Effects of erythropoietin
on cell populations and macromolecular syntheses
in foetal mouse erythroid cells

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SUMMARY

The effects of erythropoietin on maturation of erythroid cells were studied in short-term cultures of foetal mouse liver. Erythropoietin-treated cultures had about 50% more cells than untreated cultures after 24 h. The increase occurred in basophilic, polychromatic and orthochromatic erythroblasts as well as in reticulocytes. A striking feature of erythropoietin-treated cultures was the formation of macro-erythroblasts and macrocytes. Autoradiographic studies indicated that a maturation division was omitted in the formation of these cells and that macrocytes might be derived directly from polychromatic erythroblasts or earlier stages. These studies also indicated that there might be no more than three divisions during the normal development of orthochromatic erythroblasts from proerythroblasts in vitro. The mitotic index of proerythroblasts was raised in erythropoietin-treated cultures within 1–2 h. Moreover, erythropoietin caused a marked elevation of DNA synthesis in proerythroblasts within the first hour of culture but effects on DNA synthesis in other cell compartments were not pronounced. In contrast, an increase in RNA synthesis was noted in proerythroblasts and basophilic and polychromatic erythroblasts; it was greatest in the two former compartments. The stimulation was noticeable, especially in the basophilic compartment, within 2 h but increased progressively throughout the first 5–6 h. Erythropoietin did not induce an increase in total protein synthesis in cells of the normal series but macro-erythroblasts exhibited proportionately more grains.

INTRODUCTION

In the whole animal the main effect of erythropoietin may be to stimulate maturation from the erythropoietin-responsive cell (ERC) (Filmanowicz & Gurney, 1961) but there is also evidence that it accelerates maturation of recognizable erythroid cells (Blackett, 1968; Borsook et al. 1968). The maturation of recognizable erythroblast cells can be studied in vitro and erythropoietic cells in culture can be stimulated by erythropoietin to make haemoglobin (and also DNA and RNA) at a faster rate (Krantz, Gallien-Lartigue & Goldwasser, 1963; Paul & Hunter, 1969). Moreover, erythropoietin stimulation, when erythroblasts are undergoing maturation, results in the formation of macrocytes (Borsook et al. 1968; Brecher & Stohlman, 1962). The present report attempts to clarify the effects of erythropoietin on the committed erythroblast

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population by investigating proerythroblast proliferation, erythroblast maturation and the origin of macrocytic cells in cultured mouse foetal erythropoietic tissue.

**MATERIALS AND METHODS**

**Sources of materials**

All radioisotopes were obtained from the Radiochemical Centre, Amersham, Bucks. [Methyl-\(^3\)H]thymidine was obtained at a specific activity of 22–23 Ci/mmole, [5-\(^3\)H]uridine at a specific activity of 5 Ci/mmole and [4,5-\(^3\)H]leucine at 29-8 Ci/mmole. Colchicine (Baird and Tatlock Ltd., London) was used at a concentration of 10\(^{-4}\) M. Two batches of erythropoietin were employed. A batch of erythropoietin (Step 3, Lot K 147048, National Blood Resources Program of the U.S. Heart Institute, specific activity of 2-70 u./mg), derived from plasma of phenylhydrazine-treated sheep, was used at a concentration of 0-2 units per ml of medium containing 0-5 to 1-0 \times 10^6 cells. A batch of human urinary erythropoietin (A-1-TaLSL, Haematology Research Laboratories, Children’s Hospital of Los Angeles, specific activity 26-4 u./mg) was used in the majority of the experiments and gave a stimulation of haem synthesis of 240 % in 24 h when used at a concentration of 0-6 units per ml. Cells were suspended in Waymouth’s MB 752/1 medium containing 10 % foetal bovine serum (Flow Laboratories, Irvine, Scotland). Chick embryo extract (EE 100) was obtained from Difco Laboratories, Surrey, England. Plasma was prepared from heparinized mouse blood obtained by cardiac puncture.

**Preparation of cultures for cytology and autoradiography**

For cytological experiments, cultures were made from single cell suspensions prepared from 13-5-day Swiss (Porton) mouse foetal livers as previously described (Cole & Paul, 1966), except that ferric chloride was omitted from the Waymouth’s medium. Unless stated otherwise, cultures were incubated in 5 % CO\(_2\) in air in screw-capped test-tubes containing approximately 10^6 cells in 1 ml of medium. In a few experiments the buffer used was 5 mm HEPES (N-2-hydroxyethylpiperazine-N’-ethanesulphonic acid) in which case the cultures were incubated in air at 36-5 °C. Cells were counted at the start and end of each experiment with a haemocytometer. Cell diameters were measured with a micrometer eyepiece. Preparations for cytology were made with a cytocentrifuge and stained with May–Grunwald Giemsa as previously described (Paul, Conkie & Freshney, 1969).

Cultures to be examined by autoradiography were pulsed for 1 h with 0-5 \(\mu\)Ci/ml of [\(^3\)H]thymidine or [\(^3\)H]uridine or 10 \(\mu\)Ci/ml [\(^3\)H]leucine. At the end of the labelling period samples of cell suspensions were centrifuged, without washing, on to serum-coated slides using a cytocentrifuge. After fixation in methanol they were extracted in ice-cold 0-2 N perchloric acid for 10 min and washed for 1 h in cold running water. The slides were coated with Kodak AR 10 stripping film and exposed at 4 °C for 2–20 days. They were then developed in
Erythropoietin and foetal mouse erythroid cells

Kodak D19 developer and fixed and washed. Finally, after air-drying, they were stained with May–Grünwald Giemsa at pH 5.75.

[^3H]Leucine incorporation into total foetal liver cell populations during a 1 h pulse with 5 \( \mu \)Ci/ml, was measured by suspending hot 0.2 N perchloric acid insoluble material in 0.5 ml 10 % sodium dodecyl sulphate, an aliquot of which was added to toluene-based scintillator containing 33 % Triton-X100 (Lennig and Co. Ltd., Croydon, Surrey) and counted in a Beckman LS-100 scintillation counter. Haem synthesis was measured by incorporation of \(^{59}\)Fe as previously described (Paul & Hunter, 1969).

Preparation of cultures for time-lapse cinemicrographic studies

Coverslips were prepared by sticking to each a nickel mask (Smethurst High-Light Ltd., Bolton, Lancs.) with numbered windows (Carter, 1967) with a 0.3 % solution of Formvar in ethylene dichloride. Cell suspensions were prepared as described above and plasma clot cultures were made by mixing equal amounts of cell suspensions, embryo extract and mouse plasma in a Petri dish. A small aliquot of the mixture was then transferred to a coverslip carrying a nickel mask and spread out. As soon as clotting had occurred the coverslip preparation was inverted onto a slide tissue culture chamber (Sterilin Ltd., Richmond, Surrey) containing approximately 0.18 ml of Waymouth’s culture medium. The edges of the coverslip were then sealed with paraffin. Observations were made at 37 °C using a Zeiss phase-contrast microscope with a 16 mm reflex Bolex camera. After incubation and filming, cultures were fixed by adding methanol (NMR, B.D.H.) very gently, drop by drop. Each preparation was then air-dried and stained with Jenner–Giemsa. The mask facilitated subsequent identification and examination of the field, which had been followed by cinemicrography and made it possible to identify with certainty the cell types which had resulted from events observed.

RESULTS

Changes in cell populations during culture of 13.5-day mouse foetal liver cells

The change in the distribution of the major groups of cells during culture is shown in Table 1. Most of the hepatocytes disappear after 24 h in vitro. The ‘others’ category includes nucleated erythrocytes, derived from the yolk sac, smear cells and macrophages. None of these increases during the experimental period.

After 5 days without erythropoietin only orthochromatic erythroblasts and reticulocytes are found, together with macrophages and some fibroblasts.

Table 2 shows total cell numbers after 24 h of culture with or without erythropoietin. The apparent proliferation due to erythropoietin is certainly underestimated because in culture there is some cell loss due to death of hepatocytes and fragility of non-nucleated cells.

Fig. 1 shows the numbers of different kinds of erythroblasts relative to
Table 1. Distribution of 13·5-day mouse foetal liver cells in vitro

<table>
<thead>
<tr>
<th>Duration of culture (h)</th>
<th>Erythropoietic cells (%)</th>
<th>Hepatocytes (%)</th>
<th>Others (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>73</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>24</td>
<td>86</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>24 + erythropoietin*</td>
<td>90</td>
<td>2</td>
<td>8</td>
</tr>
</tbody>
</table>

Each set of figures is the mean of 200 cells classified from each of five slides.
* Urinary erythropoietin; 0·6 u./ml.

Table 2. Mean and standard deviation of cell counts after 24 h in vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h cell count</th>
<th>Apparent proliferation due to the effect of erythropoietin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>88 (2)</td>
<td>—</td>
</tr>
<tr>
<td>0·2 u./ml step III sheep erythropoietin</td>
<td>131 (2)</td>
<td>148 %</td>
</tr>
<tr>
<td>Nil</td>
<td>96 ± 9 (7)</td>
<td>—</td>
</tr>
<tr>
<td>0·6 u./ml human urinary erythropoietin</td>
<td>143 ± 12 (7)</td>
<td>149 %</td>
</tr>
</tbody>
</table>

Results are expressed as a percentage of the initial cell count. Figures in parentheses represent the number of estimates made.

a representative sample of 100 cells in the original (1 h) population. Atypical cells were classified within the compartment which they most closely resembled as judged by the nucleus to cytoplasm ratio and the degree of haemoglobinization.

When erythropoietin is present, proerythroblasts diminish by 40 % while basophilic erythroblasts increase by 50 % during 24 h of culture. Without erythropoietin there is no change in the proerythroblast compartment whilst the level of basophilic erythroblasts is reduced by 25 %. In the presence of erythropoietin, polychromatic cells increase by 140 % while orthochromatic cells increase by 230 %. In erythropoietin-treated cultures many of the orthochromatic cells and some of the polychromatic cells are atypical and are classified as ‘macro-erythroblasts’ (see below). Without erythropoietin, there is little change in the numbers of polychromatic cells while orthochromatic cells increase by 50 %. In both cultures at 24 h there is a reduction in the numbers of reticulocytes, suggesting that the actual production of anucleate cells is concealed by lysis of fragile cells.

Hormone-induced atypical cell classes

Atypical nucleated erythroid cells will be referred to as macro-erythroblasts and atypical non-nucleated cells will be called macrocytes; they probably corre-
Erythropoietin and foetal mouse erythroid cells

Fig. 1. Population changes in cultured erythropoietic cells. The mean cell number and standard deviation (indicated by bars) of each cell type at 24 h, with or without erythropoietin, was calculated from the differential count and the cell number per ml. Findings from 12 experiments were used. Typical cells are indicated by open columns above the line, atypical cells (macro-erythroblasts and macrocytes) by cross-hatched columns below the line. Pr, Proerythroblasts; B, basophilic erythroblasts; Po, polychromatic cells; O, orthochromatic cells; R, reticulocytes.

spond to the line 2 cells of Borsook et al. (1968) and Lord (1968). The least mature macro-erythroblasts are identified as polychromatic macro-erythroblasts; characteristically they vary in size. They are as large as basophilic erythroblasts or proerythroblasts (10–15 μm in diameter) but the nucleus-to-cytoplasm ratio is similar to that in polychromatic erythroblasts and the cytoplasm is polychromatic. Chromatin condensation is not as advanced as in polychromatic erythroblasts.

Orthochromatic macro-erythroblasts are up to 13 μm in diameter. The nucleus-to-cytoplasm ratio is similar to that in orthochromatic erythroblasts and the cytoplasm is well haemoglobinized with occasional vacuoles. The nucleus is often eccentric or partially extruded, and shows some condensation of chromatin, but rarely to the extent seen in orthochromatic erythroblasts.

Hence, compared with normal (line 1) cells, there appears to be an asynchronous maturation of nucleus and cytoplasm in macro-erythroblasts, similar to that seen in macrocytic anaemia. This criterion allows a distinction to be made between orthochromatic macro-erythroblasts and the very similar, although smaller, orthochromatic erythroblasts, and between orthochromatic
macro-erythroblasts and the similar-sized, but more strongly eosinophilic and highly pyknotic, nucleated erythrocytes from the yolk sac. After nuclear extrusion the orthochromatic macro-erythroblast becomes a macrocyte of up to 12 \( \mu \text{m} \) in diameter. These cells, apart from their greater size, resemble reticulocytes very closely. Electron-microscopic observations reveal numerous polysomes and a profusion of less-dense haemoglobin particles. There is no trace of nuclear remnants but there are increased numbers of mitochondria and micro-pinocytotic vesicles compared with normal reticulocytes.

A Price-Jones curve of cell diameters in the orthochromatic compartment was prepared by making micrometer measurements. The results are shown in Fig. 2. Twenty-four-hour control cultures show a 10 \( \mu \text{m} \) mode (mean diameter 9.4 \( \mu \text{m} \)) and only a few cells of diameter greater than 11 \( \mu \text{m} \). Erythropoietin-treated cultures have a biphasic frequency distribution with modes at 10 and 13 \( \mu \text{m} \). This indicates the existence of two distinct populations of orthochromatic cells.

Further information was obtained from autoradiographs of cultures labelled with \([^{3}H]\)thymidine. One ml aliquots of a single cell suspension of 13.5-day mouse foetal liver in Waymouth’s medium were pulsed with 10 \( \mu \text{Ci} \) of the isotope for 30 min. The cells were centrifuged, washed in balanced salt solution and resuspended in fresh medium, without isotope. They were incubated at 37 °C in 5 \% \( \text{CO}_{2} \) in air for a further 24 h either with or without erythropoietin. In each culture the diameters of labelled and unlabelled orthochromatic cells were measured in stained preparations. The results are shown in Table 3. Erythro-
Table 3. Diameters of labelled and unlabelled orthochromatic cells
24 h after a 30 min [methyl-3H]thymidine pulse

<table>
<thead>
<tr>
<th>Urinary erythropoietin concentration</th>
<th>Nil</th>
<th>0.6 units/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labelled cells (%)</td>
<td>14</td>
<td>42</td>
</tr>
<tr>
<td>(mean and standard deviation)</td>
<td>13.0 ± 0.9</td>
<td>12.1 ± 1.8</td>
</tr>
<tr>
<td>Unlabelled cells (%)</td>
<td>86</td>
<td>58</td>
</tr>
<tr>
<td>(mean and standard deviation)</td>
<td>10.1 ± 1.2</td>
<td>9.7 ± 1.5</td>
</tr>
</tbody>
</table>

The diameters of 200 orthochromatic cells were measured and scored as labelled or unlabelled in each culture.

Table 4. The production of macro-erythroblastic cell types from 13.5-day mouse foetal liver cell suspension after 24 h of culture with sheep step III or human urinary erythropoietin

Results are expressed as % of total cells.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Erythropoietin concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nil</td>
</tr>
<tr>
<td>Polychromatic macro-erythroblasts (%)</td>
<td>2</td>
</tr>
<tr>
<td>Orthochromatic macro-erythroblasts (%)</td>
<td>4</td>
</tr>
<tr>
<td>Macrocyes (%)</td>
<td>3</td>
</tr>
</tbody>
</table>

poietin-treated cultures had three times as many labelled orthochromatic cells as control cultures, and the labelled cells had a mean diameter similar to that of orthochromatic macro-erythroblasts. The mean diameter of unlabelled cells shows that these were predominantly orthochromatic erythroblasts.

The properties of macro-erythroblasts and macrocytes are unaffected by the source of the erythropoietin, as shown in Table 4. Moreover, in a modified Waymouth's medium containing 0.15 μM vitamin B12, 8 μM folic acid, 0.8 μM folinate and 0.01 mM ferric chloride (alone, or bound to homologous transferrin) there was no reduction in the numbers of erythropoietin-induced macro-erythroblasts formed after 24 h of culture.

Origin of erythropoietin-induced macro-erythroblasts and macrocytes

After 24 h of incubation with erythropoietin a 1 h [3H]thymidine pulse (5 μCi/ml) does not result in labelling of either orthochromatic erythroblasts or macro-erythroblasts (although all other erythroblasts become labelled). This finding is consistent with these cells, being non-dividing. However, in cultures
Table 5. Appearance of labelled DNA in orthochromatic erythroblasts and erythropoietin-induced erythroblasts 24 h after a short (0-5 h) preincubation with $[^3H]$thymidine

<table>
<thead>
<tr>
<th>Duration of culture (h)</th>
<th>Mean and standard error of the grain count per labelled cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pr</td>
</tr>
<tr>
<td>1 Control</td>
<td>53 ± 6-7</td>
</tr>
<tr>
<td>4 Erythropoietin</td>
<td>53 ± 5-1</td>
</tr>
<tr>
<td>6 Control</td>
<td>36 ± 4-2</td>
</tr>
<tr>
<td>24 Control</td>
<td>20 ± 5-3</td>
</tr>
<tr>
<td>48 Erythropoietin</td>
<td>20 ± 3-2</td>
</tr>
<tr>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>—</td>
</tr>
</tbody>
</table>

Mean and standard error of the pooled data from all cultures.

Number of labelled cells in parentheses.
—, Absence of cells.

* PoM, Polychromatic macro-erythroblasts; OM, orthochromatic macro-erythroblasts; other abbreviations as in Fig. 1.

given a $[^3H]$thymidine pulse 30 min before addition of erythropoietin the orthochromatic macro-erythroblasts appearing after 24 h of incubation are labelled. To determine the origin of these labelled erythroblasts, cultures, preincubated for 30 min with $[^3H]$thymidine were washed and incubated in fresh medium without isotopes for a further 48 h, either with or without erythropoietin. Samples were taken at 1, 4, 6, 24 and 48 h, from which autoradiographs were prepared. Grain counts were made to determine which cells might have given rise to macro-erythroblasts. From the results in Table 5 it can be seen that proerythroblasts incorporate more label than basophilic cells which, in turn, incorporate
Erythropoietin and foetal mouse erythroid cells

Incubation time after addition of 0.6 u/ml erythropoietin

Fig. 3. Distribution of grain counts in the proerythroblast compartment after 1, 4 and 6 h of culture with 0.6 units/ml urinary erythropoietin.

slightly more label than polychromatic erythroblasts. Orthochromatic erythroblasts are unlabelled in the first 6 h. The mean amount of isotope in basophilic erythroblasts and polychromatic erythroblasts does not change during the experiment, whereas in proerythroblasts the labelled DNA is progressively diluted out; on average only 38% of the grains per proerythroblast remain after 24 h of incubation while the labelling index remains at 90 to 100%. This is consistent with the proerythroblasts having undergone one or two divisions with random assortment of labelled DNA strands; it also suggests that there may be a prolongation of S phase, relative to other phases of the cell cycle.

The labelling indices in proerythroblast and basophilic erythroblast compartments are remarkably high, whether erythropoietin is present or not, following a 1 h pulse (Table 6). The implication is that almost all the cells in these compartments are capable of synthesizing DNA at the time of the pulse (making the usual assumption that [3H]thymidine incorporation diminishes greatly immediately after its removal from the medium). Hence, either the majority of the cells are in S phase or there is a large amount of unscheduled
DNA synthesis. If the cells are in S phase, presumably they have been synchronized by the culture procedure, are cycling very fast with negligible G1, G2 and M phases or S phase is prolonged relative to the other phases.

An examination of the distribution of grain counts in each cell compartment shows that in proerythroblasts (Fig. 3) there is a trend towards fewer grains per cell with increasing time in culture. The accumulation of cells with 1–10 grains per cell which is seen after 6 h in culture with erythropoietin is not apparent in the control cultures or in basophilic or polychromatic erythroblast compartments, either with or without the hormone. This suggests that erythropoietin specifically stimulates a higher rate of cell division in the proerythroblast compartment. The difference between the pooled grain counts of basophilic erythroblasts and polychromatic macro-erythroblasts in Table 5 is not significant (0.20 < P < 0.5) and the figures for polychromatic erythroblasts and orthochromatic macro-erythroblasts are very similar. On the other hand, when examined by Student’s t test, the differences between the grain counts in the polychromatic and orthochromatic macro-erythroblast compartments are found to be highly significant (P < 0.0025). These results therefore suggest that polychromatic macro-erythroblasts may be derived by division and maturation of proerythroblasts, or from basophilic erythroblasts, by maturation without division. Similarly, orthochromatic macro-erythroblasts may be derived by division and maturation of basophilic erythroblasts or polychromatic macro-erythroblasts, or they may arise directly from polychromatic erythroblasts by maturation without division. After 24 h in culture, virtually all the polychromatic macro-erythroblasts are derived from labelled precursors as are 31–46% of the orthochromatic erythroblasts and 60–75% of the orthochromatic macro-erythroblasts both in the presence and absence of urinary erythropoietin (results not shown). The fact that some of these cells are unlabelled suggests that they must arise from orthochromatic erythroblasts, from the unlabelled fraction of polychromatic erythroblasts or from the small fraction of basophilic erythroblasts and proerythroblasts not in S.

Further evidence that macro-erythroblasts may be derived by maturation without division was obtained from experiments in which cell division was blocked with $10^{-4}$ M colchicine (results not shown). During the first 8 h of culture with colchicine, both with and without erythropoietin, there was an accumulation of metaphase figures in immature cells resulting in 8% of the total erythropoietic population exhibiting metaphase figures. In cells cultured without colchicine only very few metaphase figures were seen. After 24 h of culture, macro-erythroblasts were present in cultures treated with erythropoietin and colchicine in the same proportion as in cultures treated with erythropoietin alone. The number of metaphase figures, mainly in immature cells, was then 5%. Although cells can escape into the next replicative cycle in the presence of colchicine, cell division is invariably retarded and yet the production of macro-erythroblasts in these experiments was unaffected. It may be noted that Paul &
Hunter (1968) reported that $10^{-4}$ M colchicine had no effect on the erythropoietin-induced stimulation of haemoglobin synthesis in the same experimental system pulsed after 7.5 h in culture.

**Effect of erythropoietin on mitosis in erythroid cells**

Foetal liver erythroid cells exhibit many mitotic figures during the first few hours of culture. The mitotic index then diminishes rapidly. In cultures treated with erythropoietin the number of mitoses recorded per standard microscopic field is increased by a factor of 1.5–2.5 (Fig. 4). Since it seemed unlikely that cells undergoing mitosis within 2 or 3 h in culture had also gone through a complete S phase in culture some experiments were performed in which the cells were pulse-labelled with $[^3H]$thymidine at the beginning of culture and then observed by time-lapse cinemicrography. These studies revealed that the cells
undergoing mitosis in the first few hours were unlabelled (whether treated with erythropoietin or not) whereas those undergoing mitosis later were. Accordingly it has to be assumed that the cells which underwent mitosis in the first few hours had completed DNA synthesis by the time they were put into culture. Erythropoietin apparently enables some of these cells to complete the cell cycle and enter mitosis; in its absence it must be assumed that some cells become arrested or delayed in G2 or S. Taken in conjunction with the evidence that DNA synthesis occurs in almost all immature cells in the culture it must be assumed that, even in the presence of erythropoietin, some cells which synthesize DNA in culture may not subsequently go through mitosis. This leads to the consideration that maturation may not be closely linked to cell division and that the macro-erythroblasts and macrocytes may originate from cells in which cellular material has doubled but cell division has not occurred.

The synthesis of macromolecules

An estimate of the rate of DNA synthesis can be obtained from autoradiographic grain counts following a 1 h pulse of $^{3}H$thymidine. Between 87 and 96% of the grains are over the nuclei of erythropoietic cells. The total grain counts per culture following each pulse during incubation for 6 h with and without erythropoietin yields an overall pattern similar to that obtained by Paul & Hunter (1968) by measuring isotope incorporated into extracted DNA. Reticulocytes and orthochromatic erythroblasts are unlabelled. Proerythroblasts have more grains per cell than basophilic erythroblasts, which, in turn, have slightly more grains than polychromatic erythroblasts (Table 6); this could be due to different rates of DNA synthesis, different rates of de novo TMP synthesis, different pool sizes or a combination of these. The total number of grains in each labelled compartment was estimated

<table>
<thead>
<tr>
<th>Pulse (h)</th>
<th>Treatment</th>
<th>% total cells</th>
<th>Labelling index (%)</th>
<th>Mean grains/labelled cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pr  B  Po</td>
<td>Pr  B  Po</td>
<td>Pr  B  Po</td>
</tr>
<tr>
<td>0–1</td>
<td>Nil</td>
<td>11  34  22</td>
<td>82  82  36</td>
<td>27  15  10</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>11  33  21</td>
<td>95  78  55</td>
<td>38  19  12</td>
</tr>
<tr>
<td>3–4</td>
<td>Nil</td>
<td>8   34  21</td>
<td>86  85  50</td>
<td>22  20  15</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>16  33  19</td>
<td>100 92  57</td>
<td>38  20  14</td>
</tr>
<tr>
<td>5–6</td>
<td>Nil</td>
<td>7   33  27</td>
<td>84  84  49</td>
<td>26  19  18</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>8   36  23</td>
<td>100 83  52</td>
<td>34  18  14</td>
</tr>
</tbody>
</table>

Each result is the mean from two experiments.

Table 6. Autoradiographic grain counts over erythropoietic cells, following 1 h pulses with $^{3}H$thymidine in the presence or absence of 0·6 units/ml urinary erythropoietin (abbreviations as in Fig. 1)
Erythropoietin and foetal mouse erythroid cells

Fig. 5. Rate of synthesis of DNA in erythroid precursors in the presence and absence of human urinary erythropoietin (0.6 U/ml). ■, Proerythroblasts in control cultures; ●, basophilic erythroblasts in control cultures; ▲, polychromatic erythroblasts in control cultures; ○, proerythroblasts + erythropoietin; ◆, basophilic erythroblasts + erythropoietin; △, polychromatic erythroblasts + erythropoietin. Grains were counted over 200 cells at each of the pulse times shown, in each of two experiments. The product of the percentage of cells in each class and the mean grain count per cell in each class provided an estimate of the total number of grains per cell compartment for a population of 100 cells.

From the data in Table 6. As shown in Fig. 5, erythropoietin has little effect on thymidine incorporation into basophilic erythroblasts or polychromatic erythroblasts but it provokes a marked increase of incorporation into proerythroblasts; the 78% overall stimulation in the first hour is due entirely to increased labelling of proerythroblasts. After 4 h the proerythroblast compartment in erythropoietin-treated cultures has 325% more grains than in controls, due to a combination of increased cell numbers in this compartment, a higher labelling index and relatively more intense labelling of individual cells. The number of proerythroblasts in the stimulated culture returns to control level 6 h after addition of erythropoietin, possibly because of maturation to basophilic erythroblasts.

[^H]Uridine incorporation provides a measure of the rate of RNA synthesis in each erythropoietic cell compartment. Of the grains, 86–95% are found over the nuclei of erythropoietic cells, and the total grains counted per culture, with and without erythropoietin, demonstrate a stimulation during each pulse. This
behaviour closely resembles that reported by Paul & Hunter (1969), who measured RNA synthesis by scintillation counting of extracted nucleic acids. Reticulocytes are unlabelled and orthochromatic erythroblasts have a grain count close to background. As shown in Table 7, proerythroblasts have more grains per cell than basophilic erythroblasts, which, in turn, have more grains per cell than polychromatic erythroblasts. The total number of grains in each compartment was calculated from differential and grain counts. As shown in Fig. 6, there is an erythropoietin-induced increase in [3H]uridine incorporation in all three compartments, reflecting a twofold increase in the mean grain count per erythroblast during the 3–4 and 5–6 h pulse intervals. During the 1–2 h pulse the proerythroblast compartment is not stimulated whereas the basophilic and polychromatic compartments possibly show an increase although this is of dubious significance.

These findings appear to conflict with those of Djaldetti, Preisler, Marks & Rifkind (1972) to the extent that these authors found an earlier stimulation of RNA synthesis in their cultures; following a 15 min pulse this was confined to proerythroblasts. Moreover, Chui, Djaldetti, Marks & Rifkind (1971) did not observe increased DNA synthesis. However, the reduced decline of DNA synthesis in their erythropoietin-stimulated cultures may represent the same phenomenon. The reason for these minor discrepancies is not clear but is presumably related to the way in which the cultures are manipulated.

Protein synthesis was measured first by incorporation of [3H]leucine after 24 h in vitro to determine whether erythropoietin causes an increased synthesis of all proteins, rather than specifically stimulating haemoglobin synthesis. The results in Table 8 demonstrate only a very slight increase in total protein synthesis after 24 h of culture with the hormone. The level of incorporation of [3H]leucine is considerably greater than could be accounted for by haemoglobin synthesis as measured by haem synthesis in the same system; haemoglobin

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Table 7. Autoradiographic grain counts over erythropoietic cells, following 1 h pulses with [3H]uridine in the presence or absence of 0-6 units/ml urinary erythropoietin (abbreviations as in Fig. 1)

<table>
<thead>
<tr>
<th>Pulse (h)</th>
<th>Treatment</th>
<th>% total cells</th>
<th>Labelling index (%)</th>
<th>Mean grains/labelled cell</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pr</td>
<td>B</td>
<td>Po</td>
</tr>
<tr>
<td>1–2</td>
<td>Nil</td>
<td>11</td>
<td>22</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>11</td>
<td>24</td>
<td>19</td>
</tr>
<tr>
<td>3–4</td>
<td>Nil</td>
<td>8</td>
<td>29</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>12</td>
<td>27</td>
<td>20</td>
</tr>
<tr>
<td>5–6</td>
<td>Nil</td>
<td>7</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Erythropoietin</td>
<td>14</td>
<td>28</td>
<td>22</td>
</tr>
</tbody>
</table>
Fig. 6. Rate of synthesis of RNA in erythroid precursors in the presence and absence of urinary erythropoietin. Symbols as in Fig. 5. Grains were counted over 200 cells at each of the pulse times shown, in each of two experiments. The results are expressed for a population of 100 cells as in Fig. 5.

Table 8. Protein synthesis measured by incorporation of [3H]leucine; results are expressed in terms of the number of cells present in the initial culture

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmoles leucine/10^6 cells/h during a 24-25 h pulse</th>
<th>pmoles of globin equivalent to leucine incorporated</th>
<th>pmoles haem/10^6 cells/h during a 24-25 h pulse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>1288</td>
<td>76</td>
<td>5.33</td>
</tr>
<tr>
<td></td>
<td>1174</td>
<td>69</td>
<td>3.65</td>
</tr>
<tr>
<td>0.6 units/ml urinary erythropoietin</td>
<td>1250</td>
<td>74</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>1316</td>
<td>77</td>
<td>11.03</td>
</tr>
</tbody>
</table>

Each result is the mean of duplicate cultures in each of two experiments. HEPES buffer, pH 7.2, was used in the culture medium in these experiments instead of CO₂/bicarbonate.
Table 9. Protein synthesis in haemoglobinized cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Polychromatic macro-erythroblast</th>
<th>Polychromatic erythroblast</th>
<th>Orthochromatic macro-erythroblast</th>
<th>Orthochromatic erythroblast</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nil</td>
<td>39 ± 16</td>
<td>20 ± 7</td>
<td>24 ± 8</td>
<td>12 ± 4</td>
</tr>
<tr>
<td>0·6 units/ml urinary erythropoietin</td>
<td>43 ± 19</td>
<td>20 ± 9</td>
<td>26 ± 12</td>
<td>13 ± 4</td>
</tr>
</tbody>
</table>

Results are expressed as mean and standard deviation of the grains per cell. Grains over 100 haemoglobinized cells were enumerated per culture in each of two experiments. In this experiment the cultures were maintained in a medium containing HEPES buffer instead of CO₂/bicarbonate.

![Graph showing protein synthesis in haemoglobinized cells](image)

Fig. 7. Overall rates of protein synthesis in the haemoglobinized compartments, with and without erythropoietin. The results quoted are the product of the differential counts recorded in Fig. 1 and the grains per cell recorded in Table 9.

Protein synthesis may account for no more than 5–15% of total protein synthesis in these cultures. Hence, increased incorporation of leucine due to increased haemoglobin synthesis would scarcely be greater than the variation among samples. Subsequently autoradiographs of cells incorporating 10 μCi/ml [³H]-leucine, after 24 h in culture with or without erythropoietin, were prepared and exposed for a sufficient time to allow the estimation of grain counts over cells assumed to be synthesizing mainly haemoglobin, i.e. cells in the polychromatic and orthochromatic compartments (Table 9). (Proerythroblasts had approximately 20 times the number of grains per cell found in polychromatic macro-
Erythropoietin and foetal mouse erythroid cells

469
erythroblasts.) Polychromatic macro-erythroblasts and orthochromatic macro-
erythroblasts have twice as many grains per cell as the corresponding erythroblast
cells. Since these erythropoietin-induced cell types are present in greater numbers
in the stimulated cultures than in the control cultures (Table 4), this results in
an increased rate of protein synthesis in the haemoglobinized cell compartments
(Fig. 7) in cultures treated with erythropoietin; this increase is only a little
lower than the overall increase of haemoglobin synthesis as measured by $^{59}$Fe
incorporation. The findings lead to the same general conclusion as reached by
Chui et al. (1971), that the main effect of erythropoietin is to cause an increase
in the absolute number of haemoglobin-synthesizing cells and that, without
erythropoietin, already committed erythroblasts continue their development but
erythropoiesis is not sustained. However, these authors did not report the
findings of macro-erythroblasts and macrocytes in their cultures and did not
observe the resulting increase in haemoglobin synthesis per cell.

It may be noted that, since protein synthesis diminishes as maturation pro-
ceeds, a paradoxical situation is possible in which increased maturation could
result in an overall decrease in protein synthesis in the whole culture.

**DISCUSSION**

It should be pointed out that, in these studies, one is looking mainly at the
later stages of erythropoiesis from proerythroblasts onwards. Unless proerythro-
blasts themselves are erythropoietin-responsive cells the most important site of
erthropoietin action may not be observed. The tissue culture system is defective
in that it is not self-sustaining. The emergence of giant cells and the reduction
of the number of proerythroblasts raises the possibility that some factors,
possibly nutritional, may be missing from the medium. Moreover, the physio-
logical level of plasma erythropoietin is probably in the range of 0-02–0-14 units/
ml (Goldwasser, 1966; Schooley & Garcia, 1965), and the use of 0-2 units/ml of
step III erythropoietin and 0-6 units/ml of a cruder erythropoietin in direct con-
tact with erythropoietic cells *in vitro* may represent a dose which is much greater
than normal and which produces the type of stress response occurring *in vivo*
following erythropoietin stimulation (Brecher & Stohlman, 1962). Nevertheless,
the results of these experiments do indicate that it is not necessary to postulate
the initiation of any new event by erythropoietin.

The effects of erythropoietin on the immature cells of these populations are
twofold. First, it promotes an increased rate of DNA synthesis and mitosis in
proerythroblasts (Figs. 4, 5) and an increased rate of RNA synthesis in pro-
erythroblasts, basophilic erythroblasts and polychromatic erythroblasts as com-
pared with controls (Fig. 6). The enhanced synthesis of DNA and mitosis in
proerythroblasts leads to an increased flow from the proerythroblast compart-
ment to the basophilic erythroblast compartment (Fig. 1). The result is main-
tenance of the latter compartment achieved, to some extent, at the expense of
The proerythroblast compartment (as judged by a comparison with similar cultures containing no hormone). There appears also to be some recycling within the erythropoietin-stimulated proerythroblast compartment as shown by the accumulation of cells with a low grain count (Fig. 3) 6 h after a short [3H]-thymidine pulse.

Secondly, erythropoietin causes basophilic erythroblasts to mature, probably without division, to form atypical cells which are larger than normal haemoglobinized erythroblasts, demonstrate a nucleo-cytoplasmic asynchrony and extrude their nuclei to produce macrocytes which, apart from size, are indistinguishable from reticulocytes. These two effects of erythropoietin are summarized in Fig. 8.

It is possible that some proerythroblasts also may divide and mature to form polychromatic macro-erythroblasts, but this is perhaps less likely since there is no reduction in the numbers of haemoglobinized macro-erythroblasts formed in the presence of colchicine.

The 50% increase in total cell number, provoked by erythropoietin, is accompanied by a 240% increase in the rate of haemoglobin synthesis (measured by incorporation of $^{59}$Fe into haem). Therefore, increased haemoglobin synthesis is not due simply to an increase in the number of cells synthesizing haemoglobin. However, as Table 9 shows, each macro-erythroblast synthesizes protein at twice the rate of each normal erythroblast. If one assumes that a macroerythroblast is equivalent to two normal erythroblasts and a macrocyte to two normal reticulocytes, then from the data in Fig. 1 we can calculate that the total ‘cell equivalents’ in the polychromatic, orthochromatic and reticulocyte compartments is 41 for the initial cultures and 175 for erythropoietin-treated cultures (18 + 14 + 9 and 30 + 27 + 4 + 2 [15 + 25 + 17] respectively). This is more than enough to account for the increase in haemoglobin synthesis. The calculation is necessarily a gross approximation and should be corrected for the relative
rates of haemoglobin synthesis in the individual cells which are not at present known.

Therefore the increased rate of haemoglobin synthesis appears to be due both to an increase in the number of cells synthesizing haemoglobin and to an increased rate of synthesis per cell. The increased rate of synthesis per cell would seem to be related to an increased amount of cellular material per cell. If, as is suggested by the data in Table 5, the amount of DNA per cell is greater in macro-erythroblasts than in erythroblasts, the rate of haemoglobin synthesis per genome may not be increased greatly by erythropoietin and its main effect may be to increase the number of ‘cell equivalents’.

These findings illuminate the manner in which erythropoietin acts in vitro and also explain some apparently discrepant results. It seems likely that the differences between control cultures and cultures treated with erythropoietin may not be due entirely to stimulation of DNA, RNA and haemoglobin synthesis by erythropoietin above control levels (assumed to correspond to those in vivo) but also to a slowing down of erythropoietin-dependent processes in control cultures and their maintenance in the presence of erythropoietin. Hence, the immediate increase in mitosis and DNA synthesis in erythropoietin-treated cultures as compared with controls may reflect a continuation of processes which have been initiated in the host and which are erythropoietin-dependent. Mitosis apparently stops more rapidly than DNA synthesis in culture; consequently there is probably an accumulation of some cells with premitotic amounts of RNA, DNA and proteins which results in increased rates of RNA and haemoglobin synthesis per cell as compared with control cultures. These findings are in accord with those previously recorded by Paul & Hunter (1969).

In the cultures used in the present experiments and also the Paul & Hunter experiments, there was a fairly large proportion of proerythroblasts. As the present experiments show, these may require to go through DNA synthesis before maturing, whereas basophilic erythroblasts may mature into orthochromatic macro-erythroblasts without division.

In view of the evidence that about seven cell divisions are usually involved in the maturation of proerythroblasts to reticulocytes (Tarbutt, 1967), it is interesting that in this system there is no evidence for more than two or three maturation divisions between proerythroblasts and erythrocyte, and the number could be smaller.

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