Polycythemia Vera Blood Burst-forming Units-Erythroid Are Hypersensitive to Interleukin-3

C. H. Dai,* S. B. Krantz,* R. T. Means, Jr.,* S. T. Horn,* and H. S. Gilbert*

*Departments of Medicine, Department of Veterans Affairs Medical Center and Vanderbilt School of Medicine, Nashville, Tennessee 37232; and *Albert Einstein College of Medicine, Bronx, New York 10461

Abstract

Because polycythemia vera (PV) is a clonal hematopoietic stem cell disease with a trilineage hyperplasia, and interleukin-3 (IL-3) stimulates trilineage hematopoiesis, we have studied the response of highly purified PV blood burst-forming units-erythroid (BFU-E) to recombinant human IL-3 (rIL-3). Whereas the growth of normal blood BFU-E in vitro rapidly declined by 40 and 60% after 24 and 48 h of incubation without 50 U/ml of rIL-3, the growth of PV BFU-E declined by only 10 and 30% under the same conditions, demonstrating a reduced dependence on rIL-3. A reduced dependence of PV BFU-E on recombinant human erythropoietin (rEP) was also present. Dose-response experiments showed a 117-fold increase in PV BFU-E sensitivity to rIL-3, and a 6.5-fold increase in sensitivity to rEP, compared to normal BFU-E, whereas blood BFU-E from patients with secondary polycythemia responded like normal BFU-E. Endogenous erythroid colony (EEC) formation, which is independent of the addition of rEP, was reduced by 50% after erythroid colony-forming cells were generated from PV BFU-E in vitro without rIL-3 for 3 d, whereas rEP-stimulated erythroid colonies were unaffected. These studies demonstrate a striking hypersensitivity of PV blood BFU-E to rIL-3, which may be the major factor in the pathogenesis of increased erythropoiesis without increased EP concentrations. (*J. Clin. Invest.* 1991. 87:391-396.) Key words: myeloproliferative disease • cytokines • erythroid progenitors • erythropoietin

Introduction

Polycythemia vera (PV)¹ is a clonal disease of the hematopoietic stem cell which is characterized by a trilineage hyperplasia of the marrow, leukocytosis, thrombocytosis, and erythrocyto-

sis (1). However, the control of red cell production has been principally studied because polycythemia is the primary clinical manifestation. It has been shown that the increased erythropoiesis is not the result of increased erythropoietin (EP) production, but reflects the proliferation of the abnormal clone in the absence of increased EP (1, 2). When marrow cells from PV patients were cultured, endogenous erythroid colonies (EEC) formed without the addition of EP, and were 9-37% of the erythroid colonies seen with EP (3). Zanjani et al. (4), Golde et al. (5), and Casadevall et al. (6) provided evidence that erythropoiesis in PV is hypersensitive to EP, whereas other investigators suggested that it might be independent of EP (7, 8). Recently PV CFU-E have been shown to have a normal number of low-affinity EP receptors, but to lack high-affinity EP receptors, which indicates that any enhanced EP sensitivity is unlikely to be due to enhanced EP binding to these cells (9).

In the last year, we have developed a method for enriching normal human blood burst-forming units-erythroid (BFU-E) to 57% purity, with a range of 45-79%, and have characterized the growth requirements for these cells (10). As with human marrow cells (11, 12), interleukin-3 (IL-3) is needed early in the course of BFU-E development in vitro while EP is needed later (10). Because this method allows us to obtain highly purified BFU-E, and because IL-3 is a cytokine that acts upon early hematopoietic cells to provide trilineage growth enhancement (13), which is seen in PV, we have purified PV BFU-E and have studied the response of these cells to IL-3 and EP. These investigations show that PV blood BFU-E are much more hypersensitive to IL-3 than to EP and this may account for the increased growth and differentiation of PV erythroid cells in the absence of enhanced EP concentrations.

Methods

Blood samples. 400 ml of peripheral blood was obtained from normal adults and from patients meeting established criteria for PV and secondary polycythemia (14), who signed consent forms approved by the Vanderbilt Committee for the Protection of Human Subjects and the Department of Veterans Affairs Research and Development Committee. Four of the PV patients were treated only with phlebotomy, whereas one received hydroxyurea, 500 mg every other day, in addition to phlebotomy. Two of the patients with secondary polycythemia had severe chronic obstructive pulmonary disease; two had renal disease (cyst and stones) with increased serum erythropoietin levels; and one had unilateral hydronephrosis associated with previous surgery for carcinoma of the prostate. The blood was collected in sodium heparin (Upjohn Co., Kalamazoo, MI) at a final concentration of 20 U/ml.

BFU-E purification. This method has been previously described (10). In brief, the mononuclear cells were separated over Ficoll-Hypaque (FH; 1.077 g/cm³, Pharmacia Fine Chemicals, Piscataway, NJ; Winthrop-Breton Laboratories, New York, NY) at 400 g for 25 min at 24°C. For PV samples, either buffy coat cells were suspended in an equal volume of alpha minimum essential medium (α MEM) (Sigma Chemical Co., St. Louis, MO), or the blood was diluted with one-half

Address reprint requests to Dr. Sanford B. Krantz, Dept. of Medicine, Vanderbilt Medical Center North C-3101, Nashville, TN 37232-2287.

Received for publication 24 January 1990 and in revised form 12 September 1990.

1. Abbreviations used in this paper: AB⁻, antibody-negative cells; BFU-E, burst-forming units-erythroid; D-BSA, deionized BSA; D-PBS, Dulbecco's PBS; ECFC, erythroid colony-forming cells; EEC, endogenous erythroid colonies; EP, erythropoietin; FH, Ficoll-Hypaque; IL-3, interleukin-3; IMDM, Iscove's modified Dulbecco's medium; IMDM-P, Iscove's modified Dulbecco's medium used for panning; 2-ME, 2-mercaptoethanol; α MEM, alpha minimum essential medium; PV, polycythemia vera; rEP, recombinant human erythropoietin; rIL-3, recombinant human interleukin-3.

J. Clin. Invest.

© The American Society for Clinical Investigation, Inc.

0021-9738/91/02/0391/06 \$2.00

Volume 87, February 1991, 391-396

volume of Dulbecco's PBS (Sigma Chemical Co.) containing 13.6 mM sodium citrate (D-PBS) (Sigma Chemical Co.), before separation by FH (9). The interface mononuclear FH cells were collected, washed twice with D-PBS at 600 g, 24°C, for 15 min and were resuspended in 20 ml of D-PBS. The cell suspension was overlaid on 30 ml of 10% BSA (Armour Pharmaceutical Co., Kankakee, IL) in D-PBS and centrifuged at 180 g, 24°C, for 10 min to remove platelets. This procedure was repeated and the cells were washed thrice with α MED before suspending them in α MED at 1×10^7 /ml.

The cells then were incubated with one-half volume of neuraminidase-treated and one-half volume of IgG-coated sheep erythrocytes for 10 min in a 37°C water bath (10). After centrifugation at 130 g, 24°C, for 10 min, the cells were placed in ice for 60 min and were then dispersed and separated over FH at 400 g, 24°C, for 15 min. The interface mononuclear cells were washed thrice with α MED and resuspended at 2×10^7 /ml in Iscove's modified Dulbecco's medium (IMDM) (Sigma Chemical Co.), containing 36 mM NaCl instead of NaHCO_3 , with the pH adjusted to pH 7.3 using NaOH. This IMDM was used for "panning" (IMDM-P) and had 1.0% deionized (15) BSA (D-BSA) with 2×10^7 cells per ml.

The cells then were mixed with an equal volume of CD34/anti-My10 murine hybridoma tissue culture supernatant (16, 17) at 3°C and were incubated for 30 min at 3°C using an end-over-end rotator. The cells were washed thrice with IMDM-P containing 0.25% D-BSA (washing medium) using the cell protection washing procedure previously described (10). The cells were resuspended at 3×10^7 /ml in IMDM-P, containing 1% D-BSA, for incubation with a threefold excess of immunomagnetic microspheres (Dynabeads M-450 coated with goat anti-mouse IgG; Dynal Inc., Great Neck, NY) at 3°C over 50 min using an end-over-end rotator. The cell-bound microspheres were attached to the tube wall by a magnet (model MPC-1; Dynal Inc.) and the free cells were removed by washing the tube four times with 3 ml of washing medium plus two times with IMDM-P at 3°C. After removal of the magnet, the cells that bound microspheres were gently pipetted for 30 s with 1.5 ml of IMDM-P containing 130 U/ml of chymopapain (Chymodiactin; Boots Company Inc., Lincolnshire, IL) and 0.02 M EDTA (Fischer Scientific Co., Pittsburgh, PA), at 37°C, and were incubated for another 30 s at 37°C. The magnet was applied and the cells released from the microspheres were collected and mixed with 1.5 ml heat-inactivated FCS (fraction 1). This procedure was repeated another three times (fractions 2–4). The cells from fractions 2–4 were pooled as My10⁺ cells and the cell protection washing procedure was employed before resuspending the cells in IMDM containing 0.1% D-BSA.

The My10⁺ cells were incubated overnight, in 5 ml IMDM containing 20% heat-inactivated FCS, 10% pooled human heat-inactivated AB serum, 1% D-BSA, 50 U/ml recombinant human IL-3 (rIL-3) (10⁶ U/mg protein; AMGen Biologicals, Thousand Oaks, CA), 2 U/ml recombinant human EP (rEP), (128,000 U/mg; Ortho Pharmaceutical Corp., Raritan, NJ), 10 μ g/ml porcine insulin, (26.3 USP U/mg; Calbiochem-Behring Diagnostics, La Jolla, CA), 5×10^{-5} M 2-mercaptoethanol (2-ME), (Eastman-Kodak Co., Rochester, NY), penicillin at 500 U/ml and streptomycin at 40 μ g/ml, in 25 cm² tissue culture polystyrene flasks (Becton-Dickinson, Oxnard, CA) at 37°C, in a 5% CO₂/95% atmosphere incubator.

The nonadherent day 1 cells were collected and suspended in IMDM-P containing 1% D-BSA. Nine murine monoclonal antibodies to CD15/anti-My1, CD33/anti-My9, CD45R/anti-My11, CD16/anti-My23, CD56/anti-My31 (17–22), CD7/anti-Leu9, CD20/anti-Leu16 (Becton-Dickinson), CD2/anti-OKT*1, and CD11b/anti-OKM*1 (Ortho Pharmaceutical Corp.) were added as previously described (10) and the cell suspension was incubated for 30 min at 3°C with end-over-end rotation. The cell protection washing procedure was used and the cells were resuspended in IMDM-P containing 1% D-BSA before being mixed with two series of immunomagnetic microspheres: one coated with affinity purified goat anti-mouse IgG (Dynal, Inc.) and another (Dynabeads M-450, Tosylactivated; Dynal, Inc.) coated with affinity purified goat anti-mouse IgM (μ -chain specific; Cappel Laboratories, West Chester, PA). The cells then were incubated for 60 min, at 3°C,

with end-over-end rotation and those which did not bind microspheres were collected by magnetic separation. These cells were overlaid on FH and centrifuged at 600 g for 5 min at 24°C to remove dead cells. The interface mononuclear cells were collected and the cell protection washing procedure was employed before resuspending the cells in IMDM containing 0.3% D-BSA. The cell concentration and the viability of the cells were measured by hemocytometer and dye exclusion using 0.2% Trypan blue dye (23). The cells were kept at 3°C until use (day 1 AB⁺FH cells).

Erythroid colony-forming cell (ECFC) purification. ECFC were generated in vitro from partially purified BFU-E and are defined as cells that give rise to single colonies of 2–49 hemoglobinized cells after 7 d of culture with EP (24, 25). The blood BFU-E were purified by the method of Sawada et al. (24) as modified by Means et al. (9). Briefly, heparinized peripheral blood was centrifuged over FH, treated with citrated PBS/BSA, and depleted of T-lymphocytes as described above. The remaining mononuclear cells underwent overnight adherence in plastic flasks in the presence of 20% heat-inactivated FCS and 0.4% giant cell tumor conditioned medium (Gibco Laboratories, Grand Island, NY) under conditions already described.

The following morning (day 1), the nonadherent cells were collected by gentle washing with 37° α MED. These cells then were suspended at a concentration of 70×10^6 /ml in 25 μ l CD11b/anti-OKM*1 (20 μ g/ml), 25 μ l CD2/anti-OKT*11 (10 μ g/ml; Ortho), 50 μ l CD45R/anti-My11, and 50 μ l CD16/anti-My23 (17–19, 21) for 60 min at 3°C. The cells were washed thrice and incubated in 100-mm plastic tissue culture dishes, which had previously been coated with affinity purified goat anti-mouse IgG (Boehringer Mannheim Biochemicals, Indianapolis, IN), for 90 min, 3°C, to remove residual granulocytes, monocytes, granulocyte-macrophage progenitors, T- and B-lymphocytes, and natural killer cells.

The remaining day 1 cells were collected in IMDM and cultured at 3.0×10^5 /ml in 0.9% methylcellulose (Fisher Scientific Co., Pittsburgh, PA) with 30% heat-inactivated FCS, 1% BSA, 10^{-4} M 2-ME, 500 U/ml penicillin, 40 μ g/ml streptomycin, 50 U/ml rIL-3, 10 U/ml recombinant human insulin (26 U/mg; Eli Lilly & Co., Indianapolis, IN), and rEP 2 U/ml for 4 d at 37° in a high humidity 5% CO₂ incubator.

On day 5, the cells were collected in α MED and separated over 10% BSA as described above. Adherent cells were removed by incubation in plastic flasks for 1 h at 37°C with 20% heat-inactivated FCS. The non-adherent cells were collected and incubated at 37°C, 1 h, in IMDM with 0.1% BSA to remove any surface-bound growth factors (26). The cells then were layered onto 2 ml FH and centrifuged at 600 g, 3°C, for 5 min. The interface cells were washed and collected in IMDM.

These cells were cultured for 3 d at 37°C in a 5% CO₂ incubator at a concentration of 3.0×10^5 /ml in IMDM with 30% heat-inactivated FCS, 1% BSA, 10^{-4} M 2-ME, 500 U/ml penicillin, 40 μ g/ml streptomycin, 10 U/ml recombinant human insulin, and rEP 2 U/ml. rIL-3, 50 U/ml, was added to half the culture wells.

On day 8, the cells were collected, separated over 10% BSA, and incubated in IMDM with 0.1% BSA as on day 5. Cells which had been exposed to rIL-3 for all 7 d were handled separately from those which had been exposed to rIL-3 days 1–5 only and both were assayed for ECFC.

BFU-E assay. The purified day 1 AB⁺FH cells were plated at 100–200 cells/ml in a 0.5 ml mixture containing 20% heat-inactivated FCS, 10% heat-inactivated pooled human AB serum, 1% D-BSA, 5×10^{-5} M 2-ME, 1.5 mM aminocaproic acid (Elkins-Sinn, Inc., Cherry Hill, NJ), penicillin-streptomycin, 10 μ g/ml insulin, 2 U/ml rEP, 50 U/ml rIL-3, 2 mg/ml human fibrinogen, (grade L, coagulability 90% of total protein content; KabiVitrum, Stockholm, Sweden), 0.2 U/ml thrombin (Parke-Davis, Morris Plains, NJ) and IMDM in 24-well flat-bottomed tissue culture plates (Linbro; Flow Laboratories, Inc., McLean, VA). In some experiments, which concerned the effect of delayed addition of rEP and rIL-3 on BFU-E development, the day 1 AB⁺FH cells were plated into 0.5-ml plasma clots without rEP or rIL-3 and then 0.2 ml IMDM containing 20% FCS and 1.4 U rEP, or 35 U rIL-3, was overlaid on the clots on the indicated days.

Figure 1. The effect of delayed addition of rEP (A) or rIL-3 (B) on blood BFU-E development from two normal (---) and two PV (—) subjects. The BFU-E were plated in 0.5-ml plasma clots at concentrations of 150–200 cells/ml with 50 U/ml of rIL-3 (A) or 2 U/ml of rEP (B). At the indicated times, 0.2 ml of IMDM, containing 20% FCS and 1.4 U rEP (A) or 35 U rIL-3 (B) was overlaid on the clots. The cells were incubated for 15 d and then fixed and stained with benzidine-hematoxylin. The number of erythroid bursts observed in clots plated on day 1 with both rEP and rIL-3 was taken as 100% expression. Each value is the mean of triplicates. The BFU-E purity was $62 \pm 4\%$, $47 \pm 9\%$ (normal); $61 \pm 9\%$ and $89 \pm 5\%$ (PV) (mean $\pm$ SD). One PV patient (O) received hydroxyurea 500 mg every other day in addition to phlebotomy while the other was treated by phlebotomy alone.

The cultures were incubated in a 5% CO₂/95% atmosphere, at 37°C, in a fully humidified incubator for 15 d. At the end of the incubation, the clots were removed and transferred to glass slides, fixed in 5% glutaraldehyde and then stained with benzidine-hematoxylin (15). Enumeration of BFU-E was performed as described by Clark and Housman (27). Examination of purified PV day 1 AB⁺FH cells for ECFC in six cultures has shown that $3.8 \pm 1.3\%$ of the erythroid progenitors were ECFC.

ECFC assay. Day 8 cells were assayed for ECFC by the plasma clot method of McLeod et al. (15). Cells were cultured at 10^3 /ml in 0.2-ml aliquots, using 48-well tissue culture plates, for 7 d at 37°C in a 5% CO₂ high-humidity incubator. The plating mixture contained 15% heat-inactivated FCS, 15% heat-inactivated pooled AB serum, 1% D-BSA, penicillin and streptomycin, 1 U/ml rEP, 2 mg/ml fibrinogen, 0.2 U/ml thrombin, 0.2 M aminocaproic acid, and IMDM. Clots were fixed and stained as described above. EEC formation is defined as the growth of erythroid colonies without the addition of rEP (3–5, 28). Statistical comparison was by *t* test.

Results

Dependency of normal and PV blood BFU-E on rEP and rIL-3. Purified BFU-E were plated into plasma clots with the omission of either rEP or rIL-3, and the omitted factor was added at 24-h intervals. In the presence of 50 U/ml rIL-3, 90% of normal BFU-E formed erythroid bursts after an absence of rEP for 48 h, and 50% after an absence of rEP for as long as 120 h (Fig. 1 A). PV BFU-E could be deprived of rEP for two additional days and still retain an equivalent capacity to form erythroid bursts

(Fig. 1 A). Whereas the blood BFU-E from one PV patient did not show EEC formation in this experiment, a replicate blood from the same patient did have EEC (Fig. 2).

When normal BFU-E were plated with 2 U/ml rEP and deprived of rIL-3 for 24 and 48 h erythroid burst formation was markedly reduced to 63 and 40% of BFU-E that had rIL-3 present the full time (Fig. 1 B). However, 90% of PV BFU-E continued to form erythroid bursts when deprived of rIL-3 for 24 h and 70% survived normally when deprived of rIL-3 for 48 h (Fig. 1 B). PV BFU-E appeared less dependent on large concentrations of IL-3 and EP than normal BFU-E.

Sensitivity of normal and PV blood BFU-E to rEP and rIL-3. Fig. 2 A shows the dose-response relationship for normal and PV BFU-E which were incubated with a wide variety of rIL-3 concentrations and 2 U/ml of rEP. An enhanced number of PV bursts occurred with increasing concentrations of rIL-3 when compared to normal burst formation. The rIL-3 concentration necessary for 50% expression of PV erythroid bursts was 0.004 ± 0.001 U/ml compared to 0.467 ± 0.162 U/ml for normal erythroid burst development ($P < 0.005$) demonstrating a 117-fold increase in the sensitivity of PV BFU-E to rIL-3. Almost twice as many erythroid bursts developed in the absence of rIL-3 from PV BFU-E, compared to normal BFU-E ($P < 0.01$), but a similar plateau occurred for the response of both classes of cells at 25 U/ml. The increased number of PV erythroid bursts, compared to normal erythroid bursts, at all concentrations of rIL-3 from 0.001 to 1.0 U/ml, was significant at each level with *P* values from 0.018 to 0.00001. The erythroid bursts

Figure 2. Comparison of rIL-3 (A) and rEP (B) dose-response curves in 0.5-ml plasma clot cultures of blood BFU-E from three normal (---) and four PV (—) subjects. The BFU-E were plated at 100–150 cells/ml with rEP 2 U/ml (A) or rIL-3 50 U/ml (B). Each point is the mean value of triplicates and is expressed as a percentage of the value obtained with the highest concentration of rIL-3 or rEP. The purity of the BFU-E was $42 \pm 8\%$, $51 \pm 3\%$, $40 \pm 11\%$ (normal); $76 \pm 11\%$, $81 \pm 6\%$, and $59 \pm 5\%$ (PV) (mean $\pm$ SD). One PV patient (O) received hydroxyurea 500 mg every other day in addition to phlebotomy while the others were treated by phlebotomy alone.

in PV were slightly larger than normal erythroid bursts, but no further difference in BFU-E size was present with increasing concentrations of rIL-3. A similar hypersensitivity of PV BFU-E to rIL-3 was observed in the presence of only 0.1 U/ml rEP and in this case the size of the erythroid bursts increased with increasing concentration of rIL-3 (data not shown).

The sensitivity of normal and PV BFU-E to increasing concentrations of rEP, in the presence of 50 U/ml rIL-3, is shown in Fig. 2 B. A left shift of the dose-response curve for rEP was also present with PV BFU-E, but it was far less than that seen with rIL-3. The rEP concentration necessary for 50% expression of erythroid bursts was 0.036 ± 0.015 U/ml in PV compared to 0.233 ± 0.111 U/ml with normal BFU-E ($P < 0.05$), a 6.5-fold increase in sensitivity. Without the addition of rEP $6 \pm 1\%$ PV erythroid bursts were evident after 15 d of culture compared to $0.7 \pm 1\%$ normal bursts ($P < 0.01$), but both groups of cells reached a plateau response at 1 U/ml rEP. Both the PV and the normal erythroid bursts were larger with increasing concentrations of rEP up to 0.5 U/ml. A similar dose response relationship of normal and PV BFU-E, size of erythroid bursts, and tendency for increased burst size with increased rEP was seen with increasing concentrations of rEP in the presence of 0.5 U/ml of rIL-3 (data not shown).

Sensitivity of secondary polycythemia blood BFU-E to rEP and rIL-3. BFU-E from five patients with secondary polycythemia were cultured in plasma clots with increasing concentrations of rIL-3 (Fig. 3 A) or rEP (Fig. 3 B). The rIL-3 concentration (0.54 ± 0.38 U/ml) and the rEP concentration (0.15 ± 0.07 U/ml) necessary for 50% expression of erythroid burst development were not significantly different ($P > 0.1$) from those necessary for a similar development of normal erythroid bursts. However, these concentrations were significantly different from those necessary for 50% erythroid burst development by PV cells (rEP, $P < 0.05$; rIL-3, $P < 0.05$).

Relation of rIL-3 to EEC formation by PV ECFC. EEC formation was evaluated for ECFC generated for 7 d with rIL-3, and ECFC cultured for 4 d with rIL-3 and then 3 d without this cytokine. ECFC from three different patients with PV were studied, plus a repeat from one patient, and all showed EEC formation (Fig. 4). However, the cells that had been deprived of rIL-3 from day 5 to day 8 had a 50% reduction in EEC growth. The reduction in EEC growth was localized to the EEC and was not related to a generalized cellular toxicity because the removal of rIL-3 had no effect on the growth of cells incubated with rEP (Fig. 4). The purity of ECFC ($51.7 \pm 21.5\%$ from cells exposed to rIL-3 days 1–8, and $52.5 \pm 23.7\%$ from cells exposed

to rIL-3 days 1–5) was unchanged. Normal ECFC were not studied because their EEC growth is usually $< 0.5\%$ (9, 24).

Discussion

Studies on the control of proliferation and differentiation of PV cells generally have used highly impure blood or marrow cells and have focused on the response to EP. However, it has recently become possible to purify early erythroid progenitor cells without the need for specialized equipment using immunomagnetic microspheres and monoclonal antibodies. In addition, the genes for a variety of hematopoietic growth factors, which act on hematopoietic progenitor cells at earlier stages of development, have been cloned and expressed, and these cytokines are now available to study their role in pathologic processes. Because PV manifests a trilineage hyperplasia, and IL-3 enhances trilineage hematopoiesis, we have studied the response of highly purified PV BFU-E to rIL-3.

The studies reported here show that PV blood BFU-E can be enriched to $73 \pm 13\%$ with a range of 59–89%. The method is virtually the same as that used for the purification of normal human BFU-E except that the blood was either diluted with an equal volume of α MED or the buffy coat was removed and used for FH sedimentation to reduce the viscosity due to an increased concentration of red cells.

Whereas normal human erythroid burst formation was markedly reduced in number when the usual large concentration of rIL-3 was not added in the first 24–48 h of incubation, PV BFU-E had virtually complete survival when deprived of rIL-3 for 24 h and a comparatively modest decline after 48 h of deprivation. Because we thought that one possible reason for this might be a hypersensitivity to extremely low concentrations of IL-3 produced by some of the cells, or present in the medium, we performed rIL-3 and rEP dose-response curves for normal and PV BFU-E. A 6.5-fold increase in sensitivity of PV BFU-E to rEP, compared to normal BFU-E, was demonstrated with highly purified BFU-E. However, a 117-fold increase in sensitivity of purified PV BFU-E to rIL-3 was even more striking. It is possible that this might be due to the increased erythropoiesis that is evident in PV and for that reason similar experiments were performed with BFU-E from patients with secondary polycythemia. However, these BFU-E behaved in vitro like normal BFU-E.

Some investigators have reported an enhanced sensitivity of PV BFU-E to very low concentrations of Ep, but a normal sensitivity as the concentration of Ep is increased (29, 30). How-

Figure 3. rIL-3 (A) and EP (B) dose-response curves for blood BFU-E from five secondary polycythemia patients. The BFU-E were plated at 100–150 cells/ml with rEP 2 U/ml (A) or rIL-3 50 U/ml (B). The shaded areas represent the range of response for normal BFU-E shown in Fig. 2. Each point represents the mean value from three clots and is expressed as a percentage of the value obtained with the highest concentration of rIL-3 or rEP. The purity of the secondary polycythemia BFU-E was $61 \pm 6\%$, $51 \pm 5\%$, $40 \pm 6\%$, $30 \pm 2\%$, and $51 \pm 11\%$. All five subjects were treated by phlebotomy alone.

Figure 4. Role of IL-3 in EEC growth. Partially purified PV blood BFU-E were cultured in methylcellulose at 37°C with rIL-3 (A, C) for 7 d (day 8 cells) to generate ECFC. These ECFC were compared to ECFC generated from BFU-E that were cultured with rIL-3 for 4 d and then without rIL-3 for an additional 3 days (B, D). Three experiments from three separate patients with PV, plus one repeat (patient 2B) are shown. Each pool of generated ECFC was cultured at 10^3 /ml for 7 d in quadruplicate 0.2-ml plasma clots without rEP (A, B) or in the presence of 1 U/ml rEP (C, D). EEC growth from cells exposed to rIL-3 for only 4 d was significantly decreased ($P < 0.02$ for patient 1, $P < 0.05$ for patient 2A, and $P < 0.01$ for patients 2B and 3), whereas no significant effect on EP-responsive ECFC was apparent. Results are expressed as mean $\pm$ SD. All three patients were treated solely by phlebotomy.

ever, these experiments were performed in the past with little purification of the EP-responsive cells. In this case, the denominator, the number of BFU-E actually present amongst PV and normal hematopoietic cells, may not be accurate as the growth and differentiation of the BFU-E might be altered by the numerous contaminant accessory cells and their products. With highly purified BFU-E, this variability is greatly reduced and this may be the explanation for the variability in results from different laboratories.

Because PV BFU-E maintain a higher degree of responsiveness to rIL-3 between days 5 and 7 of growth in vitro, compared to normal BFU-E, we explored the relation of this phenomenon to the development of EEC. If rIL-3 and rEP act cooperatively in the development of these cells during the period when sensitivity to both factors is present, then the presence of one factor might create a lesser need for the other. This possibility is enhanced by the observation that PV erythroid bursts increased in size when increasing concentrations of rIL-3 were added to the BFU-E in the presence of a low concentration of rEP. Indeed, when PV BFU-E were grown without rIL-3 during days 5–8, a 50% decrease in EEC formation was evident indicating that the increased period of sensitivity to rIL-3 is a major factor contributing to this phenomenon. In the presence of maximum concentrations of rEP, no effect of rIL-3 deprivation was evident for ECFC development. However, it is possible that the increased period of rIL-3 responsiveness may also account for the hypersensitivity of PV CFU-E to rEP when limiting amounts of rEP are present (4).

We have previously shown that PV CFU-E lack high-affinity EP receptors. Recently, Fraser et al. (31) reported that IL-3 down modulated EP receptors on murine spleen CFU-E. We have shown that rIL-3 has no effect on the growth and development of highly purified normal human CFU-E (25), but it is possible that the hypersensitivity to rIL-3 and the persistent responsiveness of PV erythroid progenitors to rIL-3, for as long as 6 d of maturation, might allow a down-modulation of the high-affinity EP receptors on these cells, which could persist until day 8 of incubation when the ECFC were collected and examined for EP receptors by 125 I-Ep binding experiments. Experiments are currently underway in our laboratory to determine if incubation of PV BFU-E with rIL-3 accounts for the pattern of EP receptors that has been described.

The precise cause for the rIL-3 hypersensitivity of PV blood BFU-E is not known. One likely explanation is that these BFU-E are 2 d younger and slightly more immature. The 2-d extension of PV BFU-E sensitivity to rIL-3 may provide an additional 2-d period of reduced dependence on rEP. However, Robak (32) has performed an extensive phenotypic study of PV blood BFU-E looking at the antigenic characteristics of these cells with nine monoclonal antibodies. No qualitative differences between PV and normal blood BFU-E were found, but the percentage of normal BFU-E reacting with antibody to early and late differentiation antigens was somewhat increased compared to PV BFU-E.

Additional mechanisms are possible. PV BFU-E may have an enhanced number of IL-3 receptors or an increased binding affinity for IL-3. This can be studied by autoradiography using 125 I-rIL-3. Prior autoradiographic studies with highly purified normal human BFU-E have shown a reduced number of EP receptors that increase as these cells mature to CFU-E (10). Similar studies should provide information on the relation between IL-3 sensitivity and the development of IL-3 receptors. Other possibilities for the IL-3 hypersensitivity are an enhanced sensitivity of the second messenger system, the production of another cytokine that potentiates the effect of rIL-3 (33), or the lack of production of a normal inhibitor that counteracts the activity of rIL-3 (34, 35). The present system provides a means for the investigation of most of these alternatives. Nevertheless, these studies indicate that the prominent abnormality in PV is an exquisite sensitivity of the blood BFU-E to IL-3 which could represent the primary pathogenetic event in this condition.

Acknowledgements

The authors thank Dr. Curt I. Civin for his generous gift of murine monoclonal antibodies to CD34/anti-My10, CD15/anti-My1, CD33/anti-My9, CD45R/anti-My11, CD16/anti-My23, and CD56/anti-My31, without which this work would not have been possible, and Mrs. Pat Hofmann for her assistance in preparing this manuscript.

This work was supported by Veterans Health Services and Research Administration funds, and grants DK-15555, 2 T32 DK07186, RR-95, and RR-50 from the National Institutes of Health and The Joe C. Davis Hematology Research Fund. Dr. Means is an Associate Investigator of the VA Career Development Program.

References

- Adamson, J. W., P. J. Fialkow, S. Murphy, J. F. Prchal, and L. Steinmann. 1976. Polycythemia vera: stem cell and probable clonal origin of the disease. *N. Engl. J. Med.* 295:913–916.

2. Prchal, J. F., J. W. Adamson, S. Murphy, L. Steinmann, and P. J. Fialkow. 1978. Polycythemia vera. The in vitro response of normal and abnormal cell lines to erythropoietin. *J. Clin. Invest.* 61:1044-1047.
3. Prchal, J. F., and A. A. Axelrad. 1974. Bone marrow responses in polycythemia vera. *N. Engl. J. Med.* 290:1382.
4. Zanjani, E. D., J. D. Lutton, R. Hoffman, and L. R. Wasserman. 1977. Erythroid colony formation by polycythemia vera marrow in vitro. Dependence on erythropoietin. *J. Clin. Invest.* 59:841-844.
5. Golde, D. W., N. Bersch, and M. J. Cline. 1977. Polycythemia vera. Hormonal modulation of erythropoiesis in vitro. *Blood.* 49:399-405.
6. Casadevall, N., W. Vainchenker, C. Lacombe, G. Vinci, J. Chapman, J. Breton-Gorius, and B. Varet. 1982. Erythroid progenitors in polycythemia vera. Demonstration of their hypersensitivity to erythropoietin using serum-free cultures. *Blood.* 59:447-451.
7. Eaves, A. C., G. Krystal, J. D. Cashman, and C. J. Eaves. 1988. Polycythemia vera: in vitro analysis of regulatory defects. In *Regulation of Erythropoiesis*. E. D. Zanjani, M. Tavassoli, and J. L. Ascensao, editors. PMA Publishing Corp., New York.
8. Eridani, S., J. M. Dudley, B. M. Sawyer, and T. C. Pearson. 1987. Erythropoietic colonies in a serum-free system: results in primary proliferative polycythemia and thrombocythemia. *Br. J. Haematol.* 67:387-391.
9. Means, R. T., Jr., S. B. Krantz, S. T. Sawyer, and H. S. Gilbert. 1989. Erythropoietin receptors in polycythemia vera. *J. Clin. Invest.* 84:1340-1344.
10. Sawada, K., S. B. Krantz, C.-H. Dai, S. T. Koury, S. T. Horn, A. D. Glick, and C. I. Civin. 1990. Purification of human blood burst-forming units-erythroid and demonstration of the evolution of erythropoietin receptors. *J. Cell. Physiol.* 142:219-230.
11. Sieff, C. A., S. G. Emerson, A. Mufson, T. G. Gesner, and D. G. Nathan. 1986. Dependence of highly enriched human bone marrow progenitors on hematopoietic growth factors and their response to recombinant erythropoietin. *J. Clin. Invest.* 77:74-81.
12. Emerson, S. G., S. Thomas, J. L. Ferrara, and J. L. Greenstein. 1989. Developmental regulation of erythropoiesis by hematopoietic growth factors: analysis on populations of BFU-E from bone marrow, peripheral blood, and fetal liver. *Blood.* 74:49-55.
13. Sieff, C. A., C. M. Niemeyer, D. G. Nathan, S. C. Ekern, F. R. Bieber, Y. C. Yang, G. Wong, and S. C. Clark. 1987. Stimulation of human hematopoietic colony formation by recombinant gibbon multi-colony-stimulating factor or interleukin 3. *J. Clin. Invest.* 80:818-823.
14. Berlin, N. I. 1975. Diagnosis and classification of the polycythemia. *Semin. Hematol.* 12:339-351.
15. McLeod, D. L., M. M. Shreeve, and A. A. Axelrad. 1974. Improved plasma culture system for production of erythrocytic colonies in vitro. Quantitative assay method for CFU-E. *Blood.* 44:517-534.
16. Civin, C. I., L. C. Strauss, C. Brovall, M. J. Fackler, J. F. Schwartz, and J. H. Shaper. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J. Immunol.* 133:157-165.
17. Strauss, L. C., S. D. Rowley, V. F. La Russa, S. J. Sharkis, R. K. Stuart, and C. I. Civin. 1986. Antigenic analysis of hematopoiesis. V. Characterization of My-10 antigen expression by normal lymphohematopoietic progenitor cells. *Exp. Hematol.* 14:878-886.
18. Civin, C. I., and M. R. Loken. 1987. Cell surface antigens on human marrow cells: dissection of hematopoietic development using monoclonal antibodies and multiparameter flow cytometry. *Int. J. Cell Cloning.* 5:267-288.
19. McMichael, A. J., P. C. L. Beverley, S. Cobbold, M. J. Crumpton, W. Gilks, F. M. Gotch, N. Hogg, M. Horton, N. Ling, I. C. M. MacLennan, D. Y. Mason, C. Milstein, D. Spiegelhalter, and H. Waldmann, editors. 1987. *Leukocyte Typing III: White Cell Differentiation Antigens*. Oxford University Press, Oxford. 1050 pp.
20. Strauss, L. C., C. Brovall, M. J. Fackler, J. F. Schwartz, J. H. Shaper, M. R. Loken, and C. I. Civin. 1986. Antigenic analysis of hematopoiesis. IV. The My-11 hematopoietic cell surface antigen is expressed by myelomonocytic and lymphoid, but not erythroid, progenitor cells. *Exp. Hematol.* 14:935-945.
21. Knapp, W., B. Dorken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. Kr. von dem Borne, editors. 1989. *Leukocyte Typing IV: White Cell Differentiation Antigens*. W. Knapp, editors. Oxford University Press, Oxford. 1156 pp.
22. Lanier, L. L., A. M. Le, C. I. Civin, M. R. Loken, and J. H. Phillips. 1986. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. *J. Immunol.* 136:4480-4486.
23. Zaentz, S. D., J. A. Luna, A. S. Baker, and S. B. Krantz. 1977. Detection of cytotoxic antibody to erythroblasts. *J. Lab. Clin. Med.* 89:851-860.
24. Sawada, K., S. B. Krantz, J. S. Kans, E. N. Dessypris, S. Sawyer, A. D. Glick, and C. I. Civin. 1987. Purification of human erythroid colony-forming units and demonstration of specific binding of erythropoietin. *J. Clin. Invest.* 80:357-366.
25. Sawada, K., S. B. Krantz, E. N. Dessypris, S. T. Koury, and S. T. Sawyer. 1989. Human colony-forming units-erythroid do not require accessory cells, but do require direct interaction with insulin-like growth factor-I and/or insulin for erythroid development. *J. Clin. Invest.* 83:1701-1709.
26. Sawada, K., S. B. Krantz, S. T. Sawyer, and C. I. Civin. 1988. Quantitation of specific binding of erythropoietin to human erythroid colony-forming cells. *J. Cell. Physiol.* 137:337-345.
27. Clark, B. J., and D. Housman. 1977. Characterization of an erythroid precursor cell of high proliferative capacity in normal human peripheral blood. *Proc. Natl. Acad. Sci. USA.* 74:1105-1109.
28. Reid, C. D. L. 1987. The significance of endogenous erythroid colonies (EEC) in haematological disorders. *Blood Rev.* 1:133-140.
29. Eaves, C. J., and A. C. Eaves. 1978. Erythropoietin (Ep) dose response curve for three classes of erythroid progenitors in normal human marrow and in patients with polycythemia vera. *Blood.* 52:1196-1210.
30. Lacombe, C., N. Casadevall, and B. Varet. 1980. Polycythemia vera: in vitro studies of circulating erythroid progenitors. *Br. J. Haematol.* 44:189-199.
31. Frazer, J. K., J. Nicholls, C. Coffey, F.-K. Lin, and M. V. Berridge. 1988. Down-modulation of high-affinity receptors for erythropoietin on murine erythroblasts by interleukin 3. *Exp. Hematol.* 16:769-773.
32. Robak, T. 1988. Antigenic characteristics of erythropoietin dependent and independent erythroid progenitors (BFU-E and CFU-E) in polycythemia vera and idiopathic myelofibrosis defined by monoclonal antibodies. *Arch. Immunol. Ther. Exp.* 36:733-747.
33. Eid, J., R. F. Ebert, M. S. Gesell, and J. L. Spivak. 1987. Intracellular growth factors in polycythemia vera and other myeloproliferative disorders. *Proc. Natl. Acad. Sci. USA.* 84:532-536.
34. Cashman, J. D., C. J. Eaves, and A. C. Eaves. 1988. Unregulated proliferation of primitive neoplastic progenitor cells in long-term polycythemia vera marrow cultures. *J. Clin. Invest.* 81:87-91.
35. Pluthero, F. G., M. Shreeve, D. Eskinazi, and A. A. Axelrad. 1989. An antagonist to IL-3 produced by mouse cells. *Blood.* 74:74a. (Abstr.)