Regulation of Hematopoiesis IV: The Role of Interleukin-3 and Bryostatin 1 in the Growth of Erythropoietic Progenitors From Normal and Anemic W/W' Mice


We and others have established a role for T lymphocytes and their products in the regulation of erythropoiesis. Interleukin-3 (IL-3) is a multipotential lymphokine with burst-promoting activity that is produced by activated T lymphocytes. In the anemic, stem cell--defective W/W' mouse we have described the absence of a functionally active thymocyte population that in normal animals enhances erythroid progenitor growth and stem cell self-renewal. In studies reported here we find that W/W' mouse marrow responds to exogenous IL-3 by increased erythroid progenitor cell growth. The BFU-E and CFU-E from anemic donors are more sensitive to IL-3 than are those in +/- marrow. We have recently observed a stimulatory effect of bryostatin 1 (a macrocyclic lactone derived from a marine invertebrate) on normal erythrocyte progenitors in human bone marrow progenitor assays. To test the effects of this molecule on murine normal and anemic W/W' cells we grew these cells in the presence of increasing doses of bryostatin 1. Bryostatin mimics the stimulatory action of IL-3 on W/W' bone marrow. Polyclonal antibody directed against murine IL-3 blocks the stimulatory effect of bryostatin on erythropoiesis. Otherwise inactive thymocytes from W/W' mice in coculture with W/W' bone marrow showed stimulation of erythropoiesis in the presence of bryostatin. We believe that bryostatin may in part act by stimulating T lymphocytes to release physiologic concentrations of lymphokines.

INTERNELUKIN-3 (IL-3) has been shown to have profound effects on many of the hematopoietic progenitor cells in vitro. IL-3 induces 20-α-hydroxysteroid dehydrogenase release in cultures of nu/nu splenic lymphocytes. IL-3 is produced by a tumor cell line (WEHI-3) and lectin-stimulated spleen T lymphocytes. Recombinant cDNA clones of IL-3 have been established, and the purified gene product has been produced both in bacterial plasmids and mammalian Cos cells. In vitro treatment of normal murine tissues with IL-3 results in the stimulation of proliferation of multipotential granulocyte, macrophage, eosinophil, megakaryocyte, and mast cell lineages. Of particular interest to us was the finding in vitro that IL-3 stimulates the growth of murine early erythroid progenitors (BFU-E) while inhibiting the growth of later cells in that series (CFU-E). Large doses of IL-3 administered in vivo to normal mice increase the growth of myeloid and erythroid progenitors and mature cell types. Mice pretreated with human lactoferrin respond to IL-3 in vivo in synergy with other growth factors (L-cell-derived colony-stimulating factor [CSF-1] and recombinant GM-CSF) to increase numbers and cycling rates of various hematopoietic progenitor cells (BFU-E, CFU-GM, CFU-GEMM). This lymphokine is requisite for the normal regulation of hematopoiesis.

A macrocyclic lactone, bryostatin 1, isolated from a marine invertebrate (Bugula neritina), has been recently shown by our group to modulate human hematopoiesis in vitro. This agent also has demonstrated antineoplastic activity. Its effects on human hematopoiesis involve proliferation of granulocyte/macrophage (CFU-GM) colonies in the nanomolar range and early (BFU-E) and late (CFU-E) erythroid lineages in the picomolar range. Thus bryostatin can also act as a multipotential stimulator of hematopoiesis.

The W/W' mouse has been used in numerous studies of the regulation of hematopoiesis. The animal has been shown to have a hematopoietic stem cell (HSC) defect. We have found that the abnormality in this animal, characterized by a reduction in HSC numbers and a circulating macrocytic anemia, is due in part to a malfunctioning thymocyte subset termed the anti-θ-sensitive regulatory cell (TSRC). This lymphoid accessory population has been demonstrated to modulate the growth of normal erythroid progenitor cells (CFU-E and BFU-E) in vitro. Since the disorder in the W/W' mouse involves a deficiency in the T-lymphoid cells that might produce IL-3, we tested the effect of adding multipotential growth stimulators such as IL-3 and bryostatin to W/W' erythropoietic cultures.

MATERIALS AND METHODS

Animals. We used male and female WBB6F, mice aged 6 to 8 weeks (Jackson Laboratories, Bar Harbor, ME). The hematologically normal (+/+ ) and W/W' (HSC-TSRc-deficient macrocytic anemic) animals were offspring of WB/B6 (W/W') and C57BL/6J (W'/W') parents.

Growth factors. IL-3 and bryostatin 1 were isolated and purified as previously described. Both factors were diluted to appropriate concentrations in McCoy's (GIBCO, Grand Island, NY) medium and filter sterilized.

Cell suspensions. For harvesting of bone marrow cells and thymocytes, mice were killed by cervical dislocation. The femurs and tibias of each mouse were removed, and bone marrow cells were obtained by the cutting off of the ends of the bones and flushing the interior cavities with McCoy's medium by using a 25-gauge needle attached to a syringe. For studies involving thymocytes, cells were obtained by perfusing thymus tissue through a stainless steel 60-mesh wire screen by using a 5-mL syringe and McCoy's medium. Cells were centrifuged at 600 g for ten minutes and resuspended in McCoy's medium at appropriate concentrations, after which counts...
were performed by using a Coulter counter (Coulter Electronics, Hialeah, FL).

**Progenitor cell assays.** The plasma clot method was used for CFU-E and BFU-E assays as previously described.\(^{26,27}\) For CFU-E, bone marrow cells in 0.01 mL McCoy's medium were plated in a 0.1-mL plasma clot with 0.02 mL McCoy's medium, 0.02 mL fetal bovine serum (FBS, GIBCO), 0.01 mL 10% bovine serum albumin (Sigma Chemical Co, St Louis), 0.01 mL L-asparagine (Sigma) in McCoy's at 0.2 mg/mL, 0.04 units of erythropoietin (Amgen, Thousand Oaks, CA) in 0.01 mL McCoy's medium, 0.5 units thrombin (Parke-Davis, Morris Plains, NJ) in 0.01 mL McCoy's, and 0.01 mL bovine citrated plasma (BCP, GIBCO). For BFU-E, bone marrow cells in 0.01 mL McCoy's medium were plated in a 0.1-mL plasma clot with 0.01 mL McCoy's medium, 0.03 mL FBS, 0.01 mL of 10% BSA, 0.01 mL L-asparagine in McCoy's at 0.02 mg/mL, 0.2 units erythropoietin in 0.01 mL McCoy's, 0.5 units thrombin in 0.01 mL McCoy's medium, and 0.01 mL BCP. Added IL-3, bryostatin, and thymocytes in McCoy's replaced part or all of the volume of McCoy's medium in the assays. For studies using less than 30% FBS, FBS was diluted with (BFU-E) or replaced by McCoy's medium (CFU-E). Clots were placed in micro test plates (Falcon Labware, Oxnard, CA) and incubated at 37°C in 5% CO\(_2\)/95% air for two days (CFU-E) or seven days (BFU-E). Clots were then placed on microscope slides, fixed with 5% glutaraldehyde (Fisher Chemical Co, Fair Lawn, NJ) in 0.01 mol/L phosphate buffer (GIBCO), washed with distilled water for 20 minutes, stained by the modified benzidine method,\(^{28}\) and scored for erythroid colonies (≥8 cells) and bursts (≥50 cells). Experiments described in Figs 1 and 3 were performed with 20% FBS. Experiments in Tables 1 and 2 and Fig 5 were performed without added FBS.

**Treatment of cells with anti-IL-3 antibody.** Anti-IL-3 antibody was obtained by immunizing rabbits with IL-3 as previously described.\(^{29}\) This antiserum neutralizes the action of IL-3. Antibody (300 µg/mL) was added to cultures of progenitor cells in the presence of 200 U/mL IL-3 or 10\(^{-10}\) mol/L bryostatin 1. The results were compared with the effect of antibody that had been heat inactivated (for 30 minutes at 60°C).

**RESULTS**

The effects of increasing doses of IL-3 on both W/W\(^{v}\) and normal +/+ mouse erythroid progenitor cell growth are summarized in Fig 1. IL-3 approximately doubled CFU-E growth when added to cultures of W/W\(^{v}\) bone marrow. As previously reported,\(^{15}\) when using IL-3 for normal murine bone marrow we could not demonstrate stimulation of CFU-E growth. IL-3 had no additional burst-promoting activity above the baseline seen in normal marrow (BPA) when added to cultures containing normal (30%) amounts of FBS (we screen lots of FBS for optimum BPA, data not shown), but it supports BFU-E formation in bone marrow of both W/W\(^{v}\) and +/+ mice when added to cultures containing suboptimal (3%) FBS (Fig 2). However, +/+ marrow is less sensitive to IL-3 in this experiment. These data indicate that IL-3 can support or inhibit early and late erythroid progenitor cell growth, respectively, in normal animals but only enhances the growth of W/W\(^{v}\) progenitors.

Likewise, when bryostatin was added to cultures from W/W\(^{v}\) bone marrow, we observed a doubling of BFU-E growth (from 110.86 ± 11.77 to a maximum of 236.88 ± 24.35) (Fig 3). Bryostatin at concentrations greater than 10\(^{-11}\) mol/L (ie, 10\(^{-9}\) mol/L) appears to be toxic to progenitor cell growth. We have similar effects for human progenitors.\(^{19}\) At 10\(^{-10}\) mol/L bryostatin, CFU-E are not stimulated significantly above control. Bryostatin 1 enhanced +/+ CFU-E growth only at 10\(^{-10}\) to 10\(^{-12}\) mol/L (Fig 3). Bryostatin 1, like IL-3, increased BFU-E growth from W/W\(^{v}\) bone marrow, whereas burst formation from +/+ marrow was not supported by bryostatin (Fig 4).

The similarity of the results obtained when IL-3 and bryostatin were added to CFU-E cultures of W/W\(^{v}\) bone marrow suggested that the two molecules may be working through a similar mechanism. We first examined this possi-
cultured in concentrations from 10^{-12} to 10^{-16} mol/L per clot for 3% final concentration of FBS. A quantity of 5 x 10^5 containing a...normal mice. Purified bryostatin 1 was added to cultures of cells plated.

As a control, heat-treated antibody did not block the stimulatory effect of bryostatin 1 on W/W CFU-E.

Table 1. Combined Effects of IL-3 and Bryostatin 1 on CFU-E From W/W' Donors Without Added FBS

<table>
<thead>
<tr>
<th>IL-3 (U/Clot)</th>
<th>Bryostatin 1 (mol/L)</th>
<th>CFU-E/5x10^5 Cells Plated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>---</td>
<td>---</td>
<td>7.25 ± 1.57</td>
</tr>
<tr>
<td>---</td>
<td>10^{-11}</td>
<td>41.25 ± 11.74</td>
</tr>
<tr>
<td>1.2</td>
<td>---</td>
<td>46.87 ± 12.26</td>
</tr>
<tr>
<td>20.0</td>
<td>---</td>
<td>37.62 ± 9.54</td>
</tr>
<tr>
<td>20.0</td>
<td>10^{-11}</td>
<td>31.62 ± 10.70</td>
</tr>
</tbody>
</table>

*Means ± SEM for three experiments.

Fig 5. Effect of IL-3 antibody on CFU-E. A polyclonal neutralizing antibody to IL-3 was added directly (300 μg/mL) to cultures of W/W" cells either alone (AB); exposed to heat treatment first (60°C for 30 minutes HTRx AB) or with IL-3 or 10^{-11} bryostatin (−11 M BRYO). Controls included IL-3 alone, BRYO alone, and HTRx AB + IL-3 or BRYO. The bars represent the mean of at least three experiments performed at different times with an SE of less than 10%. SE4, 5 x 10^5 cells plated.

DISCUSSION

These experiments demonstrated that IL-3 or bryostatin 1 increased CFU-E growth in vitro when added to cultures of W/W" bone marrow. IL-3 has a slight inhibitory effect on normal marrow CFU-E as previously shown, whereas bryostatin 1 can enhance CFU-E growth from normal marrow containing bryostatin 1 greatly increases the CFU-E growth above that seen with marrow alone or even with just bryostatin (Table 2), whereas 10^6 added W/W" thymocytes had little effect on the growth of CFU-E from +/+ bone marrow in the presence of bryostatin (Table 2). This is consistent with the hypothesis that W/W" T cells respond to bryostatin by the release of physiologic amounts of an IL-3-like molecule and this growth factor would stimulate W/W" but not +/+ CFU-E. Unfortunately, bone marrow with or without added thymocytes could not be shown to condition a medium in the presence of bryostatin (which was removed by a G-25 column). No IL-3 could be detected from this medium when using a titrated thymidine assay using IL-3-dependent cells (data not shown).

Table 2. Effect of 10^6 W/W" Thymocytes on the Action of Bryostatin 1 on CFU-E Growth From W/W" or +/+ Bone Marrow

<table>
<thead>
<tr>
<th>Bone Marrow</th>
<th>Bryostatin 1 (mol/L)</th>
<th>Number of Added W/W&quot; Thymocytes</th>
<th>CFU-E/5 x 10^6 BM Cells Plated*</th>
</tr>
</thead>
<tbody>
<tr>
<td>W/W&quot;</td>
<td>---</td>
<td>---</td>
<td>147.25 ± 7.56</td>
</tr>
<tr>
<td>10^{-11}</td>
<td>---</td>
<td>174.38 ± 8.89</td>
<td></td>
</tr>
<tr>
<td>10^{-7}</td>
<td>---</td>
<td>208.88 ± 6.35</td>
<td></td>
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<tr>
<td>---</td>
<td>10^6</td>
<td>176.88 ± 5.68</td>
<td></td>
</tr>
<tr>
<td>10^{-11}</td>
<td>10^6</td>
<td>305.00 ± 7.22</td>
<td></td>
</tr>
<tr>
<td>10^{-7}</td>
<td>10^6</td>
<td>414.63 ± 7.33</td>
<td></td>
</tr>
<tr>
<td>+/+</td>
<td>---</td>
<td>149.50 ± 10.47</td>
<td></td>
</tr>
<tr>
<td>10^{-11}</td>
<td>---</td>
<td>215.50 ± 7.58</td>
<td></td>
</tr>
<tr>
<td>10^{-7}</td>
<td>---</td>
<td>65.88 ± 9.18</td>
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<td>10^6</td>
<td>184.25 ± 6.70</td>
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<tr>
<td>10^{-11}</td>
<td>10^6</td>
<td>187.75 ± 10.19</td>
<td></td>
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<tr>
<td>10^{-7}</td>
<td>10^6</td>
<td>92.50 ± 8.45</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: Bm, bone marrow.

*Mean ± 1 SEM for three experiments.

†P < .05 when compared with controls.
row only at low concentrations. IL-3 stimulated BFU-E growth from +/+ and W/WV bone marrow (from zero) only under suboptimal conditions (3% FBS). The effect was seen only at reduced (3%) FBS concentrations, most likely because burst growth can be maximally stimulated with serum BPA. The presence of additional amounts of growth factors (ie, IL-3 or bryostatin) would not be stimulatory and could even be inhibitory due possibly to a concentration-dependent, negative-feedback mechanism for the growth of early or, as has been suggested, late normal erythroid progenitors.\(^5\) We have suggested previously that lymphocytes in coculture can either enhance or suppress erythropoiesis, possibly by release of a growth factor that in a concentration-dependent fashion can either inhibit or stimulate erythroid growth.\(^5\)

Our results indicated that IL-3 stimulated both CFU-E and BFU-E proliferation in the stem cell–defective animal and stimulated early but not late erythroid growth in the normal mouse. This suggested that the erythroid lineage deficiency in the W/W\(^*\) mouse may be related to inherent defects in the production or metabolism of growth factor(s). Compensation for this problem can be provided in vitro by bryostatin 1. Our studies have shown that this antineoplastic agent also has a stimulatory effect on CFU-E from W/W\(^*\) bone marrow and that this effect can be blocked with a neutralizing anti–IL-3 antibody. Thus, the response to bryostatin in this system may be due to the extracellular release of IL-3. We have not been able to detect the release of IL-3 by a tritiated thymidine assay; however, our assay may not detect minimal amounts of IL-3 that might stimulate CFU-E growth either in vitro or, more importantly, in vivo. As it has been previously demonstrated, T lymphocytes have a stimulatory role in the regulation of erythropoiesis\(^5\) as a result of growth factor production (eg, GM-CSF and IL-3); W/W\(^*\) thymocytes were apparently defective in this respect.\(^5\) It seemed possible that the cells involved in growth factor release may be T lymphocytes present in the cultured bone marrow. This hypothesis was substantiated by our findings that normally inactive W/W\(^*\) T lymphocytes could be activated by bryostatin 1 to increase W/W\(^*\) CFU-E growth above that observed with bryostatin alone (Table 2). If bryostatin 1 acts via production of IL-3, it may stimulate W/W\(^*\) T lymphocytes to produce IL-3, which in turn stimulated the growth of W/W\(^*\) BFU-E. If IL-3 is produced in response to the exposure of the W/W\(^*\) thymocyte to bryostatin, the produced IL-3 in the cocultures of +/+ bone marrow and W/W\(^*\) thymocytes should not stimulate \((10^{-11}\text{ mol/L})\) and could possibly inhibit \((10^{-7}\text{ mol/L})\) erythroid colony growth by release of IL-3. This is what we observed (Table 2). At the present time, we cannot rule out a direct stimulatory effect of bryostatin on the progenitors. In fact, we have evidence for myeloid progenitors in humans that such a direct effect can occur.\(^9\)

We conclude that IL-3 stimulated the growth of CFU-E from W/W\(^*\) bone marrow and that bryostatin 1 provides support for W/W\(^*\) BFU-E. Normal animals respond to IL-3 in vitro by the stimulation of early erythroid progenitors (BFU-E) but a decrease in later cells in the lineage (CFU-E). This effect may serve as a regulatory mechanism for proliferation of finite numbers of progenitors in the normal mouse. The different responses to IL-3 in the two litters imply that the anemic mouse may have altered growth factor production or metabolism. At the present time we cannot dissect specifically the mechanism of the alteration, (ie, missing or defective target cells, defective or downmodulated receptors, or concentration-dependent levels of the growth factor that results in the altered erythroid growth). At the biochemical level bryostatin has been shown to activate protein kinase C (PKC),\(^22\)\(^24\) and this enzyme has been shown to exist in a variety of forms.\(^35\)\(^38\) This enzyme is important in growth-related signal transduction mechanisms.\(^39\) Furthermore, the PKC activator, the phorbol diester 12-0-tetradecanoylphorbol-13-acetate has been found to induce growth factor expression in regulatory T cells (IL-2, GM-CSF, IL-3). Bryostatin has also been recently shown to induce IL-2.\(^40\) Therefore, it is possible that the W/W\(^*\) and +/+ cells could be stimulated by bryostatin to release a growth factor (ie, an IL-3–like molecule) with erythropoietinating activity that in turn could correct the defect observed in the erythroid lineage of W/W\(^*\) mice.

REFERENCES


Regulation of hematopoiesis-IV: The role of interleukin-3 and bryostatin 1 in the growth of erythropoietic progenitors from normal and anemic W/Wv mice

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