077 g/mL) and centrifuged for 20 minutes at 1,000 g. Interface cells were washed and resuspended in Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal calf serum (FCS).

Marrow cells were incubated with fluorescein isothiocyanate–conjugated $F(ab')_2$ fragments of antibody WEM-G11 for 45 minutes at 4°C and then centrifuged through an underlayer of FCS to remove unbound monoclonal antibody.

Monoclonal antibody WEM-G11 and FACS sorting. The production and purification of monoclonal antibody WEM-G11 has been described elsewhere.[16] This antibody has been shown to react specifically with promyelocytes, myelocytes, and more mature cells of the neutrophil series but not lymphocytes, monocytes, or erythroid cells.[13] The cells were labeled with WEM-G11 and analyzed and sorted with a Becton Dickinson (Sunnyvale, CA) FACS-11 instrument modified to sort on three parameters. The cells were collected into 50% FCS in IMDM by using siliconized collection tubes. The cells were counted and in all experiments were >90% viable as determined by eosin exclusion. Approximately 50% to 60% of the cells sorted were recovered. Cytocentrifuge smears of cell fractions were prepared and stained with May-Grünwald and 4% Giemsa.

Bone marrow cultures. Semisolid agar cultures were performed in 35-mm Petri dishes. The growth of human granulocyte-macrophage clones was stimulated by the addition of purified recombinant

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human GM-CSF, purified murine G-CSF, or partially purified human GM-CSF (CSFα) or G-CSF (CSFβ). All preparations of CSF were pretested and found not to exhibit high-dose inhibition. The cells were added to a mixture of 1 vol of double-strength IMDM, 1 vol of FCS, and 2 vol of 0.6% Bacto-agar (Difco, Detroit). One-milliliter volumes of the cell suspension in agar medium were added to the culture dishes. The number of cells cultured was 10^3/mL. The cultures were incubated at 37°C in a humidified atmosphere of 10% CO_2 in air. Replicate cultures were scored by using a dissection microscope at intervals between days 1 and 14 of incubation. Colonies were defined as clones of more than 40 cells, whereas total clone counts included all clones of two or more cells.

CSF preparations. Recombinant human GM-CSF was synthesized in *Escherichia coli* and purified as previously described.[17] Purified murine G-CSF was prepared from mouse lung–conditioned medium as previously described.[18] Preparations of CSFα and CSFβ were fractionated from medium conditioned by the human bladder carcinoma cell line U5637 by using phenyl-Sepharose chromatography as previously described.[19] The specific activity of the various CSF preparations was determined in agar cultures of normal human marrow cells by assigning 50 U/mL to the concentration stimulating the formation of half-maximal numbers of granulocyte-macrophage colonies. Murine G-CSF was purified to homogeneity as assessed by amino acid sequencing and had a specific activity of 6×10^8 U/mg protein; recombinant human GM-CSF was purified to homogeneity as assessed by quantitative amino acid analysis and had a specific activity of 3×10^7 U/mg protein.

Pulse stimulation of fractionated cells. An equal aliquot (approximately 50 μL) was taken from the counted, FACS-fractionated cell populations and placed in sterile centrifuge tubes. Varying concentrations of G-CSF or GM-CSF were added, and the total volume was increased to 200 μL by the addition of IMDM and 10% FCS. The cells were incubated for 45 minutes at 37°C in a fully humidified atmosphere of 10% CO_2 in air.

The cells were then spun through an underlayer of FCS (5 mL) and all the supernatant removed. IMDM and 10% FCS (200 μL) were used to resuspend the cells, which were then cultured in CSF-stimulated and unstimulated agar cultures. The washing and culture procedure involved dilution of the initial CSF by at least 10^{-4}.

RESULTS

Fractionation of normal bone marrow cells. Light-density normal human bone marrow cells were sorted by using the three parameters of 0° (low-angle) light scatter, 90° (high-angle) light scatter, and fluorescence as previously described.[13] Cells with high 0° and low 90° light scatter characteristics were separated into negative and positive populations on the basis of fluorescence. The fluorescence-negative population (blast cell fraction) consisted of 29.8% ± 8.8% (mean ± SD for eight normal marrow samples) undifferentiated blast cells, 5.4% ± 5.1% promyelocytes, 15.1% ± 7.1% lymphocytes, 32.4% ± 8.2% monocytoid cells, and 17.3% ± 11.1% nucleated RBCs. The fluorescence-positive population (promyelocyte-myelocyte fraction) consisted of 12.5% ± 5.4% promyelocytes, 25.4% ± 7.7% myelocytes, 57.8% ± 12.2% metamyelocytes, 1.4% ± 1.4% monocytes, 1.7% ± 1.1% nucleated RBCs and <1% undifferentiated blasts and lymphocytes. Therefore, in terms of cells capable of proliferation, the promyelocyte-myelocyte fraction consisted of promyelocytes and myelocytes with essentially no other cells capable of division.

CSF pulse stimulation of promyelocytes-myelocytes. Figure 1 shows the results of a typical experiment in which promyelocytes-myelocytes were pulse stimulated by 8,000 units GM-CSF (upper panel) or G-CSF (lower panel). The cells were pulse stimulated for 45 minutes, washed, and cultured in agar. The number of clones in replicate cultures was recorded throughout the incubation period.

Pulse stimulation by 8,000 units GM-CSF resulted in total clone numbers equivalent to the number of clones in cultures continuously stimulated by 400 units GM-CSF (Fig 1). GM-CSF pulse–stimulated cells were also subsequently stimulated continuously by 400 units GM-CSF. This allowed quantitation of the absolute numbers of clonogenic cells in pulse-stimulated cultures and controlled for any cell losses that may have occurred during the washing procedure. In control, unstimulated cultures, the total number of clones generated was only about 10% of the number generated in CSF-stimulated cultures.

Promyelocytes-myelocytes were also pulse stimulated by 8,000 units G-CSF (Fig 1). Four groups of cells were again examined: G-CSF pulse–stimulated cells were cultured with and without G-CSF, and nonpulsed cells were cultured with and without 400 units G-CSF. As with promyelocytes-myelocytes that were pulse stimulated by GM-CSF, the number of clones generated by G-CSF pulse stimulation was equivalent to the number in cultures stimulated continuously by G-CSF. Moreover, the data indicate that equivalent numbers of promyelocytes-myelocytes ($210/10^3$) respond to

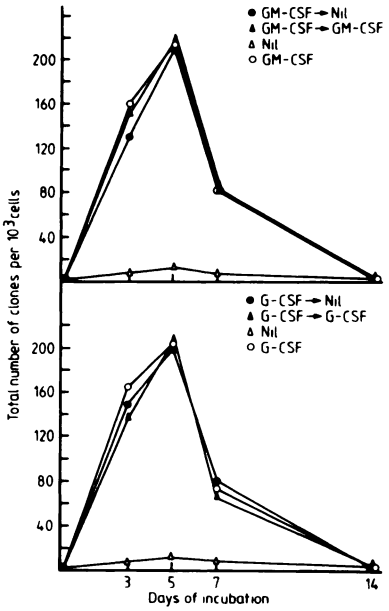

Fig 1. Total number of clones generated in cultures of normal human promyelocytes-myelocytes throughout 14 days of incubation. Each point represents the mean of quadruplicate determinations. A quantity of 10^3 cells/mL were cultured and stimulated by 400 units CSF or cultured without CSF (as indicated). Two groups of cells were pulse-stimulated by 8,000 units CSF for 45 minutes, washed, and then cultured either with 400 units CSF or without CSF. The upper panel shows the results when using purified, recombinant human GM-CSF and the lower panel, when using purified murine G-CSF.

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pulses of either growth factor or to the continuous presence of either growth factor (compare with Fig 3). Similar results were obtained in five other experiments using both GM-CSF and G-CSF.

Experiments were performed to examine the onset of clonal proliferation in CSF pulse–stimulated cultures compared with non–pulse-stimulated cells. There was no difference either in the onset of proliferation or in the fate of clones in CSF pulse–stimulated cultures compared with continuously stimulated cultures. In all situations the onset of clonal proliferation remained asynchronous, and the lag period was not shortened by CSF pulse stimulation (data not shown).

CSF dose-response relationship. Experiments were performed to determine the comparative 50% maximum biologic response for CSF pulse–stimulated *v* continuously stimulated cells.

Standard CSF dose-response curves (continuous stimulation) were established for each bone marrow sample by using serial dilutions of GM-CSF and G-CSF (Fig 2). In parallel, promyelocytes-myelocytes were pulse stimulated by varying concentrations of CSF, and the cells were then cultured either with or without CSF (Fig 3). This corrected for the four- to tenfold variation in CSF responsiveness observed with bone marrow samples from different individuals and therefore allowed accurate quantitation of the number of units of CSF to which pulse-stimulated cells had been exposed.

The results shown in Figs 2 and 3 are for experiments performed in parallel on the same marrow sample. Figure 2 shows the dose-response relationship for normal promyelocytes-myelocytes stimulated continuously by GM-CSF or G-CSF. Two hundred units of either CSF stimulated 230 clones/10^3 cells. This represented a concentration of 6.7 ng/mL for GM-CSF and 0.34 ng/mL for G-CSF. Fifty units of either CSF stimulated approximately 120 clones/10^3 cells.

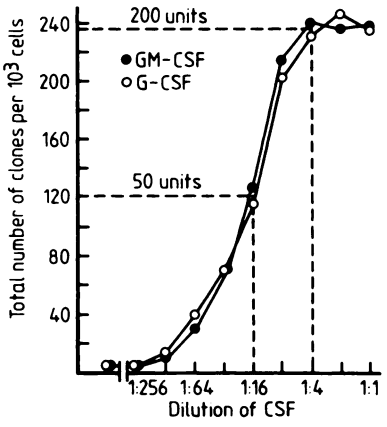

Fig 2. Dose-response relationship for promyelocytes-myelocytes stimulated by purified recombinant human GM-CSF (3×10^7 U/mg protein) and purified murine G-CSF (6×10^8 U/mg protein). Serial dilutions of CSF were used to stimulate cultures containing 10^3 cells/mL. Results were scored after five days of incubation and are means of replicate cultures. The number of clones stimulated by 200 units CSF (6.7 ng/mL GM-CSF and 0.34 ng/mL G-CSF) and 50 units CSF are indicated.

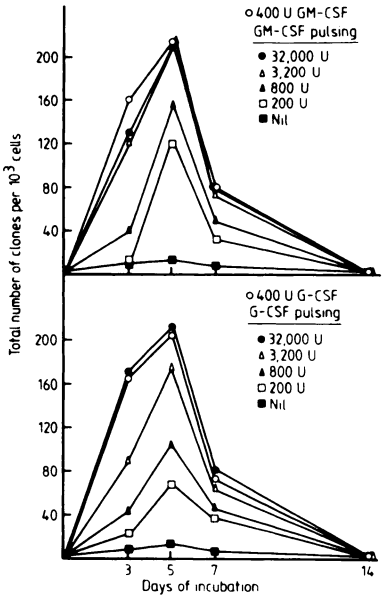

Fig 3. Total number of clones for promyelocytes-myelocytes pulse stimulated by varying concentrations of CSF. Results are for purified recombinant human GM-CSF (upper panel) and purified murine G-CSF (lower panel). Each point is the mean of quadruplicate cultures containing 10^3 cells/mL. The cells were pulse-stimulated for 45 minutes by 32,000 units, 3,200 units, 800 units, and 200 units CSF (as indicated); washed; and cultured in agar without CSF. For comparison, the total numbers of clones are also shown for cultures of nonpulsed cells stimulated by 400 units CSF (13.3 ng/mL GM-CSF and 0.67 ng/mL G-CSF).

Figure 3 shows the results of one experiment in which the cells were pulse stimulated by varying concentrations of GM-CSF or G-CSF. The numbers of clones present in cultures stimulated continuously by maximal doses (400 units) of GM-CSF or G-CSF (concentration, 13.3 and 0.67 ng/mL, respectively) are also shown. Pulse stimulation by 32,000 units GM-CSF or 8,000 or 3,200 units GM-CSF generated total numbers of clones equivalent to the maximum number in cultures stimulated continuously by 400 units GM-CSF. Cells pulse stimulated by 800 units GM-CSF generated approximately 75% of the maximum number of clones, whereas 200 units GM-CSF gave approximately 50% of the maximal number of clones compared with control cultures. In four similar experiments the percentage of the maximal numbers of clones generated by GM-CSF pulse stimulation was 95% ± 21% (mean ± SD) with 32,000 units, 87% ± 16% with 8,000 units, 96% ± 6% with 3,200 units, 72% ± 17% with 800 units, and 50% ± 22% with 200 units GM-CSF. Thus for GM-CSF pulse stimulation, the 50% maximum biologic response was achieved with 200 units GM-CSF (6.7 ng/mL) compared with a 50% response with 50 units GM-CSF when continuously present (Fig 2).

Similar results were obtained when promyelocytes-myelocytes were pulse stimulated by G-CSF (Fig 3, lower panel). When the cells were pulse stimulated by 32,000 units G-CSF in four experiments, the number of clones generated was 97% ± 18% of the maximum. Cells pulse stimulated by 8,000 units G-CSF generated 83% ± 17% clones, 3,200 units gave 88% ± 15%, 800 units gave 75% ± 23% and 200 units

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G-CSF gave 56% ± 11% clones. Thus for G-CSF pulse stimulation the 50% maximum biologic response was achieved with 200 units G-CSF (0.34 ng/mL) compared with a 50% response with 50 units G-CSF when continuously present (Fig 2).

Size of clones in pulse-stimulated cultures. Although the number of clones generated by CSF pulse-stimulated cells was comparable to the number in cultures stimulated continuously by CSF, the size of the clones was very different. Figure 4 shows the size distribution of the clones in relation to time. Clones in cultures stimulated continuously by CSF showed a progressive increase in size (panels B and D). In contrast, the majority of clones generated in CSF pulse-stimulated cultures were only two cells in size, with some clones four to ten cells in size (panel A). The small number of clones of up to 15 cells persisted for ten days in cultures of cells pulsed with CSF (not shown), whereas this was never observed in nonstimulated cultures (panel C). This suggested that for at least a small proportion of promyelocytes-myelocytes (perhaps the most CSF responsive) a single dose of CSF was able to transfer a proliferative signal to the progeny of promyelocytes-myelocytes.

Failure of blast cell fractions to respond to pulse stimulation. The enriched blast cell fractions obtained from the same six normal bone marrow samples were pulse-stimulated by 8,000 units GM-CSF and 8,000 units G-CSF. One such experiment is shown in Fig 5. Unexpectedly, there was no evidence of proliferation when blast cell fractions were pulse-stimulated by either GM-CSF or G-CSF for 45 minutes and then cultured in agar despite the fact that this

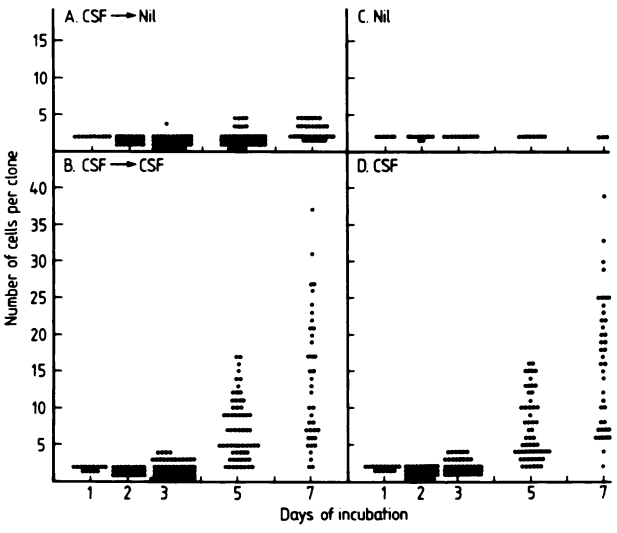

Fig 4. Size distribution of clones generated by promyelocytes-myelocytes. A quantity of 10^3 cells/mL were cultured and the size of clones recorded throughout the culture period. In panel A, the cells were pulse-stimulated by 3,200 units CSF and then placed in cultures lacking CSF. In panel B the cells were pulse-stimulated by 3,200 units CSF and then placed in cultures stimulated by 400 units CSF. In panel C the cells were not exposed to CSF in vitro. In panel D the cells were not pulse-stimulated but were placed in cultures stimulated by 400 units/mL CSF. Results shown are for cells stimulated by G-CSF. Similar results were obtained for cells stimulated by GM-CSF. Each point represents a clone.

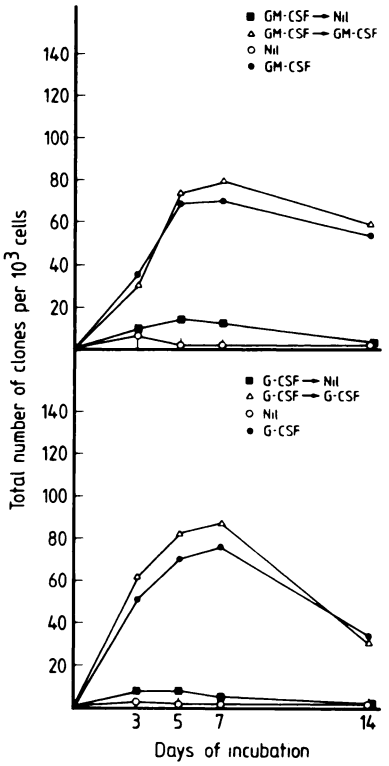

Fig 5. Total number of clones formed by blast cell-enriched fractions throughout 14 days of incubation. A quantity of 10^3 cells/mL were cultured and replicate cultures examined at each time point. The cells were pulse stimulated by 8,000 units GM-CSF (upper panel) and 8,000 units G-CSF (lower panel) and cultured either with or without 400 units CSF. Control cells were not pulse stimulated and were cultured either with or without 400 units CSF.

population did proliferate in the continuous presence of GM-CSF (80 clones/10^3 cells) or G-CSF (87 clones/10^3 cells).

DISCUSSION

In this study populations of enriched normal human promyelocytes and myelocytes were obtained by using FACS and the monoclonal antibody WEM-G11. Earlier studies have documented the transient clonal proliferation of these cells in vitro when stimulated by G-CSF.[14,15] Proliferation of some of these cells, however, was consistently observed in the absence of CSF and CSF-producing cells, and in clone transfer experiments approximately 30% of the clones continued to proliferate after transfer to cultures lacking CSF.[14] In the present study, one possible explanation for this limited proliferation was explored. Promyelocytes-myelocytes were pulse-stimulated by CSF for 45 minutes and then examined in unstimulated clonal cultures where cells continued to proliferate for up to five to ten days after pulse stimulation.

As expected from earlier studies,[14,15] proliferation after pulse stimulation was observed when the cells were stimulated either by G-CSF or GM-CSF. Proliferation was usually limited to one cell division, but on occasion clones of up to 15 cells in size were observed, which suggested that a

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single proliferative signal was able to be transmitted to daughter cells. A dose-response relationship for this phenomenon was also evident. Cells pulse stimulated by 3,200 units CSF (or greater) generated approximately 100% of the clones observed in conventional cultures containing 400 units CSF. Cells pulse stimulated by 800 units of either GM-CSF or G-CSF generated approximately 75% of the clones, and pulse stimulation by 200 units CSF generated approximately 50% of clones. The dilution factor involved in these pulsing experiments ensured that the carryover of CSF could not account for these results. These results suggested that CSF may not be required continuously to exert its biologic effect on promyelocytes-myelocytes and indicated that these normal cells were able to "remember" a CSF signal for prolonged periods after CSF withdrawal. These observations may explain the limited proliferation previously reported with these cells[13,14] where up to 10% clone formation in the absence of CSF in vitro might be expected if the cells had been previously exposed to as little as 1 to 10 units CSF in vivo.

It has been concluded previously that CSF is required continuously for proliferation of murine and human granulocyte-macrophage progenitor cells.[6,7] This conclusion is based on the inability of murine progenitor cells to survive more than 24 hours in the absence of CSF.[5,6] In support of the observations in this paper, however, continued proliferation has been documented for murine cells after the withdrawal of CSF,[20] with up to 30% of single, washed, granulocyte colony cells proliferating once or twice after transfer to cultures lacking CSF.[21] Similarly, studies using human marrow cells also documented the requirement of CSF for proliferation, but again, 30% of the clones continued to proliferate after transfer to cultures lacking CSF.[22]

A possible explanation for the pulse stimulation results on normal cells may relate to the known kinetics of G-CSF–receptor complexes. First, saturation analysis of the binding of G-CSF to normal and leukemic murine and human cells at both 0°C and 37°C indicated that a 50% biologic response occurred at a concentration where only 5% to 10% of receptors were occupied.[19,23-25] This implies that, with very high concentrations of G-CSF (as were used in the present experiments), there is a possibility of a "reservoir" of G-CSF–receptor complexes that would continue to deliver signals. This effect could be enhanced by the very slow rate of dissociation of bound G-CSF and the relatively slow rates of internalization and degradation of bound G-CSF (for example, the half-life of 50 minutes for internalization means that a cell with fully occupied receptors would still contain 10% of its occupied receptors at the cell surface after three hours at 37°C). Indeed the doses of CSFs used and the preincubation times (45 minutes) used were chosen on the basis of the known saturation kinetics for G-CSF binding to responsive cells[19,23-25] to maximally occupy all available receptors.

The inability to detect proliferation in the normal blast cell population after pulse stimulation may relate to the lower responsiveness of these cells to CSF[13,14] or to more rapid degradation of CSF-receptor complexes. It is also possible that a single pulse exposure to CSF is inadequate for these cells but that proliferation may have been evident after repeated pulse stimulation by CSF.

The observation of normal promyelocyte-myelocyte proliferation after pulse stimulation by CSF has relevance for future clinical studies of the action of CSF because the serum half-life of these molecules may bear little relationship to the biologic half-life at the target cell level.

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