Development of erythroid colony-forming cells in rat fetal spleen: apparent lack of sensitivity to an in vivo corticosteroid excess as compared to fetal liver

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Summary

The development of splenic erythroid colony-forming cells from rat embryos in the last 4 days of intrauterine life was examined after 2 and 7 days in a methylcellulose culture system. The number of 2- and 7-day erythroid colonies decreased sharply between, respectively, days 20 and 21 of gestation and days 19 and 20. Concomitantly, a maturation of proerythroblasts and basophilic erythroblasts to mature erythroblasts was detected on smears of splenic cellular suspensions.

The effect of a corticosteroid excess induced by a maternal laparotomy was tested on spleen and liver cultures from the same control or experimental fetuses. The ratio of the number of 2-day to the number of 7-day erythroid colonies did not differ in experimental and control splenic cultures, but in liver cultures was significantly lower at days 19 and 20 in experimental than in control cultures.

Key words: erythroid colonies, fetal spleen, rat embryo, corticosteroids, liver.

Introduction

At the end of intrauterine life in the rat, the liver remains the major site of red blood cell production, as in normal physiological circumstances the antenatal bone marrow contains few differentiated erythroid cells (Lucarelli, Porcellini, Carnevali, Carmena & Stohlman, 1968; Nagel, Nagel & Jacquot, 1981). Little is known about fetal splenic erythropoiesis: in spite of its considerable growth, the spleen probably does not release red blood cells before term (Nagel, Félix & Nagel, 1982). Therefore, to find out more about the erythropoietic potentiality of the spleen during the last four days of fetal life, we studied the changes in the numbers of erythroid colony-forming cells after 2 and 7 days in a methylcellulose culture system.

It is well known that circulating corticosteroids influence the evolution of the fetal hepatic erythron (Nagel & Jacquot, 1968, 1969; Jacquot & Nagel, 1976; Billat, Nagel, Nagel & Jacquot, 1980). Moreover, the number of erythroid burst-forming units (BFUe) and colony-forming units (CFUe) may be modulated in vitro by glucocorticoids (Golde, Bersh & Cline, 1976; Zalman, Maloney & Patt, 1979; Leung & Gidari, 1981; Billat, Félix & Jacquot, 1982; Roodman, Lee & Gidari, 1983). We therefore stressed pregnant rats by performing a laparotomy (Nagel & Jacquot, 1968). To discover the effect of the resulting corticosteroid excess on the fetal erythropoiesis, we removed the fetuses 24 h later, cultured their spleen and liver cells and observed the numbers of erythroid colonies after 2 and 7 days.

Materials and methods

Animals

Wistar rats (CF strain form the CNRS) were used. They were housed in a constant-temperature room with a 12 h day/12 h night cycle. They had free access to water and food (UAR commercial rat food). Coitus was assessed by the presence of spermatozoa in the morning vaginal smear. In this strain of rat, delivery generally occurs during the night between days 21 and 22 of pregnancy or in the morning of day 22.

Surgery

Laparotomy was performed under ether anaesthesia. This involved cutting through the skin, muscle wall and
peritoneum and then suturing, without touching the vis-
cera. The rat was killed 24 h later.

Chemicals
Bovine hemin (type I, Sigma Chemical Co., St Louis, MO) was
dissolved in 0.2 M-KOH and diluted with the sup-
plemented alpha medium to a concentration of 0.01 M and
then neutralized with 1 N-HCl to pH 7.5 as described by
Ross & Sautner (1976). The hemin solution was then
sterilized by filtering (0.45 μm, Millipore Corporation,
Bedford, MA).

Erythroid colony assays
Cell suspensions
Pregnant rats were killed by decapitation. Of the younger
fetuses (18 and 19 days), twelve to sixteen from three litters
were used for each experiment. Of older fetuses (20 and 21
days), only six to eight from two litters were sampled. Fetal
spleens and livers were removed aseptically, placed in
sterile ice-cold alpha medium (Gibco Bio-Cult-Ltd) and
gently disrupted. The suspension was then filtered through
a nonoxidizable steel sieve (50 μm mesh) which retains
particles, connective cells and parenchymatous cells. The
exact volume was measured and the concentration of the
haematopoietic cells in the filtrate was determined after a
fivefold dilution in 2.86% acetic acid solution to remove
anucleate red blood cells.

Erythroid colony cultures
We used the technique of Iscove, Sieber & Winterhalter
(1974) for the culture of CFUe and BFUe on methyl-
cellulose as modified by Urabe & Murphy (1978). The
culture medium contained the following: 1.25 ml of 2% methy-
cellulose solution (A7 M premium, Dow Chemical
Corporation) in alpha medium containing 1.25 eryth-
ropoietin units (Step III, Connaught Laboratories, Toronto,
Canada); 0.250 ml of a solution of 10% bovine serum
albumin (BSA) (grade V, Sigma Chemical Co., St Louis,
MO) in alpha medium; 0.025 ml of a 200 mM 1−1 solution of
L-glutamine in water; 0.025 ml of a commercial Kanamycin
solution (Kanamycin solution×100, Gibco); 0.025 ml of a
commercial penicillin and fungzone solution (antibiotic-
antimycotic solution, 100, Gibco); 0.750 ml of heat-
inactivated fetal calf serum (Gibco, batch 207548); 0.050 ml
of 7.5% sodium bicarbonate in water; 0.025 ml of β-
mercaptoethanol and 0.050 ml of hemin solution
(2×10−4 M). The BSA for this medium was prepared and
dionized according to the technique of Murphy & Sullivan
(1978). The final concentration of cells in the culture
medium was adjusted to 0.5×10^6 cells per ml. This concen-
tration suboptimal as far as the average colony size after 7
days of incubation is concerned was used because it made it
possible to culture haematopoietic spleen cells from the
youngest fetuses, taken at day 18 when the organ is just
beginning to develop. The suspensions were mixed care-
fully, and 0.2 ml samples were distributed in the wells of
microtire plates (Falcon Plastics, Oxnard, CA) and in-
cubated at 37°C in a humified atmosphere containing 5% CO2.
Hemin was incorporated into the culture medium as
an aid in enumeration of colonies, as it has been reported to
be a potent stimulator of erythroid colony growth in culture
(Porter, Meints & Messner, 1979) and to enhance the in
vitro growth of primitive erythroid progenitor cells
(Monette & Holden, 1982; Holden, Steinberg, Matzinger &
Monette, 1983).

Erythroid colonies
After 2 days of culture, erythroid colonies (2-d-EC) con-
taining eight or more cells were counted in each well,
without staining, as described by Iscove & Sieber (1975).
Such colonies can be considered to be CFUe. After 7 days
of culture, colonies appear as individual well-haemo-
globinized colonies of medium size. At the cellular concent-
ration that we used, they do not develop in bursts
characterizing BFUe. Therefore, at day 7 we enumerated
colonies comprising 16 or more cells and designated them
7-day erythroid colonies (7-d-EC).

Cytological determinations
To study the normal pattern of change in the percentage of
erthropoietic cells in spleen-cell suspensions, smears were
made in three different experiments at each stage and
stained with May-Grünwald Giemsa. The following cell
groups were distinguished: proerythroblasts and basophilic
erythroblasts, mature erythroblasts (polychromatophilic
and acidophilic) and 'other cells' of the white series or
consisting of undifferentiated cells. For each experiment
200 cells were classified, so that the mean percentage
reported at each stage is that of 600 cells.

Expression of the results
In each experiment, 2-d-EC and 7-d-EC were scored in
eight to ten wells. Results are given as means ± s.e.m. (per
2000 haematopoietic cells).

The numbers of CFUe and 7-d-EC may fluctuate from
one experiment to another carried out at the same stage,
but the ratio of the number of CFUe to the number of
7-d-EC remains remarkably stable. Therefore this para-
meter was used for comparison of the results for control
fetuses with those for fetuses submitted to an excess of
corticosteroids.

Student's t-test was used to analyse the data.

Results

(A) Changes with age in normal fetal spleen

(1) Erythroid cells in haematopoietic cell
suspensions

As shown in Fig. 1, the percentage of erythroid cells in
the total haematopoietic cells in the suspension
decreases between days 18 and 20 of gestation. It then
increases and is the same at day 21 as at day 19.

(2) Proerythroblasts plus basophilic erythroblasts

The percentage of proerythroblasts plus basophilic
erythroblasts in the erythroid population is roughly
stable in fetuses at days 18 and 19 and then decreases
(Fig. 2).

Using the values presented in Fig. 1 as cor-
rection factors, we calculated the percentage of
Erythropoietic activity in rat fetal spleen

Fig. 1. Changes with fetal age of the percentage of erythroid cells in haematopoietic cell suspensions from rat spleens. The star shows the mean of the three experiments at each age.

proerythroblasts plus basophilic erythroblasts in the whole haematopoietic cell suspension to be 19.8%, 11.7%, 5.7% and 2.5% at days 18, 19, 20 and 21 respectively.

(3) Mature polychromatophilic and acidophilic erythroblasts
Changes in the proportion of mature erythroblasts in the total erythroid population can be deduced from Fig. 2: the values are 73.6%, 73.9%, 81.7% and 94.3% in fetuses 18, 19, 20 and 21 days old, respectively. As a fraction of the whole haematopoietic cell suspension (see Fig. 1) the respective values are 53.2%, 32.0%, 25.3% and 41.5%.

(4) Numbers and relative proportions of 2-d-EC and 7-d-EC in haematopoietic cells (Fig. 3)
Between days 18 and 19 of gestation the numbers of 2-d-EC and 7-d-EC per 2000 haematopoietic cells both decrease slightly; the ratio 2-d-EC/7-d-EC is unchanged. Between days 19 and 20, the number

<table>
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<th>Fetal age (days)</th>
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<td>17.4</td>
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<td>21</td>
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<table>
<thead>
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<th>Fetal age (days)</th>
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<th>7-d-EC</th>
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<td>420</td>
</tr>
<tr>
<td>19</td>
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of 2-d-EC remains roughly stable whereas that of 7-d-EC decreases sharply; the ratio between the two is therefore much higher at day 20 than at days 18 and 19. In 21-day-old fetuses, the number of 2-d-EC is markedly lower and that of 7-d-EC also decreases again; the ratio 2-d-EC/7-d-EC reaches its highest value. Calculation on the basis of the percentages in Fig. 1 gives the numbers of 2-d-EC and 7-d-EC per 2000 erythroid cells (Table 1). The number of 2-d-EC increases between days 18 and 20 and thereafter drops sharply, whereas that of 7-d-EC decreases from day 19. Table 2 shows the evolution of 2-d-EC and 7-d-EC giving the average number of haematopoietic cells extracted per spleen at each fetal age. The rate of increase of the number of 2-d-EC and 7-d-EC progenitors is greatest between 19 and 20 days and 18

Fig. 3. Changes with fetal age of 2-d-EC and 7-d-EC numbers (±s.e.m.) per 2000 haematopoietic cells from rat spleens. The mean value of the four or five experiments for each age is represented by a star. Ratios: number of 2-d-EC/number of 7-d-EC given for each experiment; in bold types, mean ratio for each age.
and 19 days respectively; thereafter, it decreases sharply in both cases.

(B) *Effect of maternal laparotomy (corticosteroid excess) on the numbers of 2-d-EC and 7-d-EC* (Fig. 4)
Maternal laparotomy took place 24 h before the sampling of the fetuses. Splenic and hepatic haematopoietic cells from experimental fetuses were cultured in parallel with similar cells from normal control fetuses.

In 19-day fetuses, there is no difference between the ratio 2-d-EC/7-d-EC found in experimental and control splenic cultures. However, in liver cultures from the same experimental fetuses, this ratio is dramatically lower. Similar results were obtained with 20-day-old fetuses: the ratio 2-d-EC/7-d-EC does not differ in experimental and control splenic

![Graph](image-url)

**Fig. 4.** Effect of an *in vivo* excess of corticosteroids on the number of splenic (circles) and hepatic (triangles) 2-d-EC and 7-d-EC per 2000 haematopoietic cells at days 19, 20 and 21 of intrauterine life. Each circle or triangle represents the mean ± s.e.m. of two separate studies. Open circles or triangles, fetuses from normal mothers (controls); filled circles or triangles, fetuses from laparotomized mothers (experiments). Ratios: 2-d-EC/7-d-EC. *, Significantly different (*P* < 0.01) from the corresponding control culture (fetuses from normal mothers).
The results found at day 19 are perfectly reproducible fetuses as it did in the younger ages. In liver cultures, the ratio found in experimental fetuses no longer differs from that in control fetuses as it did in the younger ages. The statistical analysis of the data indicates that the ratio 2-d-EC/7-d-EC in liver cultures of 19- and 20-day fetuses from laparotomized mother is modified by a significant decrease of 2-d-EC as well as a significant increase of 7-d-EC, as compared to liver cultures of control fetuses from normal mothers.

Discussion

(A) Normal pattern of splenic erythropoiesis during the antenatal period

Between 18 and 20 days, the decrease of the percentage of erythroid population among splenic haematopoietic cells has to be related to the expansion of the white cell line: in fact, as was shown in a previous paper, the spleen does not supply circulating red blood cells during this antenatal period (Nagel, Félix & Nagel, 1982).

Within the erythroid cell line, between days 20 and 19, there is an especially pronounced increase in the proportion of mature erythroblasts. This increase is more obvious if the whole haematopoietic population is considered. This suggests an extensive maturation process between these two ages.

As far as the number of 2-d-EC and 7-d-EC is concerned, the results per 2000 haematopoietic cells show that at days 18 and 19, the pattern of evolution of 7-d-EC in the fetal spleen follows that of 2-d-EC fairly closely. Thereafter a decrease in 7-d-EC takes place between days 19 and 20 24 h before that seen by 2-d-EC. This evolution is more obvious if the number of colonies is counted in the erythroid population alone, and also when it is assessed per organ.

Thus, the whole of the data suggests the following pattern: the maturation of 7-d-EC progenitors begins between days 18 and 19, and is 24 h later followed by the maturation of CFUe. Concomitantly from day 20, the proportion of mature erythroblasts is considerably enhanced. It therefore seems that a wave of erythropoietic activity occurs in the spleen just before term. A further increase in the erythropoietic activity of the spleen in the rat was described by Lucarelli, Howard & Stohlman (1964).

(B) Splenic and hepatic changes in 2-d-EC and 7-d-EC in fetuses subjected to an excess of corticosteroids

The results found at day 19 are perfectly reproducible at day 20; they clearly demonstrate that an in vivo-induced corticosteroid excess does not affect the ratio 2-d-EC/7-d-EC in splenic cultures, but does in liver cultures from the same experimental or control fetuses. In 19-day fetuses, as in 20-day ones, we found roughly the same number of 2-d-EC and 7-d-EC in experimental and control splenic cultures. In liver cultures from fetuses of both ages, however, we observed fewer 2-d-EC and more 7-d-EC in experimental than in control cultures. Therefore we believe that an excess of corticosteroids induced in vivo probably leads to fewer CFUe and more 7-d-EC progenitors in liver at days 19 and 20. Such an inhibitory effect of endogenous glucocorticoids on CFUe has been reported by Leung & Gidari (1985a, b) in 15-day-old fetal liver cultures, in agreement with other observations concerning the in vitro effect of glucocorticoids on erythroid colony formation by human fetal liver (Hassan, Ibrahim & Rieder, 1982; Roodman, Lee & Gidari, 1983) and by murine adult bone marrow (Singer, Samuels & Adamson, 1976; Gidari & Levere, 1979).

As far as earlier progenitors are concerned, in vitro studies demonstrated that glucocorticoids inhibit the growth and/or differentiation of both BFUe and CFUe in 15-day-old mouse fetal liver cultures (Leung & Gidari, 1982). The present data suggest that in the rat, at the end of intrauterine life, maternal laparotomy increases hepatic but not splenic early erythroid progenitors at the time of development at which they are most actively proliferating (day 19) in the spleen, whereas late progenitors are reduced in the liver. This is most simply explained if corticosteroids inhibit the differentiation of the earliest erythroid progenitors, but an additional effect in stimulating their proliferation is not excluded. The precise biochemical mechanism underlying the inhibitory action(s) of corticosteroids on erythroid progenitors is unknown and a number of intriguing questions still remain. The first consideration is the controversial effect of glucocorticoids on erythropoiesis: in vivo and in vitro studies have shown both stimulation and inhibition (bibliography in Hassan, Ibrahim & Rieder, 1982). A second observation of interest is relevant to the different populations of late erythroid progenitors with age-related decreasing erythropoietin sensitivity during ontogeny (Rich & Kubanek, 1980) and also to the variable erythropoietin sensitivity of BFUe reported by Gregory (1976), Linch, Knott, Rodeck & Huehns (1982), Meytes, Ortega, Ma, Wald, Shore & Dukes (1983) and Peschle et al. (1981), for example. Otherwise, if glucocorticoid receptors appear to be involved in the inhibitory effects of glucocorticoids on erythroid progenitors (Leung & Gidari, 1985b), variable stimulatory effects...
of corticosteroids may reflect the variable concentrations of nonerythroid cells sensitive to glucocorticoids which can modulate the formation of erythroid colonies in vitro. A critical analysis of these possibilities, however, must await the isolation of purified populations of erythroid progenitor cells.

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References


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