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Interleukin 1 regulates hematopoietic activity, a role previously ascribed to hemopoietin 1

(colony-stimulating factor/hemopoiesis/early hemopoietic factors/synergistic factors)

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ABSTRACT **A murine *in vitro* assay was developed to measure potentiation of a proliferative response to suboptimal concentrations of the hematopoietic regulatory molecule granulocyte/macrophage colony-stimulating factor by an immature bone marrow population. The assay, designated the 5-fluorouracil bone marrow proliferation assay, was used to characterize potentiating activity in serum-free culture supernatants of the human tumor cell line HBT 5637. Molecular and biochemical analyses indicated that the HBT 5637-derived potentiating activity could be attributed to interleukin 1α. Serologic analysis using a monoclonal antibody against purified recombinant interleukin 1α proved conclusively that the potentiating activity in HBT 5637 serum-free supernatants is due to interleukin 1α. From these data, the activity of interleukin 1α seems to be the same synergistic activity formerly ascribed to hemopoietin 1.**

Colony-stimulating factors (CSF) are hematopoietic growth factors that cause proliferation and differentiation of immature colony-forming precursor cells to mature blood cells (1). Four subclasses of CSF can be identified by their stimulation of neutrophils, eosinophils, granulocyte/macrophages (GM), or macrophage colony formation. Macrophage colony-stimulating factor (CSF-1) is a subclass of the colony-stimulating factors specific for regulating the growth, survival, and differentiation of the mononuclear phagocytic lineage (2). GM-CSF has multilineage activity, in that it gives rise to granulocyte, macrophage, and erythroid cell lineages (3). Other CSF capable of regulating committed lineage-specific precursor cells (granulocyte-CSF; erythropoietin) in addition to another multilineage factor, interleukin (IL) 3, have been characterized (3–7).

Recently, another hematopoietic activity has been described by Stanley and co-workers (8, 9). This activity, designated hemopoietin 1, acted synergistically with the lineage-specific factor CSF-1 on early progenitor cells, which cannot yet respond to CSF-1 alone. Hemopoietin 1 was present in medium conditioned by the human bladder tumor cell line 5637 (HBT 5637) (8).

Therefore, we began studies to determine whether any evidence suggested the existence of other lineage-specific potentiating factors or, alternatively, whether a single factor could potentiate primitive hematopoietic cell responses to all CSF.

We report the development of a quantitative murine proliferation assay, the 5-fluorouracil (5-FUra) bone marrow proliferation assay (FUraBM assay), which measures potentiation of responses to limiting concentrations of GM-CSF or CSF-1 on immature bone marrow cells from 5-FUra-treated mice. We give biochemical as well as serologic evidence that interleukin 1α (IL-1α) and interleukin 1β (IL-1β) have hematopoietic potentiating activities and that IL-1α is the primary potentiating activity for responses to GM-CSF and CSF-1 in HBT 5637 culture supernatants. Furthermore, because hemopoietin 1 and IL-1α share several biochemical characteristics, HBT 5637-derived hemopoietin 1 and IL-1α are probably identical.

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MATERIALS AND METHODS

FUraBM Assay for Potentiating Activity. A single cell suspension was prepared from the femurs and tibias of C3H/HeJ mice injected i.v. 24 hr earlier with 150 mg of 5-FUra per kg of body weight. Samples were serially diluted into 96-well microtiter plates with or without suboptimal levels of either murine recombinant GM-CSF (rGM-CSF; 0.5 ng/ml) or human recombinant CSF-1 (rCSF-1; 10 ng/ml). Cells at 6×10^4 per well were added to a final sample volume of 100 μl. After a 72- to 77-hr incubation at 37°C in 5% CO_2/95% air, the cultures were incubated with 2 μCi of ^{3}H-labeled thymidine (New England Nuclear, 70–80 Ci/mmol; 1 Ci = 37 GBq) overnight. The cultures were harvested onto glass fiber filters, and the incorporated tritium was measured by liquid scintillation counting. One unit of activity is defined as the amount of sample that induced 50% maximal response.

Preparation of HBT 5637-Conditioned Medium. When cells (American Type Culture Collection) had grown to confluence in T175 flasks (Nunc), the medium was removed, and the cells were recultured in 125 ml of fresh, serum-free RPMI 1640 supplemented with the antibiotics penicillin (50 units/ml), streptomycin (50 μg/ml), and gentamycin (50 μg/ml). Eight-day conditioned medium was removed and was concentrated 10× on an Amicon hollow fiber dialyzer/concentrator (model DC10).

Salting out Chromatography. Crude HBT 5637 concentrate was precipitated onto Sepharose CL-6B-200 (Sigma) as described by Nicola (10), except that all solutions were buffered to pH 7.5 with 20 mM Tris.

Phenyl-Sepharose CL-4B. Hydrophobic chromatography was done as described by Nicola (10), except that the solutions were buffered to pH 7.5 with 20 mM Tris.

Determination of Isoelectric Point by Buffer Electrofocusing. This procedure was done as described by Prestidge and Hearn (11).

Monoclonal Antibody (mAb) to IL-1α. mAb was generated and characterized (P.J.C., unpublished work). Briefly, a BALB/c mouse was immunized s.c. in the footpads with 1 μg of purified rIL-1α (12) emulsified in complete Freund's

Abbreviations: CSF, colony-stimulating factor(s); GM, granulocyte/macrophage; 5-FUra, 5-fluorouracil; FUraBM assay, 5-FUra bone marrow proliferation assay; CFU, colony-forming unit(s); IL, interleukin; r, recombinant; CSF-1, macrophage colony-stimulating factor; mAb, monoclonal antibody; HBT, human bladder tumor cell line; rGM-CSF, recombinant granulocyte/macrophage colony-stimulating factor.

adjuvant; then the animal dosage was boosted with three successive s.c. immunizations of 2 μg of rIL-1α protein, and a final i.v. injection of IL-1α (2 μg) was administered 3 days before spleen cell fusion. Resulting hybrids were tested for IL-1α reactivity. One positive hybridoma, designated 2F4, was isolated, cloned by limiting dilution, isotyped, and ascites fluid was generated (13). The mAb was purified by passage over a MAPS II–protein A column (Bio-Rad). The 2F4 mAb specific for IL-1α had no crossreactivity to IL-1β.

Immunoprecipitation. IL-1α was immunoprecipitated as described by Mochizuki *et al.* (14).

CSF-1 Activity. This activity was measured either by a traditional colony assay or by a recently developed proliferation assay. The bone marrow-derived macrophages used in the proliferation assay were obtained as described (2); the resulting bone marrow-derived cells were harvested and assayed similarly to that described for the FUraBM assay but with the following changes: (*i*) the cell concentration was 1–2 × 10^5 cells per ml, and (*ii*) cultures were labeled overnight and were processed for [^{3}H]thymidine incorporation after a 24-hr total incubation time.

Colony Assays. Assays were done as described by Bradley and Hodgson (15). After 7–8 days of incubation, colonies containing ≥50 cells were counted. The cultures were also checked on day 14 for high-proliferating potential colonies described by Bradley and Hodgson (15).

Murine rGM-CSF. Using the full-length LBRM-33-5A4 (American Type Culture Collection) cDNA clone, a plasmid for expression in yeast was constructed (6, 16). GM-CSF that was secreted in yeast cells and was purified to homogeneity after sequential reversed-phase HPLC on reversed-phase columns as described (17, 18).

Human Recombinant Interleukins. IL-1α and IL-1β were cloned from a human peripheral blood monocyte library and expressed in *Escherichia coli* (19). Both forms of IL-1 were purified to homogeneity as described (12).

IL-1 Bioactivity. Either the murine IL-1 conversion assay or the thymocyte mitogenesis assay described (12, 19, 20) was used to measure bioactivity. Specific activity of IL-1α and IL-1β in the conversion assay is 10^9 units/μg and 10^8 units/μg, respectively. Specific activity of both forms of IL-1 is lower by a factor of ≈1000 in the thymocyte mitogenesis assay.

Inhibition of IL-1 Bioactivity. Inhibition was measured in an IL-1 conversion assay (20). Antibodies were tested for their ability to inhibit IL-1-dependent IL-2 production by the EL-4 thymoma 6.1 cell line (a gift of H. R. McDonald, Ludwig Institute for Cancer Research, Lausanne).

GM-CSF Bioactivity. A bone marrow proliferation assay and the colony assay previously described (14) were used to determine GM-CSF activity; specific activity of GM-CSF in the colony assay is 10^8 CFU-C per mg per ml.

RNA Preparation and Analysis. Total cellular RNA extraction by the guanidinium-cesium method, polyadenylylated mRNA purification, and electrophoresis on formaldehyde gels were done by standard protocols (21). Capillary blots (Hybond-N, Amersham) were hybridized with RNA probes and stringently washed as described (19). The IL-1α probe was transcribed from a Gemini system (Promega Biotec, Madison, WI)-prepared subclone of the 660-base-pair (bp) *Hin*dIII/*Hin*cII fragment of p10A, and the IL-1β probe was transcribed from a Gemini system-prepared subclone of the 570-bp *Sac* I/*Pru* II fragment from pIL-1-14 (19).

RESULTS

From observation that HBT 5637 produced a variety of hematopoietic regulatory molecules (1, 2, 8), we tested supernatants in the FUraBM assay. Serum-free HBT 5637-conditioned medium alone induced no response, whereas with GM-CSF, substantial potentiating activity was seen. Therefore, we began studies to characterize the HBT 5637 potentiating activity.

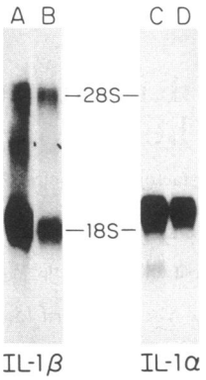

FIG. 1. RNA blot analysis of HBT 5637- and lipopolysaccharide-stimulated peripheral blood macrophage RNA. Two μg of HBT 5637 (lanes B and D) and peripheral blood macrophage (lanes A and C) poly(A)$^+$ RNA was run on duplicate blots and probed for IL-1β (lanes A and B) or IL-1α (lanes C and D). The positions of 18S and 28S rRNA were determined by direct visualization of the unbaked blot with a shortwave UV lamp. RNA was obtained from HBT 5637 cultures 48 hr after serum stimulation and from adherent macrophage cultures prepared and stimulated with 10 μg of lipopolysaccharide per ml for 12 hr as described (18).

Initially, HBT 5637 poly(A)$^+$ mRNA was screened by RNA blot analysis for expression of a number of CSF and lymphokine mRNAs. As anticipated, the HBT 5637 cells grown in the presence of serum produce abundant granulocyte CSF mRNA (4, 22), but, surprisingly, they also produce mRNA for IL-1α and IL-1β (Fig. 1). IL-1 mRNA levels were comparable with those seen in activated macrophages. Thus, it was important to test IL-1 effects in the FUraBM assay.

Table 1 shows the results of FUraBM assay, including the potentiating activity of IL-1α, IL-1β, and HBT 5637-conditioned medium. Clearly, HBT 5637-conditioned medium, IL-1α, and IL-1β potentiated proliferative responses to 0.5 ng of rGM-CSF per ml, whereas each alone had negligible proliferative activity. A 60- to 70-fold increase in GM-CSF bioactivity occurred with the HBT 5637 potentiating activity.

Table 1. Potentiating activity of IL-1α, IL-1β, and HBT 5637 conditioned medium in the FUraBM assay

Sample	GM-CSF	FUraBM assay, bioactivity units/ml*	IL-1 conversion, bioactivity units/ml*
Control	+	—	—
IL-1α	−	—	1.53×10^9
IL-1α	+	4.5×10^5	ND
IL-1β	−	—	2.63×10^8
IL-1β	+	3.1×10^4	ND
Crude HBT 5637 CM	−	22	2.6×10^4
Crude HBT 5637 CM	+	1518	ND
Purified HBT 5637 CM	−	31	4.53×10^4
Purified HBT 5637 CM	+	2100	ND

rIL-1α and rIL-1β were used at 10 μg/ml. HBT 5637 crude conditioned medium was concentrated 10×. The purified material was purified by salting out chromatography, and the granulocyte CSF was removed by phenyl-Sepharose chromatography as described. rGM-CSF was added where indicated at 0.5 ng/ml. CM, conditioned medium. ND, activity not determined.

*IL-1 activity was measured in the conversion assay, and the potentiating activity was monitored in the FUraBM assay; the method for determining activity units for both assays is described in the FUraBM assay section.

The low level of FUraBM assay activity measured without GM-CSF is likely due to potentiation of low levels of CSF, such as granulocyte CSF, in the concentrated HBT 5637 supernatants (4, 23). Although these CSF could be detected by the 5-FUra-treated bone marrow, using normal marrow no detectable CSF activity was seen in serum-free crude HBT 5637 supernatants.

IL-1α and IL-1β at concentrations of 10 μg/ml had large (4.5×10^5 units/ml and 3.1×10^4 units/ml) potentiating activity, respectively. The difference between specific activities of IL-1α and IL-1β is consistent with receptor binding studies where the affinity of the murine IL-1 receptor for human IL-1α is 10× higher than that for human IL-1β (24).

To confirm that the potentiating activity of IL-1 and HBT 5637 conditioned medium in the FUraBM assay was, indeed, measuring a hematopoietic response, the factor activities were tested in 5-FUra bone marrow colony assays. Both IL-1α and the HBT 5637 potentiating activity potentiated colony responses to rGM-CSF, natural CSF-1, and rCSF-1 (Fig. 2 *A*, *B*, and *C*). The number of cells capable of forming colonies was increased 2- to 5-fold with IL-1α or the HBT 5637 potentiating activity. When these cultures were analyzed 14 days after culture initiation, the only cultures with large (>0.5 mm) colonies were those that contained both the potentiating activity and a CSF source. We consider these cells capable of forming large colonies with high-proliferative potential similar to the immature progenitor cells described by Bradley and co-workers (15, 25).

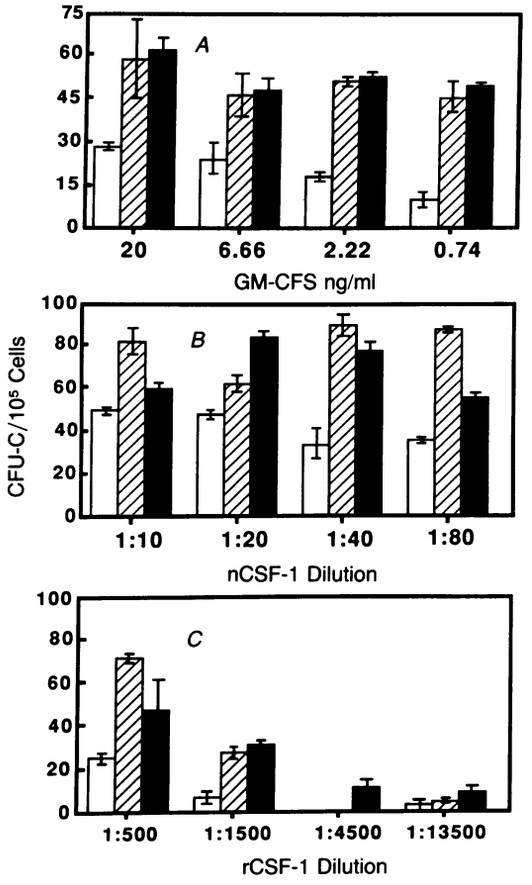

FIG. 2. Colony-forming unit (CFU-C) assay. The colony-forming ability of marrow from C3H/HeJ mice previously injected i.v. with 5-FUra in response to (*A*) GM-CSF only (□), GM-CSF plus IL-1α (□), or GM-CSF plus HBT 5637-conditioned medium (CM) (■); (*B*) natural CSF-1 (nCSF-1) only (□), nCSF-1 plus IL-1α (□) or nCSF-1 plus HBT 5637-CM (■); (*C*) rCSF-1 (□), rCSF-1 plus IL-1α (□), or rCSF-1 plus HBT 5637-CM (■). IL-1α was used at a concentration of 33 ng/ml. The source of nCSF-1 was L929 CM partially purified by DEAE ion-exchange chromatography to a concentration of ≈4 μg/ml. rCSF-1 was from yeast crude CM that had a rCSF-1 concentration of 1 μg/ml. Control yeast crude CM possessed no colony-stimulating activity. Data represent the mean of triplicate cultures ± SD.

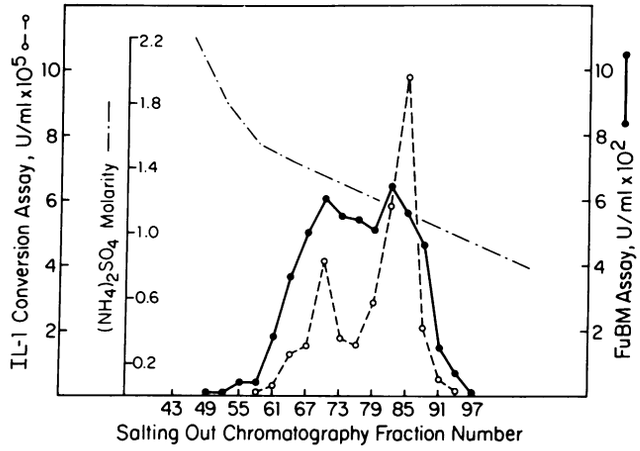

FIG. 3. Salting out chromatography. Concentrated (10×) HBT 5637-conditioned medium was chromatographed as described. IL-1 bioactivity (○); FUraBM assay bioactivity (●). U, units; FuBM, FUraBM.

IL-1α and HBT 5637 potentiating activity could also induce increased colony formation in the presence of saturating levels of GM-CSF (data not shown), thus indicating that the potentiating activity does not act via a secondary accessory cell that promotes increased GM-CSF production. In other experiments (data not shown) we observed that IL-1 increased colony formation in a dose-dependent manner. Maximal effects (4- to 5-fold that with 20 ng of GM-CSF per ml alone) in marrow 1 day after 5-FUra treatment were seen with 33–66 ng of IL-1α per ml.

Because IL-1α increased 5-FUra bone marrow colony formation and potentiated GM-CSF responses in the FUraBM assay, we began studies to establish whether HBT 5637 cells produced a specific, non-IL-1, potentiating activity. Concentrated serum-free HBT 5637 conditioned medium was fractionated by ammonium sulfate salting out chromatography (Fig. 3), phenyl-Sepharose chromatography (Fig. 4), and buffer electrofocusing (Fig. 5). These procedures separate proteins by the biophysical properties of differential solubility, hydrophobicity, and charge, respectively.

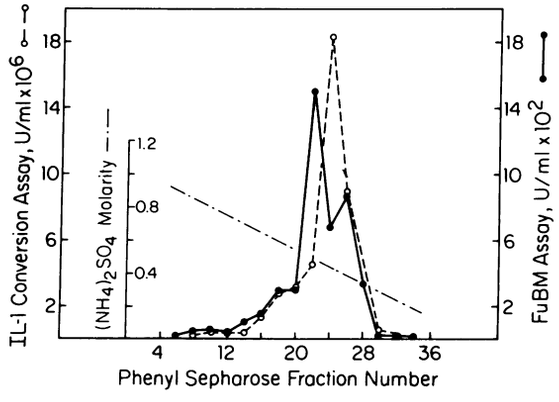

FIG. 4. Phenyl-Sepharose hydrophobic chromatography. The starting material was pooled peak potentiating activity fractions from salting out chromatography. FUraBM assay (●) and IL-1 conversion (○). The chromatography and bioassays for potentiating activity and IL-1 were done as described. U, units; FuBM, FUraBM.

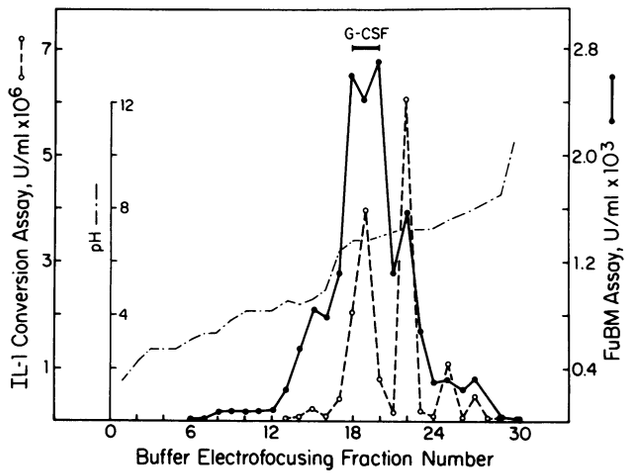

FIG. 5. Buffer electrofocusing chromatography that resolves proteins based on their isoelectric point was done as described using concentrated (10×) HBT 5637-conditioned medium. IL-1 conversion activity (○); FUraBM assay (●); U, units; FuBM, FUraBM.

Biological activity after chromatography was monitored by the FUraBM and the IL-1 conversion assays. Results of the chromatographic procedures depicted in Figs. 3, 4, and 5 revealed that the HBT 5637 potentiating activity could not be separated from IL-1 activity. Salting out chromatography effectively concentrated the colony-stimulating bioactivities (measured in human colony assays), as well as the potentiating activity (Fig. 3). The technique did not, however, resolve the bioactivities away from the bulk of the protein (data not shown). Hydrophobic chromatography on phenyl-Sepharose (Fig. 4) effectively removed the bioactivity from other CSF, as measured in a human bone marrow colony assay (data not shown), but, again, the potentiating and IL-1 activity copurified. Neither was buffer electrofocusing chromatography able to resolve IL-1α from the potentiating activity (Fig. 5). The first buffer electrofocusing potentiating activity peak measured by the FUraBM assay appears broad and is greater than the second due to the copurification of granulocyte CSF (measured in a murine colony assay). Probably IL-1 potentiated the response to granulocyte CSF—consequently resulting in an additive GM-CSF plus granulocyte CSF response.

Table 2. Specific inhibition of IL-1α-dependent IL-2 production by mAb 2F4

Antibody	Conc., μg/ml	cpm ± SEM	% inhibition*
	Inhibition of IL-1α		
Media	—	46,900 ± 6,428	—
2F4	10	17,295 ± 570	71
	1	23,654 ± 1,788	56
Isotype control	10	58,500 ± 11,030	0
	1	56,547 ± 2,343	0
	Inhibition of IL-1β		
Media	—	45,509 ± 4,333	—
2F4	10	55,711 ± 2,599	0
	1	45,847 ± 2,303	0
Isotype control	10	45,448 ± 5,003	0
	1	39,052 ± 3,871	16
Background (no IL-1)	—	5,335 ± 710	

rIL-1α and rIL-1β were used at 0.2 ng/ml. Media or supernatants containing the anti-IL-1α mAb, 2F4, or an isotype control antibody, 15A4-8C12, were added to the IL-1α or IL-1β at various concentrations (10 and 1 μg/ml). cpm values represent the mean of triplicate samples ± the SEM.

*Inhibition was determined using the IL-1 conversion assay. Percent inhibition was defined as follows:

$$1 - \left[\frac{\text{experimental cpm} - \text{background cpm}}{\text{maximum cpm} - \text{background cpm}}\right] \times 100,$$

where maximum cpm is cpm obtained with IL-1 and medium, and background cpm is cpm obtained with medium only.

Table 3. Immunoprecipitation of potentiating activity with IL-1α mAb 2F4

Sample	mAb (10 μg/ml)	% bioactivity precipitated* FUraBM assay	% bioactivity precipitated* IL-1 thymocyte assay
IL-1α (1 μg/ml)	2F4	98	96
IL-1α (0.1 μg/ml)	2F4	100	99
IL-1β (1 μg/ml)	2F4	5	0
HBT 5637 PA	Isotype control	5	0
HBT 5637 PA	2F4	100	100

The IL-1α and IL-1β used were purified recombinant proteins (19). The HBT 5637 potentiating activity (PA) was partially purified by salting out chromatography and phenyl-Sepharose as described. The 2F4 mAb, specific for IL-1α, is an IgG_1 isotype. The IgG_1 isotype control, AY-C12, is specific for an irrelevant antigen.

*IL-1 was measured in a thymocyte mitogenesis assay. The PA was monitored by the FUraBM assay. Specific activity of IL-1α was 1.5×10^5 IL-1 units/μg and 3.3×10^4 FUraBM assay units/μg. IL-1β had a specific activity of 2.6×10^4 IL-1 units/μg and 6.6×10^3 FUraBM assay units/μg. The HBT 5637 sample contained 3.4×10^3 IL-1 units/ml and 420 FUraBM assay units/ml.

The IL-1 conversion assay could be measuring a second, non-IL-1 activity. We therefore used a mAb to IL-1α to confirm that the bioactivity measured in both assays could be attributed to IL-1α. Specificity of the anti-IL-1α mAb 2F4 used in the precipitation experiments is shown in Table 2. The 2F4 mAb reacts specifically against IL-1α protein (inhibiting 71% and 56% of the response at 10 and 1 μg/ml, respectively) and not against IL-1β (0% inhibition at 10 and 1 μg/ml). The isotype control antibody did not significantly alter either the IL-1α- or the IL-1β-dependent IL-2 production by EL4 cells.

The 2F4 anti-IL-1α mAb was tested for its ability to precipitate HBT 5637 potentiating activity. As shown in Table 3, mAb 2F4 quantitatively precipitated both IL-1α at 1 μg/ml, and HBT 5637 potentiating activity. The IgG_1 isotype control did not precipitate significant (5%) amounts of the HBT 5637 potentiating activity. The antibody alone was neither stimulatory nor inhibitory (data not shown). 2F4 mAb was confirmed as specific for IL-1α because it did not efficiently precipitate IL-1β (5%). 2F4 mAb could also precipitate the 5-FUra bone marrow colony potentiating activity from HBT 5637 conditioned medium (data not shown).

DISCUSSION

A biological assay was developed to monitor activities that potentiated the proliferative response of immature bone marrow cells from 5-FUra-treated mice to suboptimal levels of GM-CSF. These cells from 5-FUra-treated mice were used because, as demonstrated earlier, they are depleted of proliferating mature hematopoietic cells and are enriched for more slowly cycling stem cells (15). Consequently, this bone marrow population does not respond to levels of GM-CSF that are biologically saturating under normal conditions. A normal marrow half-maximal proliferative response requires 500 pg of GM-CSF per ml (23 pM). At this GM-CSF concentration background thymidine incorporation response of 1000 cpm with 5-FUra-treated bone marrow is negligible.

We assumed that any potentiating activity would more rapidly induce differentiation of an immature population to respond to GM-CSF, which would then translate into increased proliferation in cultures (with potentiating activity and GM-CSF). Our assay data support this hypothesis. As anticipated, the potentiating activity also increased the number of colony-forming cells at different concentrations of rGM-CSF and CSF-1. Alone, the potentiating activity stimulated neither proliferation nor colony formation. Thus, measurable effects of the potentiating activity required lineage-specific CSF (GM-CSF or CSF-1).

The potentiating activity we have characterized from HBT 5637 cells is biochemically and serologically indistinguishable from that of IL-1α. Moreover, purified HBT 5637 potentiating activity can effectively compete for binding of radiolabeled IL-1α to murine bone marrow cells (S. Dower, personal communication).

We have further determined that IL-1β also possesses potentiating activity. Although the HBT 5637 cell line synthesizes mRNA for IL-1β, our serologic data indicated that the expressed product does not contribute to the observed potentiating activity, possibly due to low levels of expression or, alternatively, due to incorrect post-translational processing.

Our data suggest that IL-1α is identical to the protein purified by Jubinsky and Stanley (9), and designated hemopoietin 1; the cellular source is identical, and the observed M_r and the hemopoietin 1 sensitivity to organic solvents is consistent with this identification as IL-1α (9, 12). Finally, the potentiating activity from HBT 5637 cells, IL-1 and hemopoietin 1 all increase the frequency of colony formation in a FUra bone marrow colony assay (25).

IL-1 plays an active role in inflammatory responses, bone reabsorption, and immune regulation (26, 27). Our results reveal yet another role for IL-1—namely, regulation of early hematopoietic events. Consistent with a regulatory role for IL-1 in hematopoiesis is our recent observation that non-T lymphocyte hematopoietic cells bind IL-1 (D.Y.M. and R.J.T., unpublished work).

In our system IL-1 potentiates CSF responses of normally nonresponsive immature hematopoietic cells. Mechanistically, IL-1 may act via accessory cells by inducing production of other regulatory factors such as IL-3. Or alternatively, IL-1 may modulate receptors for regulatory factors. Hemopoietin 1 (IL-1α) appears to be involved in upregulation of CSF-1 receptors (8). There is also evidence that IL-1 increases IL-2 receptor numbers in T lymphocytes (28, 29). A dual requirement for IL-1 and secondary regulatory factors might confer a greater degree of specificity and restrict potential pleiotropic effects of IL-1.

The physiologic role of IL-1 in early hematopoietic events is unclear. Although proof for a physiologic role of IL-1 is lacking, our results suggest a therapeutic role for IL-1 in a variety of hematologic disorders. Finally, IL-1 with other hematopoietic factors may be efficacious in reconstituting the hematopoietic system after cytoreductive cancer therapies such as radiation and chemotherapy.

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