Granulocyte-Macrophage Colony-Stimulating Factor Reactivates Fetal Hemoglobin Synthesis in Erythroblast Clones From Normal Adults


Reactivation of fetal hemoglobin (HbF, α2γ2) synthesis was previously reported in normal human adult erythroblast colonies ("bursts") generated by erythroid progenitors (BFU-E) in fetal calf serum-supplemented (FCS') semisolid cultures stimulated with erythropoietin (Ep). Our studies focused on the reactivation of HbF synthesis in normal adult erythroid bursts generated by peripheral blood mononuclear cells (PBMCs) seeded in FCS' methylcellulose culture. Reactivation is almost totally suppressed when (a) PBMCs are grown in optimized FCS' culture, or (b) PBMCs are first stringently depleted of monocytes and then plated in FCS' medium (ie, BFU-E growth in FCS' Mo culture). In both experimental conditions, the proliferation of lymphocytes and macrophages interspersed among colonies is drastically reduced, and the cloning efficiency of granulocyte-macrophage (GM) progenitors is sharply diminished. In either case, addition of biosynthetic GM colony-stimulating factor (GM-CSF) induces a dose-related increase of HbF synthesis up to the level in FCS' culture, with even more elevated values on delayed addition of Ep. A dose-related increase was also observed in erythroblast clones generated by highly purified BFU-E. These results suggest that reactivation of HbF synthesis in normal adults is at least in part mediated by GM-CSF. Furthermore, they imply intriguing hypotheses on the mechanism of perinatal Hb switching. Finally, they raise the possibility of reactivation of HbF synthesis in β-thalassemia and sickle cell anemia by GM-CSF therapy.

In the perinatal period fetal hemoglobin (HbF, α2γ2) is subtotally replaced by HbA (α2β2) and some HbA2 (α2δ2). Thereafter, HbF (less than 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 "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), the 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 also 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.

The mechanism underlying reactivation of γ-globin synthesis in normal adult bursts grown in FCS' cultures has not yet been identified. FCS is generally conceded to contain a variety of hematopoietic growth factors and/or to enhance proliferation of accessory cells releasing endogenous hematopoietins: Thus, FCS addition obscures both methodology and interpretation of results. On this basis, we have made intensive efforts in our laboratory to optimize a FCS' culture system for cloning human hematopoietic progenitors. The current system allows adequate BFU-E proliferation and differentiation, at least up to the level observed in FCS' clonogenetic culture, but allows little proliferation of accessory cells (ie, lymphocytes and monocytes-macrophages interspersed among colonies). Inhibition of accessory cell growth was also observed in FCS' Mo culture (ie, on preliminary, stringent removal of monocytes from peripheral blood mononuclear cells [PBMCs] and subsequent cloning in FCS' medium).

We report that erythroid bursts from normal adult peripheral blood show a dramatic decrease of relative γ-chain synthesis when grown in FCS' or FCS' Mo conditions, as compared with control FCS' dishes. Furthermore, addition of recombinant GM-CSF to FCS' or FCS' Mo cultures induces a dose-related reactivation of HbF synthesis in the erythroblast colonies. Reactivation was also observed when highly purified progenitors were plated in FCS' conditions (200 cells per dish) with graded amounts of GM-CSF.

MATERIALS AND METHODS

Human Subjects

Peripheral blood was obtained from 18 normal adult volunteers who had given fully informed consent.

Recombinant Human Hematopoietins

We used the following glycosylated human recombinant hematopoietins: Ep (Amgen, Thousand Oaks, CA), GM-CSF and G-CSF

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Cell Culture Systems

Preparation of Cells and Medium

**Standard FCS culture.** PBMCs obtained by a standard Ficoll gradient were seeded (3 x 10^7 cells/mL per plate [Falcon 1008, Becton Dickinson, Lincoln Park, NJ]) in 0.9% methyl cellulose and 40% FCS (Flow, Irvine, England) in Iscove’s modified Dulbecco’s medium (IMDM, Gibco, Paisley, England), supplemented with 1.5 IU/mL Ep and \( \alpha \)-thioglycerol (10^{-5} \text{mol/L}).

**FCS culture.** PBMCs were seeded (3 x 10^7 cells/mL per plate unless otherwise specified) in 0.9% methyl cellulose in IMDM, which was supplemented not only with Ep and \( \alpha \)-thioglycerol as in FCS culture, but also with the following ingredients to replace FCS: (a) bovine serum albumin (BSA, >98% to 99% purified, Sigma, St Louis, MO, or Serva, Heidelberg, FRG) (10 mg/mL); (b) iron-saturated pure human transferrin (Behring Institute, Schoppito, Italy) (0.7 to 1.0 mg/mL); (c) human low-density lipoproteins (Sigma) (40 \mu g/mL); (d) a mixture of bovine insulin (10 \mu g/mL), sodium pyruvate (10^{-5} \text{mol/L}), L-glutamate (2 x 10^{-3} \text{mol/L}), L-glutamine (4 x 10^{-4} \text{mol/L}), and nucleosides (adenosine, cytidine, guanosine, and their deoxy derivatives uridine and deoxothyminide [10 \mu g/mL each]).

**FCS Mo cultures.** Monocytes were removed from PBMCs by a two-step procedure including (a) two 60 minute cycles of plastic adherence in standard culture conditions; (b) treatment of cells with discontinuous Percoll (Biochrom KG, Berlin, Germany) (40% to 60% Percoll) to remove adherent cells as described for FCS Mo—culture, but also with the following ingredients to replace FCS: (a) bovine serum albumin (BSA, >98% to 99% purified, Sigma, St Louis, MO, or Serva, Heidelberg, FRG) (10 mg/mL); (b) iron-saturated pure human transferrin (Behring Institute, Schoppito, Italy) (0.7 to 1.0 mg/mL); (c) human low-density lipoproteins (Sigma) (40 \mu g/mL); (d) a mixture of bovine insulin (10 \mu g/mL), sodium pyruvate (10^{-5} \text{mol/L}), L-glutamate (2 x 10^{-3} \text{mol/L}), L-glutamine (4 x 10^{-4} \text{mol/L}), and nucleosides (adenosine, cytidine, guanosine, and their deoxy derivatives uridine and deoxothyminide [10 \mu g/mL each]).

**FCS Mo cultures.** Monocytes were removed from PBMCs by a two-step procedure including (a) two 60 minute cycles of plastic adherence in standard culture conditions; (b) treatment of cells with discontinuous Percoll (Biochrom KG, Berlin, FRG) gradient (3 mL fractions: density 1.054, 1.066, 1.077). Low-density cells from fractions 1 and 2 were collected and seeded in 2 mL of the same medium containing 2 mg/mL BSA and incubated for 60 minutes at 4\( ^\circ \)C with appropriate amounts of the following MoAbs: OKT3, OKT4, OKT8, OKT11, OKT16, OKM5, OKM14 (Ortho, Raritan, NJ, USA), Leu-7, Leu-12, Leu-14, Leu-19, Leu-M1, Leu-M3 (Becton Dickinson). After three washes with cold IMDM, the cells were incubated three times for 40 minutes at 4\( ^\circ \)C with a 10-fold excess of immobilomagnetic monodisperse microspheres coated with sheep antibody to mouse IgG and IgM (Dynabeads M-450, diameter 4.5 \mu m, 1.3 x 10^{10} particles/mg, Dynal, Oslo, Norway). The beads together with rosetting cells were then retained along the tube wall with a magnet for 60 seconds. Thereafter, the supernatant fluid containing negative cells was recovered. The negative cells were washed twice in cold IMDM, resuspended in 0.5 mL IMDM, counted, and then seeded at 2 x 10^5 cells/mL per plate as described for standard FCS culture. For each antibody used, residual positive cells were <0.1%.

Hematopoietins. GM-CSF and other hematopoietin(s) were added at the dosage specified either in the starting medium or at different days of culture.
Table 1. IEF Analysis of Relative γ-Globin Chain Synthesis in Pooled Normal Adult Erythroid Bursts Grown in Either Standard FCS* or Optimized FCS+ Culture

<table>
<thead>
<tr>
<th>Culture System</th>
<th>Experiment No.</th>
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<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>1–5</td>
<td>FCS</td>
<td>FCS</td>
<td>FCS</td>
</tr>
<tr>
<td>BFU-E colonies per plate</td>
<td>65 ± 2.8</td>
<td>56 ± 2.4</td>
<td>92 ± 2.0</td>
<td>107 ± 8.6</td>
<td>97 ± 1.5</td>
<td>122 ± 3.5</td>
<td>111</td>
<td>86</td>
<td>81</td>
</tr>
<tr>
<td>Cells x 10^3 per burst</td>
<td>10.0 ± 0.9</td>
<td>8.7 ± 0.8</td>
<td>5.8 ± 0.5</td>
<td>12.2 ± 1.1</td>
<td>20.3 ± 1.8</td>
<td>25.8 ± 2.1</td>
<td>12.7</td>
<td>8.4</td>
<td>5.4</td>
</tr>
<tr>
<td>Late erythroblasts (%)</td>
<td>96 ± 1.2</td>
<td>95 ± 3.7</td>
<td>96 ± 2.3</td>
<td>98 ± 1.2</td>
<td>89 ± 3.4</td>
<td>96 ± 2.3</td>
<td>80</td>
<td>NE</td>
<td>100</td>
</tr>
<tr>
<td>Hb per cell (pg)</td>
<td>30 ± 2.5</td>
<td>28 ± 1.9</td>
<td>23 ± 1.7</td>
<td>27 ± 2.4</td>
<td>21 ± 0.9</td>
<td>21 ± 1.3</td>
<td>34</td>
<td>30</td>
<td>31</td>
</tr>
<tr>
<td>γ/γ + β Synthesis (%)</td>
<td>10</td>
<td>&lt;2</td>
<td>30</td>
<td>&lt;2</td>
<td>28</td>
<td>3</td>
<td>18</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>CFU-GM colonies per plate</td>
<td>NE</td>
<td>NE</td>
<td>12 ± 3.0</td>
<td>3 ± 2.0</td>
<td>NE</td>
<td>38 ± 9</td>
<td>30 ± 16</td>
<td>23.0 ± 2.2</td>
<td>8.1 ± 1.6</td>
</tr>
</tbody>
</table>

Abbreviation: NE, not evaluated.

Five representative experiments (left) and overall results from 15 experiments (right side, last two columns) are shown.

Colony scoring and analysis was done at days 14 and 16 for FCS* and FCS+ cultures, respectively. Experiments 1, 2, and 3 were performed in triplicate (values are mean ± SEM); experiments 4 and 5 were performed in duplicate (values are means). In experiments 1 and 3, the various parameters were also evaluated on other days (on day 12 or 11 in FCS* or FCS+ cultures respectively, as well as on days 13, 14, and 16 in both conditions). Results on relative γ-chain synthesis, no. 1:13,11, 10,7 in FCS* and NE 5, 3, ≤2 in FCS; no. 3:34, NE,28, 22 in FCS* and 11,6, NE, 3 in FCS+. The last two columns are the mean ± SEM of all 15 experiments from nine different subjects (five of them were also donors for the 14 experiments shown in Table 2). Further details are provided in the Materials and Methods and Results sections.
Table 2. IEF Analysis of Relative γ-Globin Chain Synthesis in Pooled Normal Adult Erythroid Bursts Grown in FCS - Mo Culture (i.e., by Plating PBMCs After Stringent Removal of Monocytes) as Compared With Standard FCS Dishes

<table>
<thead>
<tr>
<th>Culture System</th>
<th>Experiment No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>1–14</th>
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<tbody>
<tr>
<td></td>
<td>FCS</td>
<td>FCS-Mo</td>
<td>FCS</td>
<td>FCS-Mo</td>
<td>FCS</td>
<td>FCS-Mo</td>
<td>FCS</td>
</tr>
<tr>
<td>BFU-E colonies per plate</td>
<td>93 ± 3.1</td>
<td>51 ± 0.8</td>
<td>100 ± 5.2</td>
<td>53 ± 1.5</td>
<td>81</td>
<td>50</td>
<td>48</td>
</tr>
<tr>
<td>Cells × 10^9 per burst</td>
<td>9.5 ± 0.9</td>
<td>12.1 ± 1.0</td>
<td>32.5 ± 2.7</td>
<td>29.8 ± 0.9</td>
<td>5.4</td>
<td>9.7</td>
<td>8.8</td>
</tr>
<tr>
<td>Late erythroblasts (%)</td>
<td>98 ± 2.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>98 ± 0.6</td>
<td>100</td>
<td>98</td>
<td>65</td>
</tr>
<tr>
<td>Hb per cell (pg)</td>
<td>33 ± 1.8</td>
<td>32 ± 2.2</td>
<td>26 ± 1.9</td>
<td>32 ± 1.6</td>
<td>31</td>
<td>27</td>
<td>23</td>
</tr>
<tr>
<td>γ/γ + β Synthesis (%)</td>
<td>18 ± 2</td>
<td>27 ± 2</td>
<td>9 ± 2</td>
<td>9 ± 2</td>
<td>15</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>CFU-GM colonies per plate</td>
<td>24 ± 2.0</td>
<td>4 ± 1.2</td>
<td>23 ± 1.3</td>
<td>1 ± 0.6</td>
<td>30</td>
<td>3</td>
<td>20</td>
</tr>
</tbody>
</table>

Five representative experiments (left side) and overall results from 14 experiments (right side, last two columns). Colony scoring and analysis was on day 14 for both FCS and FCS-Mo cultures. Experiments 1 and 2 were performed in triplicate (values are mean ± SEM), experiments 3, 4, and 5 were performed in duplicate (values are means). The two last columns are the mean ± SEM of all 14 experiments from nine different subjects (five of them were also donors for the 15 experiments in Table 1). Further details are provided in the Materials and Methods and Results sections.
GM-CSF REACTIVATES HbF SYNTHESIS IN ADULT BURSTS

Fig 1. IEF analysis of globin chain synthesis in pooled erythroid bursts from a normal adult blood sample, grown in FCS'Mo' or FCS' culture supplemented with graded amounts of GM-CSF. Lane a, control FCS' culture; lane b, FCS'Mo' culture; lane c, FCS' culture; lanes d through f, FCS'Mo' culture supplemented with 1, 10, or 100 ng/mL GM-CSF, respectively; lanes g through i, FCS' culture supplemented with 1, 10, or 100 ng/mL GM-CSF, respectively; lane l, cord blood control sample.

content per cell). On the other hand, the number of CFU-GM colonies was consistently higher in FCS' versus FCS' dishes, suggesting a reduced production of endogenous GM-CSF in FCS' culture. Indeed, the latter condition is associated with a dramatic drop of background accessory cells (ie, cells interspersed among colonies), which may release GM-CSF in the culture: In FCS' culture, the number of background lymphocytes and monocytes-macrophages was between 10⁷ and 10⁸ and 0.5 to 2 × 10⁷ per dish, respectively, whereas in FCS' culture lymphocytes were virtually absent and monocytes-macrophages were markedly reduced (10² per dish). As described in the Materials and Methods section, the lymphocytes in FCS' culture comprised more than 90% T cells, largely CD4⁺, as well as less than 10² Leu-19⁺ LGL.

In a second set of experiments, we evaluated the effect of stringent removal of monocytes from PBMCs on γ-globin synthesis in erythroid bursts grown in FCS' medium (FCS'Mo' conditions). Table 2 shows the results of five representative experiments and the mean ± SEM values in 14 independent experiments. We always observed a dramatic decrease of γ-chain synthesis in FCS'Mo' as compared with FCS' control plates. No consistent changes were observed for the other parameters, except for a decreased number of GM colonies in FCS'Mo' culture. Again, the decline of GM colonies was coupled with a dramatic decrease in background cells (ie, virtual absence of lymphocytes and less than 10² monocytes-macrophages per dish), suggesting a decrease in endogenous GM-CSF production.

In two other experiments, we analyzed the relative γ-globin synthesis and HbF content in pooled bursts from FCS'Mo' or FCS' culture as compared with standard FCS' dishes (Table 3). The relative level of both parameters was sharply lower in FCS' and FCS'Mo' culture than in corresponding FCS' dishes. Parallel experiments showed that in both pooled bursts and single BFU-E colonies relative γ-synthesis values were much lower in FCS'Mo' than in FCS' conditions.

Effect of GM-CSF and Other Growth Factors in FCS' and FCS'Mo' Cultures

Various growth factors were added to FCS' cultures to analyze their effect on γ-globin synthesis (Figs 1 and 2). Ep alone did not significantly modulate γ-globin synthesis at any tested concentration (0.15 to 10 IU/mL). Negative results were also obtained with the combined addition of plateau level of Ep and graded amounts of other growth factors (ie, interleukin-1α [IL-1α], IL-1β, IL-2, G-CSF) (Fig 2 and results not shown). G-CSF induces a dose-dependent increase of GM colonies, but has no detectable effect on γ-globin synthesis.

In contrast, five separate experiments in five normal adults indicated that combined treatment with plateau level
of Ep and graded amounts of GM-CSF induced a dose-related increase of relative γ-synthesis (from 3.5 ± 1.5 to 16.6 ± 4.8 at 100 ng/mL GM-CSF, ie, up to the level in control FCS plates, 16.6 ± 5.4) (Fig 2). GM-CSF addition did not induce a significant effect on the clonogenesis of erythroid colonies (number of bursts per dish and cells per burst) or on their maturation (HB content per cell, percentage of mature erythroblasts). On the other hand, exogenous GM-CSF caused a slight increase in the number of GM colonies from 9.7 ± 2.3 to 22.5 ± 5 (control value in FCS cultures was 20 ± 4.9).

In a second series of experiments (Fig 3), GM-CSF treatment in FCS-Mo culture induced a similar dose-related increase of γ-globin synthesis up to the level in FCS controls (mean ± SEM values from five separate experiments in five normal adults: FCS+, 18 ± 6.2; FCS-Mo+, 4.4 ± 1.3; GM-CSF+ treated with 10 ng/mL GM-CSF, 20.2 ± 4.9). In this culture condition, GM-CSF addition did not significantly affect maturation of erythroid colonies. Conversely, it induced a slight increase in burst size (number of cells per burst) and a significant increase in the number of bursts: A positive correlation existed between mean γ-synthesis level and burst number (r = .90, P < .05). However, when results from single experiments were analyzed, a significant correlation was observed in only one case (r = .92, P < .01), one case showed a nonsignificant correlation (r = .77, .05 < P < .10), and the remaining three experiments showed no correlation (P > .10). The number of GM colonies increased from 2.0 ± 0.6 to 15.8 ± 2.6 in the presence of 100 ng/mL GM-CSF as compared with 20 ± 3.1 in FCS control cultures.

**Effect of GM-CSF Combined With Delayed Ep Addition**

Because GM-CSF essentially acts on early erythroid progenitors (BFU-E),19,20 whereas Ep mainly affects late progenitors (CFU-E),21,22 we investigated the effect of early GM-CSF and late Ep treatment. Thus, GM-CSF was added on day 0 of culture and Ep on day 0, 3, or 6: γ-synthesis was then evaluated on day 14 (Ep treatment on day 0 or 3) and 16 (Ep added on day 6) in mature erythroblast clones (Fig 4). On delayed addition of Ep, GM-CSF induced a striking increase of relative γ-synthesis, more pronounced than that observed with Ep treatment on day 0 and in control FCS+ cultures. In all cases, the HB content per cell and the percentage of late erythroblasts was relatively constant. Ep addition on day 3 enhanced the number of both erythroid and GM colonies, whereas treatment on day 6 induced an increase of GM colonies versus a sharp decrease of erythroid bursts: The significance of these fluctuations is uncertain.

**Effect of GM-CSF on Highly Purified Progenitors**

In a final series of experiments, we evaluated the relative γ-globin synthesis in erythroid bursts generated by purified normal adult blood BFU-E highly enriched from PBMCs and then plated at concentrations as low as 200 cells per dish in FCS+ cultures treated with different amounts of GM-CSF. Control dishes were seeded with nonpurified PBMCs (3 × 10⁵ cells per dish) in FCS+ or FCS-Mo+ conditions. Figure 5 shows the mean values ± SEM of relative γ-globin synthesis in the pooled mature bursts from six separate experiments in six different donors. The control values ranged from 14.3 ± 1.9 in FCS+ culture to 2.4 ± 1.5 in FCS-Mo+ dishes. In the cultures with highly purified progenitors, the smallest GM-CSF dose (0.1 ng/mL) induced the lowest γ-synthesis value (8.0 ± 2.8), whereas larger amounts (1, 10, or 100 ng/mL) were associated with higher γ-synthesis levels (18.3 ± 3.5, 14.5 ± 1.8, 13.7 ± 4.0). At all GM-CSF dosages, the number of bursts was similar (mean values >10 <15 colonies per dish), whereas that of GM clones was very low (<5 colonies per dish). Small erythroid clusters were occasionally observed without exogenous GM-CSF, but it was virtually impossible to analyze their globin synthesis. The erythroid maturation was optimum in all experiments (ie, the percentage of late erythroblasts was always greater than 65% to 70%).

**DISCUSSION**

These studies focused on the effect of GM-CSF on relative γ-synthesis in normal adult erythroid bursts. Three series of
GM-CSF REACTIVATES HbF SYNTHESIS IN ADULT BURSTS

Fig 2. IEF analysis of γ/γ + β synthesis (percentages) in pooled erythroid bursts from normal adult PBMCs in different culture conditions: from left to right, (a) control FCS culture treated with plateau concentration of recombinant human Ep only (1.5 IU per dish); (b) FCS culture supplemented with either graded doses of Ep alone or plateau level of Ep combined with graded amounts of recombinant human cytokines, i.e., IL-1α, IL-1β, or G-CSF (middle panel); (c) FCS culture supplemented with plateau level of Ep combined with graded doses of recombinant human GM-CSF (right panel).

Other parameters are (top to bottom) Hb content per cell, percentage of late erythroblasts, number of erythroid bursts per dish, number of cells per burst, number of CFU-GM colonies per dish. Control and GM-CSF data are mean ± SEM values from five separate experiments in five different donors. Representative single experiments for the other growth factors (middle). In FCS cultures background cells were composed of 10 to 10 lymphocytes and 0.5 to 2 x 10 macrophages per dish. In FCS cultures, treated or not with GM-CSF, background cells were composed of < 10 macrophages per dish and virtually no lymphocytes.

experiments were sequentially performed to (a) develop clonogenic cultures of normal adult BFU-E with low HbF synthesis levels, (b) evaluate the effect of GM-CSF and other hematopoietins on HbF synthesis in these culture systems, and (c) investigate the mechanism of HbF reactivation induced by GM-CSF.

We first developed new clonogenic culture conditions for normal adult blood BFU-E. In these conditions, PBMCs are either grown in FCS medium (FCS culture) or first stringently depleted of monocytes and then grown in FCS medium (FCS Mo culture). In the presence of plateau amounts of Ep, both systems allow optimum proliferation and differentiation of BFU-E. More important, the relative γ-chains synthesis in mature erythroblasts is much lower in FCS or FCS Mo conditions (2% to 4%) than in FCS cultures (18% to 20%). A similar difference was observed for HbF content.

In both FCS- and FCS-Mo culture, the number of GM colonies is markedly lower than in control FCS dishes: We suggest that this decrease results from the reduced level of endogenous GM-CSF in the FCS- and FCS-Mo system, as compared with standard FCS cultures. In this regard, GM-CSF may be released by a variety of accessory cells (e.g., T lymphocytes,20 LGLs,21 and monocytes-macrophages24). FCS- cultures seeded with high-density PBMCs (3 x 10 cells/mL) contain a large number of T cells, LGLs, and monocytes-macrophages, interspersed among colonies: therefore, in these cultures addition of Ep alone may allow efficient burst formation and erythroblast maturation, owing to release of endogenous GM-CSF (and possibly other
hematopoietins, mainly IL-3) by accessory cells. Indeed, the absolute GM-CSF requirement of adult BFU-E can be demonstrated only in unicellular FCS culture. In the culture systems we describe (FCS and FCS' Mo'), the growth of accessory cells is drastically reduced: This presumably causes a reduced production of endogenous GM-CSF, which in the presence of Ep still allows burst formation but curtails the growth of GM colonies. Along these lines, we considered the hypothesis that in FCS' and FCS' Mo' culture the reduced production of endogenous GM-CSF may be responsible for the marked decrease of relative γ synthesis, as compared with FCS' culture.

To verify this possibility, we added GM-CSF or other recombinant hematopoietins combined with plateau levels of Ep to normal adult blood BFU-E grown in FCS' or FCS' Mo' cultures. Addition of GM-CSF induced a dose-dependent increase in HbF synthesis, whereas the other growth factors (IL-1α or IL-1β, IL-2, G-CSF) had no effect. Increasing amounts of Ep (≤10 IU/mL) did not cause an increase in γ-chain synthesis. We cannot exclude the possibility, however, that "pharmacological" doses (ie, >10 IU/
GM-CSF REACTIVATES HbF SYNTHESIS IN ADULT BURSTS

Fig 5. Analysis of γ/γ'β synthesis (percentages) in pooled erythroid bursts: (left) PBMCs were grown in FCS+ or FCS+Mo- culture with a plateau level of Ep; (right) BFU-E highly purified from PBMCs (the number of BFU-E + CFU-GM in the final cell population assayed with plateau levels of IL-3 and GM-CSF ranged from 10% to 17%), were cultured in FCS+ condition with a plateau level of Ep and graded amounts of GM-CSF. (Bottom) Number of erythroid bursts and GM colonies either in FCS+ and FCS+Mo- cultures seeded with 3 × 10^5 PBMCs per dish (left) or FCS+ dishes seeded with 200 cells purified from PBMCs (right). Mean ± SEM values from six separate experiments are shown.

mL) may have some effect, in view of studies indicating that Ep reactivates HbF synthesis in baboons.26,27

We also investigated the mechanism(s) underlying the reactivation of HbF synthesis induced by GM-CSF. Theoretically, at least four alternative or complementary mechanisms may exist: (a) a defective maturation of erythroblasts in GM-CSF-treated dishes, (b) recruitment by GM-CSF of a cohort of BFU-E with a higher potential for HbF synthesis, (c) an indirect effect of GM-CSF on erythroid progenitors through hematopoietin(s) release by background accessory cells and/or GM colonies, and (d) a direct effect of GM-CSF on erythroid cells.

The first two mechanisms probably can be excluded. Indeed, the maturation block hypothesis is incompatible with control data on Hb content per cell and percentage of mature erythroblasts: these indicate an equivalent maturation of erythroblasts in the analyzed FCS+ and FCS+Mo- cultures at all GM-CSF dosages as well as in control FCS+ dishes.

The recruitment hypothesis cannot be advocated in FCS+ experiments, because the number of bursts per dish is not modified by GM-CSF treatment. In FCS+Mo- cultures, a positive correlation existed between mean values of burst number and relative γ-chain synthesis at various GM-CSF dosages. However, the 61% increase in burst number can hardly explain the 4.6-fold rise of γ-chain synthesis. More important, analysis of this correlation at the single-experiment level revealed that in only one case in five was the correlation significant, whereas in three cases the increase in one parameter did not correlate with an increase in the other. Thus, we may conclude that in FCS+Mo- culture the modest increase in burst number induced by GM-CSF is not a relevant factor to induce the marked reactivation of HbF synthesis. This conclusion is also in accord with results on relative γ-chain synthesis at single burst level in FCS+Mo- culture.

By exclusion, we imply that the increase in γ-chain synthesis is mediated by the action of GM-CSF on erythroid precursors, directly and/or indirectly, through hematopoietin(s) released by accessory cells and/or GM colonies.

In FCS+ and FCS+Mo- culture, GM-CSF addition does not induce an increase in background accessory cells and causes a modest increase in GM colonies, particularly limited in FCS+ conditions: These results are not easily reconciled with the indirect action hypothesis. More important, the action of GM-CSF on HbF reactivation is also observed in cultures of highly purified progenitors (200 cells per plate), despite the absence of background accessory cells and a very low number of GM colonies. These experiments clearly suggest that the action of GM-CSF is, to a major extent, direct. However, we cannot exclude the possibility that other cytokines may interact with GM-CSF to enhance its effect on HbF reactivation, particularly in experiments with delayed addition of Ep.

GM-CSF exerts its stimulatory action on erythropoiesis at
the BFU-E level.\textsuperscript{19,20} An effect on later progenitors (CFU-E) or morphologically recognizable precursors has not yet been documented. Thus, we suggest that GM-CSF induces in vitro the reactivation of HbF through an action at the level of BFU-E (ie, through modulation of their HbF synthesis program). Other corollaries are also worthy of consideration. By extrapolation from these in vitro experiments, we suggest that GM-CSF mediates in vivo, at least in part, the reactivation of HbF synthesis during adult stress erythropoiesis and hematopoietic regeneration.\textsuperscript{4}

Our observations may also reflect on the debated issue of the perinatal globin switch. In this regard, the stem cell potential for HbF synthesis gradually decreases in different BFU-E--derived erythroid clones.\textsuperscript{4} Experiments involving transplantation of fetal hematopoietic cells in the adult marrow environment strongly suggest that the switch is at least partially mediated by a time-related mechanism intrinsic to the stem cell and the BFU-E.\textsuperscript{28} Because GM-CSF is abundantly produced by the placenta,\textsuperscript{29} the switch may also be mediated partially by perinatal decline of placental release of GM-CSF and perhaps other cytokines (ie, by a time-related mechanism extrinsic to the stem cell).

These studies report the in vitro reactivation of HbF synthesis in normal adult erythroblasts by treatment with GM-CSF, (ie, a hematopoietin that physiologically modulates the proliferation and differentiation of erythroid cells in vitro\textsuperscript{19,20,20} and in vivo).\textsuperscript{20-32}

Reactivation of HbF synthesis in adult erythroblasts was obtained previously by treatment with antiblastic agents (ie, 5-azacytidine, hydroxyurea, bromodeoxyuridine) in vivo and/or in vitro.\textsuperscript{33-35} Patients with \(\beta\)-thalassemia or sickle cell anemia treated with these agents showed an enhanced \(\gamma\)-chain production\textsuperscript{36,37} of potential therapeutic significance;\textsuperscript{38} prolonged treatment, however, is hardly acceptable in view of possible oncogenic side effects.

A crucial issue raised by our observations is the possibility of reactivating HbF synthesis in \(\beta\)-thalassemia or sickle cell anemia patients by treatment with recombinant GM-CSF, which is currently used in phase I or II clinical trials in patients with suppression of hematopoiesis.\textsuperscript{39,40}

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