We have analyzed the reactivation of fetal hemoglobin (HbF) synthesis under rigorous in vitro conditions, i.e., in mature erythroblasts generated by erythroid burst-forming units (BFU-E) stringently purified from normal peripheral blood and grown in fetal calf serum (FCS)-free semisolid or liquid phase culture. In clonogenic dishes, graded amounts of c-kit ligand (KL) were added together with saturating levels of erythropoietin (Ep) and variable amounts of interleukin-3 and granulocyte-macrophage colony stimulating factor (IL-3/GM-CSF), i.e., high or low level, or no IL-3/GM-CSF addition. In all conditions, KL induced a sharp, dose-dependent increase in the percentage of F cells and HbF content from nearly normal levels (<10% and <2.5%, respectively, at 0.1 and 1 ng/mL) up to 40% to 50% and 10% to 15% at 100 to 200 ng/mL. This increase was not associated with significant differences of burst number or stage of maturation at the time of analysis (as evaluated on the basis of percent mature erythroblasts and Hb content per cell). However, the KL-induced reactivation of HbF synthesis was strictly and directly correlated with a sharp increase of colony size, i.e., cell number per burst. Addition of large amounts of IL-3 and GM-CSF (10 to 100 U and 1 to 10 ng/mL, respectively) significantly potentiated the KL-induced reactivation of HbF, as compared with low levels (0.1 U and 0.01 to 0.1 ng) or no addition of these growth factors: this increase was highly significant at low KL doses (i.e., 1 to 10 ng/mL). Single-burst analysis showed that the KL-induced HbF reactivation occurs homogeneously in the erythroid colonies within each of these culture conditions. We have analyzed the effect of KL in liquid phase BFU-E culture treated with the IL-3/GM-CSF/Ep combination at sequential times until terminal erythroid maturation: KL causes a sharp increase in the percentage of F cells and HbF content in all stages of maturation, whereas the IL-3/GM-CSF potentiate this effect at low KL levels. The KL-induced HbF reactivation is seemingly related to an enhanced proliferation of erythroid progenitors in the erythropoietic differentiation pathway.

IN THE PERINATAL PERIOD, fetal hemoglobin (HbF; α2γ2) is subtotally replaced by adult hemoglobin (HbA; α2β2) and some HbA2 (α2δ2). Thereafter, HbF (<1% of total Hb) is restricted to F cells, which represent less than 6% of red blood cells (RBCs). In a variety of postnatal conditions, particularly in rapid marrow regeneration (stress erythropoiesis), HbF synthesis may be reactivated up to 10% to 20% relative γ-globin content. A similar reactivation has been observed in vitro: in fetal calf serum-supplemented (FCS+) semisolid cultures treated with erythropoietin (Ep), nonpurified erythroid burst-forming units (BFU-E) from normal adults generate erythroblast colonies (bursts) with a marked enhancement of relative γ-chain synthesis (ie, 10% to 20%), as compared with corresponding in vivo levels (<2% to 3%). Evaluation of globin production in single BFU-E-derived clones showed that all normal adult bursts synthesize a significant amount of γ-chains. These results, coupled with a similar analysis of single bursts from yolk sac, embryonic or fetal liver, and cord blood, indicate that postembryonic BFU-E are always bipotent for HbF and A synthesis. The HbF potential obviously prevails in fetal life, but is gradually and almost totally replaced by the program for HbA (and some A2) production in the perinatal period. However, the potential for significant HbF synthesis is maintained in all postnatal BFU-E.

It is noteworthy that in the erythroblast differentiation pathway the synthesis of γ-chains peaks earlier than the production of γ-globin in fetal, perinatal, and adult life. It follows that modulation of HbF/A synthesis and content should be evaluated in erythroblast populations at a comparable maturation stage. The mechanism(s) underlying reactivation of γ-globin synthesis in normal adult bursts grown in FCS+ cultures has been intensively investigated. We reported that the HbF reactivation is markedly diminished in FCS− culture conditions, which show nearly normal levels of γ-chain synthesis. This phenomenon, confirmed by other investigators, may be attributed to HbF-reactivating factors either present in FCS+ or released by accessory cells in FCS− culture. In this last regard, addition of exogenous granulocyte-macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3) to FCS− semisolid cultures induces reactivation of HbF synthesis up to the level observed in FCS+ dishes.

In standard FCS+ cultures seeded with unpurified adult blood BFU-E, accessory cells produce a variety of cytokines (eg, IL-3 and GM-CSF) at levels of biologic significance. It seems necessary, therefore, to evaluate the effect of exogenous cytokines on HbF reactivation in the absence of accessory cells releasing endogenous growth factors. In this regard, we have reported methodology allowing complete purification and abundant recovery of early hematopoietic progenitors from normal adult peripheral blood (PB). The present studies describe the effect of c-kit ligand (KL), combined with a saturating level of Ep and variable amounts of IL-3/GM-CSF, on HbF production in mature erythroblasts generated by “pure” adult blood BFU-E in FCS− semisolid...
or liquid-phase culture. HbF levels were evaluated in terms of percentage of F cells and HbF content by immunofluorescence and high-performance liquid chromatography (HPLC) analysis, respectively. In addition, a variety of parameters were monitored to rigorously control these experiments, i.e., erythroblast maturation and Hb content per cell, as well as burst number and colony size in semisolid culture and cell and progenitor number in liquid phase conditions.

MATERIALS AND METHODS

Hematopoietic growth factors. Recombinant human IL-3 (specific activity, 2 to 4 x 10^8 U/mg) and GM-CSF (1.7 x 10^7 U/mg) were supplied by Genetics Institute (Cambridge, MA). Recombinant human Ep (1.1 x 10^8 U/mg) and KL (1 x 10^8 U/mg) were provided by Amgen (Thousand Oaks, CA) and Immunex (Seattle, WA), respectively.

PB. Adult PB was obtained from healthy adult male donors after informed consent. Blood (450 mL) was collected in preservative-free citrate/phosphate/dextrose/adrenaline (CPDA-1) anticoagulant. A buffy coat was obtained by centrifugation (Beckman J6M/E, 1,400 rpm/1.077) (Pharmacia Fine Chemicals, Piscataway, NJ). PBMCs were resuspended in IMDM containing 20% heat-inactivated FCS (GIBCO) and treated with three anti-CD34 MoAbs (HPCA-I, 30 µL/l; Dickininson, Oxnard, CA). Cells were then incubated with immunomagnetic IgG and IgM (Dynabeads M450, diameter 4.5 µm; Dynal, Oslo, Norway). The beads, together with rosetting cells, were then retained along the tube wall with a magnet and the supernatant fluid containing negative cells was discarded and the cells washed with 1 mL of IMDM/BSA. The rosetting cells were counted and cultured either in semisolid medium for clonogenic assays or in liquid suspension.

Progenitor purification. Cells were purified by a four-step procedure slightly modified from Gabbianelli et al. Briefly, (IA) adult PB buffy coats were separated over a Ficoll-Hypaque density (d) gradient (d = 1.077) (Pharmac Fine Chemicals, Piscatway, NJ). PB mononuclear cells (PBMCs) were collected, washed twice, and suspended in Iscove's modified Dulbeco's medium (GIBCO), Grand Island, NY). (II) PBMCs were resuspended in IMDM containing 20% heat-inactivated FCS (GIBCO) and treated with three cycles of plastic adherence. (II) Cells were washed and resuspended in IMDM with 10% FCS, and separated by centrifugation (600 g for 30 minutes at 20°C) on a discontinuous Percoll (Biochrom KG, Berlin, Germany) four-step gradient (d = 1.052, 1.056, 1.060, 1.065). (III) Low-density cells (d = 1.052 and 1.056), containing the majority of hematopoietic progenitors, were collected, washed three times in IMDM supplemented with bovine serum albumin (BSA; 2 mg/mL), Fraction V, 96% to 99% purified; Sigma, St. Louis, MO), and incubated for 60 minutes at 4°C with appropriate amounts of the following monoclonal antibodies (MoAbs): OKT3, OKT4, OKT8, OKT11, OKT16, OKM1, and OKM5 (Ortho, Raritan, NJ); and Leu7, Leu9, Leu11, Leu12, Leu14, Leu19, Leu11, and LeuM3 (Becton Dickinson, Oxnard, CA). Cells were then incubated with immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M450, diameter 4.5 µm, 1.3 x 10^7 particles/mg; Dynal, Oslo, Norway). The beads, together with rosetting cells, were then retained along the tube wall with a magnet and the supernatant fluid containing negative cells was recovered. (IV) After overnight incubation at 37°C in IMDM/20% FCS, cells were incubated for 60 minutes at 4°C in the presence of an appropriate amount of two anti-CD34 MoAbs (HPCA-I, 30 µL/l x 10^6 cells [Becton Dickinson], and B1-3C5, 10 µL/l x 10^6 cells [Sera Lab]), washed three times in cold IMDM/BSA (2 mg/mL), and incubated for 90 minutes at 4°C in the same medium containing immunomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M450). The beads, together with rosetting cells (CD34+), were then retained along the tube wall with a magnet; the supernatant fluid containing negative cells was discarded and the beads washed with 1 mL of IMDM/BSA. The rosetting cells were counted and cultured either in semisolid medium for clonogenic assays or in liquid suspension.

Methylcellulose culture. For FCS+ cultures, PBMCs were cultured at a concentration of 3 x 10^5 cells/mL/dish (two plates per point) in 0.9% methycellulose, 40% FCS, and 3 U/mL Ep in IMDM supplemented with alpha-thioglycerol (10^-4 mol/L) (Sigma) at 37°C in a 5% CO2/5% O2/90% N2 humidified atmosphere. Step IV–purified hematopoietic progenitors (1 x 10^3 cells/mL/dish) were plated in the presence of saturating level of Ep (3 U/mL), variable amounts of GM-CSF and IL-3, and graded concentrations of KL.

For FCS+ cultures, in most experiments FCS was substituted by BSA (10 mg/mL), pure human transferrin (0.7 to 1 mg/mL), human low-density lipoproteins (40 µg/mL), insulin (10 µg/mL), sodium pyruvate (10^-4 mol/L), l-glutamine (2 x 10^-3 mol/L), rare inorganic elements, nucleosides, and human recombinant hematopoietic growth factors (HGFs) (0.01 U/mL IL-3, 0.001 ng/mL GM-CSF, and 3 U/mL Ep = 10 ng/mL KL).22 The cells were split when they reached 7 x 10^5 cells/mL. Cultures were incubated in a fully humidified atmosphere of 5% CO2/5% O2/90% N2.

F cell analysis. The percentage of erythroblasts containing HbF was evaluated by indirect fluorescence as described previously.23 Briefly, cells from pooled or single bursts were cyt centrifuged on a glass slide, fixed for 5 minutes at room temperature in acetone: methanol (9:1, vol/vol), washed three times with phosphate-buffered saline (PBS), once with PBS containing 2 mg/mL BSA, and incubated for 40 minutes at 37°C with a 1:20 dilution of an antihuman HbF MoAb (Labometrics, Milan, Italy). The slides were washed twice with PBS, once with PBS/BSA, incubated for 30 minutes at room temperature with a 1:20 dilution of F(ab')2 antiserum IgGs (Dakopatts, Copenhagen, Denmark), and extensively washed in PBS. The slides were then mounted in PBS/glycerol (50:50, vol/vol) and observed under an Axioskop Zeiss microscope equipped for fluorescence. As a negative control, cells were incubated with mouse IgG instead of anti-HbF and processed as above.

Relative HbF content. HPLC separation of globin chains was performed according to Leone et al with minor modifications. Briefly, cell lysates were separated on chromatographic columns (Merck LiChrospher 100 CH8/2, 5 µm; E. Merck, Darmstadt, Germany) using as eluents a linear gradient of acetonitrile/methanol/0.155 mol/L sodium chloride (pH 2.7, 68:4:28 vol/vol/vol) (eluent A), and acetonitrile/methanol/0.777 mol/L sodium chloride (pH 2.7, 21:38:41 vol/vol/vol) (eluent B). Gradient was from 19% to 50% eluent A in 60 minutes at a flow rate of 0.8 mL/min. The optimal absorbance of the different globins was evaluated at 215 nm, because the absorbance coefficients of the different chains are identical at this wavelength.

Total Hb content per cell was evaluated as previously described.13

Morphology analysis. Cells were harvested at different days, smeared on glass slides by cytopsin centrifugation, and stained with May-Grünwald Giemsa.

RESULTS

Preliminary studies on PBMCs (ie, nonpurified PB BFU-E) grown in standard methylcellulose cultures (Fig 1 and results not shown) confirmed that in FCS+ medium erythroid bursts exhibit a high level of F cells (35% to 40%) and HbF content (20% to 25%), whereas colonies generated in FCS– conditions show markedly lower values (<5% and <2%, respectively). Interestingly, the number of bursts was similar in both FCS+ and FCS– cultures, whereas their size was apparently more elevated in FCS+ than in FCS– conditions.

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A large series of experiments was then performed to investigate HbF synthesis and content in normal adult erythroid bursts generated by BFU-E stringently purified from PB and grown in FCS$^-$ culture. The clonogenic and immunophenotypic features of the pure progenitor population have been described. Briefly, this homogeneous population comprises early erythroid (BFU-E), GM (CFU-GM), and multipotent (CFU-GEMM) progenitors, which are largely quiescent and exhibit a CD34+/lin-/CD45RO+$^+$ phenotype. The purified PB BFU-E apparently represents the counterpart of early bone marrow BFU-E, which is admittedly CD34+33-, whereas more differentiated bone marrow progenitors are CD34+/33+/CD45RO$^+$.

As shown in Fig 2A, addition of graded amounts of KL to FCS$^+$ methylcellulose culture of stringently purified BFU-E (100 cells/dish) causes a slight, nonsignificant increase of burst number, but also a striking increase in the size of BFU-E colonies, particularly in the range of 1 to 100 ng/mL KL, ie, from fewer than 5,000 cells per colony to approximately 50,000 cells per colony. Accordingly, the maturation of KL-treated bursts is delayed when compared with control bursts. To compare KL-treated and control bursts at the same stage of erythroblast maturation, bursts from control plates were picked up for analysis starting from day 16, whereas those from KL-treated dishes were analyzed from days 19 to 20.

In both groups, the percentage of mature erythroblasts was routinely greater than 50% and the Hb content per cell was greater than 20 pg (Fig 2A).

It has been recently indicated that, in clonogenic culture of purified bone marrow progenitors, KL induces an increase of not only the size but also the number of colonies. In a series of experiments larger than those reported in Fig 2A, we similarly observed that, in clonogenic FCS$^+$ culture of stringently purified PB progenitors, KL addition induces a mild but significant increase in BFU-E colony number, as well as a sharp increase in CFU-GM colony number (Fig 2B).

In KL-treated dishes, analysis of HbF production showed a dose-dependent increase in the percentage of F cells (from <10% up to 40% to 50%, mean values) and HbF content (from <2.5% up to 10% to 15%) (Fig 2A). It is of interest that the reactivation of HbF synthesis induced by KL occurs in the range of 1 to 100 ng/mL, ie, in strict parallel with the increase in BFU-E colony size (a highly significant, direct correlation exists between the percentage of F cells and cell number and burst values: $r = .986$, $P < .01$) (Fig 2A and legend).

A second series of experiments was performed in FCS$^-$ liquid phase culture of purified (step III) adult BFU-E supplemented with small amounts of IL-3 and GM-CSF and plateau levels of Ep in the presence or absence of an adequate dosage of KL (10 ng/mL). The IL-3/GM-CSF/Ep combination induces by itself a gradual differentiation specifically along the erythroid pathway until terminal maturation, which is also observed upon treatment with these HGFs + 10 ng KL (Fig 3A and legend). It is noteworthy that endogenous HGFs are not detected at the cell-seeding concentration and amount of exogenous HGF used here.

A series of liquid suspension culture experiments is shown in Fig 3A and B. At days 14, 18, and 20 of culture, 39%, 79%, and 85% (mean values), respectively, of the cells were represented by orthochromatic erythroblasts, whereas corresponding values of F cells were 9% and 7% on days 14 and 18; the HbF content was 5% on day 20. On the other hand, cultures supplemented with the IL-3/GM-CSF/Ep combination and 10 ng/mL KL showed an enhanced proliferation and delayed differentiation and maturation along the erythroid pathway, as compared with the IL-3/GM-CSF/Ep combination alone. The final cell number was greater than 1 log higher than in cultures without KL, whereas the erythroblast maturation was delayed by more than 1 week, ie, only 14% and 37% orthochromatic erythroblasts were present at days 14 and 18 of culture, whereas terminal maturation (67% orthochromatic erythroblasts) was observed on day 24. Addition of KL induced a marked increase in the number of F cells, from 20% at day 14 up to 55% at day 24, as well as a sharp increase in relative HbF content at days 20 and 24 (16% and 19%, respectively).

These experiments confirm that the proliferative effect of KL on erythroid precursors is coupled with marked HbF reactivation, which is observed at all stages of erythroblast maturation.
Fig 2. (A) HbF reactivation in pooled erythroid bursts generated by BFU-E stringently purified (step IV) from adult PB and grown in FCS{	extsuperscript{-}}cultures (10\textsuperscript{5}/mL/dish) in the presence of large amounts of IL-3 (10 to 100 U/mL), GM-CSF (1 to 10 ng), Ep (3 U), and graded amounts of KL. Erythroid bursts were analyzed in advanced stages of maturation (ie, the percent of orthochromatic erythroblasts and Hb content per cell were routinely >50\% and >20 pg, respectively). Mean ± SEM values from six separate experiments are presented. Correlation between the percentage of F cells and cell number and colony values: r = 0.986, *P < .05. **P < .01 when compared with the control KL{	extsuperscript{-}} group. (B) Number of colonies upon addition of IL-3/GM-CSF/Ep ± KL (10 ng/mL) in a separate series of nine separate experiments (other details as in A). Mean ± SEM values are presented. **P < .01 when compared with corresponding KL{	extsuperscript{-}} group.

Two separate groups of experiments were performed in clonogenetic culture supplemented with graded amounts of KL, saturating levels of Ep, and either small amounts of IL-3/GM-CSF (Fig 4A through C) or no addition of these factors (Fig 5 and results not shown). Control experiments performed in parallel were represented by KL dose{	extendash}response curves in the presence of elevated amounts of Ep/IL-3/GM-CSF. In all groups, the percentage of mature erythroblasts was routinely >50\% and the Hb content >20 pg/cell (Figs 4A and 5). KL treatment induces a dose-dependent increase in the percentage of F cells and HbF content in the presence of small amounts of IL-3/GM-CSF (Fig 4A and B) or absence thereof (Fig 5 and results not shown). The increase of these parameters is more pronounced upon addition of large doses of IL-3/GM-CSF, with a highly significant difference at relatively low KL doses (1 to 10 ng/mL) (Figs 4A and B, and 5), thus indicating a potentiating effect of these growth factors on the KL action. As expected, addition of large amounts of IL-3/GM-CSF causes an increase of the number of BFU-E colonies, as compared with the corresponding groups treated with small doses or no addition of these GFs. Finally, single-burst analysis (Fig 4C) showed that the percentage of F cells is essentially homogenous within each experimental point, ie, within groups supplemented with large or small amounts of IL-3/GM-CSF.
DISCUSSION

The reactivation of HbF in adult life has attracted considerable attention at both basic and clinical research levels. Indeed, it represents an intriguing model of partial reverse of the HbF → HbA perinatal switch. Furthermore, reactivation of HbF in patients affected by β-hemoglobinopathies is potentially of therapeutic significance. Papayannopoulou et al originally described the reactivation of HbF in clonogenetic cultures of bone marrow and adult PB BFU-E. These observations have been confirmed by a number of investigators, but the mechanism(s) underlying this phenomenon have remained elusive. This is seemingly related to the technical limitations inherent in standard BFU-E semisolid culture experiments. In this regard, three aspects are noteworthy. (1) Pure recombinant HGFs for induction of erythroid colony growth have recently become available. (2) Because FCS contains unknown hematopoietic stimuli and/or inhibitors, the use of selected FCS batches obscures methodology and interpretation of results; therefore, optimized FCS culture systems for hematopoietic progenitor proliferation and differentiation in semisolid or liquid phase conditions have been described. (3) In the cultured hematopoietic population, few progenitors (<1% and <0.1% of bone marrow and PB cells, respectively) coexist with a large number of accessory cells releasing unknown quantities of unidentified endogenous GFs, which may mask the effect of exogenous colony-stimulating factors and interleukins. In an attempt to eliminate this bias, we have recently developed methodology for nearly complete purification of early hematopoietic progenitors from normal adult PB.

In the present studies we have analyzed the mechanism of HbF reactivation under rigorous in vitro conditions, ie, in FCS methycellulose or liquid phase cultures seeded with stringently purified adult blood BFU-E. The results indicate that KL consistently induces a marked, dose-dependent reactivation of HbF.

This effect is hardly attributable to recruitment of BFU-E with high HbF potential, because the number of bursts per plate is only to a limited extent increased by KL addition in clonogenic cultures supplemented with elevated amounts of IL-3/GM-CSF/Ep. More important, the increase in HbF synthesis and content is not linked to inadequate erythroid maturation: terminally mature erythroblasts generated in KL-stimulated semisolid or liquid phase culture show at least a 5- to 10-fold increase in the percentage of F cells and HbF...
content, as compared with similarly mature erythroblasts in cultures not treated with KL.

In clonogenetic culture, the reactivation of HbF induced by KL is strictly related to the enhanced size of erythroid bursts. Similarly, in liquid suspension culture, KL addition induces a more pronounced proliferation and delayed differentiation of erythroid progenitors coupled with HbF reactivation. A cause and effect relationship may exist between these phenomena, i.e., the additional erythroid divisions induced by KL in early erythroid differentiation might lead to the reactivation of the HbF synthesis program via unknown molecular mechanisms.

In FCS- cultures of pure adult BFU-E, the effect of IL-3/GM-CSF on HbF reactivation is significant. Indeed, addition of both factors at saturating levels potentiates the action of KL on HbF reactivation, particularly at low KL doses (1 to 10 ng/mL). This phenomenon is apparently unrelated to recruitment of BFU-E with high HbF potential, because it also occurs at single burst level in both IL-3/GM-CSF-rich and -poor cultures.

The present findings shed light on previous observations on HbF reactivation in normal adult bursts generated by nonstringently purified progenitors. The enhanced HbF synthesis induced by addition of IL-3 and GM-CSF in FCS- culture of unpurified BFU-E may be in part mediated via release of endogenous KL by accessory cells stimulated by GM-CSF/IL-3. It might be further postulated that different FCS- culture conditions dampened these phenomena.

The present in vitro studies reflect on the mechanisms underlying in vivo HbF reactivation in adult marrow regeneration. It is proposed that (1) in "stress erythropoiesis" high KL and IL-3/GM-CSF activity induces extensive proliferation of erythroid progenitors, thus leading to reactivation of their HbF synthesis program; (2) under normal steady-state conditions, low KL and IL-3/GM-CSF activity is associated with a largely quiescent BFU-E population, which undergoes a more limited proliferation in the erythroid differentiation pathway, thus generating terminal erythroblasts with very low, physiologic HbF levels.
Fig 4. (A) Hb reactivation in pooled erythroid bursts generated by BFU-E stringently purified (step IV) from adult PB and grown in FCS culture (100 cells/dish) in the presence of saturating Ep levels, large or small amounts of IL-3/GM-CSF, and graded amounts of KL, as indicated. Clonogenetic parameters are shown in bottom panels. In all culture conditions erythroid bursts were analyzed in advanced stages of maturation (i.e., the percentage of orthochromatic erythroblasts and Hb content per cell were routinely >50% and >20 pg, respectively). Mean ± SEM values from seven (large IL-3/GM-CSF dosage) and three (small IL-3/GM-CSF dosage) experiments are presented. **P < .01 when compared with corresponding groups treated with low amounts of IL-3/GM-CSF. Correlation between percent F cells and cell number per burst values in groups treated with small IL-3/GM-CSF dosage: r = 0.986, P < .01. (B) Mean ± SEM percentage of HbF in pooled bursts from three experiments as in A. **P < .01 when compared with corresponding group treated with low amounts of IL-3/GM-CSF. (C) Percent F cells in single bursts generated in one of the experiments in A (representative results).
Fig 5. Hb reactivation in pooled erythroid bursts generated by BFU-E stringently purified (step IV) from adult PB and grown in FCS-culture (100 cells/dish) in the presence of saturating Ep levels, large amounts of IL-3/GM-CSF or no addition of these HGFs, and graded amounts of KL, as indicated. Clonogenetic parameters are shown in bottom panels. In all culture conditions, erythroid bursts were analyzed in advanced stages of maturation (ie, the percentage of orthochromatic erythroblasts and Hb content per cell were routinely >50% and >20 pg, respectively). Mean ± SEM values from seven (large IL-3/GM-CSF dosage) and three (no IL-3/GM-CSF) experiments are presented. **P < .01 when compared with corresponding groups treated with no IL-3/GM-CSF. Correlation between the percentage of F cells and cell number and burst values in Ep/KL groups: r = 0.986, P < .01.

ACKNOWLEDGMENT

We thank Dr. S. Gillis from the Immunex Co (Seattle, WA) for generously providing the recombinant human e-ckt ligand.

REFERENCES

c-kit ligand reactivates fetal hemoglobin synthesis in serum-free culture of stringently purified normal adult burst-forming unit-erythroid

C Peschle, M Gabbianelli, U Testa, E Pelosi, T Barberi, C Fossati, M Valtieri and L Leone