Induction of Colonies of Hemoglobin-Synthesizing Cells by Erythropoietin In Vitro
(fetal mouse liver/erythropoietin/erythroid cells/\(^{59}\)Fe/granulocytes)

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ABSTRACT A culture method has been developed in which erythroid colonies are produced in vitro from hemopoietic cells from the livers of 13-day fetuses of C3H/Bi mice. Heme synthesis by the cultures was correlated with the presence of these colonies, and the hemoglobin produced was shown to be electrophoretically normal. The individual colonies were identified as erythroid since they were erythropoietin-dependent, positively stained by the histochemical 'Lepehne' procedure for hemoglobin, and labeled by \(^{59}\)Fe radioautography. Evidence is presented that the development of these colonies is under separate control from that of granulocytic colonies found in the same cultures.

Hemopoietic differentiation along the erythroid line begins with a single pluripotent stem cell and ends with the production of a cohort of cells whose activities are directed almost exclusively to the synthesis of a single protein, hemoglobin. Because hemoglobin is a well-characterized and easily assayed protein, it has served as a useful marker for investigation of this process. Hemoglobin synthesis has been induced in vitro by the hormone erythropoietin in mass cultures of rat (1), rabbit (2), human bone marrow (3), and mouse fetal liver cells (4, 5). If colonies of hemoglobin-synthesizing cells could be obtained in vitro, analysis of the functions of individual cells in the erythroid differentiation process would be greatly simplified.

Methods of obtaining the colonies of differentiating hemopoietic cells in vitro developed over the past few years have involved the use of semisolid agar (6–8), methylcellulose (9), or plasma (McLeod and Shreeve, to be published). Cell suspensions prepared from mouse spleen, bone marrow, and fetal liver and from rat and human bone marrow have been shown to give rise to both granulocyte and macrophage colonies. However, no evidence of erythroid colonies has been reported in any of these culture systems, even though in at least one case the medium was supplemented with erythropoietin (10).

Fetal hemopoietic cells have been shown to respond to erythropoietin in vitro by a burst of increased heme synthesis that reached a peak within 24–48 hr of exposure to the hormone (4, 5). If this response involved an increase in the number of heme-synthesizing cells, and if the progeny cells remained localized, discrete colonies of hemoglobin-synthesizing cells might be obtained in culture. Therefore, using a plasma culture technique, we systematically varied conditions in attempts to obtain erythroid colonies from cultures of hemopoietic cells from the livers of 13-day mouse fetuses.

In the present communication, we describe the production of colonies in vitro under the influence of erythropoietin, show that the cells in these colonies are engaged in hemoglobin synthesis, and present evidence that the induction of erythroid colonies in the cultures is controlled independently of the induction of granulocytic colonies.

MATERIALS AND METHODS

Mice
C3H/Bi Oci mice were bred in the Division of Laboratory Animal Science, University of Toronto. Fetuses of known age were obtained by leaving one male overnight with three or four females and on day 13 taking those mice found pregnant.

Preparation of cell suspensions
Liver cell suspensions were prepared from 13-day fetal mice by the method of Cole and Paul (4) except that the livers were trypsinized at 4 C for 2 hr instead of overnight. Bone marrow suspensions were prepared by the technique described by Till and McCulloch (11).

Culture method
Cells were suspended, at 2 x 10^4 cells per ml, in medium NCTC 109 (Microbiological Associates, Bethesda, Md.) containing the following: 10% heat-inactivated fetal calf serum (FCS, Grand Island Biological Co., Grand Island, N.Y.), 0.24 U/ml erythropoietin (Step III, Connaught Medical Research Laboratories, Toronto, Canada), 0.02 mg/ml L-asparagine, 0.83% beef embryo extract (Grand Island Biological Co.), 1% bovine serum albumin, and 10% medium conditioned with mouse kidney tubules (to be described in a later publication). 1 ml of this cell suspension was added to a 35 x 10 mm Petri dish (Falcon Plastics, Los Angeles, Calif.) containing 0.1 ml of citrated bovine plasma (Grand Island Biological Co.), and the mixture was allowed to clot. Cultures were incubated for 4 days at 37ºC in a humidified incubator with 5% CO₂ in air.

Combination stain for hemoglobin and peroxidase
For distinguishing erythroid and granulocytic colonies in the same culture, a histochemical procedure for peroxidase (12) was combined with a modification of the Lepehne technique (13) for hemoglobin.

The plasma clots were removed from the Petri dishes, spread out on a 50 x 75 mm glass slide, and blotted with
Erythropoietin-Induced Cultures of Erythroid Cells

FIG. 1. Photomicrograph of 4-day culture of C3Hf/Bi fetal liver cells. (a), Erythroid colony (×2700); (b), two granulocytic colonies on the right, two erythroid colonies on the left (×2240). Stained by the modified Lepehne procedure and counterstained with hematoxylin.

filter paper. Slides were air-dried, fixed in a 1:9 v/v mixture of neutral formalin and 95% ethanol, washed with 0.01 M phosphate buffer (pH 7), air-dried, treated with ether for 60 sec, and again rinsed with 0.01 M phosphate buffer. Staining for peroxidase was performed for 1–2 min in the peroxidase reagent of Ryt6maa (12). Excess stain was rinsed off with buffer; slides were air-dried, treated with absolute methanol for 30 sec, and air-dried. Staining for hemoglobin was then carried out for 2 min in a 1% solution of 3,3'-dimethyl-oxybenzidine (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.) in absolute methanol. Slides were immediately treated for 2 min in 30% hydrogen peroxide-70% ethanol 1:3, then rinsed for 15 sec in distilled water, counterstained for 15 sec with Harris Hematoxylin, and differentiated in running tap water. Permanent preparations were made by air-drying and mounting in Permount.

Determination of heme synthesis rates

1–2 µCi of transferrin-bound 59Fe (14) was diluted in 0.5 ml medium NCTC 109 and added to culture plates after 78 hr of incubation. After a further 18 hr, 1 ml of 0.05% trypsin (3 × crystallized, Worthington Biochemical Corp., Freehold, N.J.) was added to each plate and cultures were incubated for 5 min at 37°C. The plasma clots were then transferred to 15-ml centrifuge tubes, an additional 1 ml of 0.05% trypsin was added, and after 15 min at 37°C, fetal calf serum was added to a final concentration of approximately 2%, and heme was extracted as described previously (5).

Radioautography

Plasma clots from 59Fe-labeled cultures were transferred to slides, fixed, washed, and stained for hemoglobin, but not counterstained. Slides were then dipped in liquid emulsion (Nuclear Track Emulsion, NTB3 Eastman Kodak Co., Rochester, N.Y.), stored for 2–3 weeks, and developed. The resulting radioautographs were poststained with hematoxylin and made permanent.

Polyacrylamide gel electrophoresis

Cell lysates were prepared and hemoglobins separated by gel electrophoresis by the method of Barker (15). To ensure that only [59Fe]heme was being measured, we cut out the
of philic erythroblasts of reduced, was Lepehne features, only single.

In cultures, the number of colonies that consisted of large numbers of colonies that consisted of 10–20 cells with the morphological appearance of basophilic erythroblasts and numerous mitotic figures were found. By 4 days the number of cells per colony had increased considerably; up to 60–70 cells were often present. Most of the cells had the appearance of normoblasts (orthochromatic erythroblasts) (Fig. 1); only an occasional mitotic figure remained. By the 7th day of culture, very few erythroid colonies could be seen.

We then determined whether the Lepehne-positive colonies were in fact colonies of hemoglobin-synthesizing cells. The liver cells from 13-day fetuses were cultured at 1 × 10⁶ and 2.5 × 10⁶ cells per plate. Erythropoietin (0.24 U/ml) was added to half of the cultures at each cell concentration. Eight erythropoietin-treated and eight control cultures at the higher cell concentration were treated at various times with 1–2 μCi of transferrin-bound ⁵⁷Fe for 4 hr, and hemexextractions were performed. After 6–96 hr of culture, five erythropoietin-treated and five control cultures at the lower cell concentration were stained by the modified Lepehne technique. The total number of Lepehne-positive colonies with more than 20 cells was counted.

The rate of heme synthesis decreased during the first 24 hr of culture in both erythropoietin-treated and control cultures (Fig. 2, top). However, in erythropoietin-treated cultures, there was then a sharp increase in the rate of heme synthesis, which reached a peak by 48 hr and then gradually declined. In control cultures, the rate of heme synthesis fell and then remained low throughout. In the erythropoietin-treated cultures, the onset of heme synthesis preceded the appearance of Lepehne-positive colonies. Fig. 2 (bottom) shows that by 48 hr Lepehne-positive colonies were present in considerable numbers, and by 72 hr a peak of around 1200 colonies per plate was reached; the colony number then declined. In control cultures only very few Lepehne-positive colonies were ever found. These results establish the fact that the formation of Lepehne-positive colonies is an erythropoietin-dependent process.

To obtain independent evidence as to whether the heme synthesis detected in the cultures was performed by cells in the individual Lepehne-positive colonies, we cultured the liver cells at 1 × 10⁶ cells per plate, and after 48 hr added 0.01–0.05 μCi of transferrin-bound ⁵⁷Fe to each culture. The cells were incubated for a further 48 hr, and radioautographs were prepared. Intense radioautographic reactions were found over the Lepehne-positive colonies, whereas very little label was found over other colonies or individual cells. The fact that the radioisotope was not washed out during fixation and washing indicates that the ⁵⁷Fe localized in the cells of the Lepehne-positive colonies was in macro-

![Graph](image-url)

**Fig. 2.** Time course of the response to erythropoietin by cultured fetal liver cells of C3H/Bi mouse in vitro. Results of heme synthesis response (top) are expressed as mean ± SE (eight determinations). Each point indicates the middle of a 4-hr incubation with transferrin-bound ⁵⁷Fe. Counts of Lepehne-positive colony (bottom) are expressed as mean ± SE (five cultures).

regions of the gels corresponding to the visible hemoglobin bands, homogenized them, and eluted the hemoglobin at 4°C in 3–4 ml of distilled water for 24 hr. Heme was extracted from the aqueous supernatant, and the radioactivity was counted.

To prepare ⁵⁷Fe-labeled adult C3H hemoglobin, we injected ⁵⁷Fe[ferrous citrate, 10–15 Ci/g (Abbott Laboratories Ltd., North Chicago, Ill.), intraperitoneally into C3H/Bi mice at a dose of 3–4 μCi of ⁵⁷Fe (in 1 ml of phosphate-buffered saline) per animal. 24 hr later blood was collected, the cells were washed three times with cold phosphate-buffered saline, cell lysates were prepared and subjected to electrophoresis.

**RESULTS**

In cultures examined a few hours after initiation of the cultures, only single cells were found to be stained by the modified Lepehne procedure. By 2 days the number of single cells was reduced, and large numbers of colonies that consisted of 10–20 cells with the morphological appearance of basophilic erythroblasts and numerous mitotic figures were found. By 4 days the number of cells per colony had increased considerably; up to 60–70 cells were often present. Most of the cells had the appearance of normoblasts (orthochromatic erythroblasts) (Fig. 1); only an occasional mitotic figure remained. By the 7th day of culture, very few erythroid colonies could be seen.

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Erythropoietin

Control

1. the

2. 5

were

added,

and
gel
electrophoresis.

Labeled
normal
adult
C3H/Bi
hemoglobin
was
supposed
to
gel
electrophoresis
as
control. The
gel
regions
corresponding
to
the
three
hemoglobin
bands
from
adult
C3H
were
cut
out,

and
the

9Fe
incorporated
into
heme
extracted
from
these
bands
and
from
the
regions
above
and
below
the
bands
was
counted. As
seen
in
Fig.
3, hemoglobin
synthesized
by
fetal
liver
cells
after
4
days
of
culture
was
electrophoretically
indistinguishable
from
normal
adult
C3H
hemoglobin.

These
results
show
that
the
Lepehne-positive
colonies
that
develop
in
cultures
of
mouse
fetal
liver
cells
in
the
presence
of
erthropoietin
are
composed
of
cells
that
synthesize
hemoglobin.

An
test
was
next
made
to
determine
whether
the
production
of
erthroid
colonies
is
related
to,
or
independent
of,
the
production
of
granulocytic
colonies
in
the
same
culture.
It
had
been
shown
previously
that
the
production
of
granulocytic
colonies
in
hemopoietic
cell
cultures
was
stimulated
by
conditioned
medium
(8, 9, 17). The
work
described
above
had
shown
that
the
production
of
erythroid
colonies
was
stimulated
by
erythropoietin,
and
that
colonies
of
erythroid
and
granulocytic
type
could
be
clearly
distinguished
in
the
same
cultures.

Therefore,
four
groups
of
culture
were
set
up
in
which
(a)
standard
medium
was
used
as
control,

(b)
erthropoietin
was
added
but
conditioned
medium
omitted,
(c)
conditioned
medium
but
not
erthropoietin
medium
added,

and
(d)
both
erythropoietin
and
conditioned
medium
were
added.
Each
group
included
eight
plates
at
2.5
×
10^6

and
six
plates
at
5.0
×
10^6

per
culture.

After
78
hr,
1–2
μCi
of
transferrin-bound
9Fe
was
added
to
each
of
the
cultures
at
the
higher
concentration,
and
after
a
further
18
hr,
heme
synthesis
was
determined.

At
the
end
of
4
days
of
incubation,
all
cultures
at
the
lower
cell
concentration
were
stained
and
the
numbers
of
Lepehne-
positive
and
peroxidase-positive
colonies
in
each
group
were
determined.

The
pooled
results
from
three
such
experiments
are
given
in
Table
1. In
the
control
group
in
which
neither
conditioned
medium
nor
erythropoietin
was
included,
very
few
colonies
were
found,
and
most
of
them
could
not
be
classified.

When
erythropoietin
was
included,
there
was
a
great
increase
in
the
number
of
colonies
that
developed,
and
most
of
these
were
erthroid.

Concomitantly,
there
was
a
great
increase
in
heme
synthesis
rate
by
the
cultures.
In
the
cultures
with
conditioned
medium
but
no
erythropoietin,
the
number
and
proportion
of
granulocytic
colonies
increased
greatly,
but
there
was
no
effect
on
erthroid
colony
number
and
only
a
slight
effect
on
heme
synthesis.

When
both
erythropoietin
and
conditioned
medium
were
included,
there
followed
an
increase
in
both
erthroid
and
granulocytic
colonies
over
controls. These
results
indicate
that
the
production
of
erythroid
colonies
was
virtually
independent
of
the
production
of
granulocytic
colonies.

We
next
tried
to
culture
adult
mouse
bone
marrow
under
the
conditions
developed
for
the
production
of
erythroid
colonies
by
mouse
fetal
liver
cells.

The
Lepehne-positive
colonies
obtained
were
smaller
than
those
produced
by
fetal
liver
cells,

and
the
plating
efficiencies
were
somewhat
lower.

However,
these
colonies
were
also
erthropoietin-dependent.

When
bone
marrow
cells
were
obtained
from
mice
that
had
previously
been
made
hypoxic
by
exposure
to
a
pressure
of
0.5
atmosphere
for
2
days,
the
number
of
erythroid
colonies
increased
about
3-fold.

The
method
described
here
thus
appears
to
be
useful
for
the
production
of
erthropoietin-
dependent
colonies
of
hemoglobin-synthesizing
cells
from
adult
as
well
as
from
fetal
hemopoietic
tissue.

**DISCUSSION**

A
method
has
been
presented
for
obtaining
colonies
of
erthroid
cells
in
cultures
of
hemopoietic
cells
from
the
liver
of
13-day
C3H/Bi
mouse
gametes.

The
development
of
these
colonies
was
shown
to
depend
on
the
presence
of
the
hormone
erythropoietin
in
the
medium,
and
the
colonies
were
shown
to
be
composed
of
hemoglobin-synthesizing
cells.

Moreover,
the
conditioned-medium
dependency
of
granulocytic
colonies
(8, 9, 16)
was
confirmed.

<table>
<thead>
<tr>
<th>Table 1. Influence of erythropoietin and of conditioned medium on the formation of erythroid and granulocytic colonies, and on heme synthesis rates by 4-day cultures of C3H/Bi mouse fetal liver cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Treatment</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Erythropoietin alone</td>
</tr>
<tr>
<td>Conditioned medium alone</td>
</tr>
<tr>
<td>Erythropoietin + conditioned medium</td>
</tr>
</tbody>
</table>

* Mean values from three separate experiments with six plates per group in each experiment, rounded off to the nearest integer.
† Means of the percentages from three experiments.
‡ Mean ± SE (eight cultures per group in one of the three experiments).
Addition of erythropoietin did not result in a decrease in the number of granulocytic colonies, nor did addition of conditioned medium result in a decrease in the number of erythroid colonies. The production of erythroid colonies thus appeared to be independent of the production of granulocytic colonies in the same cultures. Although other possibilities exist, these data would be consistent with the hypothesis that the two types of colonies are derived from separate cells. Suspensions of liver cells from 13-day C3H/He mice fetuses thus may contain cells already committed as progenitors of erythroid colonies and cells already committed as progenitors of granulocytic colonies, each able to respond to its own appropriate stimulus—either erythropoietin or the active agent in conditioned medium, but not both. The response to each stimulus would be a burst of cell proliferation and differentiation of the respective class of cells.

At present, the term CFU-S is used to refer to the cell that gives rise to colonies in the spleens of supralethally irradiated mice after intravenous injection of bone marrow cells, while the term CFU-C is used in reference to hemopoietic cells that give rise to colonies (mainly granulocytic) in culture (17). With the evidence in the present paper that not only granulocytic but also erythroid colonies can be obtained in vitro, it may be operationally useful to replace the term CFU-C by two terms, CFU-G and CFU-E, the former to signify the cellular unit responsible for the production of granulocytic colonies, and the latter to refer to the cellular unit that gives rise to erythroid colonies, in culture.

Evidence from sedimentation velocity measurements (5) indicates that the CFU-S is not identical with the cell(s) that respond(s) to erythropoietin by an increased rate of heme synthesis in mass cultures of mouse fetal liver. Whether or not the CFU-E responsible for erythropoietin-dependent erythroid colony formation is identical with the erythropoietin-sensitive cell(s) in mass culture, and how the CFU-E is related to the CFU-S, are at present open questions.

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