Effect of Erythropoietin on DNA Synthesis by Erythroblasts in Vitro

By EDOUD R. POWSNER AND LAWRENCE BEMAN

ALTHOUGH erythropoietin is considered to cause an increased proliferation of erythroblasts from their stem cell precursors in vivo,7,8 its mode of action in vitro is not well established. Among the various reported effects of erythropoietin on bone marrow cultures, comparatively well confirmed are its effect on erythroblast maturation, such as increased heme synthesis, measured either as iron or glycine incorporation in isolated heme,8,11,17,22,23 increased incorporation of iron into intact bone marrow cells,10-12,16,23 and, more recently, increased stromal incorporation of glucosamine8 and increased actinomycin-inhibitable RNA synthesis15,18 in rat bone marrow cells. Less well confirmed are erythropoietin effects on cell proliferation in marrow cultures. On the one hand, various workers have reported an increased proliferative index in erythroblasts,19 an increase in the relative numbers of erythroblasts13,24 and an increase of formate incorporation into nucleic acids of bone marrow cells,16 or, more specifically, into DNA-thymidine isolated from the bone marrow cells.20 On the other hand, others have failed to find either increased formate12-27 or thymidine18-27 incorporation into DNA of bone marrow cells or increased uptake of these compounds by normoblast nuclei as measured autoradiographically.7 In this report we present data obtained by autoradiographic methods to show that erythropoietin causes an increase of DNA synthesis by erythroblasts in vitro.

METHODS

Rat marrow was washed from bone with sterile culture medium, TC109, containing 50 units of penicillin and 10 units of heparin per milliliter. The marrow was diluted with the same medium to a final concentration of 3000 to 6000 cells/mm.3. The incubation mixture was slightly modified from that reported by Goldwasser's group.14,17 To half the cultures, ESF* was added in final concentration of 0.2 units/ml. The remaining control cultures were identical except for omission of ESF. Thymidine 3H (methyl tagged), specific activity

*Lot K103-214A prepared by Armour Laboratories from anemic sheep plasma.
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700 Cur/mole, was added to a final concentration of 0.33 μc/ml. Incubation was 37 C. for 24 hours in small petri dishes. Air containing 5 per cent carbon dioxide was saturated with water vapor and allowed to flow continuously through the incubator. Following incubation, smears were prepared and fixed in methyl alcohol. Cell counts were determined before and after incubation. Autoradiographs were prepared by dipping in NTB3 and were stained with Giemsa at pH 5.75. Erythroblasts were classified according to grain count; in a few cultures grains were counted over nuclei of other cells also. The mean background was about 0.1 grain/nucleus; cells with two grains or more were considered labeled. Computations included the fraction of intact erythroblasts which were labeled and the average number of grains per labeled erythroblast. Heme synthesis, measured as radioactivity in isolated hemin, was determined in parallel cultures, identical except for the use of 59Fe (ferrous) salts (0.06 μg./ml., 5-20 mc./mg.) in place of the thymidine.

RESULTS AND DISCUSSION

The effects of ESF on both heme and DNA synthesis are summarized in the accompanying tables. Heme synthesis in these cultures is increased by the addition of ESF to the cultures (Table 1). Over the 24-hour period of incubation, the amount of heme synthesized in ESF-stimulated cultures is nearly twice that synthesized in the control cultures. For example, in culture V148, 0.94 nanomole of heme was synthesized per millimeter with ESF compared to 0.56 nanomole in the control culture. This culture contained approximately 1670 erythroblasts/mm. The heme synthesis per erythroblast was 0.56 femtomoles for the stimulated cultures and 0.34 femtomoles for the control culture. These are 47 and 28 per cent, respectively, of the heme content of the rat red blood cell. The average value reported previously by us for human marrow cultured without ESF is 0.33 femtomoles of 14C labeled heme per erythroblast. The increase in heme synthesis in the presence of ESF confirms that previously reported.

Total DNA synthesis by erythroblasts of ESF-stimulated cultures is nearly double that in control cultures (Table 1). Assuming DNA synthesis is proportional to nuclear thymidine 1H incorporation, in any given experiment, DNA synthesis is proportional to the product: grains per labeled erythroblast times the fraction of erythroblasts labeled times the total number of erythroblasts. The fraction of erythroblast nuclei labeled was nearly twice as great in stimulated as in control cultures. The number of grains per labeled erythroblast (Table 1) and the total number of erythroblasts was affected little, if at all, by the addition of ESF (Table 2). Our interpretation of these data is that ESF caused more erythroblasts to enter into DNA synthesis, but that the amount thereof per synthesizing cell was not affected.

Preliminary results with four cultures indicate that both DNA and heme synthesis are greater in ESF-stimulated cultures than in control cultures as early as 5 to 8 hours after the start of incubation. Thus the mean heme 59Fe radioactivity expressed as a fraction of the 24-hour control value in cultures sampled between 5 and 8 hours was 50 and 72 per cent for control and ESF.

*Calculation of heme synthesized assumes uniform labeling of iron by addition of 59Fe. If the added, labeled iron is used by the cells to the exclusion of endogenous iron, the results given here must be divided by 20, the ratio of the total to the added iron in this culture.
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Table 1.—Effect of Erythropoietin in Vitro

<table>
<thead>
<tr>
<th>Culture number</th>
<th>Heme synthesis</th>
<th>Per cent labeled</th>
<th>DNA synthesis in erythroblasts (Nuclear labeling by thymidine &quot;H&quot;)</th>
<th>Grains per labeled nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/he radioactivity as % of control</td>
<td>Control</td>
<td>ESF+</td>
<td>Relative labeling (% of control)</td>
</tr>
<tr>
<td>V125</td>
<td>-</td>
<td>21.8</td>
<td>41.4</td>
<td>190</td>
</tr>
<tr>
<td>V126</td>
<td>-</td>
<td>26.9</td>
<td>61.2</td>
<td>229</td>
</tr>
<tr>
<td>V127</td>
<td>159</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V138</td>
<td>256</td>
<td>16.2</td>
<td>34.0</td>
<td>210</td>
</tr>
<tr>
<td>V140</td>
<td>219</td>
<td>25.4</td>
<td>67.4</td>
<td>265</td>
</tr>
<tr>
<td>V142</td>
<td>140</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>V143</td>
<td>189</td>
<td>28.9</td>
<td>49.6</td>
<td>172</td>
</tr>
<tr>
<td>V146</td>
<td>175</td>
<td>29.5</td>
<td>53.5</td>
<td>182</td>
</tr>
<tr>
<td>V148</td>
<td>165</td>
<td>43.5</td>
<td>58.0</td>
<td>133</td>
</tr>
<tr>
<td>Mean</td>
<td>186 ± 15‡</td>
<td>27.4</td>
<td>52.1</td>
<td>197 ± 16‡</td>
</tr>
</tbody>
</table>

*Each number in this and subsequent tables designates the use of a marrow cell suspension prepared by mixing the material obtained from one or more rats.

‡± standard error of mean.

Table 2.—Effect of ESF on the Total Number of Erythroblasts in Vitro*

<table>
<thead>
<tr>
<th>Culture number</th>
<th>Number of erythroblasts before incubation (cells/mm.3)</th>
<th>Number of erythroblasts after 24 hours (cells/mm.3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V165</td>
<td>1675</td>
<td>1390</td>
</tr>
<tr>
<td>V168</td>
<td>1700</td>
<td>1881</td>
</tr>
<tr>
<td>V169</td>
<td>1079</td>
<td>1002</td>
</tr>
<tr>
<td>V170</td>
<td>1260</td>
<td>595</td>
</tr>
<tr>
<td>V176</td>
<td>1430</td>
<td>1806</td>
</tr>
<tr>
<td>V181</td>
<td>1270</td>
<td>2209</td>
</tr>
<tr>
<td>Mean‡</td>
<td>1402 ± 320</td>
<td>1514 ± 246</td>
</tr>
</tbody>
</table>

*Total number of erythroblasts of all classes calculated from the total nucleated cell count, obtained by electronic counting of an aliquot of the culture, and from the fraction of cells which are erythroblasts in stained smears of the same culture.

‡± standard error of mean.

cultures, respectively. For the same cultures, the 24-hour mean values were 100 and 196 per cent. Corresponding figures for DNA synthesis were 24 and 47 per cent in control and ESF cultures, respectively, in the 5 to 8 hours interval and 100 and 160 per cent at 24 hours. No significant difference between ESF and control cultures was observed in the samples incubated for shorter intervals.

The effect of ESF on DNA synthesis appears to be relatively specific for erythroblasts. The effect on DNA synthesis by granulocytes and lymphocytes was comparatively small (Table 3).

The effect of incubation on the various cells of human bone marrow has been presented in detail previously.* In review, there is a progressive maturation of the erythroblasts. The proerythroblasts and less mature cells initially amount to less than 5 per cent of the erythroblasts and fall to zero at 24 hours. During the incubation the total number of erythroblasts remains approximately
Table 3.—Comparison of the Effect of ESF on DNA Synthesis by Erythroblasts and Leukocytes in Vitro

<table>
<thead>
<tr>
<th>Culture number</th>
<th>Cell type</th>
<th>Control</th>
<th>ESF</th>
<th>Erythroblasts</th>
<th>Granulocytes</th>
<th>Lymphocytes</th>
<th>Control</th>
<th>ESF</th>
</tr>
</thead>
<tbody>
<tr>
<td>V126</td>
<td>Erythroblasts</td>
<td>27</td>
<td>61</td>
<td>229</td>
<td>100</td>
<td>143</td>
<td>7.7</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Granulocytes</td>
<td>14</td>
<td>14</td>
<td>5.6</td>
<td>7.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>21</td>
<td>30</td>
<td>9.8</td>
<td>6.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V143</td>
<td>Erythroblasts</td>
<td>29</td>
<td>50</td>
<td>172</td>
<td>2.4</td>
<td>2.3</td>
<td>8.9</td>
<td>8.7</td>
</tr>
<tr>
<td></td>
<td>Granulocytes</td>
<td>14</td>
<td>16</td>
<td>114</td>
<td>2.4</td>
<td>2.3</td>
<td>2.4</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>36</td>
<td>33</td>
<td>92</td>
<td>10.3</td>
<td>7.5</td>
<td>7.5</td>
<td>10.3</td>
</tr>
<tr>
<td>V146</td>
<td>Erythroblasts</td>
<td>30</td>
<td>54</td>
<td>182</td>
<td>5.8</td>
<td>7.0</td>
<td>5.8</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>Granulocytes</td>
<td>16</td>
<td>17</td>
<td>5.4</td>
<td>5.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>32</td>
<td>33</td>
<td>103</td>
<td>8.6</td>
<td>7.8</td>
<td>7.8</td>
<td>8.6</td>
</tr>
<tr>
<td>V148</td>
<td>Erythroblasts</td>
<td>44</td>
<td>58</td>
<td>133</td>
<td>16.1</td>
<td>14.2</td>
<td>16.1</td>
<td>14.2</td>
</tr>
<tr>
<td></td>
<td>Granulocytes</td>
<td>14</td>
<td>19</td>
<td>135</td>
<td>7.8</td>
<td>7.6</td>
<td>7.8</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td>Lymphocytes</td>
<td>62</td>
<td>61</td>
<td>98</td>
<td>12.0</td>
<td>5.5</td>
<td>98</td>
<td>12.0</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>179</td>
<td>114</td>
<td>109</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Incorporation of thymidine *H measured in autoradiographs prepared from smears of incubated marrow cells.
0.2 units/ml.

constant. The granulocytes also show a progressive maturation, but there is a marked fall in the total number during incubation. Data obtained in the experiments described here are similar. Thus, in rat marrow there is also a progressive maturation of the erythroblast and granulocyte population during incubation. The total number of erythroblasts remains constant while the total number of granulocytes falls. One exception to this general similarity between the two types of marrow during incubation is that in rat marrow the most mature erythroblasts observed had polychromatic cytoplasm (Fig. 1). In contrast, cells with orthochromic cytoplasm predominated after incubation of human marrow. The differential counts of smears of the two samples of rat marrow of a single culture, one prior to and the other following incubation, for 24 hours are given in Table 4. Damaged cells and nuclei which could not be otherwise identified are listed separately. These are of significance only if they include a disproportionate number of immature cells or "stem" cells of functional importance during the period of incubation. We have not found any way to determine the nature of these damaged cells but the evidence that is available suggests that the cells which are not damaged are in fact representative of the population as a whole. For example, Burke and Harris, after selecting the diluent and mixing procedure which produced the smallest number of damaged cells, obtained differential counts which, although subdivided differently, are similar to the initial one given here except that they report fewer lymphocytes and no unidentified cells. Likewise, the total fraction of pronormoblasts or less mature cells in rat marrow observed by Aschkenasy are 5 per cent, and by Cameron and Watson are 2.4 per cent.

Analysis of the effect of ESF on the separate stages in maturation of erythro-
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Fig. 1—Labeled polychromic erythroblast. The focus is adjusted for the grains, on the right, and for the cell, on the left. Smear prepared from a culture V 146 incubated for 24 hours with thymidine H.£.
Table 4.—Differential Cell Count of Rat Bone Marrow before and after Incubation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Before incubation</th>
<th>After 24 hour incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>cells/mm.³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythroblasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proerythroblasts</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Basophilic erythroblasts</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Polychromic erythroblasts</td>
<td>20</td>
<td>640 35</td>
</tr>
<tr>
<td>Granulocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Myeloblasts</td>
<td>occ.</td>
<td></td>
</tr>
<tr>
<td>Progranulocytes</td>
<td>1</td>
<td>30</td>
</tr>
<tr>
<td>Neutrophils</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Promyelocytes</td>
<td>2</td>
<td>60</td>
</tr>
<tr>
<td>Myelocytes</td>
<td>4</td>
<td>130</td>
</tr>
<tr>
<td>Metamyelocytes</td>
<td>5</td>
<td>160 30</td>
</tr>
<tr>
<td>Band form</td>
<td>8</td>
<td>290 1</td>
</tr>
<tr>
<td>Polymorphonuclear</td>
<td>2</td>
<td>60 8</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>4</td>
<td>130 1</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocytes</td>
<td>2</td>
<td>60 1</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>15</td>
<td>480 5</td>
</tr>
<tr>
<td>Plasma cells</td>
<td>occ.</td>
<td></td>
</tr>
<tr>
<td>Unidentified*</td>
<td>33</td>
<td>1060 48</td>
</tr>
<tr>
<td>Total</td>
<td>100</td>
<td>3220 100</td>
</tr>
</tbody>
</table>

*Largely damaged cells or nuclei.
1Occ. = occasional signifies 0.1 to 0.5%; a blank signifies less than 0.1%.
2Total nucleated cell count (Coulter counter) times the fractional number in each category seen in stained smears prepared at the same time.

blasts was not attempted. Any cell which was within the erythroblast compartment at the end of the experiment has been included. The reason for this approach is that even initially the immature cells, proerythroblasts or less mature cells, number less than 5 per cent of the erythroblasts. The greatest possible influence of these few cells can be seen by making the extreme assumptions that they are the only cells in which DNA synthesis is affected by ESF, that in the absence of ESF they synthesize no DNA and that in the presence of ESF they synthesize DNA and that each divides to form two labeled cells. If so, they could account for less than a 10 per cent increase in the number of DNA-synthesizing cells. In contrast, the observed increase was as high as 42 per cent (from 25.4 to 67.4 per cent labeling, culture V140, Table 1). From this it is apparent that whatever the relative response of proerythroblasts or their precursors, the more mature cells which make up the bulk, both of those present and of those labeled, must participate substantially in the increased DNA synthesis induced by ESF.

Erslev has reported that ESF causes an increase in thymidine H and formate C uptake by rabbit marrow in vitro. It is not clear whether the comparatively small increase he noted is due to the measurement of radioactivity of nucleoprotein prepared from all cells of the marrow as contrasted
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Necheles and co-workers, in a report to be published, have also described an increase in DNA synthesis by human erythroblasts in vitro. They observed a 276 per cent increase in $^{14}C$ incorporation into nucleic acid as well as an increased percentage of labeled orthochromic erythroblasts in the presence of ESF.

The failure of Thomas et al.\textsuperscript{27} to find an increase in formate or thymidine incorporation may be due to differences in the culture technics. This is suggested by their concomitant failure to find the increase in heme synthesis, since reported by us and others.\textsuperscript{11,17,22,23} Similarly, the failure of Alpen et al.\textsuperscript{2} to detect, in autoradiographs, any effect on DNA synthesis must be considered with their failure to detect a stimulation of cellular iron uptake which is widely reported.\textsuperscript{10,11,12,16,20} Again there are numerous differences in technic, among which is that we used purified sheep plasma erythropoietin while Alpen et al.\textsuperscript{2} used urinary erythropoietin. We have previously shown that at least one urinary erythropoietin is inhibitory in vitro.\textsuperscript{22} It is more difficult to reconcile the failure of Goldwasser and associates\textsuperscript{18} to observe an increase of thymidine incorporation in their marrow cultures between one-half and 9½ hours because their cultures showed an increase in heme synthesis. There is an important difference between their analysis and ours. Our autoradiographic method permits detection of biochemical events in a relatively small proportion of the cells and may be more sensitive than radiochemical analysis of DNA isolated from the entire culture.

The relationship between the in vivo and the in vitro effects of ESF are not established by these experiments. The in vivo effect, as mentioned initially, appears to be primarily on the so-called stem cell; the in vitro effects reported here are observed in the relatively mature erythroblast. In this connection it is worth noting that the stimulation of iron incorporation by ESF, an effect which we have categorized as an indication of erythroblast maturation, has been given another interpretation by Erslev.\textsuperscript{12} From his experiments with rabbit marrow cultured in vitro, he concluded that erythropoietin acts primarily on the “stem cell pool” as opposed to the recognizable normoblasts. In these experiments, he noted a greater relative effect of erythropoietin on marrow from polycytemic rabbits than on marrow from normal rabbits, and very little effect on marrow from rabbits made anemic by bleeding. In other words, the magnitude of the relative ESF effect is in inverse relationship to the relative number of normoblasts in the marrow. The bone marrow of polycytemic mice is well known for its sensitivity to ESF in vivo, and the experiments of Erslev seem to indicate that it is also relatively more sensitive in vitro. This difference in sensitivity of various marrows was not observed in his experiments when cellular thymidine incorporation rather than cellular iron uptake was measured; the relative uptake of thymidine $^{3}H$ by cells of the marrow from all three types of animals was affected by ESF to an approximately equal extent. An in vitro effect of ESF on stem cells does not seem unreasonable, but the
data of our experiments offer no confirmation. Thus, we have no comparable data on hemoglobin iron incorporation or nuclear incorporation of thymidine by erythroblasts from anemic or polycythemic marrow. On the other hand, it is clear from our results that whatever the role of the few stem cells in cultures described here, the major effects must be upon the recognizable, mature erythroblasts. Specifically, ESF stimulates nuclear incorporation of thymidine \(^{3}H\) in cells which are polychromatic at 24 hours. This increase, as we pointed out above, could not have resulted from action of ESF on "stem cells" or immature erythroblasts alone even if they constituted as many as 5 per cent of the number of erythroblasts, if all of them were stimulated to begin DNA synthesis by ESF and if doubling of the number of these cells by cell division were followed by their differentiation into polychromatic erythroblasts within the 24-hour period.

**Conclusions**

The tentative interpretations about the action of erythropoietin in vitro, based on consideration of our present studies, are (1) that erythropoietin increases heme synthesis in bone marrow cultures as a whole, and probably in individual erythroblasts as well, and (2) that erythropoietin induces DNA synthesis by erythroblasts. On the basis of these limited data we cannot make a statement about the relation between these two effects.

**SUMMARIO IN INTERLINGUA**

Le tentative interpretation del action de erythropoietina in vitro, a base (IC nostre hic-presentate studios, es (1) que erythropoietina augmenta le synthese de heme in culturas de medulla ossee in general e, probablemente, etiam in erythroblastos individual e (2) que erythropoietina induce le synthese de acido deoxyribonucleic per erythroblastos. A base de iste restringite datos nos non pote pronunciar nos quanto al relation inter le duo mentionate effectos.

**ACKNOWLEDGMENTS**

Illustrations were prepared by the Medical Illustration Service, Veterans Administration Hospital, Dearborn, Michigan. The authors wish to thank Mrs. Helen Seitz and Mrs. Irene Muzquiz for their assistance.

**REFERENCES**

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