Interleukin 3 promotes erythroid burst formation in “serum-free” cultures without detectable erythropoietin

(erythropoiesis/burst-promoting activity)

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ABSTRACT Erythroid burst-forming units (BFU-E) from mouse bone marrow were grown for 7 days in agar or serum-free methylcellulose cultures in the presence or absence of erythropoietin (Ep) and/or interleukin 3 (IL-3). It was found that IL-3, even in the absence of serum and detectable Ep, was able to stimulate the full development of many erythroid bursts. This IL-3 effect was cell-dose dependent and did not appear to correlate with Ep dose. Spontaneous bursts and those stimulated by Ep only were rare and when seen were very small relative to those produced by IL-3 or IL-3 plus Ep. When addition of IL-3 or Ep to 7-day cultures was delayed, IL-3 but not Ep was shown to maintain BFU-E. No evidence was found by radioimmunoassay that Ep was produced or released in 7-day, “serum-free” cultures of bone marrow nor was Ep activity detected in culture media except those to which it had been added deliberately.

Interleukin 3 (IL-3) began its interesting history as a factor believed to regulate early T-cell differentiation. This was based on the observation by Ihle that a factor in conditioned medium from activated T cells had the ability to induce expression of 20α-hydroxysteroid-dehydrogenase, an enzyme uniquely associated with the T-cell lineage, in splenic lymphocytes from athymic nude mice (1, 2). Ihle et al. (3) proposed that this factor, a glycoprotein of Mr 28,000, be called IL-3 and has since purified it to homogeneity (4). Activated T cells are believed to be the major source of IL-3, although WEHI-3, classified as a myelomonocytic leukemic cell line, produces it constitutively. In the short time since WEHI-3 was shown to produce IL-3, it has been found that the factor also has the ability to stimulate the growth in vitro of many kinds of hemopoietic cells (5–19) and it has been called by some a multispecify colony-stimulating factor (20). It has been shown to be identical with Schrader’s P-cell-stimulating factor (21) and with stem cell-activating factor (22).

It has been recognized for some time that early erythroid progenitors require a second factor, burst-promoting activity (BPA), in addition to the hormone erythropoietin (Ep) for their full development in culture (23–26). The ability of IL-3 to stimulate erythropoiesis has been attributed to its activity as a burst promoter in the production of differentiated erythrocytes in vitro from the burst-forming progenitor or erythroid burst-forming units (BFU-E) (17, 27). It has been reported that under some circumstances “Ep-independent” BFU-E can develop (27–34). Evaluation of the site and nature of action of both BPA and Ep have, until recently, been burdened by the lack of highly purified material and by the presence of unknown quantities of BPA and Ep in constituents of culture media. Some crude preparations of Ep have been found to contain BPA as a contaminant (35).

Now that a completely “serum-free” system has been devised (36) for the in vitro growth of erythroid bursts, it is possible to seek experimental answers previously unattainable. One question in need of an unambiguous answer is that of the requirement of BFU-E for Ep. Data are presented here to show that BFU-E develop from bone marrow from mice of several strains in response to homogeneous IL-3 even in the absence of added Ep. The development of erythroid bursts is IL-3 dose- and cell-dose-dependent even in “serum-free” medium. Delayed addition of IL-3 and/or Ep to cultures reveals that IL-3 but not Ep is an absolute requirement for BFU-E maintenance. Medium and selected medium constituents are found by RIA to be essentially free of Ep activity and there is no evidence that Ep is being produced or released in culture. We conclude that IL-3 is a sufficient stimulus in vitro for the production of erythroid bursts and suggest that requirements for Ep be reevaluated in the light of these findings.

MATERIALS AND METHODS

Mice, Marrow Preparations. (C57BL/6 ♀ × DBA/2 ♂)F1 hybrid males, hereafter called B6D2F1, 9–11 weeks old, were used for most of these studies.

Mice were killed by exsanguination and single cell suspensions were prepared from femurs (sometimes tibias also) in RPMI 1640 medium. Subsequent dilutions were based on hemocytometer counts of eosin-excluding cells.

Erythroid Burst-Forming Assay in Agar. The agar method of McLeod et al. for BFU-E (37, 38) was used in early experiments. The culture medium consists of agar dissolved in αMEM (GIBCO) and added to medium at final concentration of 0.37%/20% fetal bovine serum (FBS)/1% bovine serum albumin (BSA) (fraction V, Miles)/20 μg of L-asparagine per ml/25 μg of CaCl2 per ml in NCTC 109 (GIBCO). Gentamycin sulfate is regularly added to all media at 50 μg/ml. At least five 1.0-ml portions of this mixture with bone marrow (BM) cells and appropriate factors were placed in 35-mm tissue culture plates and incubated at 37°C in 5% CO2/95% air. After 7 days colonies were stained and enumerated.

Erythroid Burst-Forming Assay in Methylcellulose. BFU-E in methylcellulose were cultured according to the method of Stewart et al. (36).

FBS enriched. The culture medium consisted of 0.8% methylcellulose/1 mM l-glutamine/0.1 mM mercaptoethanol buffered solution (Sigma)/28 μg of CaCl2/ml/1% BSA (fraction V), to which NaHCO3 was added immediately prior to use/20% FBS, not heat activated, in αMEM medium.

"Serum-free." This culture medium was the same as the

Abbreviations: BFU-E, erythroid burst-forming units; Ep, erythropoietin; IL-3, interleukin 3; BPA, burst-promoting activity; BM, bone marrow; FBS, fetal bovine serum; BSA, bovine serum albumin; U, unit(s).
FBS-enriched medium except for the replacement of FBS with the following: 10% unfiltered lipid solution (7.8 µg of cholesterol, 8.0 µg of dipalmitoyl lecithin and 5.6 µg of oleic acid dissolved per ml of 0.08% ethanol/1.0% BSA in MEM with Hanks' salts) and 10% human transferrin, 30 µg/ml, saturated with iron (66 µM FeCl₃).

For both procedures, methylcellulose solutions after appropriate addition of factors and cells were dispensed in 0.5 ml aliquots into 6 or 7 wells of a 24-well tissue culture plate.

**BFU-E Staining and Scoring.** Erythroid bursts were stained according to the modified benzidine method of Gallicchio and Murphy (39), by adding 0.1 ml of 3% H₂O₂ (30% stock solution) to 5 ml of benzidine dihydrochloride (1% benzidine in 95% ethanol). The wells were flooded with the benzidine solution and hemoglobin-containing cells stained red-brown. Bursts with multiple units or large clusters that contained more than 50 (usually >100) benzidine-positive cells were scored as BFU-E. Their erythroid nature, particularly when Ep had not been added, was further verified by examination of unstained cultures on day 7 or 8 at 100× magnification. Erythroid bursts were identified by their general shape, individual cell size, and the presence of hemoglobin, which gave them a pink color. In addition to pure erythroid bursts, which constituted the majority of those counted, mixed bursts were also scored as positive when red cells were numerous (>100 cells) and prominent. Individual bursts were removed occasionally by means of a 5-µl pipette and cells were smeared, stained (Wright/Giemsa), and examined at 100× magnification. The presence of hemoglobinized late normoblasts again confirmed erythroid identity.

BFU-E in agar were usually seeded with 0.75 × 10⁵ (0.5 × 10²-1.5 × 10³/ml) viable cells per ml of medium. BFU-E in methylcellulose were typically plated at 1 × 10⁵ cells per ml (5 × 10⁴ cells per well) in FBS-enriched medium and 2 × 10⁵ cells per ml (1 × 10⁶ cells per well) in "serum-free" medium. The numbers of BFU-E scored from at least four wells (usually 5-7) are presented as BFU-E per 10⁵ BM cells plated or as BFU-E per well.

**Erythropoietin.** Human urinary erythropoietin was obtained from a distribution program through the National Heart, Lung, and Blood Institute [CAT-1, 1140 units (U)/mg of protein; 1 unit = that amount that produces in the fasted-rat assay a response equivalent to that resulting from administration of 5 µM cobalt chloride].

**RIA.** The culture media were measured by RIA as described by Garcia et al. (40). Supernatants were diluted as necessary and samples were assayed in duplicate (or in some cases quadruplicate).

**IL-3 and WEHI-3 Supernatant.** IL-3, purified to homogeneity (4), was kindly provided by J. N. Ihle. The product had been diluted 1:100 in RPMI 1640 medium/10% fetal calf serum and filter sterilized. It had a specific activity of 5 U/ng. WEHI-3b supernatants were collected after 48-72 hr of culture from cells grown in RPMI 1640 medium supplemented with 5% FBS and 2 mM L-glutamine. The cell line was kindly provided by J. N. Ihle.

**RESULTS**

**Better Correlation of BFU-E Number with IL-3 Concentration than with Ep Dose.** To examine the effects of IL-3 on erythropoiesis, BFU-E were grown in the presence of various doses of Ep at each of several IL-3 concentrations. Numbers of erythroid bursts did not increase regularly as the Ep dose was increased in the agar system containing FBS (Fig. 1B), in FBS-enriched methylcellulose culture (data not shown), or in serum-free methylcellulose cultures (Fig. 1A). Burst numbers did increase, however, in relation to increasing concentration of IL-3. No erythroid bursts were seen in the presence of Ep alone in the three "serum-free" experiments represented in Fig. 1A. IL-3 in the absence of Ep, on the other hand, did produce bursts in a dose-dependent fashion. Some of the same data are plotted to demonstrate this more clearly (Fig. 2). A plateau is reached in erythroid burst number at a final IL-3 dilution of approximately 1:20,000 in all cases shown here. Although the number of erythroid bursts developed in culture appears to be tightly regulated by IL-3, the addition of high doses of Ep was found to produce an amplification (see Fig. 2). The two curves representing erythroid bursts scored in the presence of relatively high doses (1.25 and 2.5 U/ml) of Ep essentially overlap, as do the curves depicting those grown in the presence of low doses (0.625 or 0.01 U/ml) or in the absence of Ep. On occasion erythroid bursts were found in methylcellulose cultures lacking IL-3 with or without Ep but never in similarly stimulated agar cultures. These data show that IL-3, not Ep, is the limiting factor in burst number.

**Cell-Dose Dependence of BFU-E Growth.** The production of erythroid bursts in "serum-free" culture with Ep only, IL-3 only, or the two together was cell-dose dependent as was spontaneous erythroid burst development (Fig. 3). When cells were plated at a concentration greater than 2 × 10⁸/ml, it was difficult to score bursts because of cell crowding. The largest bursts were seen most frequently when both Ep and IL-3 were present. The smallest ones scored as positive were either spontaneous or promoted by Ep only. They met the criterion of containing a total of at least 50 benzidine-stained cells but were smaller than any scored in cultures containing IL-3.

**Establishment of Erythroid Nature of Bursts.** Because of the lack of specificity of benzidine staining, it was important to verify that bursts were indeed erythroid, especially in
BFU-E were cultured in “serum-free” methylcellulose. Symbols represent burst determinations from one to four experiments. ●, 2.5 U of Ep/ml; ■, 1.25 U of Ep/ml; ▲, 0.625 U of Ep/ml; ○, 0.01 U of Ep/ml; ▲, no added Ep.

those cases in which no measurable Ep was present in culture. Examination of unstained colonies on day 7 or 8 left no doubt that many erythroid bursts containing hemoglobin were stimulated by IL-3 even in the absence of Ep. Plucked, smeared and stained bursts from such cultures contained many late normoblasts and mature red cells.

Effects of Delayed Addition of Ep or IL-3. A further study using the “serum-free” methylcellulose method was carried out to evaluate the importance of the time during the culture period at which Ep or IL-3 was added. Fig. 4 presents the BFU-E counts obtained 7 days after initiation of the cultures (day 0) when Ep and IL-3 were added on day 0 through day 5. Values for the day 0 factor additions confirm other findings reported above that IL-3 stimulates many bursts in the absence or presence of Ep and that few bursts are observed in the presence of Ep alone. In addition, BFU-E appeared to be maintained in cultures whether Ep was present or not, inasmuch as IL-3 or IL-3 plus Ep added 1–3 days after day 0 produced a greater number of bursts than was seen in Ep or control cultures, which were all below 5 BFU-E per 10⁵ cells. The addition of IL-3 was required early since bursts scored in cultures to which IL-3 was added later (more than 2 days after day 0) were very small and resembled those grown in the absence of IL-3 for the entire 7-day period. We believe these data underscore the importance of IL-3 not only to the initiation but also to the full development of BFU-E in vitro. Ep, by comparison, appears in this experiment not to play as pivotal a role.

Ep Content of Media and Constituents. The important problem of unintentional introduction of Ep via medium constituents has remained unresolved in many in vitro studies of erythropoiesis. We confronted this difficulty by eliminating FBS and by assaying culture medium and specific medium constituents for Ep by RIA. Medium exposed to cells for 30 min and medium to which 20% FBS was added in place of transferrin and lipids in the “serum-free” system were assayed to contain 7.7 and 8.7 mU of Ep/ml, respectively. Erythropoietin levels contained in “serum-free” media are shown in Table 1. No significant levels of Ep were measured in supernatants in contact with total or adherent BM cells cultured for 30 min or 7 days. Values in the range of 10 mU/ml are near the lower limit of sensitivity of this assay.

BFU-E were cultured in “serum-free” methylcellulose. Symbols represent burst determinations from one to four experiments. ●, 2.5 U of Ep/ml; ■, 1.25 U of Ep/ml; ▲, 0.625 U of Ep/ml; ○, 0.01 U of Ep/ml; ▲, no added Ep.

**Fig. 3.** Cell-dose dependency of bursts grown with or without IL-3 in methylcellulose cultures under “serum-free” conditions. Each point represents the mean ± SEM of the counts from at least three and typically seven 0.5-ml culture wells. ○, no added factors; ●, 1.25 U of Ep/ml added; ▲, pure IL-3 added at a final dilution of 1:40,000; ■, both Ep (1.25 U/ml) and IL-3 (1:40,000 final dilution) added.

**Fig. 4.** Kinetics of IL-3 effect on BFU-E. “Serum-free” methylcellulose cultures were plated at 10⁵ cells per well on day 0 in the presence of medium only (○); Ep only, 1.5 U/ml (■); IL-3 only, 1:32,000 (▲); or Ep plus IL-3 (▲). On days 1–5, cultures were overlaid with 0.1 ml of one of the following (arrows); medium, Ep, IL-3, or Ep plus IL-3. Data are from one experiment; 6 or 7 wells were scored for BFU-E in each case on day 7. In the process of supplementing the culture, the Ep concentration was reduced from 1.5 to 1.25 U/ml, IL-3 was reduced from 1:32,000 to 1:40,000; other medium constituents were not altered throughout the 7-day period.
Table 1. Ep content before and after culture

<table>
<thead>
<tr>
<th>CM*</th>
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<th>Day 0†</th>
<th>Day 2</th>
<th>Day 7</th>
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<td>863.9</td>
<td>887.0</td>
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</tr>
<tr>
<td>+ Total</td>
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<td>+ Adherent</td>
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<td>7.5</td>
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<td></td>
</tr>
<tr>
<td>− Total</td>
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<td>8.4</td>
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</tr>
<tr>
<td>− Adherent</td>
<td>12.3</td>
<td>7.9</td>
<td>6.6</td>
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</tbody>
</table>

BM cultures contained 2 × 10^6 cells per ml; methylcellulose was omitted. Total cells were adherent plus nonadherent; adherent cells were cultured in fresh medium after removal of nonadherent cells.

*WEHI-3b-conditioned medium, 5%.
†Assay lower limit 4 mU/ml; intra-assay variability, 10% (40).
‡Medium was assayed on days 0, 2, and 7.
§Medium never exposed to cells.
||Ep added at presumed potency of 1500 mU/ml on day 0.|

Such quantities used exogenously (without IL-3) in a BFU-E assay are without biological effect (Fig. 1). Five percent WEHI-3b-conditioned medium was used as a source of IL-3 in this experiment and it clearly provided no Ep measurable by RIA. Highly purified stock IL-3 diluted 1:200 was assayed to contain 10.2 mU of Ep/ml. Used at a typical BFU-E assay dilution of 1:40,000, the IL-3 value would represent 0.05 mU of Ep/ml of culture medium. Note that Ep (CAT-1) added at a presumed potency of 1500 mU/ml assayed below 900 mU by RIA. It has been previously determined (G. K. Clemons, personal communication) that this CAT-1 sample has only 60% of its stated potency. It is possible that the material has lost some of its activity during storage. The three values in excess of 800 mU/ml in Table 1 are probably not different from each other, as the RIA has an intra-assay variability of ≈10%.

Development of IL-3-Dependent BFU-E in Several Strains.

To determine whether the apparently Ep-independent, IL-3-stimulated BFU-E measured in these experiments were not restricted to the particular F1 hybrid used, we conducted further "serum-free" experiments using BM from mice of the following strains: C57Bl/6, DBA/2, CBA, A.SW, and A.CA. Clearly the finding was not limited to the F1 hybrid most often used in this laboratory. We conclude, therefore, that the production of bursts by IL-3 in the absence of Ep is a general, not a strain-related, finding.

DISCUSSION

The data presented here show that IL-3 under the culture conditions defined above is able to stimulate the growth and full development of erythrocytes in the absence of detectable Ep. Our findings go beyond earlier reports of "Ep-independent" bursts (27-34) in that (i) no serum, a possible source of unintentional IL-3, was present in cultures, (ii) homogeneous IL-3 was used, and (iii) "serum-free" medium samples taken before and after cell growth were shown by RIA to contain no significant amounts of the hormone. The data also provide no evidence that Ep is being produced by macrophages (41) or any other cell type in the cultures. It is impossible to rule out the presence of extremely small amounts of Ep—i.e., <4 mU/ml, in the assay shown in Table 1. However, Ep levels in that range are not effective when added to BFU-E cultures and presumably would not have influenced the results. There is also the possibility that the BSA used here contains other as yet unidentified interacting impurities. It is conceivable that IL-3 renders the early progenitor much more sensitive to Ep as is suggested by other data (17) so that very low concentrations, below those measurable by RIA, are stimulatory in vitro. Under our "serum-free" conditions we found no spontaneous bursts in the absence of added Ep or IL-3. Possibly the Ep-independent BFU-E observed by others (27-34) could be explained by the presence of IL-3 in medium constituents such as FBS or cell-conditioned medium.

It should be noted that, although our finding of bursts stimulated by IL-3 in the absence of Ep affirms the related conclusions of Metcalf and Nicola (42), it is in direct conflict with results published by Goldwasser et al. (17). Those investigators reported that IL-3 caused a 5- to 6-fold increase in bursts stimulated by Ep but that it was ineffective in the absence of Ep. Although the culture medium used by them contained 30% FBS and therefore differed from ours, we do not believe that explains our discrepant results unless the FBS contained an inhibitor. The other possible explanation lies in the concentrations of IL-3 used: most of the data reported by Goldwasser et al. were obtained at an IL-3 concentration of 200 pg/ml, which is equivalent to 1 unit. Our experiments, on the other hand, often used IL-3 at a dilution of 1:40,000 (10 U/ml). As Fig. 1 shows, the lowest concentration tested, 1:160,000 (2.5 U/ml), was effective in augmenting burst numbers in the presence of Ep but was clearly less effective than higher IL-3 concentrations. Our data from delayed-addition experiments also disagree in part with theirs. We found Ep without IL-3 ineffective, whereas they found a significant number of bursts when the hormone was added on day 0 or day 1. Except for the day 0 values when IL-3 was present from the start, Ep being added at different times, our data do not conflict with theirs, nor is there a major difference between our results and theirs when Ep was added on day 0 and IL-3 on subsequent days.

Ample evidence exists from other studies that Ep does act in vivo as a differentiation/maturation factor in red cell production (43, 44). Our major finding, that IL-3 constitutes a sufficient stimulus in vitro, was therefore surprising, particularly the data indicating that Ep did not increase the burst number when added several days after initiation of BFU-E in cultures containing IL-3 (Fig. 4). In view of the evidence that IL-3 stimulates the proliferation of several cell lineages, it seems that IL-3 acts as a multispecific colony-stimulating factor or as a growth factor for early pluripotent stem cells. As such, it could stimulate stem cells nonspecifically to proliferate and differentiate to maturity in the absence of the usual regulatory hormones such as Ep. Thus IL-3 could be acting in vitro on pluripotent stem cells and/or on progenitors committed in vivo for example by interaction with Ep.

The fact that BFU-E can be maintained for several days in culture in the absence of exogenously added Ep, together with their supposed relatively great requirement for the hormone, has led some investigators to suggest that BPA is the stimulus for the early erythroid progenitor and that Ep acts only later in the maturation progression. That the early progenitor is responsive to Ep was shown recently by Dessypris and Krantz (45) who, working with human marrow, demonstrated the ability of Ep to stimulate DNA synthesis in immature BFU-E. Using a short-term liquid preassay culture, they also showed that these erythroid progenitors were maintained by the hormone in a dose-dependent manner. Despite the possibility that cells present in the nonadherent marrow population were producing BPA during the course of the experiments, their data strongly suggest that immature BFU-E can be responsive to Ep at levels that might be important physiologically. Our data and those of others (28, 46) indicate that Ep does not maintain erythroid progenitors in mouse BM cultures.

The development of a method for growing BFU-E without FBS has been a substantial aid to studies of erythropoiesis. Stewart et al. (36) starting with "serum-free" Iscove–Guilbert–Weyman medium in which CFU-E but not BFU-E can be grown made modifications to permit good growth of BFU-E in the complete absence of serum. They found that Ep, either Connaught step III (sheep, specific activity 2-9
was effective first that showed in vivo physiology. Until work was for help in vitro studies of the early erythropoietic progenitor, is the extremely high concentrations of Ep, up to 100 times normal plasma values, that are usually required for BFU-E growth. Our finding that BFU-E growth can be achieved with the addition of no Ep confirms the issue of whether or not in vitro studies of erythropoiesis are truly representative of normal in vivo physiology. Until substantial in vivo data can be obtained that will resolve these issues, in vitro studies of IL-3 effects on hemopoiesis must be accepted as relevant.

We wish to express our gratitude to Dr. Gisela K. Clemons for establishing Ep values by RIA, to Dr. James N. Ihle for generous gifts of homogeneous IL-3, and to Drs. George Brecher and Helen Londe for help in verifying the erythroid character of bursts. This work was supported by the Office of Health and Environmental Research, Office of Energy Research, U.S. Department of Energy, under Contract DE-AC03-76SF00098 and by National Institutes of Health Grant AM-28340.