

# Increased Erythropoietin-Receptor Expression on CD34-Positive Bone Marrow Cells From Patients With Chronic Myeloid Leukemia

By Albertus W. Wognum, Gerald Krystal, Connie J. Eaves, Allen C. Eaves, and Peter M. Lansdorp

Erythropoietin-receptor (EpR) expression on bone marrow cells from normal individuals and from patients with chronic myeloid leukemia (CML) was examined by multiparameter flow cytometry after stepwise amplified immunostaining with biotin-labeled Ep, streptavidin-conjugated R-phycoerythrin, and biotinylated monoclonal anti-R-phycoerythrin. This approach allowed the detection of EpR-positive cells in all bone marrow samples studied. Most of the EpR-positive cells in normal bone marrow were found to be CD45-dull, CD34-negative, transferrin-receptor-positive and glycophorin-A-intermediate to -positive. This phenotype is characteristic of relatively mature erythroid precursors, ie, colony-forming units-erythroid and erythroblasts recognizable by classic staining procedures. Approximately 5% of normal EpR-positive cells displayed an intermediate expression of CD45, suggesting that these represented precursors of the CD45-dull EpR-positive cells. Some EpR-positive cells in chronic myeloid leukemia (CML) bone marrow had a pheno-

type similar to the major EpR-positive phenotype in normal bone marrow, ie, CD34-negative and CD45-dull. However, there was a disproportionate increase in the relative number of EpR-positive/CD45-intermediate cells in CML bone marrow. Even more striking differences between normal individuals and CML patients were observed when EpR-expression on CD34-positive marrow cells was analyzed. Very few EpR-positive cells were found in the CD34-positive fraction of normal bone marrow, whereas a significant fraction of the CD34-positive marrow cells from five of five CML patients expressed readily detectable EpR. These findings suggest that control of EpR expression is perturbed in the neoplastic clone of cells present in patients with CML. This may be related to the inadequate output of mature red blood cells typical of CML patients and may also be part of a more generalized perturbation in expression and/or functional integrity of other growth factor receptors on CML cells.

© 1992 by The American Society of Hematology.

**C**HRONIC MYELOID leukemia (CML) is characterized by an increased production of multiple types of mature blood cells derived in each patient from a single Philadelphia (Ph<sup>1</sup>)-chromosome-positive hematopoietic stem cell.<sup>1-3</sup> The production of progenitor cells in each of the myeloid pathways is characteristically increased in CML and this increase is generally translated into substantially elevated numbers of mature granulocytes and monocytes, and sometimes platelets. Although all stages of erythroid cell development detectable by colony assays are, on average, increased in numbers to an extent similar to that seen for progenitors of the other myeloid lineages, the concentration of mature red blood cells (RBCs) in the circulation is usually not increased and, in fact, many CML patients present with anemia. On the other hand, some erythroid cells in patients with CML frequently show an abnormal ability to differentiate to maturity *in vitro* in the absence of exogenous erythropoietin (Ep).<sup>4</sup> Given these anomalies affecting the development of neoplastic erythroid cells in CML, it was of interest to evaluate expression of the Ep-receptor (EpR) in this disease.

Recently we developed a procedure for detecting EpR-

positive cells in murine spleen and bone marrow by flow cytometry using biotinylated Ep (b-Ep) and R-phycoerythrin-conjugated streptavidin (SA-RPE).<sup>5</sup> The sensitivity of this method was demonstrated by its ability to detect EpR-positive cells in the spleens of phenylhydrazine-treated mice that express, on average, only 400 to 500 receptor molecules per cell, and on a subpopulation of normal murine bone marrow cells.<sup>5</sup> In the present study we show that this immunostaining procedure can be adapted to detect EpR-positive cells in appropriately selected subpopulations of normal human bone marrow and that such staining shows differences in EpR expression between bone marrow cells from normal individuals and patients with CML.

## MATERIALS AND METHODS

**Monoclonal antibodies (MoAbs).** Mouse MoAb 8G12, specific for CD34, has been described previously.<sup>6</sup> MoAb 10F7MN, specific for glycophorin-A (GPA),<sup>7</sup> and OKT9,<sup>8</sup> specific for the transferrin-receptor (TrR), were obtained from the American Type Culture Collection (Bethesda, MD). MoAb 10H5.2, specific for R-phycoerythrin (RPE), was developed in our laboratory and is similar to anti-RPE antibodies described previously.<sup>9</sup> MoAb were purified from hybridoma culture supernatants using protein-A-Sepharose<sup>10</sup> and were labeled with fluorescein isothiocyanate (FITC; Sigma, St Louis, MO) using standard procedures. Biotin-labeling of anti-RPE MoAb 10H5.2 was achieved using N-hydroxysuccinimide-LC-biotin (Pierce, Rockford, IL). FITC-labeled MoAb against the common leukocyte antigen CD45 (HLe-1) was purchased from Becton-Dickinson (Mountain View, CA).

**Biotin labeling of Ep.** Biotin labeling of human recombinant Ep purified from the culture supernatants of baby hamster kidney cells expressing an Ep cDNA<sup>11</sup> was performed as described previously.<sup>5</sup>

**Cells.** Normal bone marrow samples were obtained with informed consent from healthy individuals undergoing bone marrow harvesting for use in allogeneic marrow transplant protocols. Bone marrow was also obtained with informed consent from five CML patients in the chronic phase of their disease, as leftover material from samples obtained for clinical purposes. Peripheral blood was similarly obtained from one of these patients. All CML patients were Ph<sup>1</sup>-chromosome positive. Low-density cells were isolated

From The Terry Fox Laboratory, B.C. Cancer Agency; and the Departments of Pathology, Medical Genetics, and Medicine, The University of British Columbia, Vancouver, British Columbia, Canada.

Submitted April 26, 1991; accepted September 26, 1991.

Supported by the National Cancer Institute of Canada (NCIC). G.K. and C.J.E. are Terry Fox Cancer Research Scientists of NCIC.

Address reprint requests to Peter M. Lansdorp, MD, PhD, Terry Fox Laboratory, 601 W 10th Ave, Vancouver, British Columbia, Canada V5Z 1L3.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

© 1992 by The American Society of Hematology.

0006-4971/92/7903-0003\$3.00/0

from the bone marrow of normal donors and CML patient nos. 2 and 5 using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). Nucleated cells were isolated from the bone marrow of patient nos. 1, 3, and 5 and from the peripheral blood of no. 5 by osmotic lysis of RBCs using 10 mmol/L potassium bicarbonate, 0.1 mmol/L EDTA, and 155 mmol/L ammonium chloride buffer, pH 7.4. Low-density cells and nucleated cells were washed in Hanks-HEPES balanced salt solution containing 2% (vol/vol) fetal calf serum (FCS) and 0.05% (wt/vol) sodium azide (HFN) and resuspended at  $1 \times 10^7$  cells/mL. In some experiments, cells were washed with Dulbecco's modified Eagle's medium containing 5% FCS instead of HFN and incubated for 1 hour at 37°C to internalize cell-surface bound Ep. In other experiments, cell-surface bound Ep was removed by incubating cells for 5 minutes on ice in 0.05 mmol/L glycine-HCl, 0.15 mol/L NaCl, pH 3.5. After treatment, cells were washed twice with HFN and used for amplified immunofluorescence staining with b-Ep, SA-RPE, and biotinylated anti-RPE. Identical results were obtained with acid-treated, preincubated, and untreated cells, indicating that receptor-bound endogenous Ep did not interfere with EpR detection.

**Staining of EpR, stepwise amplification of the fluorescence signal, and counterstaining with FITC-labeled antibodies.** To stain cells expressing EpR,  $1 \times 10^7$  cells in 1 mL HFN were incubated for 1 hour at 37°C with b-Ep (1 nmol/L) or, as controls, with buffer only or with b-Ep plus 200 nmol/L unlabeled Ep to compete for specific binding. Incubation was performed for 1 hour at 37°C to achieve optimal binding.<sup>12-14</sup> Control experiments with normal and CML bone marrow were also performed for 18 hours at 4°C with identical results. After incubation, cells were washed with Hanks'-HEPES balanced salt, containing 0.1% (wt/vol) bovine serum albumin and incubated with 0.2% (vol/vol) formaldehyde in the same buffer for 50 minutes at 4°C. After fixation, the cells were washed again with HFN and incubated with 1 µg/mL of SA-RPE (Molecular Probes, Eugene, OR) for 30 minutes at 4°C. The fluorescence intensity was then amplified by three incubation cycles with b-anti-RPE, followed by SA-RPE. Each incubation lasted for 30 minutes at 4°C and was followed by a single wash with at least 4 mL of ice-cold HFN. After amplification, aliquots of stained cells were counterstained for 30 minutes at 4°C with FITC-labeled MoAb against cell-surface antigens, washed again, and analyzed by flow cytometry.

**Flow cytometry.** Quantitative fluorescence analysis was performed using a FACScan flow cytometer (Becton-Dickinson, San Jose, CA). For two-color experiments, spillover of green fluorescence into the red fluorescence detector was electronically compensated to background levels using cells stained with the respective FITC-conjugated antibodies only. Twenty thousand events were collected when cells were analyzed ungated. In some experiments,

a gate was set for cells with low right-angle light scatter properties to exclude granulocytes from the analysis. In these experiments, 10,000 events were collected. For analysis of cells with high expression of CD34, a multiparameter gate was set using high forward- and low right-angle light scatter, and high green fluorescence signals to include all hematopoietic progenitor populations, but to exclude CD34-negative cells, granulocytes, and dead cells that were stained nonspecifically by the anti-CD34-FITC antibody. Cells were considered CD34-positive when their fluorescence intensity was between  $10^2$  and  $10^3$  arbitrary fluorescence units. Flow cytometry data were analyzed using Consort 30 and Lysis data management systems (Becton-Dickinson). For analysis of EpR expression on CD34-positive cells in some normal bone marrow samples, 50,000 events were collected ungated. Fluorescence profiles of EpR-expression on CD34-positive cell populations were then obtained by reanalysis of data in a multiparameter gate for cells with low right-angle light-scatter, intermediate to high-forward light scatter, and high green fluorescence signals.

**Assay for clonogenic cells.** Colony assays for erythroid (CFU-E, BFU-E), granulocyte/macrophage (CFU-GM), and multipotent (CFU-GEMM) progenitors in normal bone marrow and CML blood and bone marrow samples were performed in methylcellulose cultures as previously described.<sup>15</sup>

**Patient characteristics.** As indicated in Table 1, white blood cell (WBC) counts of all five CML patients studied were elevated at the time when marrow samples were obtained, whereas hematocrits and hemoglobin concentrations ranged from below average to normal values. Colony assays indicated that the number of some erythroid progenitors (CFU-E and BFU-E) relative to all nucleated bone marrow cells was elevated in two patients but within or below the normal range in the others. Similar heterogeneity in relative CFU-GM numbers in the marrow of the same patients was also seen, as documented previously for CML patients in general.<sup>2</sup> Values for erythroid progenitors in the peripheral blood sample obtained from patient no. 5 were within the normal range, whereas CFU-GM and CFU-GEMM were below detectable levels (data not shown).

## RESULTS

**Expression of EpR on normal human bone marrow cells.** To analyze EpR expression during erythroid differentiation, low-density normal human bone marrow cells were stained with b-Ep and SA-RPE and counterstained with an FITC-conjugated MoAb against the cell-surface antigen CD45. Although CD45 is absent from mature RBCs and only weakly expressed on erythroid progenitor cells, it is present at intermediate to high levels on monocytes, granu-

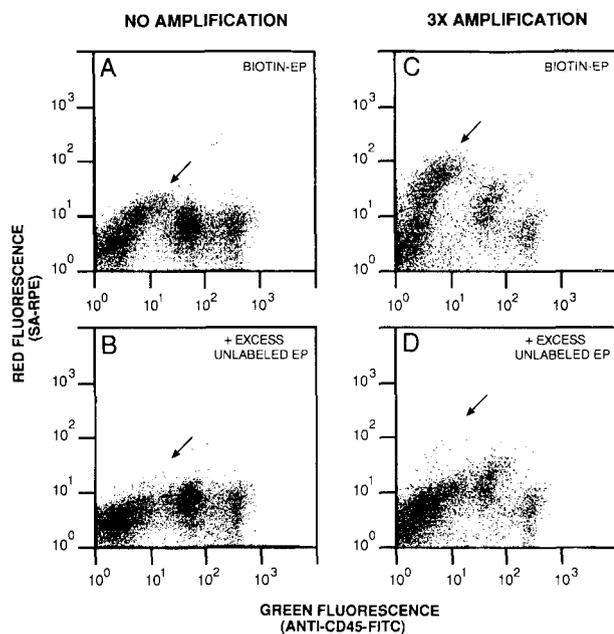
**Table 1. Blood Parameters and Bone Marrow Clonogenic Progenitor Numbers of the Five Patients With Chronic Myeloid Leukemia Used in This Study**

Patient	Hemoglobin (g/L)	Hematocrit	WBC Count ( $\times 10^9/L$ )	Progenitors per $2 \times 10^5$ Nucleated Bone Marrow Cells			
				CFU-E	BFU-E	CFU-GM	CFU-GEMM
1	149	0.44	20.2	216	37	38	0
2	104	0.31	47.5	344	224	272	8
3	124	0.36	23.8	0	2	14	0
4	129	0.39	14.0	392	151	21	0
5	84	NA	79.0	136	57	14	0
Normal*							
Mean:	148	0.43	7.8	67	56	62	1
Range ( $\pm 2SD$ )	132-164	0.36-0.50	4.4-11.3	15-305	15-208	19-201	0.04-8

\*Normal blood parameters were derived from ref 30; normal progenitor numbers were derived from ref 31.

Abbreviation: NA, not available.

locytes, and lymphocytes.<sup>16,17</sup> CD45 thus allows EpR analysis on erythroid cells without interference by nonspecific fluorescence of nonerythroid cells. Dot plots for green fluorescence (CD45 expression) versus red fluorescence (EpR expression) are shown in Fig 1A for cells from a representative normal marrow stained with b-Ep and in Fig 1B for cells from the same donor that, as a control, were stained with b-Ep plus excess unlabeled Ep. A small fraction of cells that were weakly CD45-positive (ie, CD45-dull) showed elevated red fluorescence as compared with control cells, indicating that this subpopulation consisted of EpR-positive cells. However, the specific increase in fluorescence intensity above background fluorescence was very low and did not exceed the autofluorescence in the red channel of CD45-intermediate cells. For this reason, an amplification procedure was developed to enhance the specific fluorescence intensity of the EpR-positive cells. In this procedure, the number of fluorescence molecules per cell after staining with b-Ep and SA-RPE was increased by performing sequential incubations with biotin-labeled MoAb against RPE and SA-RPE. After each incubation cycle with these two reagents, the fluorescence intensity of the EpR-positive cells increased twofold to threefold, resulting in an ~10-fold increase in fluorescence intensity for this subpopulation after three cycles (Fig 1C), while the nonspecific fluorescence of the CD45-dull and CD45-negative cell fractions remained essentially constant (Fig 1D). Background fluorescence for the CD45-positive cell fraction was also



**Fig 1.** Effect of amplification on the fluorescence intensity of low-density human bone marrow cells stained with b-Ep (A and C) or with b-Ep plus excess unlabeled Ep (B and D). The cells were analyzed after staining with SA-RPE (A and B) or after staining with SA-RPE, followed by three amplification cycles with b-anti-RPE and SA-RPE (C and D). Before analysis the cells were counterstained with FITC-labeled anti-CD45. The red fluorescence of SA-RPE of individual cells is plotted against the green fluorescence obtained with anti-CD45-FITC. Arrows indicate the position of cells that express detectable EpR.

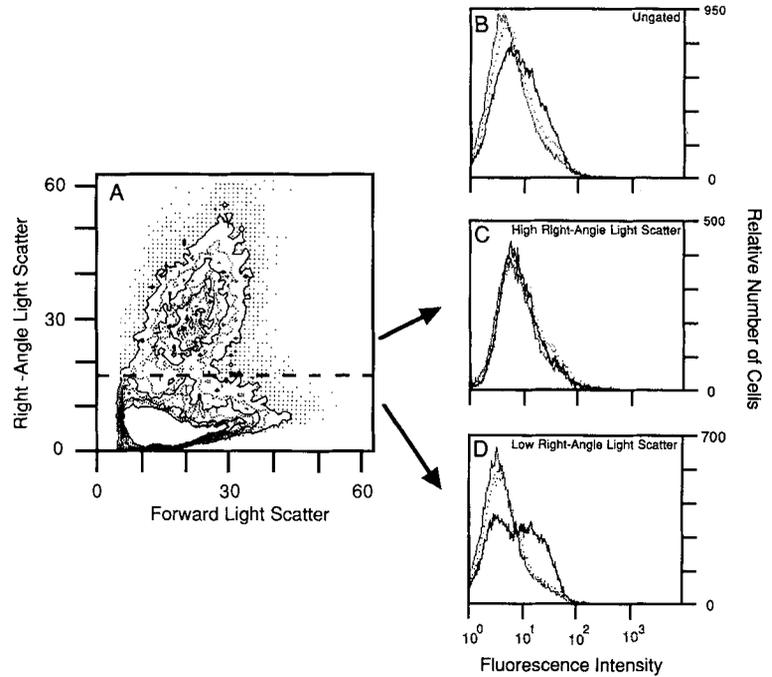
unaffected by the amplification procedure and only in the CD45-intermediate cell fraction could an approximately twofold increase of nonspecific red fluorescence be observed, possibly resulting from the interaction of b-anti-RPE MoAbs with Fc-receptor-positive cells in this cell fraction. Additional amplification cycles often led to further increases in background fluorescence of the CD45-intermediate cells and, for this reason, amplification was limited to three cycles in most experiments. As shown in Fig 1, C and D, even after amplification EpR-positive cells were not readily detectable in the cell populations that expressed intermediate and high levels of CD45, indicating that EpR expression does not occur or reach detectable levels on granulocytes, monocytes, and lymphocytes. A large fraction of CD45-negative cells and virtually all CD45-dull cells were EpR-positive, consistent with exclusive EpR expression on cells of the erythroid lineage.<sup>16,17</sup> EpR expression appeared to be strongest on the CD45-dull cell population, declined gradually with further decreases in CD45 expression, and was absent from a large fraction of the cells that were CD45-negative (Fig 1, C and D).

Analysis of cells in electronic windows for high and low right-angle light scatter, as indicated by the areas divided by the dashed line in Fig 2A, confirmed that EpR expression was not detectable on granulocytes and their precursors, which are characterized by high right-angle light scatter properties (Fig 2C). EpR positive cells were observed only amongst those cells with low right-angle scatter (Fig 2D). For comparison, the fluorescence of all cells (ungated) is shown in Fig 2B. Further analysis indicated that EpR expression occurs on cells with low forward light-scatter (ie, small cells), as well as on cells with high forward light scatter (ie, large cells) (data not shown).

The EpR-positive cells in normal bone marrow were further characterized in double-staining experiments with FITC-labeled MoAbs against the TrR and GPA, two other markers for erythroid cells. As illustrated in Fig 3, A and B, the large majority of EpR-positive cells expressed high levels of TrR and EpR-positive cells were not readily detected in TrR-negative and intermediate cell populations. Most EpR-positive cells (~80%) also expressed high levels of GPA, while a small fraction of EpR-positive cells (~20%) expressed low to intermediate levels of this erythroid cell antigen (ie, fluorescence intensity between 40 and 200 arbitrary units) (Fig 3, C and 3D).

**Expression of EpR on CML bone marrow cells.** Preliminary analysis of EpR expression on CML bone marrow cells showed a similar pattern of EpR expression as observed on normal cells, ie, EpR were detectable only on CML bone marrow cells with low right-angle light scatter properties and not on the ~90% of cells that displayed high right-angle light scatter properties (data not shown). For this reason, all subsequent analyses of EpR-expression was performed on cells with low right-angle light scatter properties only. To compare EpR expression on normal and CML bone marrow, cells were stained with b-Ep and counterstained with anti-CD45 as described previously. A dot plot of red fluorescence (staining with b-Ep and SA-RPE) versus green fluorescence (for CD45 expression) is shown in Fig 4 (C and D) with results for a normal bone marrow

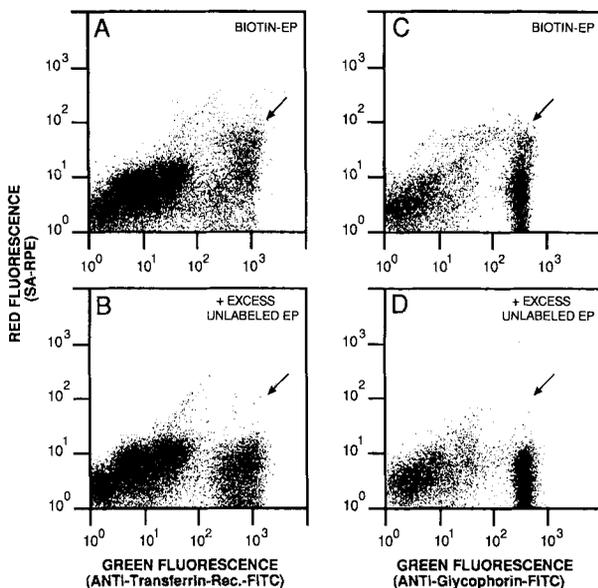
**Fig 2.** EpR expression on subpopulations of human bone marrow cells. Low-density human bone marrow cells stained with b-Ep or, as controls, with buffer only or with b-Ep plus excess unlabeled Ep were stained with SA-RPE and analyzed after three amplification cycles with b-anti-RPE and SA-RPE. (A) Contourplot of forward-versus-right angle light scatter properties of stained cells. Fluorescence data were reanalyzed in two windows for cells with low and high right-angle light scatter properties, respectively, as indicated by the areas above and below the dashed line. (Right) Fluorescence profiles for all cells (B) and for subpopulations with high (C) and low (D) right-angle light scatter properties. Cells stained with b-Ep (—); cells stained with b-Ep plus excess unlabeled Ep (· · ·); unstained cells that were put through the amplification procedure (···).



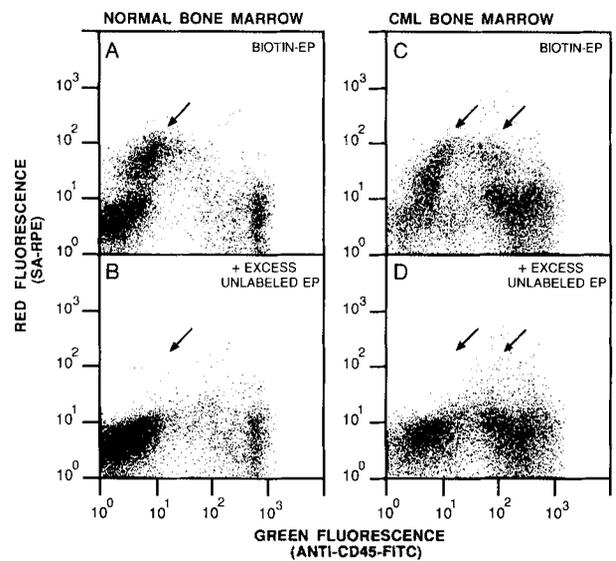
sample shown for comparison in Fig 4 (A and B). As illustrated, EpR expression on cells in the CD45-negative and -dull fraction of CML bone marrows was very similar to EpR expression on cells in this fraction of normal bone marrow. However, note that EpR-positive cells constituted a readily detectable proportion of the CD45 intermediate cells in all three CML marrows studied in this way (of which one example is shown in Fig 4, C and D) in contrast to

normal bone marrow. As for normal marrow, EpR-positive cells were not detectable in the CD45-bright population of these CML marrow samples.

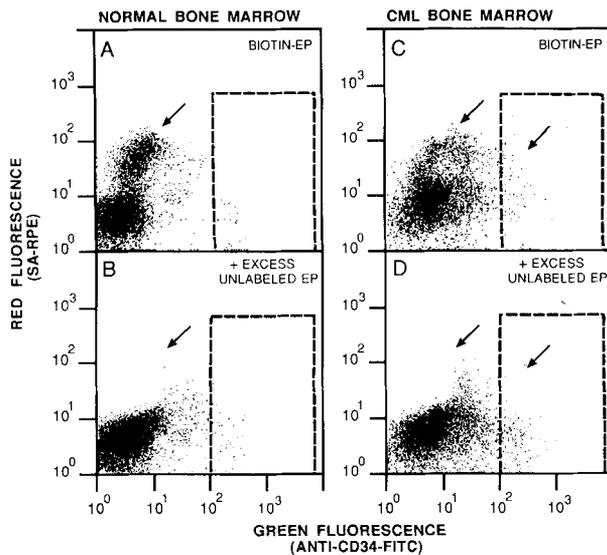
*Comparison of EpR and CD34 expression on normal and CML bone marrow.* The majority of EpR-positive cells in both normal and CML bone marrow were negative for CD34 (Fig 5), a cell-surface antigen that is expressed on primitive hematopoietic cells.<sup>18,19</sup> However, low numbers of EpR-positive cells were detectable in the CD34-intermedi-



**Fig 3.** Correlation between EpR and expression of TrR and of GPA. Normal bone marrow cells were stained as described in the legend to Fig 1, except that the cells were counterstained with FITC-anti-TrR (A and B) or anti-GPA (C and D). Arrows indicate the position of cells that express detectable EpR. Note that the majority of cells that express EpR also express GPA but that some EpR-positive cells express low to intermediate levels of GPA (C and D).



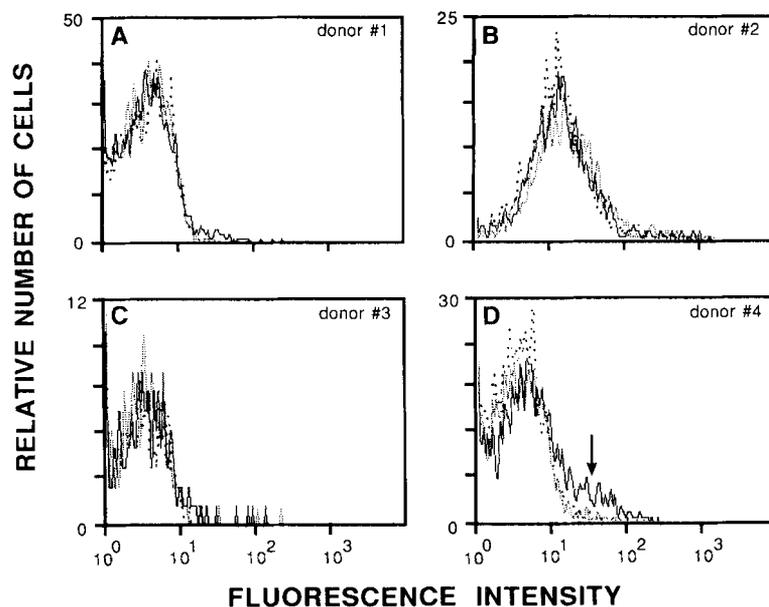
**Fig 4.** Correlation between expression of EpR and CD45 on bone marrow cells from a normal donor (A and B) and a CML patient (C and D). Low-density bone marrow cells stained with b-Ep alone (A and C) or, as control, in the presence of excess unlabeled Ep (B and D) were analyzed after staining with SA-RPE and three amplification cycles with anti-RPE and counterstaining with FITC-labeled anti-CD45. Arrows indicate the position of cells that express detectable EpR.



**Fig 5.** Correlation between expression of EpR and CD34 on normal (A and B) and CML (C and D) bone marrow cells. Cells were stained as described in the legend of Fig 4, except that they were counterstained with FITC-anti-CD34. Boxed areas indicate the window for high green fluorescence selected to analyze EpR expression on CD34-positive cells as presented in Fig 6 for normal bone marrow and in Fig 7 for CML bone marrow. Arrows indicate the position of cells that express detectable EpR.

ate subpopulation (as defined by a fluorescence intensity between 20 and 100 arbitrary units) of normal as well as CML bone marrows, despite the presence of some nonspecifically stained cells in this subpopulation, particularly in CML marrow samples (Fig 5, C and D).

Because of the low frequency of cells with high CD34-expression in normal and CML marrows (2.2% and 3.8% respectively, in Fig 5), analysis of EpR expression on these cells was performed by collecting a large number of events (1,000 or more) in a window for CD34-positive cells, as indicated by the boxed area in each dot plot shown in Fig 5.



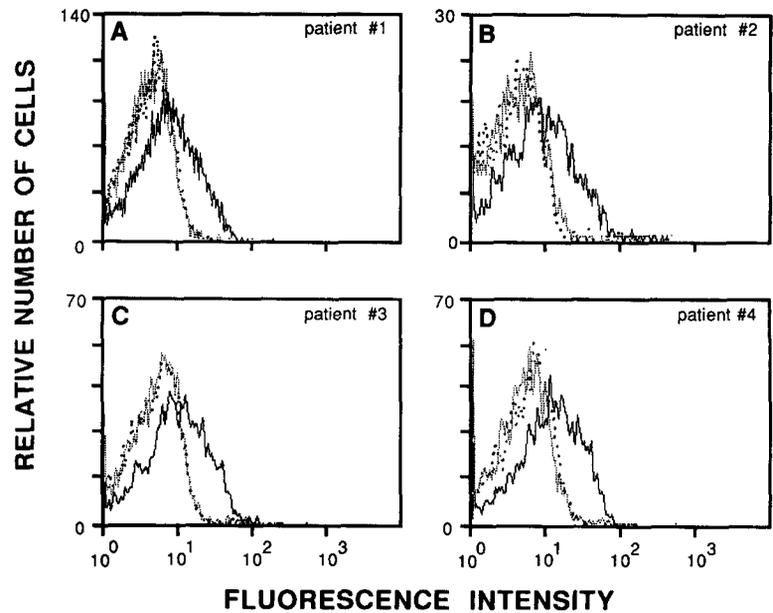
**Fig 6.** Fluorescence profiles for EpR expression on CD34-positive cells in normal bone marrow. Cells stained for EpR expression as described in the legend to Fig 1 were analyzed in a window for CD34-positive cells as indicated by the boxed area in Fig 5. Histograms are shown for the bone marrow presented in Fig 5, A and B, (A) and for three additional normal bone marrow samples (B through D). The results shown in (A) and (D) represent 2,000 and 1,000 events, respectively. The results shown in (B) and (C) were obtained by collecting 50,000 ungated events and reanalyzing the data in an electronic window for CD34-positive cells, as described in Materials and Methods. The results presented in (B) and (C) represent ~900 and ~300 events, respectively. Cells stained with b-Ep (—); cells stained with b-Ep plus excess unlabeled Ep (· · ·); unstained cells that were put through the amplification procedure (---). Arrow in (D) indicates the small subpopulation of CD34-positive cells of this donor that expressed detectable EpR.

These results are shown in Fig 6 for four normal bone marrow samples and in Figs 7 and 8A for five CML marrows. CD34-positive cells from three of four normal bone marrow donors did not express EpR at a detectable level, although in the fourth normal sample (Fig 6D) a low number of EpR-positive cells were evident. In contrast, a large fraction of the CD34-positive cells in all five CML marrows studied displayed detectable EpR (Figs 7 and 8A). The EpR-positive cells represented at least 31% to 43% of the CD34-positive cells in the respective CML marrows. These EpR-positive cells also displayed high forward and low to intermediate orthogonal light scatter properties, consistent with a blastlike phenotype (data not shown).

In most CML patients, elevated numbers of hematopoietic progenitors can be detected in peripheral blood<sup>2</sup> and these cells express the CD34 antigen.<sup>20</sup> For this reason it is of interest to know whether EpR-positive cells are also detectable in the peripheral blood of CML patients and, in particular, whether these are contained in the CD34-positive cell fraction. Analysis of light-density nucleated peripheral blood cells from CML patient no. 5 showed that circulating EpR-positive cells were not detectable (data not shown), nor were they detectable in the CD34-positive subpopulation of blood cells from this patient (Fig 8B), even though such cells were readily detectable in a sample of bone marrow taken from this patient at the same time (Fig 7A).

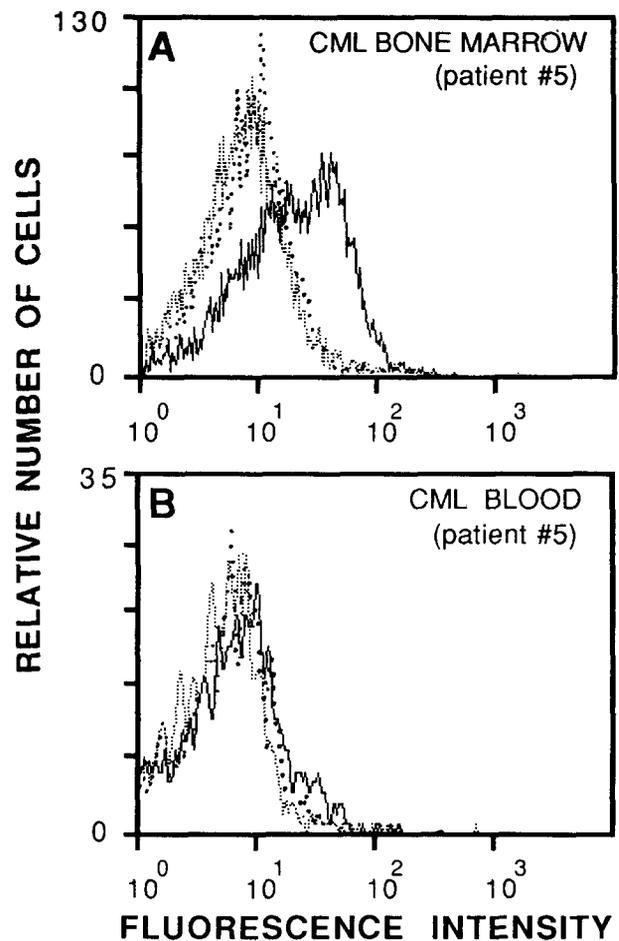
## DISCUSSION

In this study we have used biologically active biotin-labeled Ep, in combination with PE-conjugated streptavidin and multiparameter flow cytometry, to analyze EpR expression on hematopoietic cells from normal individuals and from patients with CML. The ability to detect EpR expression by flow cytometry makes it possible to compare this EpR characteristic simultaneously with other properties of the same cells, including forward and right-angle light scattering characteristics and the expression of other



**Fig 7.** Fluorescence profiles for EpR expression on CD34-positive cells in CML bone marrow. Cells were stained and analyzed as described in Fig 5. Histograms are shown for the CML cells presented in Fig 5, C and D, and for three additional bone marrow samples that were analyzed similarly (B, C, and D). The results shown represent 5,000, 1,100, 2,500, and 2,500 events, respectively.

cell-surface antigens. EpR-positive cells were consistently and readily detected in both CML and normal bone marrow cell populations after appropriate gating for low right-angle light scatter. The absence of detectable EpR on cells with high right-angle light scatter is consistent with the view that neither normal nor CML granulocytes express detectable levels of EpR. In normal bone marrow, most EpR-positive cells expressed low levels of CD45, no detectable CD34, and high levels of TrR and GPA, while a small fraction of strongly EpR-positive cells could also be identified in the GPA-intermediate cell fraction. Loken et al<sup>16</sup> have previously demonstrated that the CD45<sup>-dull</sup>, TrR-positive, GPA-positive cell fraction predominantly contains mature erythroid cells, ie, morphologically recognizable erythroblasts, whereas more immature erythroid cells (ie, CFU-E) have an intermediate expression of CD45 and have low expression of GPA. Therefore, our results suggest that most Ep-receptor-positive cells in normal bone marrow represent relatively late erythroid cells. This was confirmed by studies indicating the almost exclusive presence of basophilic and polychromatophilic erythroblasts in cell populations sorted on the basis of detectable EpR expression (data not shown). Our results also suggest that, during normal erythropoiesis, EpR expression gradually increases during early erythroid differentiation, in parallel with the loss of CD45 and CD34 and the increased expression of various erythroid-associated genes. After reaching a maximum on a TrR-positive, GPA low-to-intermediate cell stage (which possibly corresponds to the CFU-E or early erythroblast stage), EpR expression remains high when these cells mature to morphologically recognizable erythroblasts and increase their GPA expression, followed by a gradual decrease and ultimate disappearance of EpR expression on terminal maturation into enucleated reticulocytes. The observation in our study that EpR are absent from TrR-negative cells suggests that the ultimate disappearance of EpR immediately precedes or accompanies the shedding of TrR, which occurs during the maturation of



**Fig 8.** Comparison of EpR expression on CD34-positive cells in bone marrow (A) and peripheral blood (B) from a CML patient. Cells were stained as described in Fig 1. Results shown in (A) and (B) represent 5,000 and 1,200 events, respectively.

reticulocytes.<sup>21</sup> The pattern of EpR expression during terminal erythroid differentiation as elucidated in this study is in agreement with previous results involving <sup>125</sup>I-labeled Ep binding to normal human and murine erythroid cells and to cell lines "corresponding" to different stages of erythroid differentiation.<sup>12,13,22-24</sup> However, several researchers observed a more rapid evolution of EpR expression during *in vitro* differentiation of purified erythroid cells, with peak expression reached at the CFU-E stage followed by a rapid decrease thereafter, whereas our results indicate that EpR may also be strongly expressed at relatively high levels on erythroblasts. A possible explanation for this apparent discrepancy is that the incubation of erythroid cells with high Ep concentrations that was used in those studies to stimulate CFU-E differentiation may have led to rapid down-modulation of EpRs as has previously been demonstrated in other studies,<sup>25-27</sup> whereas the much lower, physiologic Ep levels to which erythroid cells are exposed in normal bone marrow could result in a slower eclipse of EpR expression during maturation *in vivo*. Alternatively, the observed differences could be related to the different experimental approaches used to analyze EpR expression.

Our results indicate that some of the EpR-positive cells in CML bone marrow are phenotypically similar to the majority of normal EpR-positive erythroid precursors, ie, they are CD45-dull and CD34-negative. However, unlike normal marrow, where a very small proportion of EpR-positive cells are CD45-intermediate and likely represent slightly less mature erythroid cells than the more abundant CD45-dull, EpR-positive cells, a large proportion of the EpR-positive cells in CML marrow are consistently found in the CD45-intermediate fraction, suggesting abnormal amplification of cells with this particular phenotype in CML.

Although most EpR-positive cells in normal and CML bone marrow were found in the CD34-negative cell fraction, evidence for alterations in EpR expression was also obtained when the small subsets of CD34-positive cells in normal and CML bone marrow samples were compared. It has been shown that the expression of CD34 on hematopoietic progenitors in CML bone marrow is very similar to its expression on normal progenitor cells, ie, highest expression occurs on the most primitive progenitors and this decreases gradually with differentiation.<sup>20</sup> As demonstrated in this study, only occasionally can EpR-positive cells be detected in the CD34-positive fraction in normal bone marrow, suggesting that most primitive hematopoietic progenitors are EpR-negative, as has recently also been reported for purified BFU-E derived from peripheral blood.<sup>28</sup> The finding that EpR are consistently expressed on a large subpopulation of CD34-positive cells in CML marrow samples was therefore unexpected. Such a result could indicate premature activation of the EpR gene, or gross

amplification of a CD34-positive, EpR-positive compartment of normal erythroid cell development. Alternatively, Ep binding kinetics or EpR processing (eg, by internalization during labeling) may be different between CD34-positive cells from normal and CML bone marrow. This latter explanation seems unlikely because azide was included during labeling to prevent internalization and because EpR were readily detectable on CD34-negative bone marrow cells from normal individuals. In addition, b-Ep binding to CD34-positive normal and CML cells was performed under conditions that have previously been demonstrated to be sufficient for achieving equilibrium binding of Ep to human and murine erythroid cells, ie, 18 hours at 4°C or 1 hour at 37°C, both in the presence of sodium azide to prevent internalization.<sup>12-14</sup> Additional characterization of CML bone marrow cells, eg, by analysis of EpR expression on purified subpopulations of CD34-positive cells, will be required to elucidate the phenotype and functional properties of the EpR-positive/CD34-positive CML cells. Unfortunately, the stringent incubation conditions required to detect the low levels of EpR expression on hematopoietic cells has thus far prevented the sorting of viable cells on basis of Ep-receptor expression for subsequent plating in colony assays.

In a study similar to ours, Visani et al<sup>29</sup> have demonstrated that primitive progenitor cells from peripheral blood and bone marrow from CML patients express the  $\alpha$ -chain of the interleukin-2-receptor (IL-2R), whereas normal bone marrow progenitor cells were IL-2R-negative. Although IL-2R expression was detectable only on cultured CML cells and not on freshly isolated cells, these results suggest that the EpR may not be the only hematopoietin receptor whose expression is deregulated on CML cells. The ability to use labeled ligand for analysis of receptor expression on phenotypically characterized cells within heterogeneous cell populations offers a new and powerful approach to evaluate the expression of receptors for other cytokines that are involved in the development of normal hematopoietic cells. In addition to providing insight into the distribution and evolution of growth factor receptors during normal hematopoiesis, such studies may lead to a better understanding of the generalized uncontrolled proliferation that is characteristic of primitive Ph<sup>1</sup>-positive cells.<sup>2</sup>

#### ACKNOWLEDGMENT

We are grateful to Cam Smith, Visia Dragowska, Sara Abraham, and Gayle Thornbury for excellent technical assistance. We also thank the technical staff of the Stem Cell Assay Service of the British Columbia Cancer Agency for processing the clinical samples used in this study and for performing the clonogenic assays. In addition, we thank Drs A. Schussele, T.G. Sparling, W. Boldt, and J.F. Dufton for making available patient material and other clinical information.

#### REFERENCES

1. Whang J, Frei E, Tjio JH, Carbone PP, Bracher G: The distribution of the Philadelphia chromosome in patients with chronic myelogenous leukemia. *Blood* 22:664, 1963
2. Eaves AC, Eaves CJ: Cell culture studies in CML, in Goldman JM (ed): *Bailliere's Clinical Haematology*, vol 1, no 4: Chronic Myeloid Leukaemia. London, UK, Bailliere Tindall, 1987, p 931
3. Raskind WH, Fialkow PJ: The use of cell markers in the study of human hematopoietic neoplasia. *Adv Cancer Res* 49:127, 1987
4. Eaves AC, Eaves CJ: Abnormalities in the erythroid compart-

ments in patients with chronic myelogenous leukemia. *Exp Hematol* 7:65, 1979 (suppl 5)

5. Wognum AW, Lansdorp PM, Humphries RK, Krystal G: Detection and isolation of the erythropoietin receptor using biotinylated erythropoietin. *Blood* 76:696, 1990

6. Lansdorp PM, Dougherty GJ, Humphries RK: CD34 epitopes, in Knapp W, Dorken B, Wilks WR, Rieber RE, Stein H, von dem Borne AEGKr (eds): *Leukocyte Typing IV*. Oxford, UK, Oxford University, 1989, p 826

7. Bigbee WL, Vanderlaan M, Fong SSN, Jensen RH: Monoclonal antibodies specific for the M and N forms of human glycoporphin. *Mol Immunol* 20:1353, 1983

8. Schneider C, Sutherland R, Newman R, Greaves M: Structural features of the cell surface receptor for transferrin that is recognized by monoclonal OKT9. *J Biol Chem* 256:8516, 1982

9. Wognum AW, Thomas TE, Lansdorp PM: Use of tetrameric antibody complexes to stain cells for flow cytometry. *Cytometry* 8:366, 1987

10. Ey PL, Prowse SJ, Jenkin CR: Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein-A-Sepharose. *Immunochemistry* 15:429, 1978

11. Powell JS, Berkner KL, Labo RV, Adamson JW: Human erythropoietin gene: High level expression in stably transfected mammalian cell lines and chromosome localization. *Proc Natl Acad Sci USA* 83:6465, 1986

12. Fraser JK, Lin F-K, Berridge MV: Expression of high-affinity receptors for erythropoietin on human bone marrow cells and on the human erythroleukemic cell line, HEL. *Exp Hematol* 16:836, 1988

13. Landschulz KT, Noyes AN, Rogers O, Boyer SH: Erythropoietin receptors on murine erythroid colony-forming units: Natural history. *Blood* 73:1476, 1989

14. Hoshino S, Teramura M, Takahashi M, Motoji T, Oshimi K, Ueda M, Mizoguchi H: Expression and characterization of erythropoietin receptors on normal bone marrow cells. *Blood* 7:156, 1989

15. Coulombel L, Eaves AC, Eaves CJ: Enzymatic treatment of long-term human marrow cultures reveals the preferential location of primitive hemopoietic progenitors in the adherent layer. *Blood* 62:291, 1983

16. Loken MR, Shah VO, Dattillo KL, Civin CI: Flow cytometric analysis of human bone marrow: 1. Normal erythroid development. *Blood* 59:255, 1987

17. Shah VO, Civin CI, Loken MR: Flow cytometric analysis of human bone marrow, IV. Differential quantitative expression of T200 common leukocyte antigen during normal hemopoiesis. *J Immunol* 140:1861, 1988

18. Strauss L, Rowley SD, LaRussa VF, Sharkis SJ, Stuart RK, Civin CI: Antigenic analysis of hemopoiesis. V. Characterization of

My-10 antigen expression by normal lymphohematopoietic subpopulations of CD34<sup>+</sup> hematopoietic cells from human bone marrow. *Exp Hematol* 14:878, 1986

19. Sutherland HJ, Eaves CJ, Eaves AC, Dragowska W, Lansdorp PM: Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. *Blood* 74:1563, 1989

20. Katz FE, Watt SE, Martin H, Lam G, Capellaro D, Goldman JM, Greaves MF: Coordinate expression of B1.3C5 and HLA-DR antigens on haemopoietic progenitors from chronic myeloid leukemia. *Leuk Res* 10:961, 1986

21. Pan P-T, Johnstone RM: Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: Selective externalization of the receptor. *Cell* 33:967, 1983

22. Sawada K, Krantz SB, Sawyer ST, Civin CI: Quantitation of specific binding of erythropoietin to human erythroid colony-forming cells. *J Cell Physiol* 137:337, 1988

23. Broudy VC, Nakamoto B, Lin N, Papayannopoulou T: Dynamics of erythropoietin receptor expression on erythropoietin-responsive murine cell lines. *Blood* 75:1622, 1990

24. Broudy VC, Lin N, Brice M, Nakamoto B, Papayannopoulou T: Erythropoietin receptor characteristics on primary human erythroid cells. *Blood* 77:2583, 1991

25. Sawyer ST, Krantz SB, Goldwasser E: Binding and receptor-mediated endocytosis of erythropoietin in Friend virus-infected erythroid cells. *J Biol Chem* 262:5554, 1987

26. Fukamachi H, Tojo A, Saito T, Kitamura T, Nakata M, Urabe A, Takaku F: Internalization of radioiodinated erythropoietin and the ligand induced modulation of its receptor on murine erythroleukemia cells. *Int J Cell Cloning* 5:209, 1987

27. Fraser JK, Nicholls J, Coffey G, Lin F-K, Berridge MV: Down-modulation of high affinity receptors for erythropoietin on murine erythroblasts by interleukin-3. *Exp Hematol* 16:769, 1988

28. Sawada K, Krantz SB, Dai C-H, Koury ST, Glick AD, Civin CI: Purification of human blood burst-forming units-erythroid and demonstration of the evolution of erythropoietin-receptors. *J Cell Physiol* 142:219, 1990

29. Visani G, Delwel R, Touw I, Bot F, Lowenberg B: Membrane receptors for interleukin 2 on hematopoietic precursors in chronic myeloid leukemia. *Blood* 69:1182, 1987

30. Williams WJ, Nelson DA, Morris MW: Examination of the blood, in Williams WJ, Beutler E, Erslev AJ, Lichman MA (eds): *Haematology* (ed 4). New York, NY, McGraw-Hill, 1990, p 9

31. Sutherland HJ, Eaves AC, Eaves CJ: Quantitative assays for human hematopoietic progenitor cells, in Gee AP (ed): *Bone Marrow Processing and Purging. A Practical Guide*. Boca Raton, FL, CRC, 1991, p 155