Human Granulocyte-Macrophage Progenitors and Their Sensitivity to Cytotoxins: Analysis by Limiting Dilution

By Kathryn E. Barber, Philip S. Crosier, Steven Gillis, and James D. Watson

Limiting dilution analysis of granulocyte-macrophage progenitor cells was performed by using adherent and T cell–depleted normal human bone marrow and the recombinant human growth factors, granulocyte colony–stimulating factor (G-CSF) and granulocyte–macrophage colony–stimulating factor (GM-CSF). Estimated frequencies for progenitor cells responding to G-CSF were one in 489 for colonies scored at day 7, and one in 1,015 for day 14 colonies. For GM-CSF the frequencies were one in 1,407 (day 7) and one in 574 (day 14). The effects of tumor necrosis factor (TNF) and lymphotoxin (LT) on the frequency of progenitors responding to either G-CSF or GM-CSF was determined. Both TNF and LT inhibited the response of cells to G-CSF, and in these cultures the frequency of progenitor cells that responded to G-CSF was reduced to less than one in 100,000 cells. In contrast, the frequency of cells able to form colonies in cultures stimulated with GM-CSF was unaltered by either cytokotoxin. This differential sensitivity to cytokotoxin suggests that either G-CSF and GM-CSF are acting on separate granulocyte progenitor populations or that TNF and LT selectively influence the biochemical pathways associated with the activation of receptors for G-CSF.

THE CYTOTOXINS tumor necrosis factor (TNF) and lymphotoxin (LT) are capable of exerting a number of effects on hematopoiesis in vitro. Although TNF and LT are distinct molecular species with different antigenic determinants, there are clear similarities in both structure and biologic properties. The cDNA molecules for human TNF and LT have been isolated and expressed. The recombinant products share 28% amino acid homology and 46% nucleotide homology, with areas of homology grouped predominantly in two regions of the molecules. These homologous regions are likely to be important in specific receptor binding, and there is evidence that TNF and LT bind to the same cell surface receptor.

We have recently reported that the suppressive effects of these molecules on granulocyte-macrophage progenitor cells are variable and dependent upon the growth factor used to stimulate granulocyte-macrophage colony formation. Colonies formed after stimulation with granulocyte colony–stimulating factor (G-CSF) are more sensitive to the suppressive effects of TNF and LT than are colonies formed after stimulation with granulocyte-macrophage colony–stimulating factor (GM-CSF). This observation suggests that progenitors responsive to G-CSF are more sensitive to cytotoxin-induced inhibition than are those progenitors responsive to GM-CSF. To determine the frequency of human bone marrow progenitors sensitive to the effects of TNF or LT a limiting dilution analysis was performed. Cultures were stimulated with either recombinant human G-CSF or recombinant human GM-CSF. We report a striking difference between the number of progenitors responsive to G-CSF when no cytotoxin is present and the number capable of proliferative response to G-CSF in the presence of either TNF or LT. This is in marked contrast to cultures stimulated with GM-CSF where neither TNF nor LT influenced the progenitor cell frequency. These findings are important in determining the mode of action of each of these hematopoietic growth factors.

MATERIALS AND METHODS

G-CSF, GM-CSF, TNF, and LT. Recombinant human G-CSF (specific activity, 1.2 × 10^8 U/mg) and recombinant human GM-CSF (specific activity, 3.7 × 10^7 U/mg) were supplied by Immunex Corp, Seattle. Recombinant human TNF (specific activity, 5.02 × 10^7 U/mg) and recombinant human LT (specific activity, 1.2 × 10^8 U/mg) were generous gifts from Genentech Inc, South San Francisco.

Human bone marrow. Human bone marrow was obtained by aspiration from a panel of healthy volunteers. This procedure had hospital ethical committee approval. The marrow was prepared for use in limiting dilution assays by dilution in Iscove's modified Dulbecco's medium (IMDM; Gibco, Grand Island, NY) supplemented with asparagine, benzylpenicillin, and streptomycin sulfate, which was followed by separation on Ficoll-Hypaque (density, 1.077 g/mL; Sigma Chemical Co, St Louis). Adherent cells were depleted by incubating the marrow cells in culture medium with 7.5% fetal calf serum (FCS, Gibco) for 60 minutes at 37°C in 36 x 12-mm polystyrene dishes (10^5 cells/2 mL). Depletion of T cells was achieved by panning where nonadherent cells were incubated with optimal concentrations of Leu-1 and Leu-5 (Becton Dickinson, Mountain View, CA), UCHT1 (P. Beverley), and OKT4 and OKT8 (Ortho Pharmaceutical Corp, Raritan, NJ) for 20 minutes at 4°C; washed; resuspended at 2 × 10^6 cells/3 mL; and incubated for 70 minutes at 4°C on 100 × 15-mm polystyrene dishes coated with goat antibody to mouse immunoglobulin (Tago Diagnostics, Inc, Burlingame, CA). The nonadherent cells were recovered by swirling the plates and gently washing five times. The number of T cells contaminating the final preparation was assessed by incubation with goat antimalate IgG–fluorescein isothiocyanate (FITC) (Tago) for 20 minutes at 4°C then analysis by FACS 440 (Becton Dickinson). The number of T cells was always <1%.

Limiting dilution assays. Limiting dilution assays were performed in 96-well microtiter plates (Nunc, Kamstrup, Denmark). Bone marrow cells were suspended in IMDM containing 0.3% agar.
(Difco, Detroit), 20% FCS (vol/vol preselected batch; Gibco), and appropriate concentrations of growth factors and/or cytotoxins. G-CSF and GM-CSF were added at final concentrations of 100 U/mL, which are saturating for growth. TNF and LT were added at final concentrations of 100 U/mL, doses that have been shown to cause suppression of granulocyte-macrophage colony formation. Cell concentrations ranged from 2.5 x 10^2/mL to 5 x 10^5/mL. Aliquots of 200 μL were plated in 24 microwells for each cell concentration. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air for 14 days. The number of wells containing one or more colonies (aggregates of 40 or more cells) were assessed at days 7 and 14.

**RESULTS**

**Frequency of granulocyte-macrophage progenitors resistant to the effects of cytotoxins.** The effects of TNF and LT on the frequency of progenitor cells that responded to G-CSF and GM-CSF were examined. In these limiting dilution experiments cultures were stimulated with either G-CSF or GM-CSF, and for each growth factor, cultures contained either no cytotoxin, TNF, or LT. Cultures were scored at days 7 and 14. Progenitor cell frequencies were calculated for each combination of growth factor and cytotoxin at both time points.

The data presented in Fig 1 are from an experiment where adherent and T cell-depleted bone marrow was stimulated with either G-CSF (panels A and B) or GM-CSF (panels C and D). Panels A and C show the results of assays scored at day 7 and panels B and D, the results of assays scored at day 14. Estimates of progenitor frequency were calculated by maximum-likelihood determinations for each growth factor/cytotoxin combination and are presented in the legend. Similar results were obtained in two limiting dilution experiments performed on bone marrow mononuclear cells that had not been further fractionated and in an additional experiment performed on adherent and T cell-depleted bone marrow. From these experiments mean estimated precursor frequencies were calculated (Table 1). Both TNF and LT inhibited the response of cells to G-CSF such that the frequency of progenitor cells responding to G-CSF was reduced from one in 489 on day 7 and one in 1,015 on day 14 to less than one in 100,000 for both populations of cells. In contrast, neither TNF nor LT significantly altered the frequency of progenitors that responded to GM-CSF.

Colonies in microcultures stimulated by GM-CSF had the

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**Fig 1.** Effect of cytotoxins on progenitor frequencies for day 7 (A, C) and day 14 (B, D) colonies stimulated by G-CSF (A, B) or GM-CSF (C, D). The data shown are from one of four reproducible experiments. The lines were drawn such that the precursor frequency, calculated from maximum-likelihood determination, corresponded to a nonresponding culture fraction of 0.37. Reciprocals of frequency estimates were as follows: ○, d7, 453 (P = .74); d14, 1,020 (P = .99). □, d7 and d14, >100,000. ○, d7, 1,381 (P = .93); d14, 452 (P = .93). □, d7, 1,365 (P = .90); d14, 480 (P = .74). △, d7, 1.313 (P = .99); d14, 481 (P = .77).
morphological appearances of pure granulocyte, pure macrophage, mixed granulocyte-macrophage, and eosinophil colonies. The relative proportions of these colony types were unaltered by either TNF or LT. The rare colony present in cultures stimulated by G-CSF in the presence of either TNF or LT was granulocytic in morphology.

**DISCUSSION**

The experiments described have given frequency estimates for late (day 7) and early (day 14) progenitor cells in adherent and T cell–depleted human bone marrow that are capable of proliferation in response to stimulation with either recombinant human G-CSF or recombinant human GM-CSF. These were one in 489 (day 7) and one in 1,015 (day 14) for G-CSF and one in 1,407 (day 7) and one in 574 (day 14) for GM-CSF. These frequencies are similar to those described for clonogenic cells in human bone marrow where light-density marrow cells were cultured with partially purified native G-CSF or GM-CSF. The linearity of the plots and demonstration that the y-axis is intercepted at value 0 for both growth factors suggests that the effects of G-CSF and GM-CSF on progenitor cells are direct rather than indirect (mediated by accessory cells).

This study demonstrates the striking difference in sensitivity to cytotoxins of progenitor cells responsive to either G-CSF or GM-CSF. Clonogenic cells that proliferate after stimulation with GM-CSF are resistant to the suppressive effects of TNF (100 U/mL) and LT (100 U/mL), with no significant alteration in the frequency of responding marrow cells. A small suppressive effect in cultures stimulated with GM-CSF is noted when TNF or LT is added in doses of 1,000 U/mL. In contrast, less than one in 100,000 cells are resistant to the effects of TNF or LT when cultures are stimulated with G-CSF.

A feature of the hematopoietic system is the overlapping functional properties of hematopoietic growth factors and apparent redundancy in the control of blood cell production. This is particularly so for G-CSF and GM-CSF, both of which stimulate the formation of granulocyte colonies and activate mature granulocyte function. The mechanisms for the differential effect observed in the experiments described in this paper are therefore of considerable interest. One interpretation of the results is that the two growth factors, G-CSF and GM-CSF, act on largely separate populations of clonogenic cells that are different in their responses to the cytotoxins TNF and LT. Cells that proliferate in response to G-CSF are responsive to the effects of TNF and LT, whereas those cells capable of proliferation after stimulation with GM-CSF are relatively unresponsive to the cytotoxins. This mechanism, implicating separate progenitor populations, could result from differences in the expression of receptors for TNF and LT between the two cell pools. The observation that progenitors giving rise to both day 7 and day 14 colonies in response to G-CSF are both extremely sensitive to cytotoxins and, by contrast, that both sets of progenitors responsive to GM-CSF are resistant suggests that the effect is not due to cytotoxins acting on a more mature cell of the same granulocyte-macrophage progenitor pool that has increased responsiveness to G-CSF as compared with GM-CSF. Additional evidence supporting this is that cell clusters (aggregates of three to 39 cells) are less sensitive to the suppressive effects of the cytotoxins than are cell colonies.

TNF and LT show considerable homology in amino acid sequence, bind to the same cell surface receptor, and share a number of biologic properties. One possibility that might account for their difference in activity in the presence of G-CSF and GM-CSF may lie in effects on biochemical pathways associated with cell surface receptors for these two growth factors. There is only limited data on the structure of receptors for colony-stimulating factors and the intracellular pathways they might influence. The receptor for granulocyte–monocyte colony-stimulating factor possesses protein tyrosine kinase activity. It is unclear whether G-CSF and GM-CSF receptors have an intrinsic protein kinase activity. Although the murine G-CSF receptor appears to have a molecular weight (mol wt) of approximately 150,000 daltons and is large enough to include an intracellular protein kinase domain, the murine GM-CSF receptor has an estimated mol wt of only 50,000 to 70,000 daltons. The intracellular domain of such a receptor may be too small to include a protein kinase component. It is possible that differences in the intracellular signaling systems activated after the binding of G-CSF and GM-CSF to their specific receptors may result in different interactions with TNF and LT. GM-CSF may be capable of antagonizing some or all of the effects of TNF and LT by acting at a postreceptor level. If the cytotoxins were to selectively influence the biochemical pathways associated with activation of receptors for G-CSF, it would not be possible to determine, from these experiments, whether G-CSF and GM-CSF were acting on the same, different, or overlapping populations of progenitor cells. There is experimental evidence from clone transfer studies that G-CSF and GM-CSF are capable of stimulating the same clone, though these experiments were conducted by using partially purified growth factors and require validation with fully purified molecules. There are at least two potential mechanisms for a selective effect of TNF and LT on biochemical pathways activated after binding of G-CSF to its receptor. The effects could be either directly inhibitory or mediated by the induction of differentiation. The cytotoxins are capable of inducing cellular differentiation, and G-CSF has been shown to be a powerful inducer of myeloid differentiation in vitro. It is possible that TNF and LT, in synergy with G-CSF, have induced a rapid differentiation of progenitor cells at the

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**Table 1. Progenitor Frequencies in Human Bone Marrow**

<table>
<thead>
<tr>
<th>Stimulus/Cytotoxin</th>
<th>Progenitor Cell Frequency</th>
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<tr>
<td>G-CSF</td>
<td>Day 7: 1:489 ± 36, Day 14: 1:1,015 ± 41</td>
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<tr>
<td>G-CSF + TNF</td>
<td>&lt;1:100,000</td>
</tr>
<tr>
<td>G-CSF + LT</td>
<td>&lt;1:100,000</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Day 7: 1:1,407 ± 124, Day 14: 1:574 ± 105</td>
</tr>
<tr>
<td>GM-CSF + TNF</td>
<td>1:1,375 ± 28</td>
</tr>
<tr>
<td>GM-CSF + LT</td>
<td>1:1,437 ± 175</td>
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</tbody>
</table>

Mean estimated progenitor frequencies for day 7 and day 14 colonies stimulated by G-CSF or GM-CSF in the absence or presence of cytotoxins. Means ± SD from three bone marrow samples are shown.

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expense of proliferation, with cell aggregates not reaching colony size or extinction of colonies before day 7.

Further investigation of the differential sensitivity of the granulocyte-macrophage system to the effect of cytotoxins may be helpful in further determining the roles of G-CSF and GM-CSF in regulating myeloid cell production.

REFERENCES


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