HEMOGLOBIN SWITCHING IN SHEEP AND GOATS

V. Effect of Erythropoietin Concentration on
In Vitro Erythroid Colony Growth and Globin Synthesis

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ABSTRACT

Erythroid colonies were generated in response to erythropoietin in plasma clot cultures of sheep and goat bone marrow cells. At low concentration of erythropoietin only hemoglobin A (beta^A globin) was synthesized in goat cultures, but at high concentrations 50% of the hemoglobin synthesized was hemoglobin C (beta^C globin). This effect of erythropoietin on the expression of a specific beta globin gene was manifested only after 72 h in vitro and followed the development of erythroid colonies. Sheep colonies behaved differently from those of goat in that little or no beta^C globin synthesis occurred even at high erythropoietin concentration. To investigate this difference, sheep marrow cells were fractionated by unit gravity sedimentation. The erythroid colony-forming cells sedimented more rapidly (3.5–6 mm/h) than the hemoglobinized erythroid precursors (1–3.5 mm/h), suggesting that the colonies were formed from an early erythroid precursor. However, the colonies formed from the sheep marrow fractions synthesized only beta^A globin even at concentrations of erythropoietin sufficient to stimulate beta^C globin synthesis in goat colonies. Morphologically, the goat colonies were larger and more mature than those of the sheep. By 96 h in vitro three-fourths of the goat colonies contained enucleated red cells compared to only 3% of the sheep colonies. Thus, erythropoietin had an equivalent effect in stimulating erythroid colony growth from the marrow of both species although there were both biochemical and morphological differences between the colonies. Hemoglobin switching appeared to require exposure of an early precursor to high erythropoietin concentration, but the results with sheep marrow suggested that the rate of colony growth and cellular maturation might also be important.

A unique opportunity to analyze the regulation of globin gene expression is provided by studying erythropoiesis in goats and certain sheep since these animals respond to an anemic stress or to injection of erythropoietin (ESF), a glycoprotein hormone which stimulates hemopoiesis, by synthesizing a new hemoglobin (4, 15–17, 29). Hemoglobin C (alpha^2beta^C) differs from the usual adult hemoglobins only in its nonalpha globin, and hence the switch represents activation of beta^A globin synthesis with concomitant suppression of the synthesis of the usual adult beta chains. During the first few months of life, newborn animals synthesize beta^C globin. Hemoglobin C may reach 20–30% of the...
total circulating hemoglobin in lambs and up to 100% in young goats, but by three months of age it normally falls to very low concentrations and is replaced by the adult hemoglobin.

The ability to stimulate hemoglobin C synthesis in vivo (9, 17, 28), and more recently in vitro (1, 3), has permitted analysis of several aspects of this switch. Gabuzda et al. (9) have found that hemoglobin C is synthesized in sheep marrow in vivo 3–5 days after injection of erythropoietin or plasma from anemic animals. This result has been interpreted to indicate that the switch is controlled in an early erythroid precursor cell. A similar conclusion has been suggested by our finding that erythropoietin-dependent hemoglobin C synthesis is detectable in goat marrow suspension cultures only after 72 h in vitro (3).

Erythropoietin appears to stimulate erythroid cell proliferation both in vivo (14) and in vitro (5, 19, 25), and may have specific effects on RNA synthesis (11, 13) and mRNA production (12). The effects of erythropoietin on the molecular and cellular control of hemoglobin C synthesis are unknown. The switch appears to be regulated by an intracellular mechanism since both hemoglobins A and C are found in the same red cell (10, 24). Control of specific beta globin synthesis does not appear to be at the translational level (cytoplasmic protein synthesis) since no evidence for the selective translation of rRNA species has been found (2, 8, 18, 23).

To quantitate the effect of erythropoietin in stimulating proliferation of an early, but presumably committed, erythroid precursor, we have grown erythroid colonies from goat and sheep marrow in plasma clot cultures (19, 26). With this technique we have been able to compare the concentration of erythropoietin required to stimulate proliferation and maturation of goat erythroid colonies in vitro with that which stimulates synthesis of beta C globin. Beta C globin synthesis appears in goat cultures only after colony growth has occurred. Sheep colonies differ from those of the goat in that little or no beta C globin synthesis occurs even at high erythropoietin concentration. The relative size and degree of maturation of sheep and goat colonies have been examined in order to provide further insight into the cellular aspects of hemoglobin switching in these animals. Furthermore, sheep marrow has been fractionated by unit gravity sedimentation in an attempt to characterize the cells capable of generating erythroid colonies.

MATERIALS AND METHODS

Cell Culture

Sheep and goats were selected for homozygosity of hemoglobin A by preliminary screening of peripheral blood hemolysates either by electrophoresis on cellulose acetate (sheep) or by column chromatography of globin chains on carboxymethylcel lulose (CM-cellulose, goats) (23). The animals were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg) and exsanguinated via a catheter placed in the femoral artery. The femurs and humeri were immediately dissected out, the ends of the bones were cut off, and the marrow was gently removed with a sterile spatula and placed in Eagle's minimal essential media (MEM). The marrow was disaggregated by flushing it through a catheter-tipped 50-ml syringe. The cells were washed once, resuspended in MEM, and dispersed into a suspension of single cells by expulsion through a 20-gauge syringe needle.

The technique utilized for generation of the clot culture was similar to that described by MacLod et al. (19). The concentrated cell suspension was diluted to a final concentration of 0.5–1 × 10⁷ cells/ml using a solution prepared as follows: 10 ml of 10 Eagle's basal medium with Hanks' balanced salts, 1 ml of 100 Eagle's basal medium amino acid solution, 1 ml of 100 mM sodium pyruvate, 1 ml of 200 mM L-glutamine, 1 ml of 7.5% NaHCO₃, and 2 ml of penicillin-streptomycin (5,000 U of each/ml) were mixed with sufficient sterile distilled water to give a final volume of 100 ml. After dilution, 1 vol of the cell suspension was mixed with 1 vol each of 0.8% beef embryo extract, 35% bovine serum albumin (BSA), and 0.2 mg/ml L-asparagine, 0.5 vol of either isologous lamb serum (sheep cultures) or heat inactivated fetal calf serum (goat cultures), and 4.5 vol of NCTC-109 (Hyland Laboratories, Los Angeles, Calif., containing penicillin and streptomycin—100 U of each/ml). Erythropoietin dissolved in 0.5 vol NCTC-109 was added to the medium to achieve the desired final erythropoietin concentration. The final proportions of the various components of the incubation medium are given in Table I.

Plasma clots were generated by adding 0.05 vol of citrated bovine plasma to 0.95 vol of the final cell suspension in culture medium and pipetting 0.1 ml into individual 6-mm diam plastic microwells. Three plastic sections, each containing six wells, were placed in a 20 × 100-mm petri dish along with a 10 × 35-mm petri dish containing 1 ml of sterile distilled water. The clot cultures were incubated in 5% CO₂–95% air with 95% relative humidity at 37°C.

Erythropoietin utilized in this study was derived from anemic sheep plasma (Lot III—4.7 U/mg supplied by Connaught Medical Laboratories, Toronto, Canada) or human urine (Lot no. H-6-TaLSL—36.3 U/mg supplied...
TABLE I

Requirements for Erythroid Colony Growth

| Components               | ml of component per ml of medium |
|--------------------------|----------------------------------|
| NCTC-109                 | 0.45                             |
| Bovine serum albumin (35%)| 0.10                             |
| Beef embryo extract (0.8%)| 0.10                             |
| L-Asparagine (0.2 mg/ml) | 0.10                             |
| Citrated bovine plasma   | 0.05                             |
| Serum (isologous or fetal calf)* | 0.05       |
| Erythropoietin (10 U/ml) | 0.05                             |
| Diluted cell suspension  | 0.10                             |

* Isologous serum was used for sheep cultures and fetal calf serum for goat cultures except as otherwise indicated.
† The amount of erythropoietin added was varied for each individual experiment.

7.2. Excess glutaraldehyde was removed after 5-min fixation by blotting with an additional filter paper strip. The filter papers were removed and the slides washed in distilled water for 10 min. After drying, the slides were stained in 1% 3,3′-dimethoxybenzidine in methanol and counterstained with Harris-Lily hematoxylin (Fisher Scientific Co., Pittsburgh, Pa.). Each clot was examined and the number of benzidine-positive colonies quantitated by counting those which contained eight or more cells.

Fractionation of Bone Marrow Cells

Sheep marrow cells were fractionated by unit gravity sedimentation on a shallow 1 to 2% bovine serum albumin gradient at 4°C (7). The “Staput” apparatus (Johns Corp., Toronto, Canada), consisting of a sequence of two reservoirs flowing into a mixing chamber with an outlet to a separatory chamber, was employed in a manner similar to the apparatus described by Miller and Phillips (21). 20 ml of 0.5% bovine serum albumin in phosphate-buffered saline (PBS, Grand Island Biological Co.) was allowed to flow from the mixing chamber into the separatory chamber followed by 1 × 10⁶ total cells (nonnucleated/nucleated = 5/1) suspended in 100 ml of PBS in 5% serum. The mixing chamber was rinsed and filled with 0.5% BSA in PBS. The gradient was generated by placing 800 ml of 1% BSA in PBS in the reservoir adjacent to the mixing cylinder and 800 ml of 2% BSA in PBS in the second reservoir, and allowing the gradient to flow through the mixing chamber and upward into the separatory chamber over a 1-h period. After the cells were allowed to sediment for 6 h, 95-ml fractions were collected by drawing off fluid from the base of the separatory chamber. The cells were recovered by centrifugation and suspended in MEM containing 5% fetal calf serum. The cell number was ascertained by counting a diluted fraction in a hemocytometer. A small aliquot of each fraction was also layered onto a slide with the Shandon cytococtrifuge, stained with benzidine-hematoxylin as described above, and the percentage of hemoglobinized cells determined. Plasma clot cultures (containing 10⁴ nucleated cells/ml) of each fraction were incubated for 96 h in the presence of varying quantities of erythropoietin. The number of benzidine-positive colonies were determined, and the synthesis of the individual globin chains was quantitated by CM-cellulose chromatography.

Radioactive Labeling of Globin

To label the cultures, 10 μCi of [1H]leucine (sp act 30–50 Ci/mmol, New England Nuclear, Boston, Mass.) in 10 μl of NCTC-109 was added to each microwell and incubation continued for 24 h. At the end of the labeling period the individual clots were removed from the microwells using a sterile spatula. The clots were digested in 0.05% trypsin ( Worthington Biochemical Corp., Free-
solution in a 15 x 60-mm petri dish. The clots were
hold, N. J.) in Ca-Mg-free PBS (Grand Island Biological
Co.). 12-14 clots were added to 5 ml of the trypsin
solution (providing a substrate for further
proteolysis), and the cells were collected by centrifuga-
tion, washed once in 0.9% saline, and lysed with 1 ml of
distilled water. The lyase was centrifuged and the result-
ing stroma-free solution was stored at -20°C. 40-60
individual 0.1-ml clots were used for analysis of the
incorporation of radioactivity into globin chains.

Analysis of Radioactive Globin by
Polyacrylamide Gel Electrophoresis

The method described by Moss and Ingram (22) was
used for the rapid analysis of sheep globin chains. A
stock urea solution was prepared by dissolving 240 g urea
(Schwarz/Mann Div. Becton, Dickinson and Co.,
Orangeburg, N. Y., ultrapure) and 77 mg dithiothreitol
(Calbiochem, San Diego, Calif.) in sufficient water to
give a final volume of 450 ml. Each of the following
solutions was prepared: (a) acrylamide: 4.24 g acrylam-
ide and 112 mg N,N'-methylene-bis-acrylamide (each
supplied by Bio-Rad Laboratories, Richmond, Calif.)
in 20 ml of stock urea; (b) gel buffer: 1.74 g Tris base
(Schwarz/Mann, ultrapure), 130 mg glycine (J. T. Baker
Chemical Co., Phillipsburg, N. J.), and 0.012 ml
TEMED (Bio-Rad) in 20 ml of stock urea; and (c) po-
lymerizing agent: 56 mg ammonium persulfate in 20 ml
stock urea. Equal volumes of acrylamide solution, gel
buffer, and the polymerizing agent were mixed, deae-
terated, and poured into gel tubes 5 mm wide by 10.5 cm
long (12 gels could be conveniently made if 9 ml of each
of the solutions were used). The gels were allowed to po-
lymerize at room temperature for 30 min and were then
preelectrophoresed at 1 mA (constant current)/gel for
0.5 h. Both the anode and the cathode chambers con-
tained buffer prepared by dissolving 15.2 g Tris base and
1.12 g glycine in sufficient stock urea to give a final vol-
ume of 350 ml.

The stroma-free lysates to be analyzed were lyophil-
ized and dissolved in 0.3 ml of reservoir buffer contain-
ing 5% sucrose and carrier sheep globin (containing
unlabeled beta^a and beta^c globins prepared from a red
cell lysate by acid-acetone extraction) at a concentra-
tion of 5 mg/ml. 0.05 ml was applied to each gel and
electrophoresis was performed for 4-5 h at 2 mA/gel.
Samples of the unlabeled globin alone were electro-
phoresed in each set of gels to serve as a marker for the
position of the globin chains. These gels were fixed and
stained in 7% acetic acid containing amido black (0.5
g/ml). After 6-8 h the gels were electrically destained.
Gels containing radioactive plus nonradioactive carrier
globin were frozen and then sliced in sections 2 mm thick.
Each section was placed in 1 ml of a mix of 9 parts NCS
(New England Nuclear) and 1 part water and heated for
2 h at 60°C to swell the gel and elute the labeled globin.
10 ml of a mix of 9 parts LSC complete and 1 part
Hydromix (both from Yorktown Research) were then
added, and radioactivity in the samples was measured at
an efficiency for 3H of approximately 20% in a Packard
Tri-Carb liquid scintillation spectrometer (Packard In-
strument Co., Inc., Downers Grove, Ill.)

Analysis of Radioactive Globin by
CM-Cellulose Chromatography

CM-cellulose chromatography was used for analysis of
globin chains to be analyzed. Heme was then extracted
by treatment with acid-acetone, and the globin obtained
was dried under a stream of N\_2. It was dissolved in 0.01
M Na\_2P\_4O\_7, pH 6.8, in 8 M urea and applied to the
CM-cellulose column. The column was eluted with a
nonlinear gradient of increasing sodium concentration (6,
23), and 1.5 ml fractions were collected directly into
scintillation vials. 10 ml of Riafluor (New England
Nuclear) were added to the vials, and double label liquid
scintillation counting was performed with an efficiency of
56% for $^3$H and 19% for $^3$C.

RESULTS

Erythroid Colony Growth

Erythroid colonies were grown from both sheep
and goat bone marrow cells in response to erythro-
poietin. The number of erythroid colonies was
quantitated by counting those which contained
more or benzidine-positive cells. At 48 h the
erythroid cells were immature in appearance with
vesicular nuclei and cytoplasm which was only
weakly stained with benzidine (Fig. 1 a). At 72 h
the colonies were larger and more deeply stained
with benzidine (Fig. 1 b). After 96 h in vitro many
of the goat colonies contained fully mature red
cells from which the nuclei had been extruded,
although there were still large colonies containing
quite immature-appearing erythroid cells (Fig. 1
c). In general the sheep colonies contained fewer
cells, and these cells were more immature in
appearance than goat colonies at equivalent times
in vitro (Fig. 1 d).

The number of colonies formed was propor-
Figure 1  Morphology of erythropoietin-dependent sheep and goat erythroid colonies. 1a. Goat colonies after 48 h in vitro. Illustrated are one 8-cell colony and one 4-cell group which would not yet be considered a colony. 1b. Goat colonies after 72 h in vitro. The colonies are larger and some of the red cells have begun to undergo enucleation. 1c. Goat colonies after 96 h in vitro. Further maturation has occurred. 1d. A sheep colony at 96 h. The colony is smaller and the cells are more immature. All cultures contain 0.5 U/ml (sheep plasma, Step III) erythropoietin. × 630.
Globin Synthesis in Goat Marrow Cultures

The effect of increasing the concentration of erythropoietin on the synthesis of the individual globin chains in goat marrow culture was analyzed as illustrated in Fig. 3. After 96 h in vitro at an erythropoietin concentration of 0.025 units/ml, \(^{3}H\)leucine was incorporated into the beta\(^A\) and alpha chain, but only a minimal amount of radioactivity was eluted in the position of the beta\(^C\) chain (Fig. 3 a). Increasing the erythropoietin concentration to 0.125 U/ml resulted in a twofold increase in incorporation of radioactivity into total globin (Fig. 4 c) and substantial synthesis of beta\(^C\) globin (Fig. 3 b). At a concentration of 2.0 U/ml there was a further increase in total globin synthesis and 54% of the \(^{3}H\)leucine incorporated into beta globins was now in the beta\(^C\) peak (Fig. 3 c). From the data in Fig. 3 we determined that, as the percent \(\beta^C/\beta^A + \beta^C \times 100\) of beta\(^C\) globin synthesis increased with increasing erythropoietin concentration from approximately 10% at low ESF concentrations to 50% at 2 U/ml, there was a proportionate decrease in the percentage of beta\(^A\) globin synthesized.

The effect of erythropoietin concentration on goat colony growth, total globin synthesis, and specifically beta\(^C\) globin synthesis was determined by the experiment illustrated in Fig. 4 a, c, and e. Even at the lowest concentration tested there were nearly maximal numbers of colonies generated (Fig. 4 a). In the absence of the hormone, generally there were fewer than 40 colonies per well, although in this experiment 70 colonies were found. Total hemoglobin synthesis increased almost three times over the 2-log range of ESF concentration (Fig. 4 c). Very little beta\(^C\) synthesis occurred at the lowest concentration of ESF (Fig. 4 e). Between 0.125 and 2.0 U ESF/ml there was a dramatic increase in the amount of beta\(^C\) formed. It reached 54% of the total beta globin synthesized at the highest ESF concentration. The ESF used in these experiments was Step III (4.7 U/mg) derived from anemic sheep plasma.

Globin Synthesis in Sheep Marrow Cultures

The response of sheep marrow cultures differed in several respects from that of the goat. Colony growth was dependent on the presence of erythro-
Globin Synthesis in Cultures of Fractionated Sheep Marrow

In sheep marrow separated in an albumin gradient, the erythroid colony-forming cells sedimented more rapidly than most of the hemoglobinized erythroid precursors (Fig. 6). Two pools of cells were obtained, the first sedimenting between 1 and 3 mm/h which contained 95% of the hemoglobinized cells, and the second sedimenting between 3.5 and 6 mm/h which contained 98% of the erythroid colony-forming cells. Clot cultures of each pool (54 microwells) were incubated in vitro for 96 h and labeled for the last 24 h with [3H]leucine. Globin synthesis in the cultures containing colonies was 100 times that found in the cultures which included previously differentiated hemoglobinized cells but lacked colonies (data not shown). Three different concentrations of ESF were tested (0.025, 0.125, and 0.5 U/ml), and betaC globin synthesis was less than 5% at each concentration both in those cultures from the pool containing the colony-forming cells and in those from the pool of hemoglobinized cells.

Comparison of Sheep and Goat Cultures

Colony number, globin synthesis, and betaC synthesis in the sheep cultures at 96 h were compared to those in goat cultures as illustrated in Fig. 4. At 96 h, sheep colonies were found to be generally smaller than goat colonies, two-thirds being in the range of 8–12 cells compared to one-third for the goat (Fig. 7). One-third of the sheep; and fetal bovine serum. Human urinary ESF (H-6-TalSL) was tested at several concentrations up to 10 U/ml. Colony growth and globin synthesis were substantial, but betaC synthesis did not occur. A representative gel separation of the globins synthesized under these conditions is indicated in Fig. 5 a.

Bone marrow was studied from a sheep already synthesizing hemoglobin C. This animal had been phlebotomized for 3 wk during which time its hematocrit was maintained at 12–14%. At the end of this period the bone marrow was cultured with increasing concentrations of erythropoietin. The relative proportions of betaA and betaC globin from clot cultures labeled for 24 h were analyzed by polyacrylamide electrophoresis. There was no difference between four successive 24-h labeling periods at the various ESF concentrations. The result for 0.5 U ESF/ml at 96 h is illustrated in Fig. 5 b.
FIGURE 4 Relationship of colony growth, total globin synthesis, and beta<sup>c</sup> globin synthesis to erythropoietin concentration. The radioactivity in globin was calculated by summing the counts per minute which eluted in the individual peaks. Inclusion of the same amount of [<sup>14</sup>C]globin carrier in each chromatographic separation permitted correction for losses during both preparation and chromatography of the globin; these amounted to 10–30%. a, c, and e: Goat cultures; b, d, and f: sheep cultures.

FIGURE 5 Polyacrylamide gel electrophoresis of sheep globin labeled during the period of 72–96 h in vitro. Details of the technique are given in Materials and Methods. The position of the individual chains was determined by electrophoresis of labeled standards. (a) Globin synthesized in the cultures grown from the marrow of a normal sheep; (b) globin synthesized in the cultures of a sheep which had been made anemic by phlebotomy in order to stimulate synthesis of hemoglobin C.
concentrations of ESF, whereas little or no beta^c globin was synthesized in sheep cultures.

**Globin Synthesis in Colonies Generated by Human Erythropoietin of High Specific Activity**

Both sheep and goat marrow were cultured in the presence of the highly purified human urinary erythropoietin. Because of the limited amount available, only one concentration was tested. At 2.0 U/ml the number of colonies and the synthesis of beta^c did not differ significantly from the results obtained with the crude hormone obtained from sheep plasma, but total globin synthesis was slightly less (data not shown). Thus, 50% of the total beta globin synthesized in goat colonies was beta^c, but no incorporation of radioactivity into beta^c globin was detected in the sheep cultures.

**DISCUSSION**

Erythropoietin seems to have several effects on erythropoiesis. One of the earliest responses is stimulation of RNA synthesis (11, 13), perhaps specifically mRNA (12), but the hormone also stimulates proliferation of committed erythroid precursors in vivo (14) and in vitro (15, 19, 26) and may enhance hemoglobin synthesis in cells already making hemoglobin (14). Our studies have shown two well-defined effects of erythropoietin in vitro...
on goat erythroid cells. These are (a) proliferation to form colonies of hemoglobinized cells, an effect which occurs at low (and high) concentrations (Fig. 4 a), and (b) stimulation of beta-c globin synthesis, an effect which occurs to a significant extent only at high concentrations of ESF (Fig. 4 e). That the same substance exerts both effects is suggested by the similar result obtained with crude (4.7 U/mg) ESF prepared from sheep plasma and highly purified (12,000 U/mg) material from human urine. A similar conclusion has been suggested by studies in which ESF of various degrees of purity has been injected into intact sheep (28).

The action of erythropoietin on cell proliferation and on specific intracellular biochemical events is partially clarified by our studies. Both effects appear to be exerted primarily on an early precursor cell. Cell proliferation in response to ESF to form multiple hemoglobinized progeny has been shown in vivo to be a property of an undifferentiated responsive cell in spleens of plethoric mice (14) and also in vitro as shown with purified fractions of undifferentiated mouse fetal liver cells (5). Sedimentation velocity analysis of rat marrow has led to the identification of a large, rapidly sedimenting cell which responds to ESF by cell division (20). We also found that the colony-forming cell from sheep marrow is rapidly sedimenting, indicating that it is a large and immature cell (Fig. 6). The sequential development of goat colonies from small clusters of weakly benzidine-positive cells with large vesicular nuclei (Fig. 1 a) to completely hemoglobinized colonies (Fig. 1 e) has further substantiated the concept that we have stimulated proliferation of an early precursor cell to give erythroid colonies.

That the action of ESF on specific biochemical events occurs on an early precursor cell has been suggested by our finding that synthesis of beta-c globin in goat cultures occurs only after the

**Figure 8** Comparison of goat and sheep colonies as to the time course of colony development, globin synthesis, and beta-c globin synthesis. Labeling of the cultures was for 24-h intervals. The erythropoietin concentration was 0.5 U/ml (sheep plasma, Step III) and the nucleated cell concentration 10⁶/ml. (a) Goat; (b) sheep.
that both hemoglobins A and C are found in the goat marrow, but we have detected little or no same red cell (10, 23), indicating that the switch must be mediated by an intracellular mechanism. Regulation at the level of mRNA translation into specific globin chains does not appear to occur (2, 8, 18, 23). Thus ESF at high concentrations seems to act on an early erythroid precursor in goat marrow determining which of the specific beta globin genes is transcribed. Alternatively, there might be selective processing of the immediate gene transcription product to produce beta\textsuperscript{A} and/or beta\textsuperscript{C} mRNA depending on the concentration of erythropoietin to which the cell is exposed. That this action of ESF becomes apparent only after proliferation of the cell to form differentiated progeny is consistent with the requirement for DNA synthesis and mitosis for hormone-dependent differentiation in other systems (27). Whether there is early accumulation of mRNA for the specific beta globins which, later in differentiation, is translated into globin or whether production of the specific beta globin mRNA continues during differentiation can only be a matter of speculation at this time.

Sheep erythroid colonies have been grown under conditions very similar to those employed for the goat marrow, but we have detected little or no beta\textsuperscript{C} synthesis. Several explanations might account for this finding. Perhaps expression of the individual beta globin genes is determined earlier in erythropoiesis in the sheep than in the goat so that the erythroid colony-forming cell is already committed to synthesis of particular globin chains. The result obtained with the phlebotomized sheep (Fig. 5 b) appears to support this notion, although the similar time course of development of beta\textsuperscript{C} synthesis in intact sheep and goats is against it (9, 15). In contrast to our results, Adamson and Stamatoyannopoulos (1) have induced the synthesis of substantial amounts of hemoglobin C in short-term suspension cultures of sheep marrow. Thus, they have demonstrated the potential for hemoglobin C activation. The reason for the difference between their results and ours is not immediately apparent.

The differing appearance of sheep and goat colonies (Fig. 1 c and d) has led us to compare more precisely several aspects of their growth. Total globin synthesis increases dramatically in goat cultures as the colonies mature (Fig. 8 a) but actually declines in sheep cultures after 48 h (Fig. 8 b). Since 100 times more globin is synthesized between 72 and 96 h in gradient separated sheep marrow fractions which have colonies, we conclude that most of the globin produced is derived from these colonies. Thus, exposure of the immature sheep erythroid precursor cell, capable of colony growth, to high concentrations of ESF is insufficient to activate the synthesis of beta\textsuperscript{C} globin. The lack of beta\textsuperscript{C} globin synthesis in sheep colonies can be correlated morphologically with their relatively small size and their failure to mature completely in 96 h to enucleated red cells (Fig. 7). Thus, although the amount of ESF added to the sheep and goat cultures is equivalent, the differing morphological and biochemical behavior of the two types of colonies suggests that the effect of the hormone on intracellular phenomenon in the two species might be different. Further studies will be needed to clarify whether this is a property of the erythropoietin preparation or intrinsic to the erythroid cells of the two species.

Expression of specific globin genes cannot be studied profitably without consideration of the processes of cellular proliferation and maturation which occur during erythroid differentiation. The biochemical action of ESF in inducing expression of specific globin genes appears to be exerted early in erythropoiesis, indicating the need to use purified populations of early precursor cells to study the molecular mechanism of this action of the hormone. It is hoped that definition of the growth behavior of the erythroid cells necessary for expression of specific globin genes will permit a search for factors responsible for the switch from fetal to adult hemoglobin in man and other species.

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