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Research Article

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The following conclusions were drawn: hemoglobin phenotype is determined at a stem cell level. Erythroid stem cells appear to undergo gradual renewal. The identity of the plasma factor which induces Hb C formation is not yet known; it is not present in plasma from nonanemic sheep, and its production is not dependent upon hemoglobin genotype. If the plasma factor turns out to be erythropoietin, then this hormone must have an important influence on the pool of erythroid stem cells.

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Erythropoietic Kinetics in Sheep Studied by Means of Induced Changes in Hemoglobin Phenotype

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ABSTRACT This investigation is concerned with the kinetics of the reciprocal relationship between sheep hemoglobin (Hb) A and Hb C formation in response to anemia. The relative synthesis of the hemoglobin types was assessed at various times in bone marrow erythroid cells incubated in vitro with ^{59}Fe. The changeover from Hb A to Hb C formation lagged by about 3 days behind the development of anemia and was complete within about 11 days. After recovery from anemia the reciprocal change back to preanemic conditions proceeded at a much slower rate, Hb C formation gradually declining to unmeasurable levels over about 25 days.

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INTRODUCTION

A change in the hemoglobin electrophoretic pattern occurs in sheep having the gene for hemoglobin (Hb) A when they become anemic (1–5). Van Vliet and Huisman (5) showed that Hb A is entirely replaced during severe anemia by a new hemoglobin type which they named Hb C. In heterozygous Hb AB sheep the relative proportion of Hb B remains constant during anemia; homozygous Hb B sheep do not show any change. These hemoglobins all possess the same alpha chain but differ at multiple amino sites in their beta chains (6–10). Thus the conversion from Hb A to Hb C formation is primarily one of a reciprocal relationship in the activity of the genes directing the synthesis of $beta^A$ and $beta^C$ chains.

The first objective of the present investigation was to define the kinetics of the relative formation of Hb A and Hb C during the onset of and following recovery from severe anemia. The relative synthesis of the two hemoglobins was measured by the use of in vitro incorporation of an isotopic label in serial bone marrow specimens. The results show a rapid replacement of Hb A by Hb C in young erythroid cells within approximately 11 days, but after recovery from anemia the return to the original preanemic state proceeds at a much slower rate. Van Vliet and Huisman (5) had observed

that the absolute concentration of Hb C in the peripheral blood remains constant for a few weeks after recovery from anemia. This finding led them to the suggestion, which we thus confirm, that the formation of Hb C continues well beyond the recovery from the anemic stimulus which first evoked its production.

Our second objective was to determine the role of a humoral factor in this change of genetic expression by giving infusions of plasma taken from anemic donor sheep to nonanemic recipients. The results demonstrate a major influence of this plasma on the induction of the changed hemoglobin phenotypic pattern, in agreement with the findings of Moore, Godley, van Vliet, Lewis, Boyd, and Huisman (11), who used human urinary erythropoietin, and those of Boyer (12), who also used plasma from anemic donor sheep. The results are considered in terms of their relevance to stem cells, the action of erythropoietin, and to previously proposed models of erythropoiesis.

METHODS

Animals. All studies of Hb C induction were conducted on three homozygous adult Hb A sheep of mixed breeds, two ewes and one ram, 50–80 kg in weight. (Sheep numbers were changed during the investigations.) Sheep of various hemoglobin types were used as plasma donors.

Production of anemia in sheep. Hemolytic anemia was produced by intraperitoneal injections of phenylhydrazine given on alternating days. The initial dose was 0.28 ml/kg of a fresh neutral 5% solution; 0.42 ml/kg was given in subsequent injections. Two to four doses were required to produce severe anemia. Bleeding anemia was produced by phlebotomy of 1 liter of whole blood every 2nd or 3rd day. Infusions of isotonic saline were given as required after phlebotomy.

Plasma infusions. 1 liter of blood was collected into acid citrate dextrose (ACD) solution from anemic or control donor sheep, immediately centrifuged, the fresh plasma withdrawn, and 750-ml volumes infused into nonanemic recipient sheep. No untoward effects of the infusions were noted.

Relative synthesis of Hb C in bone marrow erythroid cells. Serial bone marrow aspirations were collected in heparinized syringes from the sternal marrow cavity. Serial studies were done in three sheep made anemic with phenylhydrazine and allowed to recover spontaneously and in one sheep subjected to blood loss anemia and then abruptly transfused back to normal hematocrit with autologous packed red cells. Five serial studies were conducted in nonanemic sheep given plasma infusions. In four of these, the infusions were from single anemic donors, and in one control study, six nonanemic donors were used.

Incubations. The bone marrow cells, which were always diluted with peripheral blood, were incubated overnight in the presence of ^{59}Fe.[1] Incubations were carried out in Eagle's S minimal essential medium with 20% normal sheep serum. $^{59}FeCl_3$ was bound to the serum before its addition to the culture. The iron binding capacity of the serum was not exceeded. The incubations, containing 1–5 μc/ml of ^{59}Fe in the final mixture, were carried out overnight in stationery milk dilution bottles in an incubator with 5% CO_2 in humidified air at 37°C. Samples were then washed in saline, hemolyzed, and ultracentrifuged at 100,000 *g* for 1 hr at 4°C.

Hemoglobin separation. The ^{59}Fe-labeled hemoglobins were separated by starch granule electrophoresis (0.05 M veronal buffer at pH 8.6) at 15 volts/cm for 22 hr at 4°C. Volumes of 0.3-ml hemolysate were converted to cyanmethemoglobin immediately before application to the starch block. After completion of the separation, the starch block was cut into 0.5-cm sections which were quantitatively transferred to counting tubes, and the radioactivity was measured in an automatic well scintillation spectrometer. Hemoglobin was eluted from the individual sections by addition of 4.00 ml of veronal buffer and optical densities read at 540 mμ after centrifugation. The percent of relative synthesis of Hb C was derived from the Hb C radioactivity divided by the sum of Hb C and Hb A radioactivity × 100. Where no distinct Hb C radioactivity peak was seen, relative Hb C synthesis was taken to be zero.

Cyanide ion was added to the hemolysates in storage at 4°C before electrophoretic separation to prevent the possibility of heme-heme exchanges which may occur between methemoglobin molecules (13). Fractionations repeated after 3–4 months of hemolysate storage showed no change in the isotope patterns.

Miscellaneous studies. Hematocrits were done in quadruplicate by the microhematocrit method. Reticulocyte counts were done on dry preparations after vital staining with brilliant cresyl blue. Erythropoietin assays were performed by a modification of the hypertransfused mouse method (14).

RESULTS

Kinetics of induction of Hb C formation. The replacement of Hb A formation by that of Hb C is almost complete within about 11 days after the onset of anemia induced by phenylhydrazine, with a lag phase of about 3 days between the onset of anemia and the induced hemoglobin change (Fig. 1). In one sheep more intensively studied after severe blood loss, the first bone marrow study was not performed until the switchover to Hb C formation appeared complete, which was at a point 12 days after the onset of anemia, in good agreement with the results shown in Fig. 1. 3 days after the anemia had been abruptly corrected by acute transfusion

[1] Abbott Laboratories, North Chicago, Ill.

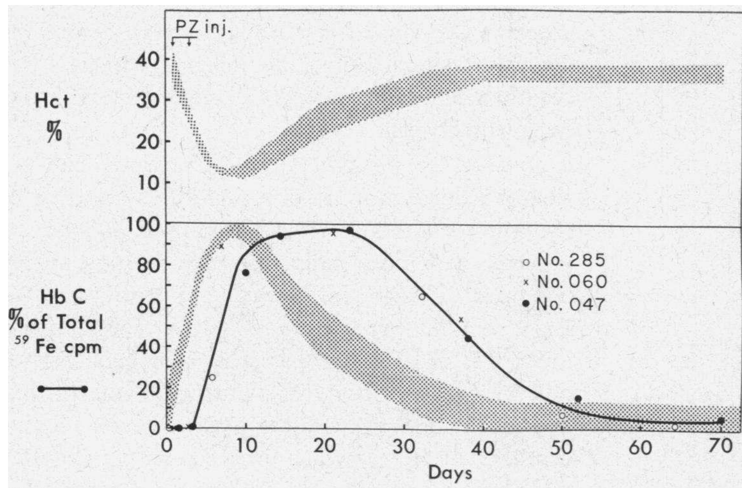

FIGURE 1 Change of hemoglobin type synthesized by homozygous Hb A sheep made anemic with phenylhydrazine (*PZ*). The illustration is a composite of studies in three sheep. The reciprocal of the hematocrit is expressed on the lower graph as "relative" anemia on an arbitrary scale from 0 to 100%. On day 25 the hematocrit exceeded 30%, a level which may be considered nonanemic in sheep. During the following 25 days Hb C formation disappeared at a linear rate, reaching levels near zero between days 50–60.

of autologous packed cells, the switchover still appeared complete. The reversion to the pre-anemic pattern then began (Fig. 2). Whether Hb A formation is actually totally absent during sufficiently severe anemia cannot be definitely concluded, since the methods used are not capable of detecting trace quantities. If any Hb A was formed at the point of maximum anemia in the study illustrated in Fig. 2, the amount was indeed minimal. Conversely, no Hb C formation was ever detected before induction of anemia. In a separate study repeated fractionation of an hemolysate also failed to reveal the presence of Hb C in the peripheral blood of a nonanemic sheep. (The limit of sensitivity of the method was about 0.1% of Hb C.) Previous suggestions that trace quantities of Hb C are demonstrable in nonanemic sheep have not documented the absence of anemia during the months preceding procurement of the blood sample (10).

Kinetics of the disappearance of Hb C formation after recovery from anemia. The lag between the recovery from anemia and the disappearance of Hb C–producing erythroid cells from the marrow was distinctly more pronounced than that found between the onset of anemia and the appearance of

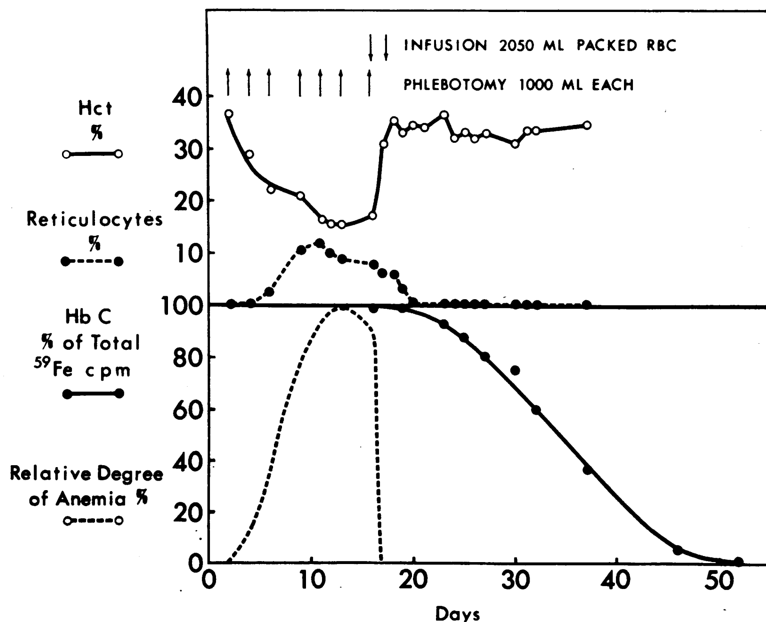

FIGURE 2 Persistence of Hb C formation in a homozygous Hb A sheep after abrupt recovery from severe anemia. Anemia was produced by phlebotomy, and the autologous packed red cells reinfused, as indicated. All ^{59}Fe was found in Hb C at the peak of anemia as well as 3 days later. After this phlebotomy and reinfusion the relative formation of Hb C declined at a rate exactly as that described in Fig. 1, following a linear slope and reaching levels near zero about 25 days after termination of anemia.

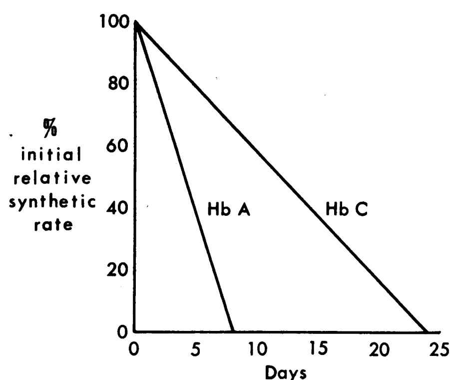

FIGURE 3 Rates of disappearance from marrow erythroid cells of Hb A and Hb C formation relative to one another. During these reciprocal changes Hb A disappears under conditions of severe anemia, whereas Hb C disappears during the nonanemic state. The slopes are −12.5% per day and −4.2% per day.

these cells (Figs. 1 and 2). Reticulocytes left the peripheral blood within 5 days of abrupt recovery from anemia, but Hb C formation declined slowly in a linear fashion over a 25–30 day period after hematocrits of 30% were exceeded. The data are not sufficient to determine whether minute quantities of Hb C are produced during the weeks that follow. The linear decline was not dependent on the rate of recovery from anemia; the same slope was observed after abrupt recovery from anemia as in spontaneous recovery. As noted, there was a delay of 3 days after abrupt recovery before the downward slope began, an interval which is not readily measurable during the more gradual spontaneous recovery.

Analysis of the kinetic relationships. The period of induction of Hb C formation is also that of the reciprocal loss of Hb A formation. This period proceeds at a more rapid rate than the postanemic reciprocal change in hemoglobin formation. In Fig. 3, the relationships are shown as simple linear functions. The phenotypic change in gene activation in the erythroid cells during anemia is expressed as the rate of disappearance in relative synthesis of Hb A, which proceeds with a negative slope of 12.5% per day and reaches completion within 8 days of its initiation. The change after anemia is expressed as the relative disappearance of Hb C formation. This follows a negative slope of 4.2% per day, only one-third the rate of the reciprocal changes during anemia. Thus the presence of increased concentrations of erythropoietin or of some other companion to anemia may influence the rate of the reciprocal change.

Humoral induction of Hb C formation. In four separate studies Hb C synthesis was readily induced in marrow erythroid cells by a series of infusions of fresh plasma taken from anemic donor sheep (Figs. 4–8). In two studies, only 2–4% of Hb C was being produced 2 days after the first

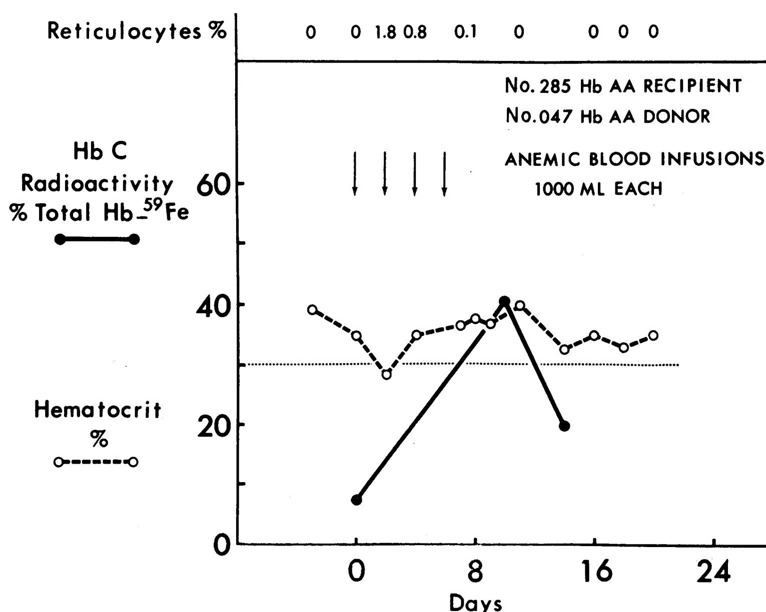

FIGURE 4 Induction of Hb C formation by infusions of whole blood taken from a donor with phenylhydrazine anemia with hematocrit 15%. The erythropoietin titre of the plasma given in the first infusion was 2.6 U/ml. The recipient had not yet fully recovered from a previous anemic episode, which explains the initial value of 9% for the relative Hb C synthesis before the first infusion. Only three marrow examinations were done.

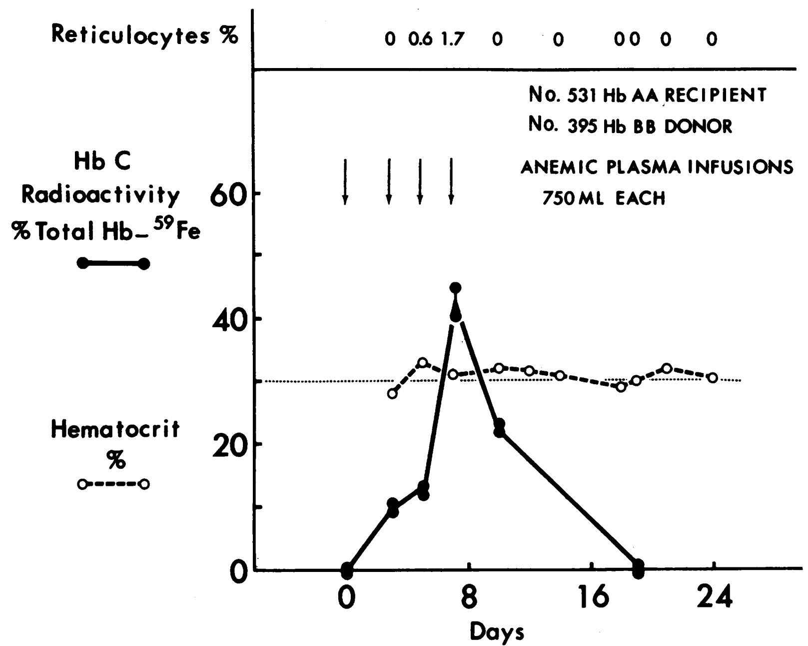

FIGURE 5 Induction of Hb C formation by infusions of plasma taken from a donor with phenylhydrazine anemia with hematocrit in the range of 12–14%. The erythropoietin titre of the plasma given in the third infusion was 4 U/ml. Duplicate determinations are indicated for Hb C radioactivity. Although definite induction was present on day 3, the value reached a maximum of 43% on day 8, declining after the last infusion.

infusion, and in a third, Hb C synthesis was 10% of the total on the 3rd day after the first infusion. The relative formation of Hb C appeared to become maximal after the second or third infusion in those studies in which a sufficient number of determinations were done, reaching 40–56% of the total 5–8 days after beginning the infusions. In three instances relative Hb C formation either

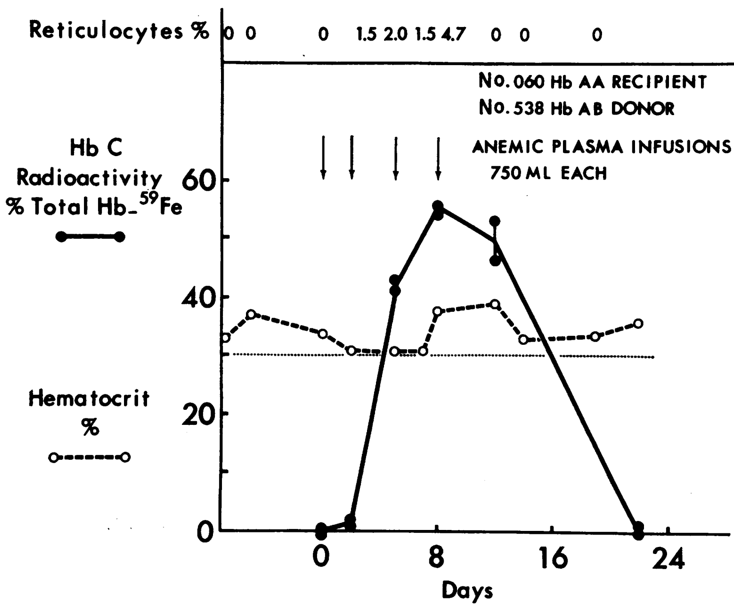

FIGURE 6 Induction of Hb C formation in a homozygous Hb A sheep given infusions of plasma from a donor with phenylhydrazine anemia with hematocrit in the range of 12–18%. The erythropoietin titre of plasma given in the third infusion was 2.7 U/ml. Only a minute amount of Hb C synthesis was evident 2 days after the first infusion, but the proportion rose steeply thereafter to 55%. Duplicate determinations are shown.

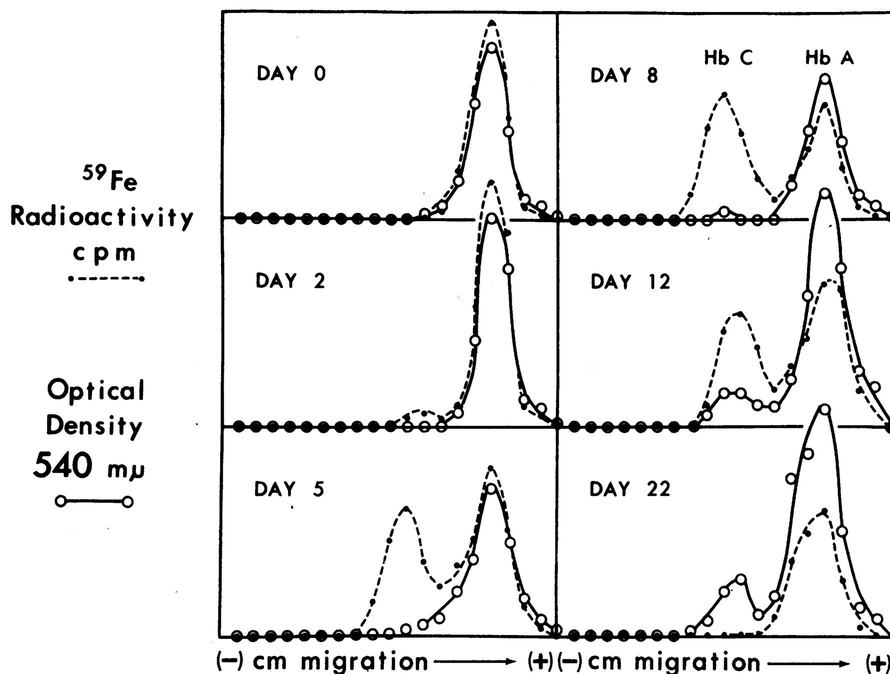

FIGURE 7 Starch-granule electrophoresis of the labeled marrow hemolysates of the study shown in Fig. 6. Note the appearance of a distinct though small radioactive Hb C peak 2 days after the first infusion. Note the appearance of an optical density Hb C peak which becomes maximal after the disappearance of Hb C radioactivity.

failed to increase or actually declined after the last infusion. The plasma with the least elevated erythropoietin titre (0.6 U/ml) produced the least relative induction of Hb C synthesis (23%); assays of plasma used in the other studies indicated higher erythropoietin concentrations in the range of 2.6–4.0 U/ml, or about 1950–3000 U per infusion.

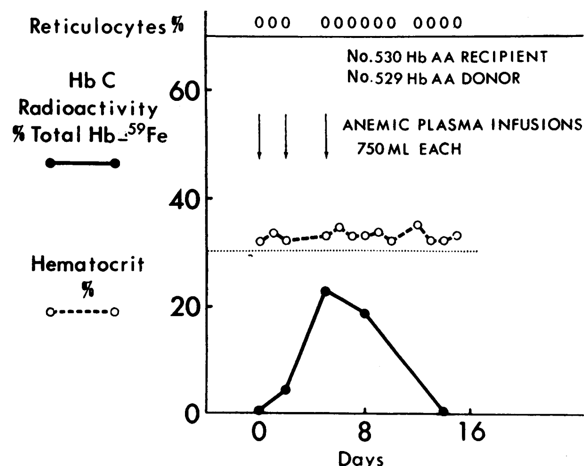

FIGURE 8 Induction of Hb C formation in a homozygous Hb A sheep given infusions of plasma from a donor with blood loss anemia with hematocrit of 16%. The erythropoietin titre of the plasma given in the third infusion was 0.6 U/ml, which appears to explain the lesser degree of induction compared to the studies shown in Figs. 4–6.

The effects of the infusions on hemoglobin phenotype in marrow erythroid cells were no longer evident 11–14 days after the last infusion, when Hb C formation again was not measurable, in contrast to the more prolonged persistence after severe anemia.

The quality of the electrophoretic separation is illustrated in Fig. 7. Optical density and radioactivity zones correlate; the peaks are well separated. The appearance of a Hb C optical density peak after localization in this zone of radioactivity is a clear demonstration that synthesis had occurred and resulted in the appearance in the peripheral blood (in which the marrow cells are diluted) of a small quantity of Hb C.

A control study was done to rule out such nonspecific effects as excitement and the anticoagulant used in plasma collection. Plasma was taken from donor sheep with normal hematocrits and adminis-

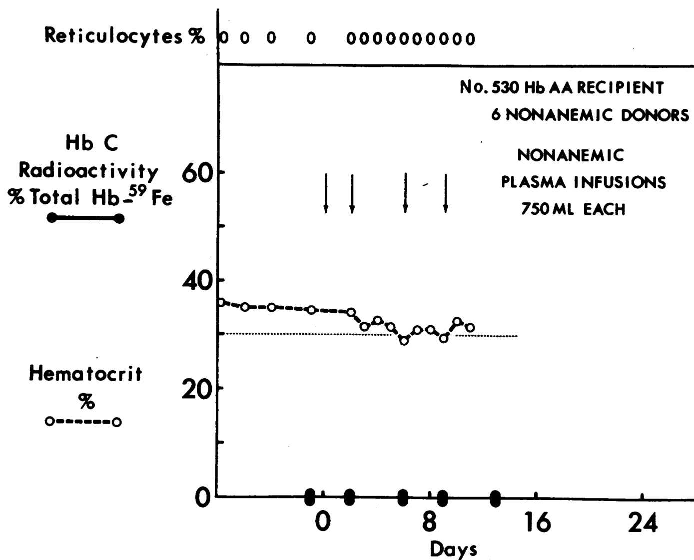

FIGURE 9 Failure to induce Hb C formation in a control study with nonanemic sheep as donors. Conditions were otherwise identical to those in the studies shown in Figs. 4–6, and 8.

tered as above to a recipient sheep (Fig. 9). No induction of Hb C formation was observed.

Three lambs were born of two homozygous Hb A ewes shortly after their course of severe phenylhydrazine anemia. Electrophoresis of hemolysates prepared from the red cells of these newborns showed a normal pattern consisting almost entirely of fetal hemoglobin.

DISCUSSION

Hemoglobin phenotype determination at a stem cell level. The effects of a strong anemic stimulus persist in Hb A sheep for about 25 days after recovery from anemia. During this period there is a slow linear decrease in erythroid cells capable of synthesizing the hemoglobin type initially induced by the anemic stimulus. This kinetic relationship strongly suggests that the determination of this hemoglobin phenotype occurs at a stage of cell development which has retained a capacity for self-renewal, that is, at a stem cell level. If determination were primarily at an erythroblast level of cell maturity, as proposed by Beale, Lehmann, Drury, and Tucker (10), the disappearance of Hb C formation should have been essentially complete within the period of erythroblast maturation into the mature erythrocyte, that is in about 5 days.

The 25 day period after recovery from severe anemia represents one of readjustment of the stem cell pool to the conditions that existed before the anemia. The slow linear decline, in fact, suggests the gradual nonrandom extinction of a cell population of limited numbers and life span. If erythropoiesis is in a steady state, an equal and reciprocal replacement with Hb A–committed stem cells accompanies the decline of those committed to Hb C. Renewal of the stem cell pool thus occurs during this recovery period. Such renewal might be explained if the pool of committed erythroid stem cells is constantly in a state of cell cycle, as suggested by Stohlman (15); as new cells replenish the pool, their commitment is to Hb A under nonanemic conditions.

Acceleration of the reciprocal change during anemia. The rate of change of hemoglobin phenotype is about three times more rapid in the presence of severe anemia than after recovery (Fig. 3). Although Hb A formation may conceivably rise immediately after the onset of the anemia, there is little doubt that its formation rapidly dwindles to insignificant proportions; the stem cell pool changes its commitment completely in favor of the newly induced hemoglobin type. If a minimum cell cycle time of 18 hr is assumed (16), it is unlikely that the first Hb C–committed stem cells go through more than one cycle before entering the erythroblast cell compartment. On the other hand, the persistence of Hb A formation during anemia is longer than one would expect solely from the disappearance of a committed pool of

erythroblasts and suggests rapid extinction of Hb A–committed stem cells.

The rapidity of the changeover may not be only a function of the stem cell. There is some evidence that the intermitotic interval of the erythroblast becomes shorter with erythropoietic stimulation (17–20); this shortened interval would contribute significantly to the rapidity of the change by abbreviating the erythroblast maturation period.

The role of erythropoietin in determination of hemoglobin phenotype. The results of these experiments together with those of Boyer (12) indicate that in response to anemia a plasma factor is produced which has the property of changing genetic expression in the erythropoietic system in sheep of a certain genotype. In a previous experiment Moore et al. (11) administered partially purified human urinary erythropoietin (300 U twice daily for 5 consecutive days by intraperitoneal injection) to a maturing lamb and noted the appearance of only about 1% of Hb C in the peripheral blood within about 13 days of the first dose. The evidence to date, while suggestive, is thus not sufficient to establish the identity of erythropoietin with the Hb C–inducing factor. However, the production of this factor is not dependent upon hemoglobin genotype.

The mechanism of action of the plasma factor may be direct or indirect. A direct theory based on the genetic model of Jacob and Monod (21) requires penetration of the cell membrane by the factor followed by derepression of the operator gene for $beta^C$ messenger RNA production linked with reciprocal repression of the $beta^A$ operator locus. If the plasma factor and erythropoietin are the same substance this postulate requires a second action for erythropoietin in addition to its known effect of stimulating erythropoiesis.

An indirect theory may also be proposed, based on the events of the cell cycle. Certain periods of the cell cycle are especially sensitive to radiation effects (22). The two points of maximal sensitivity are the late G_1 and early S phase and the late G_2 and M phase (22). The presence of erythropoietin or of other stimulants in the erythroid cell at certain sensitive points in the cell cycle may influence genetic expression by an indirect effect on DNA replication or division. The failure of relative Hb C formation to increase after the last of a series of infusions of anemic plasma in three experiments might be explained if erythroid cell synchronization were set up by the initial pulse-like stimulations, the final stimulation hitting the synchronized population at a relatively insensitive phase in the cell cycle.

The prolonged formation of Hb C after anemia cannot be attributed to persistence of erythropoietin in the circulation. The biological half-life of erythropoietin in man is 25 hr (23), and it is even shorter in smaller animals (24, 25).

The role of erythropoietin in erythropoiesis. If erythropoietin is indeed the plasma factor responsible for the induction of the changed hemoglobin phenotype during severe anemia, an important consequence regarding its site of action follows: exposure to high concentration of erythropoietin changes the character of the erythroid stem cell pool. Thus while erythropoietin has been considered to act primarily to differentiate stem cells into pronormoblasts (26), it is also possible that it acts upon the stem cell pool itself. Other evidence suggests that mature erythroblasts also may not be insensitive to its presence (17–20). Thus the hormone may influence the entire line of dividing erythroid-committed cells from the least to the most mature.

Relation to models for erythropoiesis. Lajtha (27) has proposed that erythropoietin causes an erythroid-committed dormant stem cell to differentiate into a pronormoblast, a secondary signal triggering a stem cell division to maintain the numbers of progenitor cells. The studies of sheep erythropoiesis are more suggestive of a pool of cycling committed stem cells which gradually undergo renewal, resembling the model put forth by Stohlman (15). Kretchmar (28) has proposed a mathematically based model which considers that erythropoietin is only active during the late G_1 and S phases of the cell cycle, a postulate which is attractive from the point of view of explaining the possible action of erythropoietin in changing genetic expression. However, he also assumes that erythropoietin specifically induces hemoglobin formation in the normal differentiation of stem cells into proerythroblasts, which leads to difficulty in explaining the mechanism of $beta^A$ chain production in nonanemic sheep. Investigations which have used the mouse spleen colony technique for studies of stem cell kinetics have suggested that there are erythroid-committed stem cells which, being

"erythropoietin reponsive," have first differentiated and only secondarily react to the hormone (29, 30). By means of the phenotypic changes which occur in sheep hemoglobins the data presented here concur that erythroid commitment is decided at the stem cell level. Since hemoglobin synthesis becomes active only at a more mature level of erythroid cell development, they furthermore suggest that differentiation of cells for phenotypic expression and for active synthesis of protein may be chronologically dissociated processes.

Hemoglobin phenotype changes in response to anemia similar to those in sheep have also been described in goats (31) and possibly in rats (32, 33). Investigation of such changes in genetic expression in fully developed animals may implement our understanding of the control of genetic expression and its relation to cellular differentiation both in the fetal as well as in the adult condition.

ACKNOWLEDGMENTS

Dr. Allan J. Erslev contributed the erythropoietin assays and, more significantly, provided critical and constructive advice about erythropoietic mechanisms. Dr. Elias Schwartz critically reviewed the manuscript. Expert technical assistance was provided by Constance Windus, Carl Sachs, and Halina Wolska.

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