Erythroid colony formation by fetal rat liver and spleen cells *in vitro*: inhibition by a low relative molecular mass component of fetal spleen

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Summary

Liver and spleen hematopoietic cell suspensions from 20-day-old-fetal rats were fractionated on Percoll gradients. A granulocyte-rich splenic fraction inhibited CFUe production by cultures of a CFUe-enriched liver fraction, and by cultures of unfractionated liver and spleen hematopoietic cells. Conditioned medium from the spleen cell fraction contained an inhibitor of relative molecular mass, $M_r$, 25-35 x 10^3. The sensitivity of spleen cells to the inhibitor varied with the age of the fetus from which they were derived (20-day-old < 18-and 19-day-old). No such age-dependence was found for liver cells. The inhibitor affects cycling CFUe, blocks the lethal effect of AraC, does not appear to be lineage-specific and its influence can be reversed by washing.

Key words: CFUe inhibition, fetal spleen, fetal liver.

Introduction

The liver is the major site of red blood cell production in the rat fetus during the last four days of intrauterine life. Under normal physiological circumstances, the antenatal bone marrow contains few differentiated erythroid cells (Lucarelli et al., 1968; Nagel et al., 1981) and despite its considerable growth, the spleen probably does not release red blood cells before term (Nagel et al., 1982). There is thus a specific pattern of erythropoiesis at any given fetal age. Within this pattern, the role of each of the sources of red blood cells is influenced by the microenvironment.

Both the earliest precursor cells and the various intermediate committed cell precursors appear to require growth factors to sustain their proliferation and differentiation (see: Paukovits et al., 1991 for recent review). As such factors are often produced by other hematopoietic cells or stromal cells in the tissue environment, the regulation of hematopoiesis probably involves complex interacting cell networks. Regulation may be via cell-to-cell contact and/or locally acting factors produced by stromal cells (Harrison, 1982).

We previously described the evolution of erythroid colony-forming cells in the rat fetal spleen and reported that they are less sensitive than fetal liver cells to excess corticosteroid *in vivo* (Nagel and Nagel, 1987). Thus, the spleen and hepatic CFUe of the fetal rat react differently to corticosteroids. This study investigates the erythropoietic capacities of fetal spleen and liver in terms of erythroid colony-forming units (CFUe). Spleen and liver cells were fractionated by Percoll density gradient centrifugation. A granulocyte-enriched spleen fraction (S2) inhibited CFUe when co-cultured with a liver fraction (L1). S2-conditioned medium was found to contain a 25-35 x 10^3 $M_r$ fraction which inhibited CFUe formation by L1 liver cells and by cultures of spleen and liver hematopoietic cells. The sensitivities of spleen and liver cells from fetuses of different ages to the inhibitor were assayed, the negative regulation on cycling CFUe was tested by cytosine arabinoside suicide experiments, and the inhibitor activity and specificity were analysed.

Materials and methods

Animals

All experiments were performed on Wistar rats (CF strain, from CNRS). Coitus was assessed by the presence of spermatozoa in the morning vaginal smears. Rat pups were generally born during the night between days 21 and 22 post coitus or on the morning of day 22.

Chemicals

Bovine hemin (type I, Sigma Chemical Co., St Louis, MO) was dissolved in 0.2 M KOH, diluted to 0.01 M in supplemented alpha medium (Gibco), neutralized with 1 N HCl (Ross and Sautner, 1976) and sterilized by filtration (0.45 μm, Millipore Corp., Bedford, MA).
Erythroid colony assays

(a) Cell suspensions
Pregnant rats were killed by decapitation. Six to eight 20-day-old fetuses from two litters, or eight to twelve 18- and 19-day-old fetuses from three litters, were used in each experiment. The fetal spleens and livers were removed aseptically, placed in sterile ice-cold alpha medium and gently disrupted in a Potter-Elvehjem homogenizer with a loose-fitting plastic pestle. The resulting cell suspension was passed through a stainless steel screen (50 µm mesh) to remove particles, connective cells and parenchyma cells. Aliquots of this cell suspension were diluted fivefold in 0.4 N acetic acid (to remove mature RBC) and the hematopoietic cells counted in a hemocytometer.

(b) Spleen and liver cell fractions
Cells were layered onto a discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient. The gradient components were prepared by diluting a stock solution of Percoll (Percoll 100) (9 vol. of commercial solution plus 1 vol. of 10 times PBS concentrate Bio-Merieux, Lyon, France), to 90%, 60%, 40% and 20% (Percoll 80, etc) with alpha medium.

Spleen cell fraction (S2). Alpha medium (0.8 ml) containing 2.2 x 10^6 hematopoietic cells was layered onto a gradient of (from bottom to top) 0.2 ml Percoll 100, 0.8 ml Percoll 80, 0.8 ml Percoll 60, 0.8 ml Percoll 40. The tubes were centrifuged at 500 g for twenty minutes at 4°C. The cells at the Percoll 80-Percoll 60 interface from ten separate centrifuge tubes were pooled and then washed three times with alpha medium. Cell viability was over 95% by trypan blue exclusion.

Liver cell fraction (L1). Alpha medium (0.6 ml) containing 6.4 x 10^6 hematopoietic cells was layered onto a gradient of (from bottom to top) 0.2 ml Percoll 100, 0.6 ml Percoll 80, 0.6 ml Percoll 60, 0.8 ml Percoll 40 and 0.5 ml Percoll 20. The tubes were centrifuged as above and the cells at the Percoll 60-Percoll 40 interface were harvested and washed three times with alpha medium. Cell viability was over 95%.

Preparation of conditioned medium from S2 spleen cells
A suspension of S2 cells (0.4 ml), 3 x 10^6 cells/ml in RPMI 1640 (Gibco) buffered to pH 7.4 with 5 mM Hepes was incubated at 37°C for 12 hours. The conditioned medium was removed, filtered through a 0.45 µm Millipore membrane and stored at -20°C. Control medium was the same volume of cell-free RPMI 1640 buffered to pH 7.4 and incubated in the same conditions. Conditioned media were either added to cultures (0.25 ml) or pooled and subjected to gel filtration chromatography.

Gel filtration
Conditioned media were pooled (3-4) and 1 ml samples were fractionated on a 60 by 0.9 cm column of G100 superfine (Pharmacia). The gel was equilibrated in and fractions eluted with PBS, pH 7.4. The flow rate was 5 ml/h and 1 ml fractions were collected. Protein standards, Blue Dextran 20000 (gel filtration calibration kit, Pharmacia) and glucose were all dissolved in PBS. The eluted fractions were pooled to give five fractions. These were passed through a 0.45 µm Millipore membrane and assayed for CFUe inhibition. Inhibitory activity was found only in the 25-35 x 10^3 M pools. Fractions eluted after the glucose marker (post-glucose) were used as controls. Protein was estimated by absorbance at 280 nm, assuming that 1 OD unit = 1 mg/ml or by the Lowry procedure (Lowry et al., 1951). The protein concentration in conditioned media was 175 µg/ml and that of 2 x concentrated 25-35 x 10^3 M pools was 15 µg/ml.

Erythroid colony cultures
Erythroid progenitors were cultured on methylocellulose by the technique of Iscove et al., (1974), as modified by Urabe and Murphy (1978). The culture medium was 1.25 ml 2% methylcellullose (A4M premium, Dow Chemical Corp.) in alpha medium containing 1.25 units erythropoietin (pig erythropoietin, P28 F2 512 iu/µg protein, CNTS, Paris, or 1 unit recombinant-human erythropoietin, Amgen, 100 i.u/µg protein), 0.25 ml 10% bovine serum albumin (BSA, grade V, Sigma) in alpha medium, 25 µl 200 mM L-glutamine in water, 25 µl kanamyacin (Kanamyacin solution times 100, Gibco), 25 µl penicillin and fungizone (antibiotic antimycotic solution times 100, Gibco), 0.75 ml heat-inactivated fetal calf serum (batch 01107, Lab. J. Boy. Reims, France), 50 µl 7.5% sodium bicarbonate in water, 25 µl, β-mercaptoethanol and 50 µl hemin solution (2 x 10^-4 M). The BSA was prepared and deionized according to Murphy and Sullivan (1978). The final concentration of cells was 0.5 x 10^6/ml. The volume of whole S2-conditioned medium or S2 inhibitory fraction was 0.25 ml. Controls were 0.25 ml RPMI 1640 incubated as for the conditioned medium or 0.25 ml post-glucose eluate.

Co-cultures contained 0.25 x 10^5 hematopoietic L1 liver cells and 0.25 x 10^5 hematopoietic S2 spleen cells.

Erythroid colonies

(a) 2-day erythroid colonies
Erythroid colonies containing eight or more cells after 2 days in culture were counted in each well, without staining, as described by Iscove and Sieber (1975). These colonies were considered to be CFUe.

(b) 7-day-erythroid colonies (7-d-EC)
Colonies were medium to large and well-hemoglobinized. They did not develop in BFUe-like bursts at the cell concentration used. Colonies of 32 or more cells were therefore counted and designated 7-day-erythroid colonies (7-d-EC).

Granulocyte-macrophage colonies
The culture medium for erythroid progenitors (see above) was used without erythropoietin or hemin, but supplemented with 10% PWM-activated lymphocyte-conditioned medium from adult rat spleen. After 7 days of incubation, colonies consisting of 20 or more granulocytes and monocytes were counted as CFU-Gm-derived colonies.

Assay of inhibitor

(a) Cytosine arabinoside (AraC) suicide
AraC kills cells in the S, DNA synthesis, phase. Hematopoietic liver cells from 20-day fetuses (3 x 10^6 cells/ml) were incubated for one hour at 37°C in RPMI 1640 containing 10% FCS and 20 µg/ml AraC. Controls were incubated in medium without AraC. The cells were washed once, counted and plated out in medium with or without the 25-35 x 10^3 M fraction. The number of CFUe in control and AraC-treated cells cultured without the inhibitor gave the proportion of CFUe in the S phase.

(b) CFUe cell cycling inhibition assay
Four tubes were used for each assay. Each contained 3 x 10^6 20-day hematopoietic liver cells/ml in RPMI plus 10% FCS. Two tubes contained 20% vol/vol 25-35 x 10^3 M fraction; the
other two (controls) contained medium alone. The tubes were incubated for two hours at 37°C, and AraC (20 μg/ml) was added to one tube of each pair. The cells were incubated for a further one hour, washed once, counted, plated and the number of CFUe assayed for each treatment. The proportion of CFUe in the S phase was estimated by comparing the CFUe number in the control and AraC-treated tubes of each pair.

(c) Washing experiments
The protocol described in b) above was used up to the two hours incubation at 37°C. One tube of each pair was then washed twice and AraC was added to all four tubes for one hour before assaying for CFUe.

Cytology
Cell smears were prepared and stained with May-Grunwald Giemsa. Proerythroblasts and basophilic erythroblasts, mature erythroblasts (polychromatophilic and acidophilic), mature granulocytes, lymphocytes and ‘other’ white or undifferentiated cells were identified. 200 cells were typed in each of three experiments for a total of 600 cells.

Statistics
Colonies in 4-6 wells were scored in each experiment. Results are given as means ± s.d. or s.e. (per 2000 hematopoietic cells). Data were analysed using Student’s t-test.

Results

(1) Liver (L1) and spleen (S2) cell fractions
(a) CFUe-derived colonies (Table 1)
Liver fraction L1. L1 produced 3- to 5-fold more CFUe-derived colonies than did cultures of total hematopoietic liver cells.

Spleen fraction S2. S2 and total hematopoietic spleen cells produced similar numbers of CFUe-derived colonies.

Co-cultures of L1 and S2. The co-cultures produced fewer CFUe-derived colonies (about 60%) than the calculated mean 1/2 (L1 + S2) in all three experiments.

Table 1. CFUe colonies in cultures of liver L1 and spleen S2 cell fractions and in co-cultures of L1 and S2

<table>
<thead>
<tr>
<th>Liver fraction</th>
<th>Spleen fraction</th>
<th>Co-cultures</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>S2</td>
<td>L1+S2</td>
<td></td>
</tr>
<tr>
<td>62.0±4.1</td>
<td>31.2±1.3</td>
<td>29.5±2.6</td>
<td>36.9</td>
</tr>
<tr>
<td>70.9±3.8</td>
<td>31.5±2.4</td>
<td>33.5±2.6</td>
<td>34.0</td>
</tr>
<tr>
<td>97.5±2.6</td>
<td>30.0±2.8</td>
<td>35.0±3.0</td>
<td>45.1</td>
</tr>
<tr>
<td>(Total liver): (Total spleen):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.9±2.3*</td>
<td>36.6±1.7*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All cells were from 20-day-old fetuses. Results are means±s.d. of 4-6 wells for each of three experiments. Percentage inhibition was calculated from the equation:

\[ \frac{1/2 \times (\text{CFUe} \times L1 + \text{CFUe} \times S2) - (\text{CFUe co-cultures})}{1/2 \times (\text{CFUe} \times L1 + \text{CFUe} \times S2)} \times 100 \]

*Total liver and spleen: mean±s.e. colony count of three experiments.

(b) Cytological characteristics
Smears of L1 contained more proerythroblasts and basophilic erythroblasts (19.3 ± 1.8%) than did smears of total hematopoietic liver cells (11.0 ± 0.9%, P < 0.005), but fewer polychromatophilic and acidophilic erythroblasts (63.5 ± 1.5%) than the unfractonated liver smears (76.8 ± 1.9%, P < 0.001). The L1 fraction also contained more ‘other’ cells (14.6 ± 0.7%), mainly large undifferentiated cells, than the unfractonated liver smears (7.8 ± 1.1%, P < 0.001). Mature granulocytes accounted for 2.5 ± 0.4% of the L1 fraction and 4.3 ± 0.3% of the total hematopoietic liver cell smear (P < 0.01).

Spleen S2 smears contained more proerythroblasts and basophilic erythroblasts (13.1 ± 0.3%) than did unfractonated spleen hematopoietic cells (8.3 ± 0.7%, P < 0.005). The S2 fraction contained 19.6 ± 2.7% polychromatophilic and acidophilic erythroblasts and the unfractonated spleen preparation contained 27.2 ± 2.3% of these cells (NS). The major difference between the two preparations was in their granulocyte contents: S2 contained 52.1 ± 1.9% and the unfractonated spleen preparation contained 17.3 ± 0.6% (P < 0.001), but they also differed in their ‘other’ cell contents (S2: 12.6 ± 1.3%, spleen: 38.2 ± 2.6%, P < 0.005) and lymphocyte contents (S2: 2.5 ± 0.2%, spleen: 8.9 ± 1.1%, P < 0.05).

(c) Effects of S2-conditioned medium on CFUe production by L1
Three different S2-conditioned media all significantly (P < 0.001) decreased the number of CFUe colonies in L1 cultures (Table 2).

The percent inhibitions were similar to those produced by co-culture of S2 and L1 cells (Table 1). The inhibitory effect of the 25-35 x 10^4 M fraction of S2-conditioned medium was dose-dependent (Fig. 1). This fraction also significantly (P < 0.001) inhibited CFUe-derived colonies in cultures of both L1 liver fractions and unfractonated liver hematopoietic cells (Table 3), but the percent inhibition of L1 cultures was higher than that of unfractonated liver cells. All assays were carried out with an inhibitor concentration close to 1.

Table 2. Effect of S2 conditioned medium on the number of CFUe-derived colonies in liver L1 cell cultures

<table>
<thead>
<tr>
<th></th>
<th>CFUe colonies/2000 L1 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>63.4±4.2</td>
</tr>
<tr>
<td>S2 cond. medium</td>
<td>41.0±2.6</td>
</tr>
<tr>
<td>% inhibition</td>
<td>35.3</td>
</tr>
<tr>
<td>Controls</td>
<td>72.0±4.0</td>
</tr>
<tr>
<td>S2 cond. medium</td>
<td>35.4±2.2</td>
</tr>
<tr>
<td>% inhibition</td>
<td>50.8</td>
</tr>
<tr>
<td>Controls</td>
<td>67.2±5.0</td>
</tr>
<tr>
<td>S2 cond. medium</td>
<td>39.6±1.8</td>
</tr>
<tr>
<td>% inhibition</td>
<td>41.1</td>
</tr>
</tbody>
</table>

*All cells were from 20-day-old fetuses. Controls: S2-conditioned medium replaced with alpha medium (see Materials and methods). Results are means±s.d. (colonies scored from 4-6 wells). Percentage inhibition was calculated from the equation:

\[ \frac{\text{control CFUe} - \text{S2 cond. medium CFUe}}{\text{control CFUe}} \times 100 \]
Controls: • Whole S2-conditioned medium  
  ✪ RPMI 1640  
  ✪ Post-glucose G100 eluate

Fig. 1. Dose-dependent inhibition of CFUe colony formation in cultured liver L1 cells by a 25-35 × 10^3 M_r fraction from S2-conditioned medium. Cells were incubated for 48 h with diluted 25-35 × 10^3 M_r fraction or the appropriate positive or negative control. The data are means ± s.e. of three to five experiments (4-6 wells per experiment), except for 15 µg protein/ml (mean of two experiments). Controls: • Whole S2-conditioned medium, ✪ RPMI 1640, ✪ Post-glucose G100 eluate.

### Table 3. Inhibition of CFUe colony formation in cultured liver and spleen hematopoietic cells and liver L1 cells by the 25-35 × 10^3 M_r fractions from three pools of S2-conditioned medium

<table>
<thead>
<tr>
<th>M_r fraction of S2-conditioned medium</th>
<th>% inhibition of CFUe colonies in cultures of:</th>
<th>Liver L1</th>
<th>Total liver</th>
<th>Total spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td></td>
<td>48.1</td>
<td>36.0</td>
<td>36.3</td>
</tr>
<tr>
<td>Pool 2</td>
<td></td>
<td>45.3</td>
<td>30.5</td>
<td>34.7</td>
</tr>
<tr>
<td>Pool 3</td>
<td></td>
<td>37.8</td>
<td>27.5</td>
<td>38.0</td>
</tr>
</tbody>
</table>

All cells were from 20-day-old fetuses. Control cultures contained inactive post-glucose G100 eluate. Results are percentages of CFUe colony inhibition.

(2) Responses of liver and spleen cells from fetuses of different ages to 25-35 × 10^3 M_r fraction of S2-conditioned medium

S2-conditioned medium significantly (P < 0.001) inhibited colony formation by spleen cultures from 18-19 and 20-day-old fetuses (Tables 3 and 4). The percent inhibition of spleen cells from 20-day-old fetuses was lower than that of cells from 18- and 19-day-old fetuses (Table 4).

Similarly, CFUe-derived colony production by liver cells from 18, 19-and 20-day-old fetuses was significantly (P < 0.001 or P < 0.005) inhibited (Table 5).

### Table 4. Inhibition of CFUe colonies in cultures of spleen hematopoietic cells from 18-, 19- and 20-day-old fetal rats by 25-35 × 10^3 M_r fractions of three pools of S2-conditioned medium

<table>
<thead>
<tr>
<th>M_r fraction</th>
<th>18-days</th>
<th>19-days</th>
<th>20-days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>50.6</td>
<td>42.2</td>
<td>36.3</td>
</tr>
<tr>
<td>Pool 2</td>
<td>(68.0 ± 2.3)*</td>
<td>(44.5 ± 1.5)*</td>
<td>(40.0 ± 1.3)*</td>
</tr>
<tr>
<td>Pool 3</td>
<td>(74.0 ± 3.5)</td>
<td>(62.0 ± 2.9)</td>
<td>(41.0 ± 2.1)</td>
</tr>
</tbody>
</table>

Control cultures were incubated with post-glucose G100 eluate. Results are percentages of CFUe colony inhibition.


### Table 5. Inhibition of CFUe colonies in cultures of liver hematopoietic cells from 18-, 19- and 20-day-old fetal rats by 25-35 × 10^3 M_r fractions of three pools of S2-conditioned medium

<table>
<thead>
<tr>
<th>M_r fraction</th>
<th>18-days</th>
<th>19-days</th>
<th>20-days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>29.1</td>
<td>30.7</td>
<td>36.0</td>
</tr>
<tr>
<td>Pool 2</td>
<td>(42.3 ± 1.6)*</td>
<td>(31.8 ± 0.05)*</td>
<td>(21.5 ± 0.6)*</td>
</tr>
<tr>
<td>Pool 3</td>
<td>(51.8 ± 2.1)</td>
<td>(45.6 ± 2.3)</td>
<td>(23.8 ± 1.0)</td>
</tr>
</tbody>
</table>

Control cultures were incubated with post-glucose G100 eluate. Results are percentages of CFUe colony inhibition.


However, in contrast to the spleen cultures, the overall percent CFUe inhibition in 18- to 20-day-old fetuses was not significantly different.

(3) Effects of the 25-35 × 10^3 M_r fraction

(a) On CFUe DNA synthesis and proliferation

Cells were incubated for one hour at 37°C with or without AraC before being plated with or without the 25-35 × 10^3 M_r fraction. The results in Fig. 2 are the mean ± s.e. of three experiments. There was no significant additive effects of inhibitor and AraC (P > 0.2) for either cultures of liver total hematopoietic cells (Fig. 2A) or cultures of L1 cells (Fig. 2B). The 25-35 × 10^3 M_r fraction appeared to inhibit a part of the CFUe population responsive to AraC.

AraC had a smaller lethal effect on liver total hematopoietic cells that had been incubated with 25-35 × 10^3 M_r fraction before the AraC suicide experiments (Table 6). Washing cells incubated with inhibitor before incubating them with AraC almost abolished the effect of 25-35 × 10^3 M_r fraction (Table 6).
Table 6. Inhibition of CFUe cell cycling by 25-35 × 10³ Mr fraction and the effect of washing cells incubated with inhibitor on AraC suicide of CFUe

<table>
<thead>
<tr>
<th>CFUe colonies/2000 hematopoietic liver cells</th>
<th>- Ara C</th>
<th>+ Ara C</th>
<th>% killed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unwashed control incubated without inhibitor</td>
<td>15.6 ± 0.9</td>
<td>9.0 ± 1.4</td>
<td>42.3</td>
</tr>
<tr>
<td>Unwashed cells incubated with inhibitor</td>
<td>14.1 ± 1.2</td>
<td>12.8 ± 1.3</td>
<td>9.2</td>
</tr>
<tr>
<td>Washed control incubated without inhibitor</td>
<td>17.2 ± 0.7</td>
<td>9.2 ± 0.8</td>
<td>46.5</td>
</tr>
<tr>
<td>Washed cells incubated with inhibitor</td>
<td>17.5 ± 1.1</td>
<td>11.1 ± 0.6</td>
<td>36.6</td>
</tr>
</tbody>
</table>

Results are means ± s.e. of three experiments. All cells were from 20-day-old fetuses.

Fig. 2. Effect of 25-35 × 10³ Mr fraction on cultures of hematopoietic cells subjected to AraC suicide. (A) Cultures of 20-day liver total hematopoietic cells. (B) Cultures of liver fractions: 10.5 ± 1.8. + + , after AraC treatment. Cultures without 25-35 × 10³ Mr fraction. Mean percentage ± s.e. of CFUe colony cytotoxicity (3 experiments). 8.7 ± 1.1 CFUe colonies/2000 hematopoietic cells in cultures with 25-35 × 10³ Mr fraction. + + , after AraC treatment. Cultures with 25-35 × 10³ Mr fraction. Mean percentage ± s.e. of CFUe colony cytotoxicity and/or inhibition (3 experiments). 7.7 ± 1.3 CFUe colonies/2000 hematopoietic cells in cultures with 25-35 × 10³ Mr fraction.

(b) On earlier erythroid progenitor-derived colonies after 7 days in culture (7-d-EC)
The percentage inhibition of 7-d-EC in L₁ cultured cells was 22.0 ± 0.6 (four experiments). A threefold concentrate of the 25-35 × 10³ Mr fraction produced greater inhibition (32.9 ± 0.8, three experiments). (In L₁ control cultures, 7-d-EC / 2000 hematopoietic cells were 11.0 ± 1.5. The inhibition was always significant: P < 0.01).

Inhibition of in vitro CFUe by a spleen extract

Lever and spleen total hematopoietic cells were tested at days 19 and 20 as CFU-Gm. The CFU-Gm colonies/2000 hematopoietic cells were 13.9 ± 1.3 and 11.5 ± 0.9 in liver cultures, 25.2 ± 3.2 and 14.4 ± 1.7 in spleen cultures. Incubation with 25-35 × 10³ Mr fraction altered CFU-Gm colony development (four experiments). The inhibitions were: 17.9 ± 2.9 on day 19 and 18.5 ± 2.7 on day 20 (P > 0.05) in liver cultures, 21.3 ± 2.1 on day 19 and 21.1 ± 1.8 on day 20 (P < 0.05) in spleen cultures.

Discussion
The data show that 20-day-old rat fetal spleen contains an inhibitor of hematopoietic progenitor cell proliferation that is released by granulocytes or other cells in the spleen S₂ fraction. The inhibitory effect on CFUe seems to be more marked for the liver fraction L₁ than for total liver hematopoietic cells (Table 3) and for the spleen colonies of younger fetuses (18- and 19-day-old) than for older ones (20-day) (Table 4). The formation of CFUe-derived colonies by the S₂ spleen fraction is not totally inhibited, and 10% S₂-conditioned medium does not decrease CFUe-derived colony formation by S₂ cell cultures (unpublished). This suggests that the inhibitor acts on a population of CFUe which is larger in L₁ cells and the spleens of younger fetuses, and that some CFUe are not responsive to it, especially those in total liver hematopoietic cells and the spleens of 20-day-old fetuses.

This differential responsiveness to the 25-35 × 10³ Mr fraction may be due to the state of the progenitor cells and their DNA-synthetic activity. The proportion of CFUe engaged in DNA synthesis at the time of plating out was assessed by incubation with AraC. The inhibitor affects a portion of the cycling CFUe that are sensitive to AraC. Pre-incubation with the inhibitor blocks the lethal effect of AraC on cycling CFUe, indicating that DNA synthesis is shut down within minutes of exposure to the inhibitor. The effects of the inhibitor can be reversed by washing, confirming that DNA synthesis in hematopoietic cells can be controlled even after cells have entered the S-phase of the cell cycle (Del Rizzo et al., 1990). If the suppression is indirect, the sensitivities of CFUe to the inhibitor may depend on the number of accessory cells involved. A lack of these cells could explain the unresponsive CFUe in the S₀ population.

Though the inhibitor does not appear to be specific to CFUe, since colonies derived from earlier erythroid progenitors (7-d-EC) respond to it and other cells (CFU-Gm) are probably affected by it, its effect on CFUe seems to be more marked.

The pattern of erythropoiesis in the spleens of 18- to 21-day-old fetuses (Nagel and Nagel, 1987) might help explain some of the present data. The maturation of early progenitors begins between fetal days 18 and 19 and is followed, 24 hours later, by CFUe maturation.
There is then a sharp decrease in the number of CFUe in the erythroid population, while the proportion of mature erythroblasts is considerably enhanced. These changes between days 20 and 21 might be due to inhibition of CFUe proliferation. But it is difficult to identify the cell population producing the $25-35 \times 10^3 M_f$ factor. Granulocytes that release regulators depressing CFU-Gm in humans (Boyum et al., 1976; Aardal et al., 1977; Stryckmans et al., 1977; Broxmeyer et al., 1978; Moberg et al., 1978; Philip et al., 1981; Helgestad et al., 1988) and in rat (Jovićić and Stojanović, 1988), as well as monocytes and stem cells, might be implicated. Natural suppressor cells, whose effects on hematopoiesis remain unknown, occur in the fetal liver, neonatal spleen and adult bone marrow, all of which are the dominant sites of hematopoiesis at one stage of development (Sadelain et al., 1990). At least two cell types could produce inhibitory activity: one related to monocytes, and the other to low density non-adherent cells.

A variety of molecules are known to inhibit hematopoiesis (Reviewed by: Axelrad, 1990; Broxmeyer et al., 1991; Paukovits et al., 1991), but it is not known whether some act directly on hematopoietic cells or whether they induce other effector molecules in accessory cell populations (Haworth, 1989). One, transforming growth factor beta (TGFβ), is an almost ubiquitous molecule of $25 \times 10^3 M_f$ that, directly, rapidly and reversibly inhibits the cycling early cell types in hematopoiesis. The sensitivity of hematopoietic progenitors to TGFβ may depend on their differentiation stage, even in the same lineage, and its direct effects may be modified by humoral factors released from other cell populations in response to TGFβ (Hino et al., 1988). The Vienna Bergen hemogenic regulatory peptide (HP) from normal rat marrow and mature human granulocytes rapidly and reversibly inhibits CFU-Gm colony formation, but not that of BFUe and CFUe. Negative regulatory protein (NRP) (79 $\times$ 10$^3$ $M_f$) rapidly (< 20 min) and reversibly reduces the proportion of murine marrow BFUe synthesizing DNA, but does not appear to affect CFUe or CFU-Gm. Inhibin, a $32 \times 10^3 M_f$ glycoprotein hormone that is a member of the TGFβ supergene family, suppresses in vitro erythroid colony formation by human marrow BFUe and CFUe. The bovine marrow tetrapeptide acs.d.KP inhibits the in vitro growth of human progenitors BFUe, CFUe and CFU-Gm, but the inhibition never exceeds 50%: it does not affect cycling murine cells. Its main effect is to markedly reduce the percentage of cells in DNA synthesis (Broxmeyer et al., 1991; Paukovits et al., 1991). Lastly, macrophage-derived tumor necrosis factor alpha (TNFa) inhibits CFUe, BFUe and CFU-Gm colony formation in vitro (Broxmeyer et al., 1986; Akahane et al., 1987; Roodman et al., 1987). Means et al., (1990) have shown that inhibition of human CFUe by TNF requires accessory cells that are neither adherent cells (monocytes) nor T lymphocytes. Thymic suppressor activity (Harada et al., 1985; La Russa et al., 1989) and interleukin-1-a, a macrophage-derived cytokine acting through induction of TNFa (Johnson et al., 1989, 1991), also inhibit CFUe.

The $25-35 \times 10^3 M_f$ factor described here has some effects similar to those of several hematopoietic regulators, but does not appear to be identical to any of them. Further experiments are required to identify the $25-35 \times 10^3 M_f$ factor and the way in which it inhibits CFUe.

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