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HEMOPOIETIC CELL PRECURSOR RESPONSES  
TO ERYTHROPOIETIN IN PLASMA CLOT CULTURES

William Louis Kennedy  
(Ph. D. thesis) *✓*

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Hemopoietic Cell Precursor Responses  
to Erythropoietin in Plasma Clot Cultures

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HEMOPOIETIC CELL PRECURSOR RESPONSES  
TO ERYTHROPOIETIN IN PLASMA CLOT CULTURES

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ABSTRACT

This study investigates the time dependence of the response of mouse bone marrow cells to erythropoietin in vitro. The purpose is to determine how long the immediate erythroid precursors, the "CFU-E," require the hormone in order to make colonies of eight or more cells in plasma clot cultures. The technique includes making  $^{59}\text{Fe}$ -autoradiographs of whole clots to efficiently identify erythroid colony number and size in response to timed exposures of human urinary erythropoietin (Ep). Abbreviated exposures to Ep are achieved by applying a rabbit anti-human erythropoietin immune serum to the cultures at times ranging from 0.3 hour to 30 hours following their initiation with Ep at  $t=0$ . The addition of antiserum effectively neutralizes the Ep activity for the rest of the 48-hour culture period. Experiments include studies on the Ep response of marrow cells from normal, plethoric, or bled mice. Proliferation of myeloid cell precursors (CFU-C) is also monitored in each erythropoietic state.

Results with normal marrow reveal: (1) Not all CFU-E are alike in their response to Ep. A significant number of the precursors develop to a mature erythroid colony after very short Ep exposures (as little as 18 minutes), but they account for only  $\sim 13\%$  of the total colonies generated when Ep is active for 48 hours. Ep is therefore not necessary

for their maturation through the recognizable erythron. If Ep is active more than 6 hours, a second population of erythroid colonies emerges at a nearly constant rate until the end of the culture. The response of this second colony-forming population to Ep is slow enough that even 24 h exposures are not adequate to cause the maximum activation of CFU-E seen in control cultures (continuous exposure to Ep). Full erythroid colony production requires prolonged exposure to erythropoietin. (2) The longer erythropoietin is actively present, the larger the number of erythroid colonies that reach 17 cells or more. This defines a second action of Ep on erythroid precursor cells in vitro by which amplification is increased in response to longer exposures to the hormone.

Two distinct populations of immediate erythroid precursors are also present in marrow from mice made plethoric by exposure to carbon monoxide (CO). In these mice tested 8-9 days after CO treatment, total colony numbers are equal to or below those obtained from normal mice. However, the population of "fast-responding" CFU-E (which require short Ep exposures for their expression) is consistently decreased to 10-20% of that found in normal marrow. The remaining colonies are formed from plethoric marrow at a rate equal to that found for normal marrow. With increasing Ep exposures, the number of large colonies produced increases, as in the normal case. The overall size distribution of the colonies derived from plethoric marrow remained the same as that of normal marrow after 48 h Ep exposures.

From the marrow of mice bled once or twice, total erythroid colony production is equal to or above that of normal marrow. Two populations

of colony-forming cells are again evident, with the fast-responding CFU-E being below (less than 10% of) normal levels. The lack of colonies from this group was compensated in bled mice by rapid colony production in the second population. A real increase in numbers of precursors present in this pool increased the rate of colony production in culture to twice that of normal marrow. The number of large colonies obtained from bled mice was again increased as the Ep exposure was lengthened, but this increase was not significant until Ep was left active 24 hours. This effect of Ep was exhibited after Ep exposures of 6-12 hours in normal and plethoric marrow cultures.

These results suggest the existence of two populations of Ep-responsive cells in the immediate erythroid precursor compartment of mice: (1) A CFU-E pool proper, which is on the verge of commitment into the recognizable erythron, and which requires very short Ep exposures for entry; and (2) A "pre-CFU-E" stage which feeds into the CFU-E pool (with Ep present), and makes colonies after longer Ep exposures. In addition, the pre-CFU-E response to Ep involves 1-2 self-replications before entering the CFU-E population. Amplification within the pre-CFU-E, the CFU-E, and the recognizable erythroid compartments are independent of the erythropoietic state of the donor marrow in vitro. The recognizable erythron is affected little, if any, by even the prolonged presence of Ep. Therefore, the regulation of mouse erythropoiesis by erythropoietin appears confined to the immediate erythroid precursor compartment, but requires that the hormone stimulate both proliferation and maturation of Ep-responsive precursors.



## Dedication

To my hardworking parents and wife

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## I. INTRODUCTION

### A. The existence of erythropoietin

It is now generally accepted that regulation of the mature red cell pool in mammals, birds, and fish is controlled by the plasma level of a factor called erythropoietin. The existence of this substance was first proposed (but not satisfactorily proven) by Carnot and Deflandre in 1906. Not until the 1950's was the biologic effectiveness of a humoral stimulator of erythropoiesis convincingly demonstrated in parabiotic rats (134), rabbits (43, 17, 86, 78), and human beings (161). This substance is now thought to be a glycoprotein of less than 70,000 daltons, with recent estimates of 46,000 daltons (60), and 60,000 daltons (83) for sheep and human erythropoietins, respectively. It does appear to be homogeneous and has recently been highly purified (118). Its presence in blood plasma at minute concentrations (estimated at about  $10^{-12}$  M (60, 80), has significant effects on erythropoiesis (defined as the processes which lead to production of mature red blood cells). Because of its molecular size and chemistry, its localized production in the body, and its effects on specific target cells (as we will outline later in this introduction), erythropoietin is justifiably a hormone (63). It will be referred to as such in this work and abbreviated as "Ep." Different extracts of erythropoietin contain the hormone at different concentrations, which at present are best determined by a standard polycythemic mouse assay, the bioassay. The Ep activity in the extracts used in the studies presented here were measured by this method.

As with all hormones, the targets of erythropoietin are cells--in

this case, some red blood cell precursors, possibly at several levels of maturation. Of greatest interest are early, unidentified precursors. Many properties of these cells have recently been elucidated in culture, but their specific requirements for the presence of the hormone, and the timing of the resultant cellular effects have not been so well characterized. The present work hopes (1) to identify the stage(s) of erythropoiesis which require Ep; (2) to specify more clearly the hormonal requirements of red cell precursors; and (3) to shed some light on the response of these cells in different states of erythropoietic activity.

#### B. Cycle of erythropoietic regulation

The primary function of mature red blood cells lies in their ability to deliver oxygen to tissues when it is needed, and to take away the waste carbon dioxide. The role of erythropoietin in regulation of this oxygen delivery most likely involves the following step-wise series of cellular events:

(1) An unknown cellular mechanism monitors the tissue oxygenation level in a region of the body presumably located in the kidney (149). Here a group of cells compare the supply of oxygen released from the blood stream with the instantaneous demand for oxygen by the tissues (52, 86, 87, 150a).

(2) This information is then translated into a chemical control of erythropoietin production, possibly via an increase in blood pH (115, 150a) or lower serum  $\text{CO}_2$  levels (115). Ep production and, thereby, plasma Ep levels are modulated, at least in part, by kidney cells (149, 131). Oxygen demand that exceeds supply, or a decrease in peripheral

hemoglobin saturation causes an increase in secretion of Ep (152).

Conversely, Ep production decreases when the supply of oxygen surpasses the demands of these kidney tissues.

(3) These variable plasma levels of Ep then govern the production and release of mature red blood cells into the bloodstream. To do this Ep acts directly to increase the number of erythroid precursors and induces their further maturation into enucleated red blood cells (4).

(4) The total cellular volume of these mature red blood cells circulating in the bloodstream, in turn, governs the level of tissue oxygenation.\* In general, the greater the number of red cells functioning in the blood (by their capturing and releasing normal amounts of oxygen at the right times), the more oxygen is delivered to the tissues. This completes the cycle of erythropoietin regulation by providing a feedback to the cellular mechanism of step (1), which monitors the resultant change in tissue oxygen levels.

Therefore, the normal production rate of red blood cells, which in human beings amounts to about 2 million per second or 5000 per second in mice (142), is ultimately regulated by their number functioning in the bloodstream (or, technically, by the chemical equilibrium of the circulating oxyhemoglobin). As the red cell mass exceeds that required for complete oxygenation of the tissues, red cell production falls after plasma Ep levels abate (94). If at a later time oxygen

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\*Total oxygen carried by the blood can also be increased by a shift to a different form of hemoglobin (seen in sheep and goats (124)), or by increased production of 2,3 diphosphoglycerate (shifting the oxyhemoglobin dissociation curve)(150a). These mechanisms, as Bessis and Brecher point out (14), may not be as general a response to stress as is red cell proliferation.

demand begins to outweigh red cell deliveries to the tissues, Ep levels rise, causing increased production of mature red cells. Morley and Stohlman (121, 122) suggested that the cycle just described may lead to sustained oscillations, each overshoot of Ep production resulting in accelerated shut-off of Ep production by the excess of red cells produced. Regular oscillations of reticulocytes were indeed observed in dogs.

Given this generally accepted concept of erythropoiesis, it is interesting that we know almost nothing about steps (1) and (2) above, i.e., oxygen supply:demand detection and its translation into elaboration (or activation) of erythropoietin; we know more, but still little, about step (3) concerning the regulation of red cell amplification and differentiation. We know a great deal about the physiology of oxygen delivery to the tissues, as required in step (4). For the present investigation, we have chosen to study the "middle" of the Ep feedback loop, which is described in step (3) as the regulation of red cell amplification and differentiation, for which the following foundation has been laid:

In the past, the question of presence of Ep in normals was controversial, although sporadic reports did demonstrate erythropoietin in urine (1, 3, 171) and plasma (116) of healthy human beings. Recently, however, a refined radioimmunoassay using highly purified Ep has consistently demonstrated 4-5 milliunits (International Reference Preparation) in normal human serum (55). Although this assay also identifies desialated hormone, the obligatory presence of Ep in normal blood is consistent with the observation that injection of an antiserum to Ep

produces severe anemia in normal mice (56). These facts support the notion that Ep plays a role in the maintenance of normal red cell levels in healthy subjects, a notion that has only recently been widely accepted. It means that Ep is interacting daily with specific cells in the blood, bone marrow, or spleen. The result and purpose of this interaction is eventual amplification of the mature red blood cell pool. The principal action of Ep in normal or disease states is to induce these precursors of red blood cells to progress through a well-defined series of maturation steps. At the cellular level, therefore, Ep is continually causing proliferation of unidentified erythroid precursors, coincident with their differentiation (which includes both commitment and maturation (113)) along a portion of an economical, well-ordered hierarchy from morphologically unidentifiable cells, through a "buffer zone" of committed erythroid cells, to the nearly perfect biconcave discs of the mature red blood cells.

### C. Blood cell hierarchy

#### 1. Pluripotent stem cells

At the beginning of this hierarchy is a group of stem cells which is able to populate the blood with all necessary precursors to red and white cells, megakaryocytes (platelet producers), and lymphocytes. A common stem cell for all hemopoietic cell lines was first proposed in 1909 by Maximow (109). However, he assigned the role to the small lymphocyte. Although this is not the correct cell, the common precursor notion is still correct. The precursor is still not completely identifiable under the light microscope or the electron microscope (172), but at least a part of these cells (although other groups have

been proposed (141)) is functionally characterized by their formation of spleen colonies when transplanted into lethally irradiated, syngeneic mice (164). If transplanted early enough, these cells alone are able to spare these animals from a sure death (177). Each spleen colony consisting of erythroid, myeloid, or megakaryocytic cells, either separately or mixed (105, 177), probably starts from a single cell (12, 174). However, mixed colonies may be comprised of progeny of the parent stem cell plus progeny of its daughter stem cells (168). The important features of these "colony forming units in the spleen" (CFU-S), which make them true stem cells are:

1. Their pluripotentiality for all blood cell lines (51, 177), including lymphocytes (106, 101).
2. Their ability to reproduce themselves (153).
3. Their extensive proliferative capacity (12, 164).

These three properties give CFU-S the ability to restore normal levels of all blood cells under conditions of host depletion. Whether CFU-S play a role in daily hemopoietic regulation is still being debated (18).

But it is known that they are present in extremely low numbers in the marrow and spleen of mammals (about one million in mice (142)), and there are few of them in a cycling state (5-20% according to  $^3\text{HTdR}$  suicide or hydroxyurea experiments (11, 16, 96, 120, 173)). In addition, it has recently been proposed that the CFU-S compartment normally consists of a continuum of cells moving toward decreasing capacities for self-renewal, increasing likelihood of differentiation, and increasing proliferative activity (76). Although these cells may not be involved directly in daily regulation of hemopoiesis (this being left to the

"committed cells" considered later in this introduction (18)), their proliferative activity can be increased by such stimulations as irradiation or drugs (11, 16, 96, 19, 173). However, self-renewal, differentiation, proliferation, and hemopoietic regeneration as a whole appear independent of the known regulatory influences affecting erythropoiesis (21, 16, 90, 148, 144), including changes in plasma Ep levels (35, 126, 166).

The property of CFU-S with which we will be most concerned in the research described here is the ability of this precursor to differentiate from a pluripotent stem cell into what is called a "committed stem cell," or "committed progenitor cell," i.e., a transit population of cells destined to occupy only one of the possible types of blood cell lineages. This transition is presumed irreversible and therefore cannot be the fate of every CFU-S. This would nullify its self-renewal capacity. As stated above, CFU-S differentiation toward the erythroid line in normal mice is now generally believed to be unaffected by erythropoiesis, and, in fact, appears regulated by very powerful (36), but "local" influences from the surrounding tissues (163, 167, 108, 168, 34, 35, 50, 117). The mechanism that commits some CFU-S into the erythroid line, some into granulocytes, some into megakaryocytes, some into lymphocytes, and some not at all, is not yet known. Most investigators would agree, though, that stem cells fall into these different spaces in a stochastic manner (28, 141, 165). Information on stem cell proliferation has emerged from recent in vitro techniques for studying the CFU-S "microenvironment" (37, 38, 98), but regulation of their commitment has not been obtained in vitro.

## 2. Committed progenitor cells

By whatever method, or under whatever influences, some stem cells (suggested by Lajtha (97) to be 40% of the cells per cycle) do enter one of the committed progenitor compartments for red, white, megakaryocytic, or lymphoid cells. The existence of the committed erythroid compartment is not an a priori prediction of stem cell "maturation," but its presence was first documented by Alpen and Cranmore (4). In the marrow of severely bled dogs injected with radioactive iron, they saw a rapid influx of unlabelled cells into the heavily labelled identifiable erythroid pool. This could only mean that: (1) There existed non-erythroid cells that were entering the identifiable erythroid line after radioactive iron was no longer available for labelling their heme; and (2) These new cells normally occurred in low numbers in marrow as unidentifiable, immature precursors to the more mature erythroid cells. In addition, the work of Alpen and Cranmore clearly showed that the proliferation of these precursor cells is under the control of a specific humoral factor, erythropoietin and the differentiated erythroid compartment is not self-maintaining, but is fed from an unidentifiable precursor pool. This study along with those of Alpen, et al (6), Jacobson, et al (86) and Erslev (44) helped to localize a major site of action of erythropoietin: some immediate progenitor cells of the erythroid line. At the time (1959-1960) these data led naturally to the conclusion that a multi-potent stem cell pool was being induced (by Ep) under anemic stress to differentiate directly into the erythron (5). However, it was not until the studies of Bruce and McCulloch (21) (and later Schooley, et al (145), and Stohlman,

et al (159)) that these "Ep-responsive cells" were shown to exist between the pluripotent stem cell and the earliest identifiable erythroid cells. This has recently been extensively confirmed by others with new in vitro techniques (68, 79). This "buffer zone" of cells was shown to be actively proliferating (77, 97) and this observation answered the question as to how the non-proliferative stem cells (CFU-S) could make over  $10^8$  mature red cells per day in human beings.

More information about this new compartment came when, in addition, Lajtha (99) postulated an "age structure" within this erythroid progenitor pool, and Stephenson, et al (156) formed erythroid colonies in plasma cultures. It is now obvious that such maturation levels do exist in mouse marrow, possibly numbering three roughly distinguishable states in this erythroid committed stem cell continuum. However, the existence and utility of these new levels is still tentative, since at present they are only in vitro assays which may or may not truly represent the in vivo situation. The three stages appear to be physically and functionally separable when semisolid culture techniques are used with large to medium doses of Ep being present in culture for 2 to 10 days (64, 65). It is probable, however, that not all of them are included in the erythropoietin-responsive cell pool.

The earliest committed step, designated "BFU-E" or "erythrocytic burst-forming unit" arises by day 8-14 in methylcellulose (82) or plasma clot culture (9), and may not be far removed from the pluripotent stem cell (67). (Humphries, et al (78a) have recently proposed that day 14 BFU-E are precursors to day 8 BFU-E. For our purposes, these cells will simply be called BFU-E.) There are several reasons

for this proximity. These BFU-E are: (1) clonal (162); (2) the same size (about 7.8  $\mu\text{m}$ ) and density as CFU-S (80); (3) at least bi-potent in their function, giving rise to megakaryocytes as well as erythroid cells, but no granulocytes (112, 88, 78a); and (4) their numbers present in spleen colonies correlate well with the number of CFU-S in the same colony (67). They still have a large proliferative capacity (10-12 cell divisions in culture)(82), and limited self-renewal (78a). The control of their proliferation appears to require some as yet unknown humoral factor(s) (88, 81, 78a, 66), in addition to, but different from Ep (88, 81, 78a, 66). However, the crude Ep extracts used today were the first substances known to supply the right factors, so they do contain the sufficient stimulation factor(s) for BFU-E growth when used in culture at high concentrations. The large, dispersed colonies produced (from which they get their "burst-like" appearance) initially do not contain any hemoglobin-positive cells and are therefore not recognizable as erythroid (80). Most BFU-E do not contain recognizable erythroblasts until 7 days or more in cultures with Ep present.

Most investigators obtain these recognizably erythroid bursts at 25-50 per  $10^5$  nucleated mouse marrow cells seeded (64, 79, 2). In vivo these cells have a low growth fraction (30-40% are killed by a pulse of  $^3\text{HtdR}$  (2, 79, 74)), and the total population is present in marrow and peripheral blood in unchanging numbers after bleeding or hypertransfusion (2, 79), although migration between marrow and spleen is seen in these perturbed states (2, 74). Thus, the proliferation and differentiation of these early stages of erythroid development go on continuously and are not influenced by the usual erythropoietic regulations.

Therefore BFU-E do not appear to be a part of the "Ep-responsive cell" pool (80, 81).

As these very early erythroid cells mature, they gradually lose their proliferative and self-renewal capacities (65), and obtain an ability to respond to lower levels of Ep as their cell size and density increase (65). They are now only unipotent and are designated "day 3 BFU-E," since they arise by day 3 in cultures given moderate amounts of Ep. These cells, however, may only be partially "Ep-responsive" since they also proliferate if the above-mentioned "BFU-E factor(s)" are added with minute levels of Ep (our own observations), and they respond only modestly to hypertransfusion or bleeding (65, 2). These cells give rise to a characteristic colony morphology (64), containing mature erythroid cells from only 6-7 cell divisions (about 100 mature cells).

As these cells progress a little further down the erythroid line, they, in turn, reach a position just prior to the identifiable erythron, where all are now in cycle (2, 74, 79). This subpopulation of cells, the CFU-E, can make single clusters (clones) (132) of up to 64 cells within two days in response to low levels of Ep (compared to that required by BFU-E) in semisolid culture. The mouse CFU-E is also larger than its BFU-E, being 8.2-10  $\mu$ m in diameter (80), and is thought to look like a "transitional lymphocyte" (139, 179). It has a limited proliferation capacity in culture (5-6 cell divisions). Ep is definitely required for it to progress into the identifiable erythron, since its numbers in marrow and spleen (normally 10-fold higher than BFU-E) increase 3- to 5-fold after bleeding or Ep injection, and its numbers decrease after hypertransfusion to 20-30% of normal (2, 74, 79). The

CFU-E is, at this point, the only candidate left as a fully "Ep-responsive cell." After 48 hours these colonies undergo little further proliferation, but continue toward terminal maturation and extrusion of their nuclei to form mature red blood cells. The CFU-E, the erythrocytic colony-forming unit, is the focus of this dissertation and will be described in detail later.

### 3. The recognizable erythron

After a pluripotent stem cell has been committed to the erythroid pathway for its cell production, and after it has developed past the CFU-E precursor stage by Ep, it then enters the recognizable erythron. This terminal end of the erythroid line is characterized by the presence of hemoglobin at each of its six stages (138, 140), and by the distinct cellular morphology taken on by each successive stage with a Romanowsky stain. As a basis for the following review of human erythroid cells I have used a recent medical text on hematology (104).

The recognizable erythron is comprised of:

1. Pronormoblasts
2. Basophilic normoblasts
3. Polychromatophilic normoblasts
4. Orthochromatic normoblasts\*
5. Reticulocytes
6. Mature red blood cells (erythrocytes)

The pronormoblast arises within 1 or 2 cell divisions from its CFU-E precursor (142, 154) as a large cell, 20 to 25  $\mu$ m in diameter in

---

\*The term "orthochromatic" means "true color" and implies that these cells contain a full complement of hemoglobin compared to mature red cells. However, as Bessis (13) points out, this is not a proper description of these late normoblasts, since a great deal of hemoglobin synthesis occurs after this stage. The term "orthochromatic" will be retained for the present discussion but with the above reservation.

human marrow and blood. It has a large nuclear:cytoplasmic ratio with large, multiply prominent nucleoli. All pronormoblasts are thought to be in cycle (154). Although this stage does produce some hemoglobin (142, 154), its cytoplasm is intensely basophilic (due to many polyribosomes) and its nuclear chromatin is fine.

The basophilic normoblast retains most of the basophilia of the pronormoblast, but there is an increase in heterochromatin (clumping). This cell is smaller than the pronormoblast (16-18  $\mu\text{m}$ ) with the nucleus occupying about 3/4 of the cell area. This stage undergoes one cell division (133) and contains some easily demonstrable hemoglobin (138).

The polychromatophilic stage is named for its marked increase in stainable hemoglobin giving it a metachromatic hue in its cytoplasm as the polyribosomes become diluted. It is smaller than the basophilic stage (12-15  $\mu\text{m}$ ) with the nucleus occupying less than half of that area. The chromatin is in well-defined clumps. There is no nucleolus. Hemoglobin production is proceeding rapidly when this cell stage makes the one final mitotic division (133) into the orthochromatic stage.

The orthochromatic normoblast is relatively uniform in staining appearance as the proportion of hemoglobin increases. The nucleus becomes pyknotic, looking very small and dense. The cell diameter varies from 10-15  $\mu\text{m}$  with the nucleus taking only 25% of its area. Although this cell is not capable of locomotion, the cell surface does invaginate and undergoes movements in preparation for extrusion of the nucleus. No DNA synthesis (and no cell division) occurs but RNA synthesis continues for production of hemoglobin.

Upon rather quick (10 minutes) extrusion of its nucleus, the

normoblast abruptly enters the reticulocyte stage, but normally remains in the marrow for about 24 hours. Then, possibly in response to Ep, the reticulocyte is released into capillary sinuses (129). From the time of nuclear extrusion until about 48 hours later when it will mature fully into a red blood cell, the human reticulocyte will use its last RNA to synthesize the remaining 20-25% of the red cell hemoglobin content. No RNA (or DNA) synthesis is performed by mammalian cells at this stage (47). The reticulocyte will extrude unneeded organelles to become a nearly uniform 7.5  $\mu$ m diameter biconcave disc--the mature red blood cell.

The erythron of rodents is very much the same as that just described for human erythroid cells. The progressive morphologic changes of the various stages are very similar. As in human cells hemoglobin has been detected in guinea pig pronormoblasts both in vivo (138) and in vitro (139). Also in the guinea pig it has been shown that only 60% of the polychromatophilic cells divide to enter the orthochromatic stage (133). This may also be true in humans, and suggests that the transition to the orthochromatic stage is partly random rather than totally postmitotic (133). The orthochromatic population of the guinea pig marrow is completely renewed every 20-24 hours (154). At the reticulocyte stage, however, larger differences arise between human and rodent erythropoiesis. When entering this stage mouse reticulocytes still need to synthesize 40% of the final hemoglobin content of their mature red cells, and rats need about 60% (138). In addition, the mature red cells of mice are slightly smaller than their human counterparts.

In perspective, then, the stages of erythroid differentiation can

be pictured, as in Figure 1, as a three-tiered series of connected compartments (100): the pluripotent stem cells feeding (when necessary) into a highly proliferative committed progenitor pool, which, in turn, supplies the recognizable erythron with self-maturing pronormoblasts. As the cells progress through these three compartments, their proliferative activity (as measured by  $^3\text{HTdR}$  suicide) gradually increases from almost zero for the CFU-S (in a good, "clean" mouse) to 100% for the pronormoblast stage (133). This proliferative pattern is roughly indicated at the right of Figure 1.

To progress through all of these stages of maturation it has been estimated that the various red cell stages may include as many as twelve cell divisions (142); 5-10 in the committed compartment and 3-5 in the recognizable erythron (4, 130, 154). The left column of Figure 1 also shows these data as the approximate number of cell divisions remaining for cells arriving at each stage.

#### D. The effects of erythropoietin on blood cells

##### 1. The erythropoietin-responsive cells

With this basis we can now follow the data on the effects of Ep on cells which are progressing toward the red blood cell stage. It is again instructive to start with more fundamental in vivo observations. These involve the basics behind the typical bioassay of erythropoietin as described morphologically by Filmanowicz and Gurney (48) and studied extensively by others (32, 54, 70, 176). If mice are made polycythemic by two days of transfusions of packed red cells, the sudden increase in oxygen delivery to the tissues shuts down endogeneous Ep production and eventually no more Ep-responsive cells are being induced to differentiate.

By six days after the last transfusion, almost no recognizable red cell precursors are visible in a marrow smear, and plasma Ep is decreased to undetectable levels. A similar state of low erythropoietic activity and high hematocrit can be induced by placing animals under low atmospheric pressure (low oxygen) or high carbon monoxide tension to stimulate red cell production and then to move the animals to an environment of normal oxygen tension (32, 150). They will cease Ep and red cell production. If radioactive iron is injected intravenously into these animals or the hypertransfused animals described above on day six or seven after treatment, very little of the iron will be found in their peripheral blood cells, that is, very little hemoglobin synthesis is going on at this point.

Having created animals with very low erythropoietic activity, Filmanowicz and Gurney (48) then injected an Ep extract to once again stimulate red cell production. Looking in the spleen after one injection they found a wave of erythropoiesis: Pronormoblast numbers peaked on day 1 after injection of Ep (more recent data shows pronormoblasts appear within 12-16 hours of Ep injection, indicating that the Ep-responsive cells are indeed not far removed from the pronormoblast (28, 142)). This was followed by normoblasts on day 2-3 and reticulocytes peaked on day 3-4. This orderly sequence of cell production and maturation confirmed the structure of the recognizable erythron presented earlier. In addition they found that all pronormoblasts were absent on day 3, indicating that the duration of differentiation of precursors and subsequent divisions took less than 72 hours. Also they showed an average reticulocyte survival time of two days. (More recent data shows 34-45 hours (84, 129).) So the total time from onset of precursor

differentiation to release of young red cells is somewhat less than 96 hours in these mice.

It is now known that Ep is rapidly produced at the first sign of need, reaching detectable plasma levels if mice are kept under hypoxic conditions for only 3 hours (157). Periodic peaks in Ep production have been observed during prolonged hypoxia (22), but these observations have not been repeated. Also Ep disappears from plasma very rapidly with a half-life of about one hour (160), but still manages to set in motion a wave of red blood cell production like that seen in the bio-assay. Thus the effects of Ep on cells already committed to progress into or through the erythron must be rapid. The actions of erythropoietin on these immature erythroid precursor cells were detected early (4). Its cellular effects include proliferation and differentiation of these Ep-responsive cells (135), but when and how these effects occur is not known.

The timing of the actions of Ep during increased hemopoietic demand have been analyzed in relation to the cell cycle of the Ep-responsive cell. Kretchmar (92) has constructed a computer model of erythropoietic recovery of Ep-responsive cells like the CFU-E. It is based on the assumption that Ep must be present before DNA synthesis (in G1) to be effective in derepression of the genes of a responsive cell. Therefore the fraction of these cells which become derepressed by Ep depends upon the length of G1 and on the level of Ep present. An Ep-sensitive population increases in size as G1 is lengthened (up to the "effective life of the hormone"), whereas these cells become resistant to Ep if G1 is short. Maximum response to Ep occurs when the

length of G1 (is intermediate and) approximates the hormone's "lifespan." The key regulatory mechanism then becomes a proposed feedback path governing the length of G1. This would be determined by the immediate need for differentiated cells so that the length of G1 depends on the population size of the undifferentiated pool. Evidence for this key mechanism is highly speculative. However, some studies by Morse et al (123) and Kretchmar et al (93) are compatible with a shortened G1 phase in Ep-responsive cells responding to increased demand. In vivo studies by Schooley (143) have also shown that Ep-responsive cells are indeed receptive only during G1 in polycythemic mice. The normal steady state has not yet been investigated in this regard, but extension of these data to the recognizable erythron is instructive. If maturing erythroid cells were to respond to Ep, they may be receptive in G1 only, as are the Ep-responsive cells described above. However, Alpen and Johnston (7) have found that G1 is short or nonexistent in these cells. This would make it very hard for Ep to affect the recognizable erythroid cells, lending support to data that show that Ep is not necessary for maturation of cells in the recognizable erythron.

Reports of Ep effects during other parts of the cell cycle include proposals that cells respond during a resting G1 (" $G_0$ ") phase (97), G2 (60) and S (127). However, in view of the fact that Ep-responsive cells (CFU-E) are all in cycle while still responding to Ep, a  $G_0$  response time is no longer plausible (159). Data supporting a response to Ep during G2 (60) and S phases (127) are still being debated (137).

Information on the effects of Ep at the molecular level is also available. Using purified fractions of these precursors, Djaldetti et al

(39) have shown that the earliest detectable response to Ep appears to be the formation of new RNA within 30 minutes, followed by an increase in globin mRNA after six hours of erythropoietin exposure. These results are in general agreement with Gross and Goldwasser (69). Thus the primary molecular action of the hormone may be to increase some specific RNA synthesis in specific precursor cells. The rapidity of this effect, along with data from experiments using Ep attached to glass beads in vitro (40), and on erythroleukemic cells in vitro (136), suggest that Ep interacts with the cytoplasmic membrane to produce a second messenger (60). This, in turn, leads to an increase in DNA synthesis within two hours, and hemoglobin synthesis soon after (6-10 hours) (60).

Receptors for Ep at the cytoplasmic membrane have recently been proposed by some authors as a key element in erythropoietin-sensitivity and erythropoietic regulation (25, 80, 81). Goldwasser believes that the receptors on Ep-responsive cells have a short life-span, being receptive to Ep during only a portion of the cell cycle, possibly G2 (60). This sensitive time appears to be about 1.5 hours out of the 7.5 hours in vitro cell cycle time (60). More recent data on the "age structure" (BFU-E to CFU-E) in the committed progenitor compartment have led Iscove (81) to an attractive proposal that the number of Ep receptors on Ep-responsive cells increases gradually as the cell progresses toward the recognizable erythron. This would give the more mature cells their increasing sensitivity to erythropoietin's differentiating effects as they go from BFU-E to CFU-E. Simultaneously, a decrease in sensitivity to "BFU-E factor(s)" also occurs (81). The interplay between these two effects--one trying to induce differentiation of late

forms, and the other mainly stimulating proliferation of early forms-- may act to regulate erythropoiesis by a system of checks and balances within the committed progenitor pool. Another possibility proposed earlier by Krantz (91) pictures erythropoietin as being capable of increasing the number of these cytoplasmic receptors, and thereby increasing its own targets to amplify its effects. However, in light of Iscove's recent data (81), it now seems more likely that the "BFU-E factor(s)" would be responsible for increasing the number of Ep receptors.

## 2. The recognizable erythron

The effects of Ep, if any, on the recognizable erythron are also still debated, with most arguments reviewed by Krantz and Jacobson (91). Numerous in vitro works showing Ep effects on the maturing erythron are hindered by a lack of separation of CFU-E (Ep-responsive cells) from the recognizable erythron to show the effect observed was not caused by differentiation of the precursor cells. Circumstantial evidence from in vitro systems and fetal liver cells includes observations of crude Ep extracts causing increased proliferation in the erythron (58, 71, 72, 73), increased DNA (75) and RNA (39) synthesis, increased hemoglobin synthesis (69), and early denucleation and possible bypass of the orthochromatic stage (130). This latter finding (the production of macrocytes) has also been reported in vivo (20, 158), although its occurrence may only be a secondary effect. That is, the greatly increased erythropoiesis precipitating a macrocytosis may create a local nutritional deficiency of a DNA precursor preventing a full number of divisions under stress (46). Many studies need correction, since Ep has been

shown to cause premature release (not production) of reticulocytes from marrow and spleen into the blood (128, 129).

On the other hand, it has been fairly convincingly demonstrated that Ep has little or no direct effect on erythroblast maturation. This was amply demonstrated in vivo by Alpen and Cranmore (4), where they found no change in halftime for divisions of erythroblasts nor marrow transit time after bleeding. There was also no acceleration of hemoglobin synthesis and no change in the number of divisions in the erythron. Several investigators have found a similar lack of effect on maturation (4, 44, 46, 129, 137), and on the ratio of early to late erythroblasts (5, 8, 128, 30, 151, 155). There were also reports of continued erythroid maturation after the stimulus has been withdrawn both in vivo (143, 147), and in vitro (89) by addition of anti-erythropoietin serum to the system. These studies and other in vitro studies (26, 27, 29) give strong evidence that erythropoietin is not necessary for normal red cell production past the erythropoietin-responsive cell and that it probably does not accelerate development of the erythron above the normal rate. In addition, it is only needed to increase the number of hemoglobin synthesizing cells and not the quantity of hemoglobin synthesized per cell (4, 8, 137, 42). Ep has no effect on proliferation or maturation of the other blood cell lines (45), including white cell count (62, 49, 125, 110, 85, 169, 175) or platelet count (125, 95, 169, 175) in vivo. However, recent in vitro studies do implicate Ep as a stimulator of megakaryocytic proliferation (112, 88, 78a) or differentiation (78a).

E. Statement of the significance of the problem

With the introduction of a method for culturing murine erythroid precursors by Stephenson, et al (156), it has become possible to study the response of bone marrow suspensions to moderate levels of erythropoietin extracts. They have firmly established in an improved semisolid culture system (111) that colonies of mature erythroid cells arise within 48 hours from an unrecognizable precursor cell, called the CFU-E. These clusters are clonal (132) and increase both in size (29) and in number (111, 29) as the Ep (extract) concentration is increased (until plateau levels are reached). However, in all of these studies Ep is allowed to remain present and active (82) throughout the culture period, and does not address itself to the question whether the continued presence of Ep is required. It should be recalled that on the average only 8-16 normoblasts are produced per colony, suggesting strongly that each colony is derived from a precursor through 3-4 divisions without significant self-replication of the CFU-E itself. Consequently, the need for continued presence of Ep (if verified experimentally) would indicate that Ep has an effect on later stages of CFU-E development as well as on the CFU-E itself. However, in retrospect, we find that even a colony size of 16 cells does not eliminate possible self-replication of precursors. For example, two self-replications, each daughter CFU-E producing 2 to 4 hemoglobin synthesizing cells, would also result in erythroid colonies of 8-16 cells. Whatever their eventual interpretation, the early results of this study (89) were of enough interest along these lines to continue the work.

In addition, we studied the need for prolonged presence of Ep (or

the lack of such need) not only in normal, but also in cells derived from mice with increased and decreased red cell production. The reasons for doing these studies are as follows: Should it turn out that CFU-E colonies at a given Ep level are unaffected by the origin of CFU-E from mice with normal, decreased, or increased production, this would strongly indicate that regulation of erythropoiesis physiologically occurs at the BFU-E or earlier level, with CFU-E maturation being unaffected. Conversely, if the CFU-E colonies were affected, participation of CFU-E in the actual regulation of erythropoiesis would be likely. Finally, we attempted to determine whether Ep itself or perhaps some other protein(s) needs to be present for prolonged periods for maximum production from CFU-E.

## II. MATERIALS AND METHODS

### A. Mice and marrow cell preparations

LAf-1 female mice (bred by Jackson Labs, Bar Harbor, Maine) were used as a source of bone marrow cells in all experiments. They were obtained at approximately 12 weeks of age and either used immediately or housed 6-10 per cage with feed and water ad lib. Experiments were performed with three mice of the same age ranging from 12-21 weeks for different experiments and different states of erythropoietic activity. Two to three hours prior to most experiments microhematocrits were taken. With the mice under very light ether anaesthetic, a small amount of blood was drawn from the orbital sinus just above the right eye into a heparinized microhematocrit capillary tube. After a 5 minute centrifugation at 12,000 rpm the proportion of packed red cells to whole blood was measured with a millimeter rule. Three different states of erythropoietic activity were investigated in these mice: the normal state, a plethoric state, and an acutely bled state.

#### 1. Preparation of the plethoric state

This procedure was very kindly performed for us by Dr. J.C. Schooley (Donner Laboratory, University of California, Berkeley, Ca.). Briefly, normal mice 16 (trial 1) or 12 (trial 2) weeks of age were subjected to an environment increasingly enriched in carbon monoxide for 3-4 weeks. During this time their hematocrits were periodically checked and the mice were removed into a normal air environment when their hematocrits were above 70. They remain in air for 8-9 days at which time the erythropoietic activity (as measured by a 72-hour <sup>59</sup>Fe uptake) falls to very low levels, especially in female mice (150). It is at this nadir of

erythropoietic activity that the marrow of these mice (now 20 (trial 1) or 15 (trial 2) weeks of age) was used for the present studies. On days 8 or 9 after carbon monoxide treatment hematocrits in the mice being used averaged 75 (trial 1) or 63 (trial 2), and a marrow smear showed nearly complete suppression of erythroid precursors. Normal, untreated mice at 12-15 weeks of age served as controls for this group.

## 2. Preparation of bled mice

Acute bleeding of 6-10 mice at age 12-15 weeks was performed from the heart with the last bleed occurring three days prior to culture of the marrow. All bleeding was performed under general anaesthesia (sodium pentobarbital, intraperitoneally injected at 50 mg/kg). Approximately 0.33 ml was taken from each mouse for a single bleed, or 0.28 ml followed by 0.22 ml was taken if two bleeds (on consecutive days) were used. Physiologic saline was injected intraperitoneally half-way through the bleeding and at its completion, such that the injected volume equalled that of the extracted blood. Hematocrits remained high (90% of normal) three days after a single bleed, and only decreased to about 85% of normal three days after the double-bleed procedure. Marrow smears were also prepared at this time. Normal, untreated mice of the same age served as controls for this group.

## 3. Preparation of marrow cells

When the mice were ready for use one from the first group was quickly sacrificed by cervical dislocation. The muscles were stripped from around the right femur which was then dislocated at the hip and knee joints. Care was taken to not break or open the bone prematurely, thus keeping the contents of the marrow clean and intact. The ends of

the bone were then pierced with a sterile 23 gauge needle and the marrow was flushed rapidly with 1 to 2 ml of sterile ice cold collecting medium (described below) into a test tube placed on ice. The above steps were then repeated for each mouse in that group, and a common test tube was used to pool the marrow from similarly treated mice. Each batch of cells was aseptically dispersed into a single-cell suspension by gentle passage through a 23 gauge needle, followed then by a 27 gauge needle. A small aliquot of each suspension was sucked into a white blood cell diluting pipette (with an accuracy of  $\pm 1\%$ ) and then diluted 1:10 with Turk's solution (3% glacial acetic acid, 0.01% Crystal Violet in an aqueous solution). All nucleated cells were stained and fixed during a 5 minute exposure in this solution (with continuous shaking), and the cells were then counted in a hemacytometer at 200x magnification. With no prior centrifugation, portions of the suspensions were diluted to the desired nucleated cell concentration. As little time as possible elapsed (30-60 minutes) before these cells were plated in complete plasma culture medium (described below). The above procedure was then repeated separately for the other sets of mice (if needed). The final concentration of nucleated cells in cultures from normal mice was uniformly  $4.5 \times 10^5$  per ml; that of bled mice was half of that number ( $2.3 \times 10^5$  per ml); and plethoric marrow cells were seeded at twice that number ( $9 \times 10^5$  per ml).

#### B. Culture components and procedure in vitro

##### 1. Media components

The in vitro method of McLeod, et al (111) (with some modifications) was used to culture murine CFU-E in plasma clots. For this

system the following media components are utilized:

a. Supplemented Hank's Minimum Essential Medium (Supplemented HMEM) is made as follows:

10 ml MEM with Hank's Base (concentrated 10-fold, Grand Island Biological Co. (GIBCO), Grand Island, N.Y.)

1 ml Nonessential Amino Acids (concentrated 100-fold, GIBCO)

1 ml Sodium Pyruvate (concentrated 100-fold to equal 100mM, GIBCO)

1 ml L-glutamine (concentrated 100-fold to equal 200mM, GIBCO)

0.83 ml Sodium Bicarbonate (7.5% solution, GIBCO)

plus sterile distilled water to make 100 ml total volume, and

2 ml Penicillin-Streptomycin (GIBCO solution with 10,000 U/ml and 10,000 µg/ml, respectively).

b. Fetal calf serum (from Flow Labs, Rockville, Md., except for some initial experiments for which serum from GIBCO was used) was heat-inactivated at 56°C for 30 minutes and filtered while still warm (about 40°C) through a 0.45 µm Nalgene filter (Nalge Co., Rochester, N.Y.). This procedure depleted the serum of complement and then removed any particulate matter which might hamper microscopic examination of clots. Serum growth properties were consistently but only slightly enhanced by heat-inactivation and filtering. Storage of 2-5 ml aliquots at -20°C was for no more than 2 months.

c. NCTC-109 medium (Microbiological Assoc., Bethesda, Md.) constituted a major portion of the culture medium. Penicillin (final concentration 100 U/ml) and streptomycin (final concentration 100 µg/ml) were added to this medium before use and it was stored at 4°C.

d. Beef embryo extract (Colorado Serum Co., Denver, Col.) served

as a calcium source for clot formation, and probably supplied other nutrients. The lyophilized powder (50% by volume when reconstituted) was reconstituted with Supplemented HMEM and frozen ( $-20^{\circ}\text{C}$ ) in 1 ml aliquots before use. For culture 1 ml of reconstituted extract was diluted with 5 ml NCTC-109.

e. Bovine serum albumin (BSA, Fraction V from Sigma, St. Louis, Mo.) was detoxified and diluted to a 10% solution according to McLeod, et al (111) as follows: Fifty grams of powder was added to 91 ml of sterile distilled water and dissolved by repeated mixing at  $4^{\circ}\text{C}$  for 3 hours. When all lumps were eliminated with a glass rod, 5 g of resin (Ag-401-X8 D, 20-50 mesh, from Biorad Labs, Richmond, Ca.) was added to detoxify the BSA. This mixture was stirred at  $4^{\circ}\text{C}$  every 15 minutes for one hour and let stand at  $4^{\circ}\text{C}$  for another hour. At this time the clear fluid at the top was decanted into a sterile 100 ml bottle to which 5 g of resin had been added. The previous procedure was repeated except that the bottle was left standing at room temperature during the final hour. The clear, upper portion was decanted into a 500 ml graduated cylinder. For each 15 ml of BSA 1.1 ml of 10-fold concentrated (10 x) Dulbecco's phosphate-buffered saline (PBS without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ ) was added. To this 37% BSA solution enough 1 x Dulbecco's PBS was added to make a final 10% solution. This 10% BSA was then sterilized by passage through a  $0.45\ \mu\text{m}$  Nalgene filter. Aliquots of 2 ml each could be made immediately or the solution could be stored at  $4^{\circ}\text{C}$  overnight before aliquoting. Long-term (6-month) storage was at  $-20^{\circ}\text{C}$ . Just prior to use in the culture system an aliquot was thawed and buffered with 0.05 ml sodium bicarbonate (7.5% solution). A 0.01 drop of sterile

phenol red (0.4 g/100 ml 1x PBS) was then added to each 2 ml aliquot of 10% BSA.

f. L-asparagine (N.R.C. from GIBCO) was diluted with Supplemented HMEM to a concentration of 2 mg/ml. It was then sterilized through a 0.45  $\mu$ m Nalgene filter, distributed in 0.5 ml amounts, and stored at -20°C for up to 6 months. To use it in the culture system, one 0.5 ml aliquot was diluted with 4.5 ml NCTC-109.

g. Bovine plasma with 3.8% sodium citrate was obtained from Irvine Scientific Prod., Irvine, Ca. After warming it to 37°C it was filtered by passage of 20 ml aliquots through 0.45  $\mu$ m Nalgene filters, and was stored at -20°C in 2 ml aliquots. It served as a fibrinogen source for the clot meshwork.

h. Collecting and diluting medium consisted of Supplemented MEM with Hank's base with a final concentration of 2% fetal calf serum.

Each new batch of fetal calf serum, beef embryo extract, bovine serum albumin, and bovine plasma was pre-tested in the culture system for its ability to maximize erythrocytic colony growth of marrow collected from normal mice. We were not able to pretest the culture components for maximal response on plethoric or bled marrow. The response of the plethoric and bled animals (and their cellular requirements) were assumed to be the same as that of normal marrow.

## 2. Specialized components

a. Low specific activity Ep: An extract of human urine (HUE-II) which contains 15-20 units per mg protein (International Reference Preparation (bioassayable)) was generously supplied by Dr. Joseph F. Garcia of Lawrence Berkeley Laboratory, Berkeley, CA. Its preparation from

severely anemic persons is outlined in reference number 170. It was supplied in 5 mg lyophilized quantities, diluted to 20-25 U/ml with Supplemented HMEM, and stored at -20°C. It was not sterilized, filtered, or dialyzed. From this stock solution the desired concentration (usually 2.5 U/ml) was made with NCTC-109 for use in the culture system. The final concentration of this "low specific activity Ep extract" in culture was 0.25 U/ml, except for Ep dose-response experiments discussed below.

One small sample of stock solution (50 U/ml) of HUE-II was desialated by the method of Goldwasser, et al (61). A small amount was dissolved to 0.03 M HCl and heated at 80°C for 30 minutes. Dilution to 2.5 U/ml with NCTC-109 brought the solution back to normal pH before use in culture.

A few initial studies used an erythropoietin extract obtained from phenylhydrazine-treated sheep plasma. This step III material (from Connaught Medical Research Laboratories, Willowdale, Ontario, Canada) was of very low specific activity (4.8 units per mg) and was treated the same as HUE-II.

A normal human urine extract with no detectable Ep activity was employed as a control for the crudeness of the Ep extracts being used. This normal urine protein was prepared the same as HUE-II and was treated for culture the same as was the low specific activity Ep extract.

b. High specific activity Ep: Another more purified extract of human urine origin was also used as a source of erythropoietin activity in some experiments. This "high specific activity Ep extract" was kindly supplied by Dr. Peter Dukes through the National Heart, Lung,

and Blood Institute (pool T-7-E). Its potency was found to be far below original estimates and so it was reassayed at  $150 \pm 20$  (SEM) IRP Units per mg protein [in an exhypoxic mouse bioassay]. The treatment of the lyophilized powder was the same as that of the low specific activity Ep, i.e., it was diluted initially to 50 U/ml and stored (in sealed glass ampoules) at  $-20^{\circ}\text{C}$ . Further dilutions to the desired concentration used NCTC-109 medium directly before culture. This Ep preparation was not sterilized, filtered, or dialyzed.

c. Anti-erythropoietin serum: A rabbit anti-human erythropoietin immune serum was prepared against the low specific activity Ep described above, and supplied by Drs. Joseph F. Garcia and John C. Schooley of the University of California, Lawrence Berkeley Laboratory, Berkeley, California. Descriptions of its preparation (56) and properties (146) are available. Whole serum, able to neutralize 25 units of the low specific activity Ep per ml, was diluted 1:15 in Earle's balanced salt solution and will be referred to as "anti-Ep." For most experiments it was not necessary to sterilize the anti-Ep. The anti-Ep was not heat-inactivated.

A normal rabbit serum (NRS, the anti-Ep vehicle), obtained at the same time that anti-Ep was prepared, served as a control for the many normal proteins presumably contained in the antiserum. The NRS was treated in the same way as the anti-Ep before use in the culture system.

d. Iron-59 solution: Radioactive  $^{59}\text{FeCl}_3$  was obtained from New England Nuclear (Boston, Mass.) with a specific activity of 20-22 mCi per mg iron. One or two millicuries were buffered with a few drops of saturated sodium citrate (following the addition of a pH indicator,

bromocresol green). This solution was then diluted to 20  $\mu\text{Ci/ml}$  with sterile Earle's balanced salt solution and kept at room temperature up to 6 months. Its pH should be 7.0-7.5 as determined with pH paper. For culture use the activity of the stock solution was calculated (if the time between preparation and use was 5 days or greater) and appropriate dilutions were performed to 1.0  $\mu\text{Ci/ml}$  and 0.5  $\mu\text{Ci/ml}$  in Earle's balanced salt solution. Prior binding of  $^{59}\text{Fe}$  to serum transferrin was not necessary (41).

3. In vitro procedures and plating

a. Setting up the culture conditions: The complete culture medium for this system consisted of the following quantities or some multiple of these quantities according to McLeod, et al (111):

1. 0.1 ml beef embryo extract (reconstituted and diluted)
2. 0.2 ml fetal calf serum
3. 0.1 ml bovine serum albumin (10% solution)
4. 0.1 ml L-asparagine (0.02 mg)
5. 0.2 ml NCTC-109
6. 0.1 ml cells (properly diluted with collecting medium)
7. 0.1 ml erythropoietin (usually 2.5 U/ml stock)
8. 0.1 ml citrated bovine plasma

Total = 1.0 ml

Directly before the cells were harvested from the mice the first five culture components were combined in a test tube in the above proportions and kept on ice. When marrow cells were obtained from the mice, counted and properly diluted, enough cells for all cultures of that marrow were added to the tube. This mixture (usually measuring

8-10 ml) was mixed gently and then divided into several batches and kept on ice.

To the main batch was added erythropoietin (of high or low specific activity, depending on the experiment) at time equal to zero. For ease of handling this mixture was divided into 3-5 smaller batches and bovine plasma was added to one tube. After plating of this tube into 0.15 ml clots (described below) the next small batch received bovine plasma and was plated, and so on. The cultures were allowed to clot at room temperature, taking 10-15 minutes after they are plated. At certain times after the addition of Ep and clotting, 3-5 of these clots received a 0.15 ml (250 mU) overlay of anti-Ep capable of neutralizing 7 times the Ep contained in each clot. Some of the cultures plated from the first tube were given this overlay immediately after clotting, i.e., at 18 minutes after the addition of Ep, at room temperature. Later time points for the addition of anti-Ep from the same antiserum solution as was used at 0.3 hours were typically 1, 2, 6, 12, 24, and 30 hours after the Ep had been added.

Another batch of 1 ml volume was treated with Ep in the same fashion as the main batch, but, before bovine plasma addition and plating, and within 3 minutes of the addition of Ep, it received 0.067 ml of whole antiserum. The final concentration of anti-Ep in these cultures was therefore capable of neutralizing 170 mU Ep per ml of culture medium, a 7-fold excess. These cultures received anti-Ep so quickly after Ep that they will be called "zero" hour time points for the addition of anti-Ep. The upper line of Figure 2 shows these and the later time points for the addition of antiserum in relation

to the addition of Ep and plating.

The following control cultures were included: some clots received anti-Ep on top (near time zero) but no Ep; others received a normal rabbit serum overlay (near time zero) with or without Ep; others were not given Ep or anti-Ep or NRS at any time.

b. Plating the cultures: Plating of the complete plasma culture media was performed in Multi-well Tissue Culture Plates (Falcon Plastics, Oxnard, Ca.). These plates consist of 24 (6 x 4) cylindrical wells of 17 mm diameter, 10 mm in depth, and 2.2 ml total volume. Only the central 6-8 wells were used for cultures, with all others being filled prior to plating with sterile water for humidification. To make each clot, 0.15 ml of the slightly viscous plasma culture medium was placed in the center of a well so that it formed a button of 12-13 mm diameter without touching the sides of the well. (Some did touch the walls, however. These were found to give inconsistent colony growth and so were never included in the data.) This procedure was best performed with cold medium at a hood temperature of 28°-30°C. Under these conditions the medium would optimally spread and attach to the plate, but the clotting was complete within 15 minutes without allowing the cells to settle out. Four to five clots for each dose point were distributed throughout 6-8 Multi-well plates. Depending on the size of the experiment, all of the plating was completed within 30-45 minutes after time zero. Some plates in each experiment were inspected on an inverted microscope for cell clumps. All plates were then loosely covered and incubated for 48 hours at 37°C in an atmosphere of high humidity with 4% CO<sub>2</sub> in air (except for experiments where the CO<sub>2</sub> was varied). The

middle line of Figure 2 roughly summarizes these changes in the temperature of the cellular environment: as the plating procedure progresses cells are removed from an ice bath (0°C), they are warmed to room temperature (25°-20°C) during plating, and then placed in a 37°C incubator after their first 30-45 minutes of exposure to Ep.

c. Pulsing the cultures with radioactive iron: Thirty hours after the addition of Ep (time zero), each clot received a 0.15 ml overlay of the  $^{59}\text{Fe}$  solution described earlier. Those cultures to which an overlay of anti-Ep or normal rabbit serum had already been added received  $^{59}\text{Fe}$  at a concentration of 1.0  $\mu\text{Ci/ml}$  and those without overlays received 0.5  $\mu\text{Ci/ml}$  iron solutions. The clots to which anti-Ep was added at "30 hours" (the same time  $^{59}\text{Fe}$  was to be added) were actually treated with anti-Ep at 29.5 hours after Ep and incubated at 37°C until the addition of  $^{59}\text{Fe}$  at 30 hours. In this way they were subjected to undiluted anti-Ep for at least 30 minutes.

d. Harvesting the cultures: Forty-eight hours following the addition of Ep the incubation of all clots was terminated. The plates were drained and the clots were fixed in situ at room temperature with about 1 ml of 5% glutaraldehyde in 0.01 M phosphate buffer (pH 7.0 to 7.2) for 6 minutes. For the next 2½-3 hours the clots were then gently washed with 10-12 changes of distilled water to remove the glutaraldehyde. The first three changes were completed quickly, and the rest were more or less evenly distributed over the remaining time. After the wash the clots become white disks which are easily teased off of the plate, and they were then transferred individually to a clean microscope slide using a small U-shaped spatula. The slides are placed on

a 39°-40°C warming tray, covered, and allowed to dry slowly (about 3 hours).

e. Autoradiography: An  $^{59}\text{Fe}$  autoradiograph of each slide was prepared by dipping them into warm (42°C) NTB2 Nuclear Track Emulsion (Eastman Kodak). The slides were allowed to dry, were placed in light-tight slide boxes, and exposed for 5 days at 4°C. At the end of the exposure they were developed en masse for 6 minutes in D-170 developer at 18°C. After a quick water wash at 18°C, the slides were placed in fixer for 15 minutes at this temperature and again washed in cold water.

f. Staining: It was also found necessary to stain these autoradiographs at 18°C to prevent lifting of the emulsion. The slides were first immersed in cold distilled water for 15 minutes and transferred to a cold Giemsa staining solution. This utilized a 0.01 M phosphate buffer (pH 7.0) to dilute stock Giemsa (Harleco, Gibbstown, N.J.) 1:50. Staining took 15-18 minutes at 18°C and was followed by a quick rinse in two changes of cold distilled water. The slides were allowed to dry and were then counted.

Some experiments involved comparisons between  $^{59}\text{Fe}$ -autoradiographic identification and benzidine-hematoxylin staining of erythroid cells in the plasma clots. For these studies the staining procedure of McLeod, et al (111) was followed very closely. The benzidine stain was applied to clots with or without previous  $^{59}\text{Fe}$  exposure in culture. A few clots were stained with benzidine prior to autoradiograph, and subsequently stained with hematoxylin.

g. pH Measurements: Over a period of two years the pH of some media components and complete plasma clot medium (with a 1:3 mixture of

Supplemented HMEM:NCTC-109 substituted for Ep) was periodically measured after 24 hours incubation at 37°C at various levels of CO<sub>2</sub> in air. This was done with or without cells for the following solutions:

1. NCTC-109 with penicillin (100 U/ml) and streptomycin (100µg/ml)
2. Plasma clot culture medium with bovine plasma (citrated) added
3. Plasma clot culture medium without bovine plasma (citrated)

The CO<sub>2</sub> concentrations ranged from 2.7% to 5.2% in three different incubators of different design. The measurement of these concentrations was performed chemically using a Fyrite CO<sub>2</sub> indicator (Bacharach Instr. Co., Pittsburg, Pa.), which has an unknown accuracy with a precision of about ±3% of readings taken in the range used here.

For the pH measurements a single-electrode Corning Digital 109 meter was used after calibration at 37°C with warm buffer (pH 7.00, Mallincrodt, Inc., St. Louis, Mo.). Calibration was repeated at frequent intervals when many measurements were made. Five to ten ml of the solution being tested was placed into a small tissue culture flask (25 cm<sup>2</sup>, Falcon Plastics, Oxnard, Ca.) for incubation with the cap in a loose position to allow gas exchange. After 24 hours of incubation the cap was sealed and the pH was quickly measured on each successive flask. Usually three flasks were tested for each medium at each CO<sub>2</sub> concentration.

#### C. Counting and recording methods

Inspection of the clots for iron-labelled cells and clusters of cells in stained autoradiographs was performed by scanning the slides on the movable stage of a Zeiss binocular light microscope. Most counting took place at a magnification of 160x, with closer inspection

of many cells at 400-1000x. Whether a group of cells was labelled or not was usually quite clear, although, if necessary, the number of grains contained in the area of the cluster (or cell) were counted and compared to the average of three background counts over adjacent spots of approximately the same area but containing no cells. If the counts over the cluster (or cell) numbered twice that of background or greater, the cluster was considered to be labelled. All iron-labelled clusters of 8 or more mononuclear cells were counted on each clot and called "CFU-E colonies." Likewise, all single, mononuclear, labelled cells were enumerated per clot. In addition, a sample of colonies and smaller clusters (2-7 labelled cells) on each clot was classified according to cell number. For many clusters and colonies it was necessary to determine the number of cells at 400-1000x. If adequate growth was achieved, a total sample of 200 labelled clusters and colonies was used for this purpose. If fewer than 200 clusters were available all were classified. The number of clusters with granulocytic morphology was also monitored on the same clots. These were groups of large, unlabelled, usually polymorphonuclear cells ranging in number from 2 to 30 per cluster. As a measure of their proliferation, only those clusters having 5 or more cells were tabulated as positive CFU-C descendants.

Therefore each clot supplied four pieces of data: number of CFU-E colonies, number of single, labelled cells, a frequency distribution of labelled cluster sizes, and number of granulocytic clusters of 5 or more cells. The colony data was handled as "CFU-E per clot" or "CFU-E per  $10^5$  nucleated cells seeded," with the single cells and CFU-E clusters usually being quoted on a per clot basis.

### III. RESULTS

#### A. Colony and culture characteristics

##### 1. Colony photographs

Figure 3 is a typical scene from a plasma clot culture of normal mouse marrow after 48 hours in culture. This  $^{59}\text{Fe}$ -autoradiograph shows a small erythroid colony of eight polychromatophilic normoblasts, a larger erythroid colony of about 22 (mostly polychromatophilic) cells, and a small erythroid cluster of four orthochromatic cells. In spite of the high background characteristic of the use of  $^{59}\text{Fe}$  in plasma clots, each of these groups of cells is clearly labelled with radioactive iron, whereas the surrounding (presumably non-erythroid) cells are clearly not labelled. We believe the high incidence of background in these autoradiographs is mostly a result of  $^{59}\text{Fe}$  complexes with transferrin and other proteins found in the serum components of the media. When the clots are fixed with glutaraldehyde these complexes become permanently fixed in the fibrin meshwork. The glutaraldehyde fixative itself is also an important source of "chemical fog" (background) in this system, but this fog is minimized by extensive washing of the clots with distilled water before autoradiography.

Figure 4 shows a typical, slightly more mature erythroid colony of approximately 16 cells after 48 hours culture of normal marrow. In accordance with in vivo data, showing that in rodents the rate of  $^{59}\text{Fe}$  incorporation is maximal in the early erythron (102, 4), the maturity of this orthochromatic colony is reflected in a slightly lower grain count per cell than the polychromatophilic colonies photographed from the same clot for Figure 3. However, this indication that orthochromatic

cells indeed have a slower rate of hemoglobin synthesis compared to polychromatophilic cells is not always consistent in these in vitro cultures. It is true that on the average the orthochromatic colonies take up less of the  $^{59}\text{Fe}$  label, since they are never labelled more than the polychromatophilic ones and are sometimes less well labelled. But there is no consistent difference in the labelling of these two types of colonies. This deviation from the in vivo case may be due to a deficiency of in vitro cultures for increasing hemoglobin synthesis. Such a deficiency was recently observed by Mohandas and Prenant (119). In addition, the status of iron pools in vitro is unknown, since a large amount of iron is included in culture when the marrow is flushed out. The quantification of hemoglobin synthesis in maturing erythroid cells in vitro is then difficult because of the unknown ratio between "hot" ( $^{59}\text{Fe}$ ) and "cold" (inorganic) iron and unknown transferrin situation in the cultures. There is, however, a consistent decrease in grain counts per cell for colonies where extruded nuclei are abundant as the cells become very mature. Since cell lysis sets in at this point, it is difficult to know whether the decrease in the apparent rate of hemoglobin synthesis is actually due to the advanced maturation state. The vast majority of this type of colony does incorporate enough  $^{59}\text{Fe}$  to permit detection above background levels. Therefore, the 18-hour labelling period is long enough to allow the most mature cells to take up plenty of iron. Using this method only those few colonies that reach advanced maturity in the first 24 hours of culture would remain unlabelled by the end of the culture period. When such unlabelled, apparently erythroid, colonies were counted, their number

never exceeded 1-2% of the labelled colonies present.

The morphology and labelling of the single cells pictured in Figure 4 after 48 hours of culture is very much the same as those in all clots. This type of cell was never found in clusters. Their size is generally large, with about half of the cross-sectional area taken up by an eccentric nucleus, and they show about the same level of iron uptake as the erythroid cells. However these are not believed to be erythroid cells, but possibly macrophages which have phagocytosed some  $^{59}\text{Fe}$  label. Evidence for this belief comes from their unresponsiveness to Ep in vitro (as will be shown later), and the fact that their morphology and iron labelling is identical to that of presumed macrophages grown in both methylcellulose and plasma clot cultures with high levels of Ep for 5-15 days, during which these cells proliferate extensively (our own observations).

Figure 5 is a higher magnification of the cells in the colony seen in Figure 4. This group of cells is predominantly at the orthochromatic stage of erythroid maturation, but a few of the cells contain a nucleus which is slightly larger and less condensed. The cells with larger nuclei are not likely to be preparing for mitosis, but probably have just matured from the polychromatic normoblast stage. This variation in the stage of maturation within a colony is typical of all colonies, independent of the erythropoietic state of the donor marrow. Most colonies reach the orthochromatic stage after 48 hours in culture with Ep, but a few still remain at the polychromatic stage. Within each colony there is, nevertheless, a high degree of synchronous maturation, with the majority of the cells being present at one stage of

the erythron. All colonies continue to mature if they are cultured beyond day 2 (with or without Ep), with some showing expulsion of nuclei and subsequent lysis after 3-5 days of culture.

The same colony was again photographed at high magnification in Figure 6, with the focus now on the grains of the emulsion. The radioactive-iron labelling appears uniformly distributed over the cells of the colony and the grains extend outside of the colony area, which aids their detection at lower magnifications (100-200x). Because of this added ease in counting, iron-59 was used instead of iron-55, the latter giving much more localized label with no increase in the signal-to-noise ratio.

Three clusters of unlabelled polymorphonuclear cells typical of the granulocytic colonies in these cultures were photographed in the autoradiograph of Figure 7. Their level of labelling in  $^{59}\text{Fe}$ -autoradiographs was never above background, which, combined with their morphology, makes them easily characterized as myeloid and not erythroid.

## 2. $^{59}\text{Fe}$ -autoradiography vs. benzidine for erythroid identification

Figure 8 shows an erythroid colony and a myeloid cluster photographed from a clot not labelled with  $^{59}\text{Fe}$ , but simply stained with benzidine-hematoxylin following fixation after 48 hours of culture. Benzidine does offer an advantage for making certain which single cells in the clots were truly erythroid, rather than just those able to take up  $^{59}\text{Fe}$  (which includes macrophages). On one occasion some slides were pre-stained with benzidine, followed by dipping to produce an autoradiograph with Giemsa counterstain. This process did not render any selective advantage for analysis compared to a plain Giemsa autoradiograph.

However, contrary to what is seen in  $^{59}\text{Fe}$ -autoradiographs, rarely do all of the cells of a colony "label" with a benzidine stain. In addition, the staining technique was extremely sensitive to small changes in the staining solutions, the balance between benzidine and hematoxylin, and other staining conditions so that achieving consistent contrast and stain quality was very difficult. Because of these shortcomings it was often necessary to decide subjectively whether some colonies were truly erythroid. These problems especially hampered decisions on clusters of immature cells where the hemoglobin content of the cells is low and the staining needs to be optimal.

Table I gives the results of four experiments where  $^{59}\text{Fe}$ -autoradiographs were made in parallel with benzidine-stained clots. That is, some clots received an iron-overlay and others from the same culture received no overlay. The data clearly shows that significantly more colonies are identified as erythroid clusters of 8 or more cells on  $^{59}\text{Fe}$ -autoradiographs compared to benzidine-stained slides. The explanation for this result probably lies in the greater detection sensitivity for low levels of hemoglobin production obtained with the radioactive label ( $^{59}\text{Fe}$ ) as opposed to the chemical label (benzidine). That is, colonies of lightly hemoglobinized cells have a greater chance of being identified under the radioactive label because it identifies the rate of hemoglobin synthesis rather than hemoglobin concentration. In addition, colonies which contain such low amounts of hemoglobin as to not demonstrably stain with benzidine are likely to be the immature ones. With  $^{59}\text{Fe}$  present it is these cells that would incorporate the label most rapidly and would be easily detected as erythroid on an

autoradiograph.

Also demonstrated in Table I is that the source of Ep and the CO<sub>2</sub> concentration in culture did not affect the resulting detection efficiency counts when autoradiographs were used. It is possible that (1) the <sup>59</sup>Fe, or (2) the balanced salt solution (BSS) overlay contributed to the observed difference in colony counts. Since the additional iron content of the overlays may have stimulated the erythroid growth (the citrate buffer in the added iron would be unimportant, according to Dunn, et al (41)), it was necessary to calculate how much iron was present in the clots before and after the overlay. The major sources of iron in the culture medium are the fetal calf serum and the bovine citrated plasma. (The beef embryo extract might contribute smaller amounts but will not be included in the calculations.) According to Dunn, et al (41), fetal calf serum from Flow Labs (the source used here) contains about 300 µg inorganic iron per 100 ml serum. The bovine plasma can be expected to add at least half of this amount, since human, rat, dog, and swine sera have 100 (23), 140 (33), 73 (109a), and 175 (24) µg/100 ml, respectively. Therefore the serum and plasma used in our system can be expected to contribute greater than 0.1 µg Fe to each 0.15 ml clot. The <sup>59</sup>Fe overlays, on the other hand, are derived from solutions of high specific activity (about 20 µCi/µg Fe), which is diluted initially to 20 µCi/ml BSS. Therefore the final iron concentration of the initial stock solution is about 1 µg/ml. This solution is diluted 1:20 or more for use on most cultures, leaving about 0.05 µg Fe per ml BSS. Consequently, about 0.008 µg Fe is contained in a 0.15 ml overlay of <sup>59</sup>Fe. When compared to the amount of iron likely to be

present in the cultures from the serum and plasma components, a  $^{59}\text{Fe}$  overlay represents an increase of less than 10% in the total iron content of the cultures. The effect of this small amount of iron on erythroid colony growth should be insignificant.

The presence of the BSS overlay on the cultures would also not be expected to contribute to any increase in colony counts on autoradiographs, since most overlays could only lead to some dilution of the culture components, especially Ep and fetal calf serum. The results of experiments designed to test several different types of overlays are summarized in Table II. In accord with expectations, single BSS overlays are slightly detrimental to erythroid colony growth, even if supplemented to 20% fetal calf serum. An overlay of culture medium identical to the clot material, but lacking Ep, also tends to depress the colony counts, indicating that the Ep content of the cultures is the limiting component. Additional overlays of BSS (with or without normal rabbit serum) given early in the culture period (close to time zero) do not tend to decrease the colony counts much below the levels obtained with just one overlay given late in the culture period. Thus, the overall effect of overlays is to decrease CFU-E growth approximately 10%. Therefore, the advantages of identifying erythroid colonies by  $^{59}\text{Fe}$ -autoradiographs are greater than any detrimental effects introduced by the BSS carrier solution.

### 3. Histogram of cluster sizes for normal marrow in culture

Figure 9 shows a frequency distribution of the sizes of  $^{59}\text{Fe}$ -labelled groups of cells found after 48 hours in cultures of normal mouse marrow. With Ep present this entire culture period a wide

variety of cluster (all labelled groups) and colony (8 cells or more) sizes is obtained on each clot. In addition, each of the clusters and colonies has reached at least the polychromatic or orthochromatic stage of maturation. However, as the figure shows, there are many clusters that fully matured but reached only the 2-4 cell size, while others reached the same maturity but became 10-20 times that size during culture.

Within this spread of cluster and colony sizes, there is a marked predominance in the frequency of groups comprising a binary series of 2, 4, 8, and 16 cells. The frequency of this sequence of binary cluster sizes shows that within the majority of erythroid clusters there is a significant degree of mitotic synchrony which gives rise to more or less synchronous doublings of the cluster size. When this result is combined with the small differences in maturation seen within each erythroid cluster (refer to Figure 5), we find that the bulk of erythroid amplification in these plasma clots of normal mouse marrow proceeds synchronously in both maturation and cell division. These patterns have also been seen in vitro using cinemicrophotography (31).

#### B. Time of colony appearance and disappearance

Figure 10 is a representative pattern of response of normal mouse marrow cells as they form colonies of 8 or more erythroid cells over culture periods of 1-5 days. If the cultures are fixed after only 24 hours of culture there are some erythroid colonies already formed. However, if the cells are given another 24 hours to respond, a very large number of clusters are at the 8-cell stage or larger. These mature colonies begin to lyse by day 3 of culture and others are

quickly reaching the final stages. On day 3 only a few new colonies are being formed and the total CFU-E colony count decreases as a large number of cells mature and lyse. This process continues on day 4 until very few colonies are still to be found in cultures fixed after 5 days of culture.

For the present studies 48 hours of culture is used as an optimal endpoint where cell proliferation is maximal and lysis is minimal. All cultures were allowed to grow to this optimal time limit and then fixed.

C. Linearity of response with increasing cell concentrations

Figure 11 shows that, as more cells were seeded per clot, the number of CFU-E colonies increases in proportion to the seeded numbers, independent of the method of colony identification ( $^{59}\text{Fe}$ -autoradiography or benzidine stain). The response of this normal marrow to a constant dose of Ep (0.25 U/ml) is, in fact, linear from limiting numbers of cells (under 2000 per clot) up to 135,000 nucleated cells seeded. This represents a range of  $0.14\text{--}9.0 \times 10^5$  cells per ml. Although the studies presented later for different states of erythropoietic activity used cell concentrations of  $2.3\text{--}9.0 \times 10^5$  per ml culture, they can be expected to give results which are easily related to each other. In addition, the cell dose-response line for CFU-E goes through the origin (within experimental error). This is indication, but not proof, that each of these erythroid colonies arises from a single nucleated marrow cell. This proposal is now supported by very strong genetic (132) and cine-microphotographic (31) evidence.

Also shown in Figure 11 is the linearity of the "CFU-C precursor" response seen in the same clots from which the curve labelled  $^{59}\text{Fe}$  was

derived. This line for CFU-C also covers a 64-fold range of cell concentrations.

As more cells were plated per clot there was no difference in the size of the labelled erythroid clusters.\* Data from two experiments were included in Table III, which shows the erythroid response grouped according to size of the clusters. The frequency of small, medium, or large clusters did not change significantly as the cell concentration was increased. This again indicates that direct comparisons of these parameters between experiments seeded at different cell concentrations can be made.

D. The effect of pH and CO<sub>2</sub> on CFU-E response

The buffer used in this plasma clot system (sodium bicarbonate) required adjustment of CO<sub>2</sub> levels in culture to achieve optimal pH conditions. The growth of CFU-E in this system was, in fact, found to be very sensitive to the pH of the medium. Initial observations on this subject are presented in Table IV. When the CO<sub>2</sub> concentration was lowered from 5% (which most investigators in this field are using) to 4%, a definite increase in the number of erythroid colonies per clot was observed. In addition, this increase was not related to the source of Ep, nor was it affected by the method used for identifying erythroid colonies (see Table IV).

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\*Large colonies could arise from overlap or adjacency of two smaller colonies in these clots. However, calculations show the probability of overlap of colonies is directly proportional to the number of colonies in the clot and only becomes statistically significant at  $9 \times 10^5/\text{ml}$  or greater for normal marrow. We estimate that approximately 3.2% of the expected colonies will actually be a "double" colony when 800 colonies are present in a clot. Only 1.6% of 400 colonies would be expected to overlap.

To find the optimal pH for CFU-E growth in our system, tests for pH of the primary medium component (NCTC-109) and for the pH of the complete culture medium (with or without cells) were made after 24 hours of incubation at various CO<sub>2</sub> concentrations. This data is presented in Figure 12. As expected, the pH of the media decreases as the CO<sub>2</sub> concentration in the incubator increases. The rate of this decrease is the same for NCTC-109 (alone or with cells) and the plasma clots (with or without cells), and measured about 0.1 pH unit decrease for each 1% increment in CO<sub>2</sub> content tested. The pH of the collecting (and diluting) medium is appropriately buffered to pH 7.4, since the cells are exposed to this medium at ambient CO<sub>2</sub> levels (0.33%) directly after being removed from their marrow habitat (blood pH). Handling the cells at this pH might therefore be an improvement over placing them directly into NCTC-109, which, as shown in Figure 12, has a pH above 7.6 at ambient CO<sub>2</sub> levels. Such an improvement was observed over both NCTC-109 and Alpha medium (our own observations). Complete culture medium without citrated bovine plasma would be unsatisfactory also, since its pH at ambient CO<sub>2</sub> is  $7.18 \pm 0.05$ .

To relate these pH measurements to the growth of CFU-E, a direct comparison of CFU-E numbers at three different pH (or CO<sub>2</sub>) levels was performed using <sup>59</sup>Fe-autoradiographs. For this experiment small tissue culture flasks of NCTC-109 (with and without cells) and complete culture medium (with or without cells) were placed in triplicate into incubators set at 3%, 4%, or 5% CO<sub>2</sub> concentrations. At the same time CFU-E cultures were also placed in the three incubators. Tests for pH in the flasks were performed after 24 hours and the CFU-E cultures were

terminated after 48 hours. The results of this experiment are shown by the small squares in each curve of Figure 12. The pH of the test media fell directly on the lines already established, and the CFU-E number per clot was clearly maximized at a CO<sub>2</sub> concentration of 4%. The optimal pH for growing murine CFU-E colonies in a plasma clot system is therefore approximately 7.25. This pH is appropriate when compared to the pH of venous blood in mice (7.23-7.32 (15)), to which their bone marrow cells are exposed in vivo. In addition, Paul, et al (130) found pH 7.2 optimal for growing bone marrow cells in vitro with Hepes buffer.

E. Erythropoietin dose-responses for CFU-E

1. Low specific activity erythropoietin extract (20 U/mg)

A representative CFU-E response of normal mouse marrow to graded doses of HUE-II (15-20 bioassayed units per mg protein) in culture is shown in Figure 13. At low Ep concentration in culture the number of erythroid colonies increases proportional to the logarithm of the dose of added Ep.\* However, after a maximum number of colonies is reached at 0.25 U/ml, no further increases in colony numbers were obtained with this crude Ep extract. In fact, at a concentration of 2 U/ml of culture the CFU-E response is depressed to half of maximum. This pattern of response for normal mouse marrow CFU-E has been consistently observed by others using crude Ep extracts in semi-solid cultures (64, 111, 82). The decrease in erythroid colonies at high Ep concentrations is probably

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\*The quantity of Ep indigenous to the culture components (like fetal calf serum and bovine plasma) was not included in determining the Ep doses. Since fetal calf serum can contain 0.03-0.05 U/ml (41), each clot has about 0.001 U Ep present before any is added. This amount of Ep would be expected to only shift the lowest dose points of Figure 13 slightly to the right.

due to the presence of inhibitory substances in the Ep extract, since further purification of similar extracts leads to a continuation of the plateau values (82).

The dose of the Ep extract used for the present studies was always 0.25 U/ml of complete culture medium. Dose-responses for this extract were not performed on marrow from plethoric or bled mice.

2. High specific activity erythropoietin extract (150 U/mg protein)

CFU-E colony production from normal mouse marrow as a function of the dose of the semi-purified Ep extract is very much the same as the response to HUE-II. Figure 14 shows this is an experiment that directly compares erythroid colony production at several doses of each Ep extract. The response to the high specific activity Ep extract reaches a maximum at 0.25 U/ml before dropping at higher Ep concentrations. The similarities between this pattern and that of the low specific activity extract (which has 10 times more contaminant proteins) indicate that erythropoietin is almost surely the only stimulating component in these extracts of human urine.

3. Desialated Ep, inactive urinary extract, and normal rabbit serum

Tests for effects of contaminant proteins in the Ep extracts included studies with normal urine proteins (prepared and treated before culture like the low specific activity Ep extract) and studies with the desialated form of Ep. The results of CFU-E growth with these substances present in the culture medium are presented in Table V. The mixture of normal urine proteins was the major source of protein in the low specific activity Ep extract. If either low or high specific activity Ep was included in cultures grown with urine protein, the colony

response was depressed slightly below control levels. However, as shown in the table, desialated Ep retains full CFU-E stimulating activity in vitro, equalling that of the native hormone.

To assess the effects of the anti-Ep vehicle on this CFU-E growth, some cultures were given an overlay of normal rabbit serum (NRS, not containing antibody) very soon after the exposure of the cells to Ep. In five experiments an insignificant depression of CFU-E numbers resulted, yielding 80, 65, 86, 90, and 100% of the control (Ep only) value. Such small decreases can be fully attributed to the addition of a balanced salt solution overlay to the cultures (see Table II). However, other experiments with normal marrow showed 80-100% suppression of erythroid colony growth when NRS was applied in this way. That is, only 20, 8, and 0% of the control yield was obtained in these particular studies. The explanation for this cytotoxic behavior of the anti-Ep vehicle is unknown, since the antiserum itself had no such effect on the same marrow. Thus the results of experiments with anti-Ep remained unaffected by a negative result with NRS, and closely followed data obtained with marrow which was spared this effect. The cytotoxicity was also independent of the erythropoietic state of the marrow and was not abolished by heat-inactivation (30 minutes at 56°C) of the NRS.

#### F. Effect of timed erythropoietin exposures on colony production

##### 1. Normal mice

Having established the conditions for growing CFU-E colonies in vitro, experiments were performed where the exposure of the cells to erythropoietin was abbreviated by the addition of anti-Ep at various times after Ep was added (low specific activity Ep extract). Because

these studies were difficult to control for, many cultures in each experiment were used to establish baseline responses to Ep, the culture components, and serum overlays. These data will be discussed first, and are presented for normal marrow in Figure 15. When Ep was present for the entire 48 hour culture period, the "positive" controls yielded an average of  $405 \pm 40$  (SEM) colonies per clot. Since  $6.7 \times 10^4$  cells were seeded in each clot, the number of colonies formed from normal marrow averaged about 600 per  $10^5$  nucleated marrow cells seeded.

The "negative" controls shown in Figure 15 include those cultures to which no exogenous Ep was added. If no addition of an anti-Ep overlay was made, a small but significant number of colonies do arise, numbering 5-10% of the positive controls. We attribute this spontaneous colony growth to the actions of endogenous Ep in the culture components, mainly in the fetal calf serum. Addition of antiserum or NRS to cultures with no exogenous Ep causes a slight depression in spontaneous colony growth. The slight decrease is again probably due to the salt solution overlay itself (see Table II).

Figure 16 shows how CFU-E from normal marrow responded to abbreviated exposures of Ep in four experiments done at the same time as the control levels presented in Figure 15. A positive and negative control which received no overlay are included at the right of Figure 16. As the time of exposure to active Ep is increased, the patterns exhibit the following characteristics: (1) When Ep is allowed to remain active for as little as 18 minutes, there is a significant increase in colony production above the "zero" time addition of anti-Ep in three of the four experiments. That is, some cells need only a brief exposure to

active erythropoietin to create colonies of mature erythroid cells during the next 48 hours of culture. The antiserum-BSS solution has no problems diffusing through the clots, since the actions of Ep were apparently cut short within the first 18 minutes. In addition, the temperature history of these early cultures during most of their short exposure to active Ep is the same as or below the hood temperature, i.e., 28°-30°C. Because the cultures were placed into 37°C only after some 45 minutes of Ep exposure, the temperature of cultures which received anti-Ep at time "zero" and one hour after Ep was also low while Ep was active. Therefore, the response of at least some normal marrow cells to Ep is not only rapid, but can occur in vitro at these non-physiologic temperatures. In fact, the response of these cells is high at these low temperatures. This is suggested by the fact that "cold" Ep exposures of between 0.3 and 1 hour produced colony numbers equal to those observed after nearly 6 hours of Ep exposure at 37°C. This fact is the basis for the second characteristic of these curves: (2) The level of CFU-E production achieved after brief (0.3-1 h) Ep exposures is unchanged for Ep exposures of around 6 hours. This plateau region shown in trials 3 and 4 of Figure 16 indicates that after a portion of the cells respond to short exposures of active Ep and are transformed into CFU-E producing colonies, no further colony production results in the cultures unless Ep is actively present for more than 6 hours. The plateau region then defines a "fast-responding" population which comprises no more than 20% of the total possible colony production by marrow from normal mice. (3) Hence, the rest of the colony forming units (the "slow-responding" population) requires the presence of

active Ep for times greater than 6 hours. This sub-population of CFU-E, in fact, will continue to produce erythroid colonies for the full exposure period permitted in our experiments. This fact is brought out by the almost constant rate at which the colony numbers increase as Ep is left active from 6 to 48 hours. This suggests that maximum numbers of erythroid colonies can only be achieved by more prolonged exposures to active Ep. As shown in Figure 16, only about one-half of the possible CFU-E colonies emerge if Ep is actively present during the first 24 hours of the culture period. The active erythropoietin extract is therefore necessary throughout the observed period for the maximum yield of erythroid colonies to be realized.

In addition to the above changes in the number of colonies per clot as Ep was left active for longer times, a distinct shift in the size of erythroid colonies was observed. As shown in Figure 17 for four experiments with normal marrow, more large colonies arise as the exposure to Ep is lengthened. There are very few larger colonies when Ep is left active for only 2 hours. But the response to Ep being active for the first half of the culture period (24 hours) produces a considerable number of <sup>59</sup>Fe-labelled colonies with more than 16 cells, comprising about 7% of all labelled clusters. Even more large colonies arise in control cultures where Ep was allowed to remain active for the entire culture period (48 hours in Figure 17). After this 48 hour Ep exposure about 10% of all labelled clusters have more than 16 cells. Complete data on the size of erythroid clusters as a function of Ep exposure is presented in Table VI. The rise in proportion of large colonies (17+) present is affirmed to be a rather smooth function of the time Ep is

left active. There is no significant change in the proportion of any other cluster size.

Table VII summarizes the significance of this effect that the low specific activity Ep extract had on the proliferation of Ep-responsive cells from normal mice. With no exogenous Ep added (or with normal rabbit serum overlays only), there is essentially no change in the size of erythroid clusters over the 48 hours of culture (2.1-3.0% of the clusters have greater than 16 cells). These values establish a control baseline to which values obtained with Ep can be compared. When Ep is added but is followed immediately by antiserum at "zero" time, the production of large colonies is not significantly depressed below this control level ( $1.4 \pm 0.7(\text{SD})\%$  vs.  $2.1 \pm 0.8(\text{SD})\%$  of all erythroid clusters). However, with Ep allowed to be active for the entire culture period (with or without normal rabbit serum) a highly significant increase in the proportion of clusters reaching the larger colony sizes is observed.

Therefore, longer exposures of normal marrow to Ep have simultaneously increased both the number of erythroid colonies and the relative size of the colonies. This suggests that the bulk of the erythroid colony production in cultures of normal mouse marrow is a result of actual proliferation rather than mere maturation.

A similar proliferation of myeloid cells was also observed to be a function of Ep exposure time in these cultures. Since no particular effort was made to grow granulocytes in this system, their numbers are relatively small in the two-day cultures. But by counting the clusters with 5 or more cells, a rough measure of the myeloid precursor (CFU-C)

response to Ep (and anti-Ep) was obtained. Table VIII summarizes this data for the same four experiments presented in Table V. The production of granulocyte clusters is significant when neither Ep nor anti-Ep were added to the cultures. Therefore, some myeloid precursors (like some erythroid precursors) are able to respond to factor(s) found in the serum components alone. The addition of anti-Ep, either alone or immediately after exposure to Ep, slightly suppresses this growth below the control level. But, if Ep is allowed to stay active (even in the presence of normal rabbit serum) for the entire 48 hours of culture, a significantly increased number of myeloid clusters reach the 5-cell stage or more. Therefore, protracted exposure to the crude Ep extract does increase "colony" production from the myeloid precursors in the donor marrow.

However, the erythroid and myeloid precursors differ in the kinetics of their response. For a direct comparison of erythroid and myeloid proliferation the data of Table IX should be referred to. The actual number of large CFU-E colonies (greater than 16 cells) has been calculated. Knowing how many CFU-E were present per clot and knowing what fraction of the total erythroid clusters they represented (the sum of the "8-16" and "17+" groups), one first calculates the total number of erythroid clusters (2-64 cells) per clot. Then the number of large erythroid colonies per clot is just the fraction of clusters having "17+" cells times the total number of clusters just calculated. These average numbers of large erythroid colonies, given in Table IX, along with the number of myeloid precursors, are plotted in Figure 18 as a function of the time Ep was left active. The erythroid cells

respond well to longer exposures of active Ep by making more and more large colonies per clot. However, the myeloid cells do not respond so vigorously in the 48 hour cultures. Therefore, myeloid cell response to Ep may only be a response to a general colony stimulating factor (possibly different from Ep) contained in the low specific activity Ep extract. Although some preliminary results with a semi-purified Ep extract support the view that Ep is not the stimulator of granulocyte growth in the cultures (unpublished observations), the present data does not rule out the possibility that the two cell types differ in sensitivity to the same stimulant (possibly Ep).

## 2. Plethoric mice

When the marrow collected from the femurs of chronically plethoric mice was exposed to Ep for the same time schedules as those used for normal marrow, the CFU-E response was lower than or equal to that of normal mice. As shown for the positive controls in Figure 19, plethoric marrow yielded 50-100% of colony numbers obtained from normal marrow when Ep was active the entire culture period. The magnitude of the colony production appears to depend to some extent on the hematocrit of the plethoric animals, since the number of colonies of Trial 1 (Hct =  $75 \pm 1$ ) was significantly lower than that of Trial 2 (Hct =  $63 \pm 1$ ). Also noted in all cultures of plethoric marrow was a qualitative increase in the proportion of immature colonies. These were easily recognized as labelled with  $^{59}\text{Fe}$ , but the cells and their nuclei were slightly larger than the polychromatophilic cells usually seen.

The negative controls are also shown in Figure 19 and are compared with the response from normal marrow. The plethoric state showed a

tendency to decrease the number of colonies in control cultures given no Ep nor anti-Ep. This trend indicates that spontaneous CFU-E growth, which is probably a response to Ep contained in the culture components, shifts slightly downward during plethora. However anti-Ep lowered the colony yield from plethoric marrow when given alone. As shown in Figure 19, the addition of anti-Ep without Ep caused a significant depression of colonies in Trial 1, but no change in Trial 2, while normal marrow shows a small decrease with anti-Ep. Thus, plethoric marrow reacts, on average, to anti-Ep in a way similar to normal marrow, but to a greater extent.

When the exposure of plethoric marrow cells to active Ep was terminated by anti-Ep additions to the cultures at various times, the CFU-E response was generally below normal. The patterns of Figure 20 were observed in two experiments on plethoric marrow, and, as in the normal case, a gradual increase in CFU-E colonies as Ep remains active for longer times emerged. A conspicuous change in the pattern is a general decrease in colony yield if Ep is left active for the short times of 0.3-6 hours. That is, there is a decrease in the "fast-responding" CFU-E population in plethoric marrow. The other group of Ep-responsive cells, which require active Ep for longer times, is not so depleted, and shows a normal rate of colony production as Ep is left active for longer times. These data suggest that the induction of plethora in mice causes a decrease in the number of CFU-E that require only short exposures to active Ep (0.3-6 hours), with no change in colony production by the rest of the CFU-E population. Just as for normal marrow, Ep must be left active the entire culture period to achieve maximal

CFU-E response from plethoric marrow.

Also observed in the CFU-E response of plethoric marrow was the emergence of larger colonies as the exposure to active Ep was lengthened. Table X shows data which exhibit the increase in percentage of  $^{59}\text{Fe}$ -labelled clusters reaching 17 cells or greater similar to that seen in the normal case. The increase in the proportion of these erythroid clusters is a significant response of plethoric marrow to Ep exposure. This is shown in Table XI. However, not seen in the erythroid response or normal marrow is the sharp increase in very small clusters when Ep is present 24 hours or less (refer to Table X). For these short Ep exposures this shift resulted in a distinct decrease in the proportion of clusters having 8-16 cells in addition to the decrease in large colonies of greater than 16 cells.

In vitro changes in myeloid cell proliferation from plethoric marrow were similar to those seen with normal marrow. However, the number of white cells precursors induced to proliferate from plethoric marrow was 2- to 4-fold higher than normal. These data are presented in Figure 21. They show a consistently high level of CFU-C clusters of 5 or more cells when Ep is present for all of the culture period. A 25% lower yield results if Ep is not included in cultures of plethoric marrow, a decrease similar to that found in normal marrow CFU-C numbers. The high number of white cell precursors found in plethoric marrow is probably a direct response to plethora for the marrow as a whole. With the chronic lack of erythroid cells in the marrow, the unused space may be occupied by myeloid cells, thereby raising the proportion of myeloid precursors seeded in culture.

The number of single labelled cells was also greatly increased in cultures of plethoric marrow. When Ep was active the whole culture period, the increase measured 5-fold above normal in both experiments. As with normal marrow, the number of single labelled cells showed no definite response to timed Ep exposures. The large increase in presence of this cell type parallels and may be related to the increase in CFU-C. The reason for such increases is, again, probably a reflection of the population shift toward white blood cells in the contents of the marrow.

### 3. Bled mice

When Ep was active for all of the 48 hour culture period, the production of erythroid colonies from the marrow of mice which were acutely bled once was about equal to the yield of normal marrow. However, when bled twice the colony yield doubled. These positive control levels are shown in Figure 22. This was so even though the hematocrits of both single- and double-bled mice were lowered to about the same level (46 and 44, respectively). Only after the more severe double-bleed procedure did the number of colony-formers in the marrow rise above normal levels. These results are not likely to be affected by the different number of cells seeded in the cultures of bled marrow as opposed to the normal marrow. As observed earlier (see Figure 11), normal marrow shows a linear CFU-E response within the range of cell concentrations used for bled marrow.

If neither Ep nor anti-Ep were included in marrow cultures from bled mice little difference in colony production was observed between normal and bled mice. These data are also shown in Figure 22, and indicate that spontaneous CFU-E production is relatively unaffected by

bleeding. However, when anti-Ep was added alone to marrow cultures of bled mice, spontaneous colony growth was completely eliminated in both experiments. This means that the cells that are normally able to respond extremely quickly to the low levels of Ep contained in the culture components are eliminated in bled marrow.

When abbreviated Ep exposures were used on marrow cells from mice bled either once or twice, a gradual increase in CFU-E numbers was observed as the hormone remained active for longer time periods. The data of two experiments are plotted in Figure 23. Although the response of CFU-E from the marrow of bled mice exhibits the general characteristics of the response from normal marrow, three notable changes from the normal marrow response are observed. One is the lack of colonies produced after short exposures (0.3-6 hours) of bled marrow to Ep. This population of "fast-responding" CFU-E measure an average of 5% of that seen in normal marrow, a significant decrease. Secondly, the drop in this population is compensated for by an increase in the number of colony-forming cells that require Ep for more than 6 hours, so that the control (Ep active for 48 hours) yield from bled mice is comparable to or well above that of normal mice. Thus, the third departure from normal is: the vast majority of erythroid colonies developed from stimulated marrow are more rapidly produced than their counterparts from normal marrow. This effect of Ep on erythroid colony production is exhibited in Figure 23 by an increase in the slope of the curves from bled mice. By comparison of the data between the 6 and 24 hour additions of anti-Ep for normal and bled mice, the increase in colony production per unit time that Ep is active on bled marrow averages twice that found for normal

marrow. A marked change in slope indicates that bleeding alters positively the rate of proliferation in response to Ep for cells from bled marrow. Nonetheless, Ep must still be left active for the entire culture period for maximum colony yield from bled marrow.

Table XII shows that the size of erythroid colonies emerging from bled marrow was also increased as Ep remained actively present for longer times. This is evident from the rise in proportion of large colonies (17+) found in both trials. However, unlike normal and plethoric marrow, a delay was observed before this effect was realized in cultures of bled marrow. That is, if Ep was active for only 12 hours, no large colonies emerge from bled marrow by the 48-hour endpoint, whereas, normal and plethoric marrow will produce numbers of large colonies above Ep only control levels after a 12 hour Ep exposure. Nevertheless, bled marrow cells can produce just as high a proportion of large colonies as normal marrow does by the end of a 48 hour Ep exposure. As in the plethoric case, the erythroid clusters produced in response to brief exposures of bled marrow to Ep were extremely small, and there were significant increases in the proportion of clusters having 2-4 cells. This situation gradually returned to normal levels as the time between the addition of Ep and anti-Ep was lengthened.

Table XII also shows how many single labelled cells were present in marrow cultures from bled mice. As in the normal and plethoric marrow cultures, single labelled cells showed no consistent pattern with protracted Ep exposures.

According to Table XIII, the change in the fraction of large erythroid colony sizes (the proportion reaching 17 or more cells) after

exposure of bled marrow to Ep is highly significant, and did not depend on the severity of the bleeding procedure.

The proliferation of myeloid precursor cells in control (Ep only) cultures of bled marrow showed no consistent changes from normal levels. This data is presented in Figure 24. Without exogenous Ep added CFU-C numbers again were not different from the normal response. The results suggest that the presence of CFU-C in the marrow is unaffected by erythropoietic stimulation.

The data presented in Figure 24 also show a consistent CFU-C stimulation in the presence of Ep. Again, the myeloid precursors are probably responding to a colony stimulating factor present as a contaminant of the crude Ep extract. Their response to the extract does, however, appear to be greater than the response to myeloid precursors in normal marrow. The cause for this difference is unknown.

A second look at data obtained from the normal mice used in trial 4 shows how sensitive CFU-E responses can be to timed exposures of Ep. The three mice had hematocrits of 48.2, 50.5, and 52.6, which yield an average hematocrit of  $50.4 \pm 1.2$  (SEM). This hematocrit is not significantly lower ( $p > 0.10$ ) than the  $52.6 \pm 0.1$  (SEM) found for the mice in trial 3, but the CFU-E responses of the two groups are very different. Figure 16 illustrates how different trial 4 appears at the early points of anti-Ep addition when compared to trial 3 and the other curves for normal mice. However, the variations of trial 4 are not just random, but closely resemble the properties of the curves from bled marrow, just discussed. That is, the "fast-responding" population is severely decreased in number, and the rate of colony production in the other

colony-forming population is increased. In addition, as Table VI indicates, trial 4 showed a tendency toward an increase in the proportion of erythroid clusters having 2-4 cells. This pattern was not seen in any other experiment on normal mice, but was a consistent finding for marrow cells from bled mice. Marrow smears from the mice of trial 4 (normal mice) showed a markedly stimulated marrow and confirmed the erythroid colony indications that these mice were experiencing a slight but detectable increase in erythropoietic activity.

#### 4. Comparison of different erythropoietic states in vitro

A brief comparison of two characteristic CFU-E responses in normal, increased, or decreased erythropoietic states is presented in Table XIV. Comparing the early CFU-E response (those that made colonies after only a short pulse of Ep) is best made at the 2 hour addition of anti-Ep. For the very short Ep exposures ("0" - 1 hour) we believe that the non-physiologic conditions during plating, coupled with the rapidity of the response of some cells to Ep largely contributed to large fluctuations in early colony numbers. For Ep exposures of 2-6 hours a brief plateau often emerges, but is quickly interrupted by new colony formations after 6 hours. So the 2 hour exposure appears least affected by plating conditions and later effects of Ep. For these reasons the 2 hour response (the "fast-responding" population) was measured as a proportion of the total colony yield from that marrow. From Table XIV it can be seen that these cells make up a small but significant portion (13%) of erythroid colony-formers in normal marrow. The fast-responding population in plethoric marrow, however, is very small, measuring only 3% of the total, and in bled mice their numbers are barely detectable

(0.2%). These results suggest that large changes in this small CFU-E subpopulation are a significant part of the erythropoietic response.

Also summarized in Table XIV are the observed hours of Ep exposure required by the cells to double the number of erythroid colonies per clot. These numbers are average slopes of straight lines which approximate the colony response to Ep exposures between 6 and 24 hours. No change from normal in the measure of erythroid cell proliferation rate was observed for marrow cells from plethoric mice, but bled mice produced cells which responded to Ep in vitro at twice the normal rate.

#### IV. DISCUSSION

This study has sought to characterize the immediate precursors of the (CFU-E) by investigating how long these cells need or use the hormone, erythropoietin, for their activation. Abbreviated exposures of mouse marrow cells to Ep in plasma clot cultures were achieved by application of a rabbit antiserum against a human urine Ep extract. Addition of the antiserum at various times after exposure of the cells to Ep effectively neutralized the actions of the hormone from that time on.

The basic culture technique of McLeod, et al (111) was used with some important modifications. The use of a flat culture area for the clots, followed by fixation in situ helped assure 100% retrieval of each clot. An optimal pH level for CFU-E growth was also obtained by adjustment of the CO<sub>2</sub> concentration to 4%. A critical part of the culture system was the addition of <sup>59</sup>Fe to the cultures, which aided the identification of erythroid clusters and colonies, especially the immature ones. The radioactive label consistently identified 10-15% more colonies than the peroxidase (benzidine) stain. With the modified method, accurate quantitation of erythroid colony and cluster production per clot was relatively easily achieved.

While the main thrust of this work was the time requirements of CFU-E for Ep, the experiments also reconfirmed that bioassayable Ep is indeed an obligatory requirement for growth of CFU-E in culture. CFU-E colonies of 8 cells or greater were formed in response to three different preparations of Ep from two different sources, human urine and sheep plasma. In addition, these three extracts contained Ep in specific

activities that varied 30-fold. The Ep dose-response for CFU-E production was found to be nearly identical for the two extracts from human urine having Ep concentrations of 15-20 U/mg protein and 150 U/mg protein. Attempts to detect CFU-E stimulating activity in a sample of normal urine proteins (Ep carrier proteins) were unsuccessful. In addition, no potentiating effects on marrow cells were detected when Ep extracts were used at optimal concentrations with normal urine proteins present.

On the other hand, crude Ep extract, which had been desialated, yielded 100% of the erythroid colony growth found for the original Ep preparation. A similar CFU-E response to desialated Ep was observed in methylcellulose cultures by Sieber (152a). However, desialated Ep has no detectable erythropoietic activity in vivo, probably because liver removal of desialated glycoproteins from the bloodstream is too rapid to allow much interaction with marrow cells (61). Even though marrow cells can be stimulated in vitro by desialated Ep, in addition to native Ep, it is native Ep that is most likely the predominant form to which the marrow cells are responding in plasma clot cultures. This is because radioimmunoassay of the low specific activity Ep extract, an assay which is sensitive to both Ep and desialated Ep, measures the concentration of the erythropoietic activity in the extract as equal to that found by bioassay (55). Therefore, insignificant amounts of desialated Ep are likely to be present in the crude Ep extract. It is also unlikely that significant amounts of Ep were desialated while in the semisolid cultures, since Iscove and Sieber (82) have retrieved 100% of the bioassayable Ep activity after an Ep extract was present

for 9 days in semisolid cultures. Therefore, the development of early (day 2) erythroid colonies in plasma clots appears to be a response to bioassayable Ep levels.

Without Ep added to plasma clot cultures, or if anti-Ep is added alone or immediately after Ep at the beginning of the culture period, a few erythroid colonies develop. However, if normal marrow is exposed to Ep (even at low temperatures) for as little as 18 minutes, an additional number of erythroid precursors can be triggered into colony production. Approximately 13% of all colonies obtained from normal marrow arise after Ep exposures of 1-2 hours. Although generally small colonies result from such brief exposures, their cells mature completely through the erythron and proliferate without any more need for Ep. Therefore, in the normal mouse at steady state erythropoiesis, at least some CFU-E are on the verge of commitment into the erythron. The continued presence of Ep up to 6 hours has almost no effect on the size or number of colonies arising from these immediate erythroid precursors. We shall functionally describe this population as CFU-E proper, since they are apparently a group of unrecognizable cells present at the last stage prior to the erythron.

Cultures of normal marrow begin to respond again if Ep remains active for more than 6 hours. The response is not an immediate one, but a gradual one. The number and size of erythroid colonies increases at a nearly constant rate as Ep is left active for longer times. This type of response defines the actions of Ep on a second population of Ep-responsive cells. The result is that a full erythroid colony response to Ep only occurs when Ep is allowed to remain active for the

entire culture period. A very similar response to timed Ep exposures has been observed in vivo by Schooley (143). In his studies, an anti-Ep serum was injected into hypertransfused mice at various times after an injection of Ep. Red blood cell production resulting from these abbreviated Ep exposures was measured at the end of 56 hours by a standard 72-hour  $^{59}\text{Fe}$ -uptake. The results showed a gradual increase in  $^{59}\text{Fe}$ -uptake as the Ep exposure (to several different doses) was protracted. Red cell production was significant for Ep exposures of 12 hours, but the full effect of Ep was not exhibited even after exposures as long as 24 hours. As in our in vitro studies, Ep had to remain active for almost 48 hours before control (no anti-Ep) levels were reached. Hence, erythroid colony production in vitro appears to simulate the in vivo response of "Ep-responsive" cells to brief Ep exposures quite accurately, and the in vitro system clearly defines at least two populations of immediate erythroid precursors: one that can produce colonies after only a short pulse of Ep, and a second population that needs longer exposures to the hormone before colonies emerge from it.

Since the entry of "fast-responding" CFU-E into the erythron requires only short Ep exposures, it is probable that the second population consists of precursors to the CFU-E proper. If so, the longer Ep exposures required by the second population suggests that most of these cells are undergoing some "preconditioning" before colony production. In addition, the gradual emergence of colonies from the second population indicates that they are committed into the erythron only a few at a time, and, therefore, are present in the marrow at various stages of maturation or "distance" from the erythron. Therefore, at the

time of plating, the maturation state of each cell in what we will call the "pre-CFU-E" population might be governed by how much "preconditioning" had been accomplished in vivo. Their induction into the erythron may only require that Ep be present after the cells complete the preparations. Hence, most stages of the committed progenitor compartment would be unresponsive to the hormone. In this case, many cells would be ready to enter the erythron, but only few would be called in the normal steady state. As will be discussed later, the present data from cultures without added Ep do not support this possibility.

Alternatively, the continuation of the "preconditioning" might require the presence of Ep, independent of the state of preparation of the pre-CFU-E. That is, the pre-CFU-E may be part of the Ep-responsive cell pool. If this is true, the longer the in vitro Ep exposure the larger would be the number of cells able to complete the steps needed to enter the erythron, and the larger the number of colonies that would be produced. The data presented in this study did exhibit this type of response, and allow us to roughly summarize in Figure 25 the steps taken by the immediate erythroid precursors as they progress into the erythron. As shown, Ep is probably necessary at each step.

The preparations made by erythroid precursors, whether Ep-dependent or Ep-independent, may include synthesis of necessary precursor molecules for hemoglobin synthesis. However, the data of Table VI suggest that another form of preparation may take place. As more colonies are produced in response to longer Ep exposures, a higher proportion of them are large colonies (17 or more cells). Within the two-population model shown in Figure 25, this implies either: (1) the

pre-CFU-E undergo some self-replication (A situation where both daughter cells are at the same stage of maturation as the parent. Divisions which would result in the two daughter cells being at different maturation states are not being suggested here.), or (2) no self-replication occurs early, but Ep has a direct effect of increasing proliferation within the erythron. The kinetics of production of large colonies by these two methods is compared in Figure 26.

Under circumstances where self-replication of pre-CFU-E occurs (with or without effects of Ep on the erythron), these earlier, more "unprepared" precursors might be allowed to divide a few times before entering the CFU-E state of responsiveness to Ep. The resulting colonies would be larger than those produced from the CFU-E. It is probable that this self-replication of pre-CFU-E would be Ep-dependent. For, without Ep added, a large number of small, unlabelled clusters of immature cells would be present after 48 hours of culture if the self-replication was Ep-independent. No such increase was observed in the cultures without Ep. Therefore, pre-CFU-E can rightly be considered a part of the Ep-responsive cell pool.

How much self-replication might go on in the pre-CFU-E population is not known. This question is complicated by the fact that not every CFU-E that arises has time to make 8 cells, but if one assumes that 3-4 divisions occur in the erythron, then it can be estimated that only 1-2 divisions can be occurring in the pre-CFU-E pool to give rise to the larger colonies of 16-64 cells. It is also not known whether the number of self-replications are equal for each precursor. However, given the narrow range of divisions expected from these cells (1-2),

the number of self-replications cannot be very different among them.

If, on the other hand, no self-replication of CFU-E occurred, then large colonies could arise by direct effects of Ep on the colonies that have cells in the erythron (refer to Figure 26). Longer exposures of Ep, it is implied, could increase the number of divisions in the erythron, giving rise to colonies that reach above the 16-cell range. If a direct effect of Ep on the erythron were the only method by which large colonies could be obtained, then no large colonies would be expected to emerge when all Ep activity was neutralized within a short time of the addition of Ep. But according to Table IX, large colonies did emerge in almost all cultures of normal marrow, numbering about 1-7 per clot in those given anti-Ep at "0"-6 hours after Ep. Therefore, the production of large colonies in culture does not appear to rely on direct effects of Ep on the expansion of the erythron. This notion is compatible with our earlier finding that Ep was not necessary for the maturation of erythroid cells, and strongly suggests that Ep has little or no effect on the erythron in normal mice.

An additional observation in cultures of normal marrow is the consistent occurrence of labelled clusters of two mature erythroid cells after 48 hours in culture. Most of these doublets had reached the polychromatophilic or orthochromatic stage, suggesting that they had matured through the erythron but divided only one time. This behavior is diagrammed in Figure 26. If this were the case they should be heavily labelled with  $^{59}\text{Fe}$ . Such cells were actually observed by Alpen and Cranmore (4), comprising up to 15% of cells entering the erythron in normal dogs. In plasma clots the mature doublets that were observed

were not particularly heavily labelled, but this could be a result of a deficient response in vitro systems may have to variations in hemoglobin synthesis (119).

To briefly summarize, the data from abbreviated Ep exposures of normal marrow best support the following notions about CFU-E:

- (1) At least two populations of immediate erythroid precursors exist.
- (2) The early, "pre-CFU-E" respond to Ep by self-replicating once or twice before entering the erythron.
- (3) The product of self-replication is sensitive to conversion by Ep into CFU-E.
- (4) Those cells that have matured to a stage just outside of the erythron (CFU-E) respond to very brief exposures of Ep by entering the erythron.
- (5) Cells within the recognizable erythron do not notably respond to Ep.

Figure 27 summarizes how these proposals dictate the structure and kinetics in the committed progenitor compartment. Regulation by Ep appears confined to the compartment, but stimulation by the hormone appears necessary throughout the compartment.

The model described above can be applied to states of decreased or increased erythropoiesis. In the marrow of chronically plethoric mice the number of (fast-responding) CFU-E is significantly depressed to about 20% of normal. The (less mature) pre-CFU-E population also tends to be below normal in number. The regulation of erythropoiesis in these animals, therefore, can include marked changes in both

postulated immediate precursor compartments. In the plethoric state, the more mature (CFU-E) compartment shows no indication of build-up in numbers, which one might expect when entry into the erythron is inhibited. In fact the number of these cells in the marrow is somewhat suppressed in plethoric mice. These cells may die more quickly or be removed from the marrow. But a more appealing suggestion is that, indeed, an earlier stage of CFU-E development is involved in the regulation of erythropoiesis, and reacts to lower plasma levels of Ep by a decrease in number. According to the present hypothesis of self-replication of pre-CFU-E, this group may then help regulate erythropoiesis in plethoric marrow by a decrease in the number of cells induced to self-replicate. Another alternative explanation would be a decrease in the average number of self-replications, but this is not supported by data on the size of the colonies. That is, in plethoric marrow cultures given only Ep, the proportion of colonies having more than 16 cells is the same as that found for normal marrow. Hence, the data from both normal and plethoric marrow lend support to the idea that a pre-CFU-E population is also Ep-dependent. In addition, once triggered into the erythron, CFU-E respond fully to Ep, independent of the erythropoietic state of the marrow.

The response of marrow from acutely bled mice to short exposures of Ep also supports the existence of two immediate precursor populations. Ep exposures of 6 hours or less again define a "fast-responding" population. However, it is almost eliminated from the marrow of mice bled either once or twice, since the number of cells in this group measures only about 5% of that found in normal mice. This means that the pool

of cells that are on the verge of commitment (CFU-E) were activated into the morphologically identifiable erythron soon after the bleeding and their numbers had not yet been replenished 3 days after bleeding. Such a rapid depletion of the CFU-E compartment represents the first line of defense against the consequences of blood loss, and explains why the hematocrit of these animals drops only a few points after severe bleeding.

The number of colonies produced by the less mature (pre-CFU-E) population, on the other hand, is increased by either one or two bleeds so as to replenish or exceed the total number of mature CFU-E found in the normal state. Therefore, pre-CFU-E are again involved in regulation of erythropoiesis, and they are stimulated by elevated plasma Ep levels to increase their number. However, no change from the normal case in the resulting colony sizes is observed after 48 hours with Ep. Therefore, the number of cells produced per CFU-E remains the same irrespective of the erythropoietic state of the marrow. This fact suggests that stress erythropoiesis is regulated by the rate of flux into and out of the CFU-E pool, and not by changes in the number of cells produced by individual CFU-E.

In all of the studies described above, shifts in the erythroid: myeloid ratio within the marrow of hematologically stressed animals undoubtedly occurred. Therefore, the absolute numbers of colonies produced in vitro are difficult to relate between the different erythropoietic states, since seeding fixed numbers of nucleated cells into culture does not take such shifts into account. However, the significance of effects of Ep on the pre-CFU-E and CFU-E pools remains unaffected.

Lastly, it is very likely that the "pre-CFU-E" population defined in this study has been (at least in part) previously identified by Gregory and Eaves (65) as "day 3 BFU-E." However, the Ep-responsiveness of the day 3 burst appears to only be modest, while that of pre-CFU-E is absolute. Further studies are required to determine the regulatory steps in erythropoiesis at these stages, and at the day 8 BFU-E level. Because of the high mobility of cells in the pre-CFU-E and/or CFU-E stages in culture (57), the present data cannot specify whether amplification obtained from one pre-CFU-E is changed under hematologic stress. Therefore, our studies cannot rule out the possibility that day 8 BFU-E are involved in the regulatory response to erythropoietin. However, recent studies (2, 74, 79) indicate that they are not involved.

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TABLE I

Number of colonies per clot  $\pm$  SEM

CO <sub>2</sub> Conditions	Ep source	Identified by Benzidine	Identified by Iron-59	Benzidine/Iron-59
4%	<sup>a</sup> Human urine	320 $\pm$ 28	362 $\pm$ 28	0.88
5%	Human urine	278 $\pm$ 11	315 $\pm$ 15	0.88
4%	<sup>b</sup> Sheep plasma	395 $\pm$ 35	490 $\pm$ 39	0.81
4%	Sheep plasma	443 $\pm$ 23	472 $\pm$ 22	0.94
<sup>a</sup> 20 U/mg protein				0.88 $\pm$ 0.03 SEM
<sup>b</sup> 4.8 U/mg protein				p<0.025

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XBL7812-12403

Comparison of benzidine staining and <sup>59</sup>Fe-autoradiography as methods for identifying erythroid colonies in plasma clot cultures. Data from 48 hour cultures with 0.25 U Ep per ml,  $4.5 \times 10^5$  cells/ml. The p-value compares the observed ratio to 1.0.

TABLE II  
EFFECT OF OVERLAYS ON CFU-E GROWTH

First overlay	Time after Ep (hr)	Second overlay	Time after Ep (hr)	Percent of control response
None (control)	—	BSS + <sup>59</sup> Fe	30	—
None	—	BSS + FCS + <sup>59</sup> Fe	30	92 <sup>a</sup>
None	—	<sup>b</sup> Cult. Med. + <sup>59</sup> Fe	30	92 <sup>a</sup>
BSS	0	BSS + <sup>59</sup> Fe	30	87 ± 5

<sup>a</sup> Data was obtained from one experiment.

<sup>b</sup> Culture medium overlay did not include Ep.

BSS = Balanced salt solution, FCS = Fetal calf serum

\*Colonies were identified by Benzidine stain; other data were obtained by autoradiographs

TABLE III					
		Cluster or colony size			
		(Percent of total clusters $\pm$ SEM)			
Cells plated ( $\times 10^{-4}$ )	Colonies per clot	2-4 cells	5-7 cells	8-16 cells	17+ cells
13.5	600	39 $\pm$ 9	12 $\pm$ 3	40 $\pm$ 9	8.6 $\pm$ 3
6.8	301	42 $\pm$ 4	15 $\pm$ 1	38 $\pm$ 3	4.9 $\pm$ 1
3.4	163	35 $\pm$ 4	14 $\pm$ 1	44 $\pm$ 1	6.8 $\pm$ 3
1.7	99	42 $\pm$ 2	11 $\pm$ 0.3	40 $\pm$ 2	6.8 $\pm$ 1
0.84	28	43 $\pm$ 1	12 $\pm$ 0.2	41 $\pm$ 1	3.5 $\pm$ 1
0.21	9	47 $\pm$ 6	11 $\pm$ 0.6	35 $\pm$ 6	7.6 $\pm$ 1
8.8	450	38 $\pm$ 1	17 $\pm$ 1	39 $\pm$ 1	6.0 $\pm$ 1
4.4	230	36 $\pm$ 3	16 $\pm$ 2	42 $\pm$ 2	6.7 $\pm$ 1
1.1	58	44 $\pm$ 5	15 $\pm$ 0.7	36 $\pm$ 3	5.2 $\pm$ 1

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XBL7812-12404

Size of cell inocula has no effect on resulting erythroid cluster sizes. 0.25 U Ep per ml, 48 hour cultures of normal marrow. If available, 200 clusters per clot were examined.

TABLE IV

Ep source	Method of colony identification	No. colonies with 5% CO <sub>2</sub> ÷ No. colonies with 4% CO <sub>2</sub>
<sup>a</sup> Human urine	Benzidine	0.87
Human urine	Iron-59	0.87
<sup>b</sup> Sheep plasma	Benzidine	0.84
Sheep plasma	Iron-59	0.71
Human urine	Benzidine	0.75
Human urine	Iron-59	0.80
Human urine	Iron-59	0.61
<sup>a</sup> 20 U/mg protein		0.78 ± 0.04 SEM
<sup>b</sup> 4.8 U/mg protein		p < 0.005

XBL7812-12401

Comparison of erythroid colony numbers obtained under 4% and 5% CO<sub>2</sub> culture conditions. The p-value compares the observed ratio to 1.0.

Table V  
EFFECT OF NORMAL URINE PROTEINS  
AND DESIALATED Ep ON CFU-E NUMBERS

Culture conditions	No. of CFU-E ( $\pm$ SEM)	Percent of control response
Low specific activity Ep <sup>a</sup> (control)	517 $\pm$ 60	—
Low specific activity Ep plus urine protein	420 $\pm$ 12	81
High specific activity Ep <sup>b</sup>	400 $\pm$ 10	77
High specific activity Ep plus urine protein	310 $\pm$ 30	60
Urine protein only	10 $\pm$ 10	2
No Ep, no urine protein	2 $\pm$ 2	0.4
Desialated low specific activity Ep <sup>c</sup>	—	99

<sup>a</sup> 15-20 U/mg protein, at a final concentration of 0.25 U/ml culture

<sup>b</sup> 150 U/mg protein, at a final concentration of 0.25 U/ml culture

<sup>c</sup> Data obtained from a separate experiment using a dilution  
of low specific Ep equivalent to 0.25 U/ml culture.  
Desialated Ep colony yield = 317 $\pm$ 20, control yield = 320 $\pm$ 20.

XBL792-3201

Table VI:  $^{59}\text{Fe}$ -labelled erythroid clusters from normal marrow grouped according to size. The culture conditions give the times for which Ep was actively present. For longer Ep exposures, a larger percentage of clusters reached 17 cells or larger, with little change in the other categories. Data were obtained from 3-4 clots per point  $\pm$  SEM.

Table VI  
NORMAL MICE

Size classification of  $^{59}\text{Fe}$ -labelled  
Erythroid colonies and clusters  
(With single labelled cells)

Culture conditions	Trial No.	No. cells per cluster or colony (% of total $\pm$ SEM)				Single labelled cells (per clot)
		2-4	5-7	8-16	17+	
Control, Ep only*	1	36 $\pm$ 4	17 $\pm$ 1	37 $\pm$ 3	10 $\pm$ 2	630 $\pm$ 95
	2	30 $\pm$ 10	19 $\pm$ 4	36 $\pm$ 1	13 $\pm$ 3	290 $\pm$ 130
	3	36 $\pm$ 3	14 $\pm$ 3	43 $\pm$ 1	7 $\pm$ 0.9	169 $\pm$ 12
	4	38 $\pm$ 3	12 $\pm$ 4	40 $\pm$ 2	10 $\pm$ 2	50 $\pm$ 10
Control, No Ep nor anti-Ep	1	49 $\pm$ 16	24 $\pm$ 1	25 $\pm$ 7	2 $\pm$ 2	200 $\pm$ 10
	2	32 $\pm$ 5	21 $\pm$ 3	42 $\pm$ 6	5 $\pm$ 2	104 $\pm$ 12
	3	51 $\pm$ 4	17 $\pm$ 1	30 $\pm$ 4	3 $\pm$ 0.4	190 $\pm$ 21
	4	33 $\pm$ 3	36 $\pm$ 6	29 $\pm$ 1	2 $\pm$ 1	42 $\pm$ 14
Ep + Anti-Ep (t="0")	1	78 $\pm$ 10	12 $\pm$ 8	9 $\pm$ 2	1 $\pm$ 1	490 $\pm$ 55
	2	39 $\pm$ 2	22 $\pm$ 2	37 $\pm$ 4	2 $\pm$ 2	190 $\pm$ 31
	3	48 $\pm$ 5	15 $\pm$ 1	37 $\pm$ 1	1 $\pm$ 0.6	360 $\pm$ 120
	4	39 $\pm$ 3	24 $\pm$ 2	36 $\pm$ 4	1 $\pm$ 1	350 $\pm$ 100
(t=.3h)	2	35 $\pm$ 5	19 $\pm$ 2	39 $\pm$ 1	7 $\pm$ 2	380 $\pm$ 25
	3	44 $\pm$ 4	17 $\pm$ 1	38 $\pm$ 4	2 $\pm$ 1	220 $\pm$ 38
	4	50 $\pm$ 6	12 $\pm$ 5	37 $\pm$ 2	1 $\pm$ 1	89 $\pm$ 40
(t=1h)	2	42 $\pm$ 3	22 $\pm$ 2	32 $\pm$ 2	4 $\pm$ 0.7	330 $\pm$ 80
	3	49 $\pm$ 2	15 $\pm$ 1	33 $\pm$ 2	3 $\pm$ 0.4	238 $\pm$ 60
(t=2h)	1	49 $\pm$ 14	20 $\pm$ 4	29 $\pm$ 10	2 $\pm$ 0.6	350 $\pm$ 27
	2	41 $\pm$ 8	24 $\pm$ 3	32 $\pm$ 5	3 $\pm$ 1	230 $\pm$ 36
	3	49 $\pm$ 4	10 $\pm$ 1	39 $\pm$ 3	2 $\pm$ 0.4	200 $\pm$ 55
	4	65 $\pm$ 8	4 $\pm$ 3	30 $\pm$ 1	21 $\pm$ 1	52 $\pm$ 9
(t=6h)	3	41 $\pm$ 2	14 $\pm$ 1	41 $\pm$ 2	4 $\pm$ 0.6	102 $\pm$ 19
	4	62 $\pm$ 8	16 $\pm$ 2	20 $\pm$ 2	2 $\pm$ 1	57 $\pm$ 12
(t=12h)	1	33 $\pm$ 3	28 $\pm$ 2	36 $\pm$ 3	3 $\pm$ 0.5	280 $\pm$ 25
	2	43 $\pm$ 2	20 $\pm$ 3	30 $\pm$ 2	7 $\pm$ 3	360 $\pm$ 55
	3	44 $\pm$ 1	15 $\pm$ 1	38 $\pm$ 1	3 $\pm$ 0.4	182 $\pm$ 47
	4	47 $\pm$ 1	16 $\pm$ 2	35 $\pm$ 3	2 $\pm$ 0.4	46 $\pm$ 10
(t=24h)	1	38 $\pm$ 4	21 $\pm$ 2	35 $\pm$ 2	6 $\pm$ 1	290 $\pm$ 43
	2	36 $\pm$ 5	24 $\pm$ 3	31 $\pm$ 2	9 $\pm$ 1	360 $\pm$ 29
	3	40 $\pm$ 3	13 $\pm$ 3	41 $\pm$ 1	7 $\pm$ 0.4	273 $\pm$ 90
	4	34 $\pm$ 3	14 $\pm$ 1	46 $\pm$ 4	6 $\pm$ 1	119 $\pm$ 14
(t=30h)	3	40 $\pm$ 2	13 $\pm$ 0.2	41 $\pm$ 2	7 $\pm$ 0.9	270 $\pm$ 24
	4	30 $\pm$ 1	14 $\pm$ 1	45 $\pm$ 2	11 $\pm$ 1	118 $\pm$ 21

\*Ep level = 0.25 U/ml

Table VII

NORMAL MICE

Changes in Erythroid cluster size  
after 48 hour culture with or without Ep

Anti-Ep added at t="0"?		Percent labelled clusters with >16 cells ( $\pm$ SD)				
		<u>Trial 1</u>	<u>Trial 2</u>	<u>Trial 3</u>	<u>Trial 4</u>	<u>Average</u>
No Ep	Yes	3.0 $\pm$ 2.6	1.8 $\pm$ 1.7	1.4 $\pm$ 1.4	0	1.6 $\pm$ 1.2
	No	1.5* $\pm$ 2.6	5.1 $\pm$ 3.4	2.5 $\pm$ 0.7	2.0 $\pm$ 2.0	2.8 $\pm$ 1.6
With Ep	Yes	1.0 $\pm$ 1.8	2.1 $\pm$ 2.1	1.1 $\pm$ 1.0	2.0 $\pm$ 2.0	1.6 $\pm$ 0.6
	No	10.* $\pm$ 3.6	13 $\pm$ 5.3	7.3 $\pm$ 1.6	10 $\pm$ 3.1	10 $\pm$ 2.3

p < .001\*\*

\*The same value was obtained when normal rabbit serum was added at t= 0

\*\*p-value obtained by one-tailed t-test

XBL792-3093

Effect of 48 hour Ep exposure on the frequency of large erythroid colonies (greater than 16 cells). With Ep allowed to remain active the entire culture period, a significantly higher percentage of clusters are large (greater than 16 cells). Data are means of 3-4 clots  $\pm$  SD.

Table VIII

NORMAL MICE

Changes in Myeloid proliferation  
after 48 hour culture with or without Ep (20 U/mg)

Anti-Ep added at "t=0"?		Number of Myeloid clusters per clot with >4 cells ( $\pm$ SD)				
		Trial 1	Trial 2	Trial 3	Trial 4	Average
Ep {	Yes	24 $\pm$ 10	26 $\pm$ 2	36 $\pm$ 5	46 $\pm$ 2	33 $\pm$ 10
	No	22 $\pm$ 8*	40 $\pm$ 7	55 $\pm$ 7	60 $\pm$ 11	44 $\pm$ 17
Ep {	Yes	22 $\pm$ 10	32 $\pm$ 7	48 $\pm$ 4	90 $\pm$ 10	48 $\pm$ 30
	No	56 $\pm$ 11*	70 $\pm$ 9	73 $\pm$ 12	98 $\pm$ 10	74 $\pm$ 17

p<0.05\*\*

\*The same value was obtained when normal rabbit serum was added at t=0.

\*\*p-value obtained by one-tailed t-test.

XBL794-3383

Effect of Ep exposure on myeloid proliferation. The number of granulocytic clusters arising from presumed CFU-C precursors increases significantly when Ep is active for the entire culture period. Data are means of 3-4 clots  $\pm$  SD.

Table IX  
NORMAL MICE  
 Data for calculation of number  
 of large colonies per clot  
 (Avg. of 2-4 experiments)

Culture conditions	Avg. No. colonies per clot ( $\pm$ SEM)	Avg. No. cells/cluster or colony (% of total $\pm$ SEM)				Avg. total labelled clusters	Avg. No. colonies with 16 cells
		2-4	5-7	8-16	17+		
Control, Ep only	405 $\pm$ 40	35 $\pm$ 2	16 $\pm$ 2	39 $\pm$ 2	10 $\pm$ 1	821	82
Control, No Ep No anti-Ep	26 $\pm$ 8	41 $\pm$ 5	24 $\pm$ 4	32 $\pm$ 4	3.0 $\pm$ 0.7	76	2
Anti-Ep only	18 $\pm$ 7	59 $\pm$ 13	15 $\pm$ 5	24 $\pm$ 9	1.6 $\pm$ 0.6	70	1
Ep + ant-Ep (t=0)	15 $\pm$ 7	51 $\pm$ 9	18 $\pm$ 3	30 $\pm$ 7	1.3 $\pm$ 0.3	48	1
(t=0.3h)	33 $\pm$ 13	43 $\pm$ 4	16 $\pm$ 2	38 $\pm$ 0.6	3.3 $\pm$ 2	80	3
(t=1h)	70 $\pm$ 2*	45 $\pm$ 4	19 $\pm$ 3	33 $\pm$ 0.5	3.5 $\pm$ 0.5	192	7
(t=2h)	47 $\pm$ 13	50 $\pm$ 5	15 $\pm$ 5	33 $\pm$ 2	2.0 $\pm$ 0.4	134	3
(t=6h)	34 $\pm$ 13*	51 $\pm$ 10	15 $\pm$ 0.5	31 $\pm$ 10	3.0 $\pm$ 1	103	3
(t=12h)	107 $\pm$ 15	42 $\pm$ 3	20 $\pm$ 3	35 $\pm$ 2	3.8 $\pm$ 1	276	10
(t=24h)	235 $\pm$ 27	37 $\pm$ 1	18 $\pm$ 3	38 $\pm$ 3	7.0 $\pm$ 0.7	522	37
(t=30h)	375 $\pm$ 100*	35 $\pm$ 5	14 $\pm$ 0.5	42 $\pm$ 2	9.0 $\pm$ 1	735	66

\*Data taken from 2 experiments

XBL 792-3202

<sup>59</sup>Fe-labelled erythroid clusters grouped according to size. The total number of colonies of greater than 16 cells is calculated as a function of Ep exposure time, and is shown to increase if Ep is active for longer times.

Table X

PLETHORIC MICE

Size classification of  $^{59}\text{Fe}$ -labelled  
Erythroid colonies and clusters  
(with single labelled cells)

Culture conditions	Trial No.	No. cells/cluster or colony (% of total $\pm$ SEM)				Single labelled cells (per clot)
		2-4	5-7	8-16	17+	
Control, Ep only	1	42 $\pm$ 3	11 $\pm$ 0.7	39 $\pm$ 3	8.4 $\pm$ .9	1200
	2	40 $\pm$ 4	12 $\pm$ 1	37 $\pm$ 1	11 $\pm$ 1	1500
Control, No Ep	1	47 $\pm$ 1	15 $\pm$ .5	37 $\pm$ 1	0.6 $\pm$ .6	1100
No anti-Ep	2	35 $\pm$ 4	22 $\pm$ 2	37 $\pm$ 1	1.6 $\pm$ 1.6	1200
Ep + anti-Ep (t=0)	1	62 $\pm$ 4	12 $\pm$ .4	28 $\pm$ 4	0.3 $\pm$ .3	900
	2	54 $\pm$ 4	23 $\pm$ 3	23 $\pm$ 3	0	800
(t=.3h)	1	74 $\pm$ 2	10 $\pm$ 2	16 $\pm$ 3	0.5 $\pm$ .5	800
	2	59 $\pm$ 2	16 $\pm$ 2	24 $\pm$ 4	0.7 $\pm$ .7	1000
(t=1h)	1	70 $\pm$ 9	8 $\pm$ .6	25 $\pm$ 8	0	1300
(t=2h)	1	68 $\pm$ 4	16 $\pm$ 2	17 $\pm$ 4	0	1200
	2	60 $\pm$ 2	19 $\pm$ 0.5	21 $\pm$ 4	0.3 $\pm$ 0.3	300
(t=6h)	1	74 $\pm$ 3	8 $\pm$ 4	18 $\pm$ 2	.4 $\pm$ .4	1000
	2	54 $\pm$ 3	18 $\pm$ 4	27 $\pm$ 3	1.5 $\pm$ 1	900
(t=12h)	1	66 $\pm$ 2	11 $\pm$ 1	23 $\pm$ 2	0.9 $\pm$ .2	1500
	2	60 $\pm$ 3	15 $\pm$ 5	25 $\pm$ 2	1.2 $\pm$ 1	1000
(t=24h)	1	48 $\pm$ 2	13 $\pm$ .5	36 $\pm$ 1	2.6 $\pm$ .6	1700
	2	33 $\pm$ 5	14 $\pm$ 1	45 $\pm$ 4	7.6 $\pm$ 1	1100
(t=30h)	2	31 $\pm$ 4	12 $\pm$ 1	47 $\pm$ 2	10 $\pm$ 1	1300

XBL792-3106

$^{59}\text{Fe}$ -labelled erythroid clusters grouped according to size. Marrow from plethoric mice. The culture conditions give the times for which Ep was actively present. For longer Ep exposures, a larger percentage of clusters reached 17 cells or larger, with some decrease in 2-4 cell clusters. Data were obtained from three clots per point (mean  $\pm$  SEM).

Table XI

PLETHORIC MICE

Changes in Erythroid cluster size  
after 48 hour culture with or  
without Ep

Anti-Ep added at t="O"?		Percent labelled clusters with >16 cells ( $\pm$ SD)		
		Trial 1	Trial 2	Average
No Ep	Yes	0	$2 \pm 2$	$1 \pm 1$
	No	$0.6 \pm 1$	$5 \pm 2$	$2.8 \pm 2$
With Ep	Yes	$0.3 \pm 0.5$	0	$0.2 \pm 0.1$
	No	$8.4 \pm 1.6$	$11 \pm 1$	$9.7 \pm 2$

$p < 0.01^*$

\*p-value obtained by one-tailed t-test

XBL792-3091

Effect of 48 hour Ep exposure on the frequency of large erythroid colonies (greater than 16 cells).  
For further explanation see Table VII. Data are means of three clots  $\pm$  SD.

Table XII

BLED MICE

Size classification of  $^{59}\text{Fe}$ -labelled  
Erythroid colonies and clusters  
(with single labelled cells)

Culture conditions	Trial No.	No. cells/cluster or colony (% of total $\pm$ SEM)				Single labelled cells (per clot)
		2-4	5-7	8-16	17+	
Control, Ep only	1	40 $\pm$ 2	13 $\pm$ 2	40 $\pm$ 1	7.4 $\pm$ 1.3	55 $\pm$ 10
	2	33 $\pm$ 6	12 $\pm$ 3	43 $\pm$ 8	12 $\pm$ 1.5	54 $\pm$ 11
Control, No Ep, No anti-Ep	1	48 $\pm$ 2	14 $\pm$ 3	36 $\pm$ 3	2.0 $\pm$ 0.6	
	2	37 $\pm$ 14	26 $\pm$ 4	37 $\pm$ 10	0	12 $\pm$ 4
Ep + Anti-Ep(t=0)	1	100	0	0	0	
	2	80 $\pm$ 9	16 $\pm$ 6	4 $\pm$ 3	0	290 $\pm$ 10
(t=0.3h)	1	100	0	0	0	76 $\pm$ 9
	2	64 $\pm$ 8	15 $\pm$ 8	21 $\pm$ 4	0	36 $\pm$ 2
(t=1h)	1	92 $\pm$ 8	8 $\pm$ 8	0	0	58 $\pm$ 10
(t=2h)	1	90 $\pm$ 10	10 $\pm$ 10	0	0	77 $\pm$ 9
	2	65 $\pm$ 5	24 $\pm$ 6	11 $\pm$ 2	0	22 $\pm$ 10
(t=6h)	1	82 $\pm$ 2	12 $\pm$ 6	7 $\pm$ 7	0	51 $\pm$ 13
	2	59 $\pm$ 3	17 $\pm$ 3	25 $\pm$ 7	0	25 $\pm$ 8
(t=12h)	1	65 $\pm$ 0.9	20 $\pm$ 3	15 $\pm$ 3	0	47 $\pm$ 1
	2	46 $\pm$ 4	22 $\pm$ 3	32 $\pm$ 1	0	32 $\pm$ 9
(t=24h)	1	48 $\pm$ 2	15 $\pm$ 3	34 $\pm$ 2	3.0 $\pm$ 0.6	65 $\pm$ 2
	2	32 $\pm$ 4	18 $\pm$ 2	45 $\pm$ 2	4.6 $\pm$ 0.2	20 $\pm$ 4
(t=30h)	1	44 $\pm$ 4	12 $\pm$ 2	38 $\pm$ 3	5.7 $\pm$ 1.4	83 $\pm$ 8
	2	32 $\pm$ 4	14 $\pm$ 1	45 $\pm$ 2	9.0 $\pm$ 1.2	42 $\pm$ 9

XBL792- 3105

$^{59}\text{Fe}$ -labelled erythroid clusters grouped according to size. Marrow from bled mice. The culture conditions give the times for which Ep was actively present. For longer Ep exposures, a larger percentage of clusters reached 17 cells or larger, but the shift is delayed. Decreases in 2-4 cell clusters are marked as Ep exposure is increased. Data were obtained from three clots per point (mean  $\pm$  SEM).

Table XIII

**BLED MICE**

Changes in Erythroid cluster size  
after 48 hour culture with or  
without Ep

		Percent labelled clusters with >16 cells ( $\pm$ SD)		
		Trial 1 <sup>a</sup>	Trial 2 <sup>b</sup>	Average
No Ep	Yes	0	0	0
	No	0	0	0
With Ep	Yes	0	0	0
	No	7.4 $\pm$ 1.3	12 $\pm$ 2	9.7 $\pm$ 2

p<0.005\*

<sup>a</sup>Mice were bled once

<sup>b</sup>Mice were bled twice

\*p-value obtained by one-tailed t-test

XBL792-3090

Effect of 48 hour Ep exposure on the frequency of large erythroid colonies (greater than 16 cells). For further explanation see Table VII. Data are means of three clots  $\pm$  SD.

Table XIV: Comparison of erythroid colony response for three different erythropoietic states. Compared to normal marrow, the proportion of colonies produced by the fast-responding CFU-E population decreases dramatically in marrow cultured from both plethoric and bled mice. Bled mice have significantly lower proportion in this pool than do plethoric mice. No change from normal was observed in the hours of Ep exposure required by plethoric marrow to double the number of colonies initiated between Ep exposures of 6 and 24 hours. However, bled marrow only required Ep exposures of half that of normal marrow, resulting in stepped-up colony production. Data for normal marrow was obtained from average of four experiments.

Table XIV

COMPARISON OF ERYTHROPOIETIC  
STATES IN VITRO

<u>Erythropoietic state of mice</u>	<u>Fast-Responding CFU-E population (% of total colonies)</u>	<u>Hours needed with Ep to double colony yield</u>
Normal	13 ± 4	6
Plethoric	* 2.8 ± 0.2	** 6 ± 1
Bled	* 0.2 ± 0.2	** 3 ± 0.5

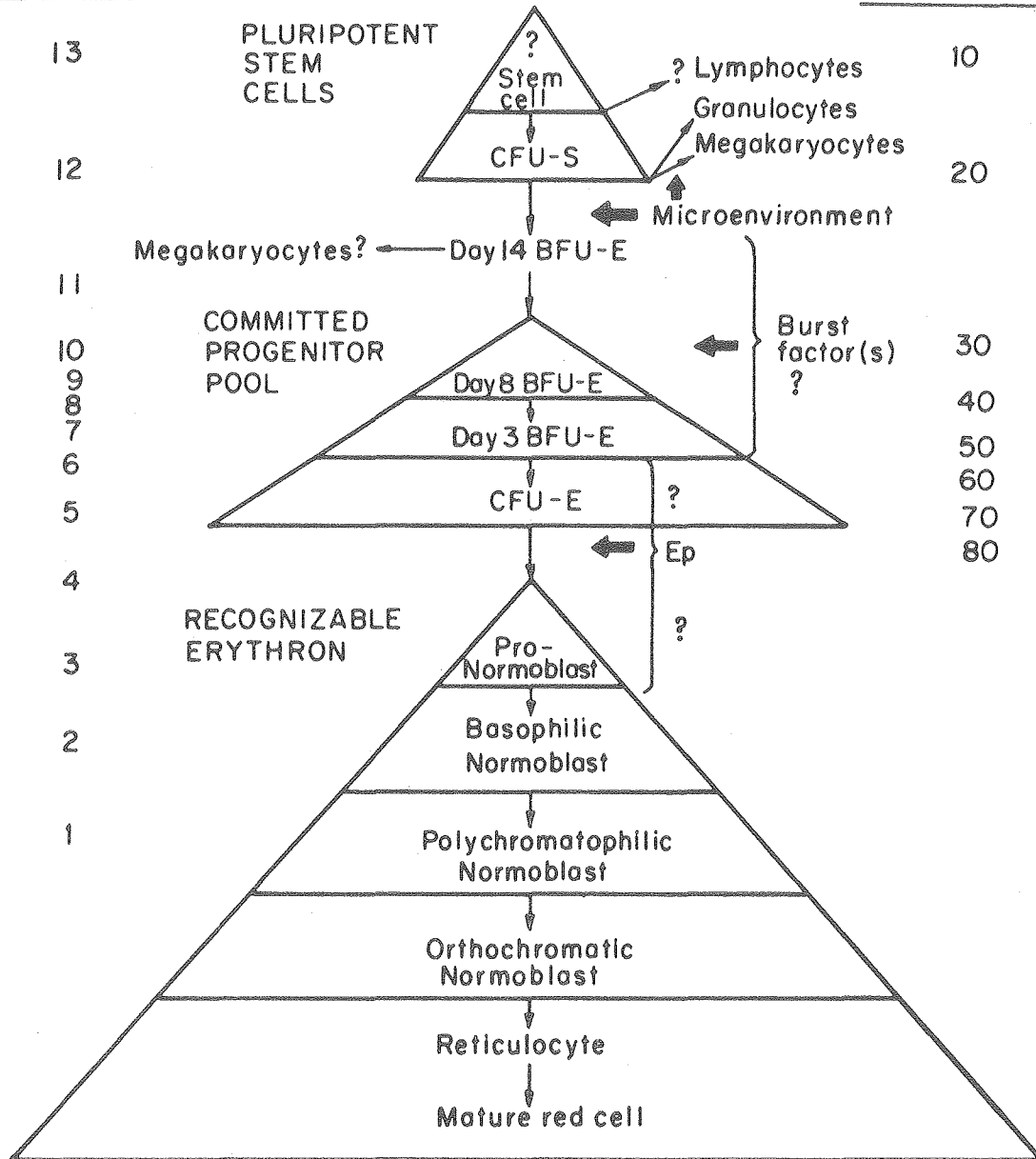
\*Difference is significant at  $p < 0.001$  level

\*\*Difference is significant at  $p < 0.05$  level

XBL792-3092

Approximate  
number of  
cell divisions  
Remaining

Approximate  
percent of cells  
in S Phase  
(%  $^3\text{HTdR}$  kill)

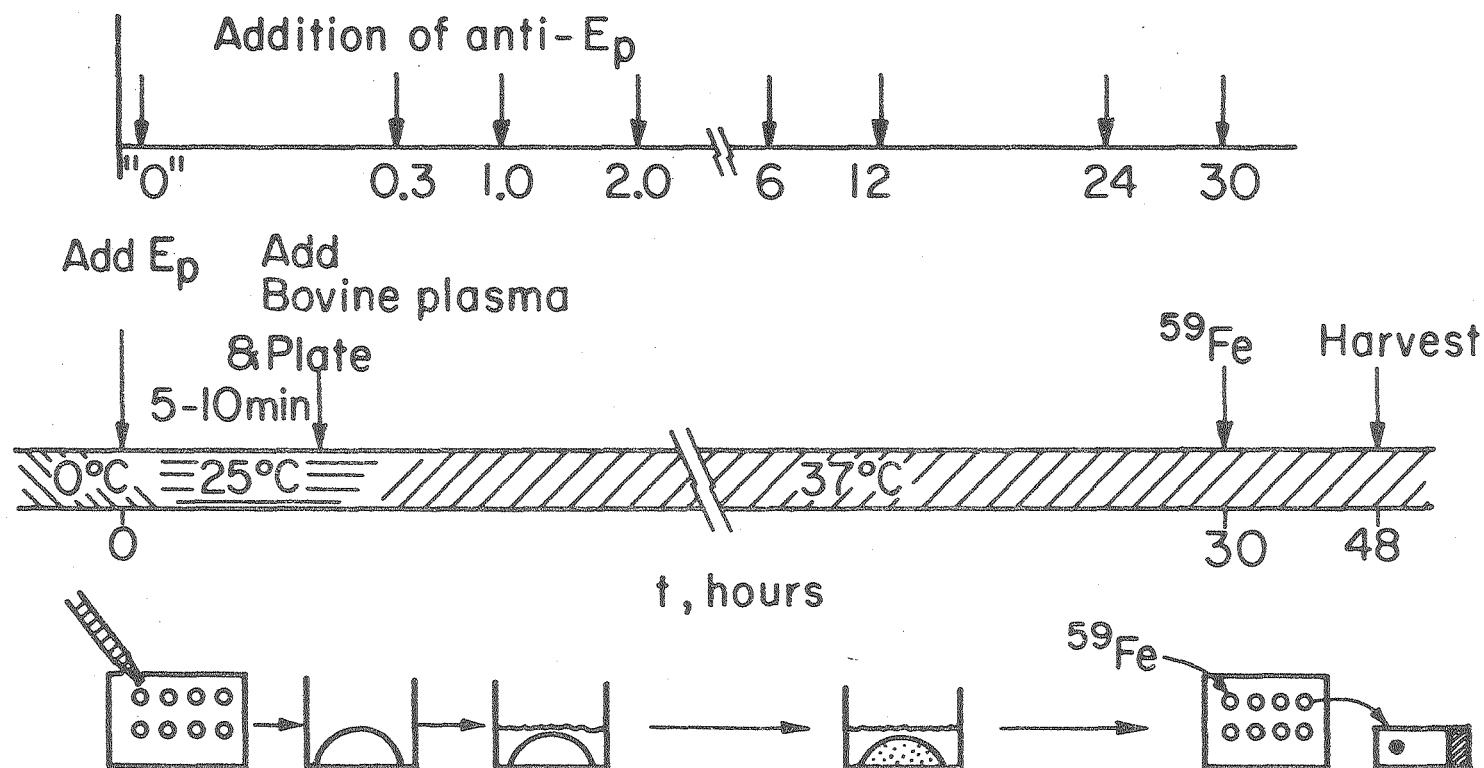


## STAGES IN ERYTHROID DIFFERENTIATION

XBL791-3038

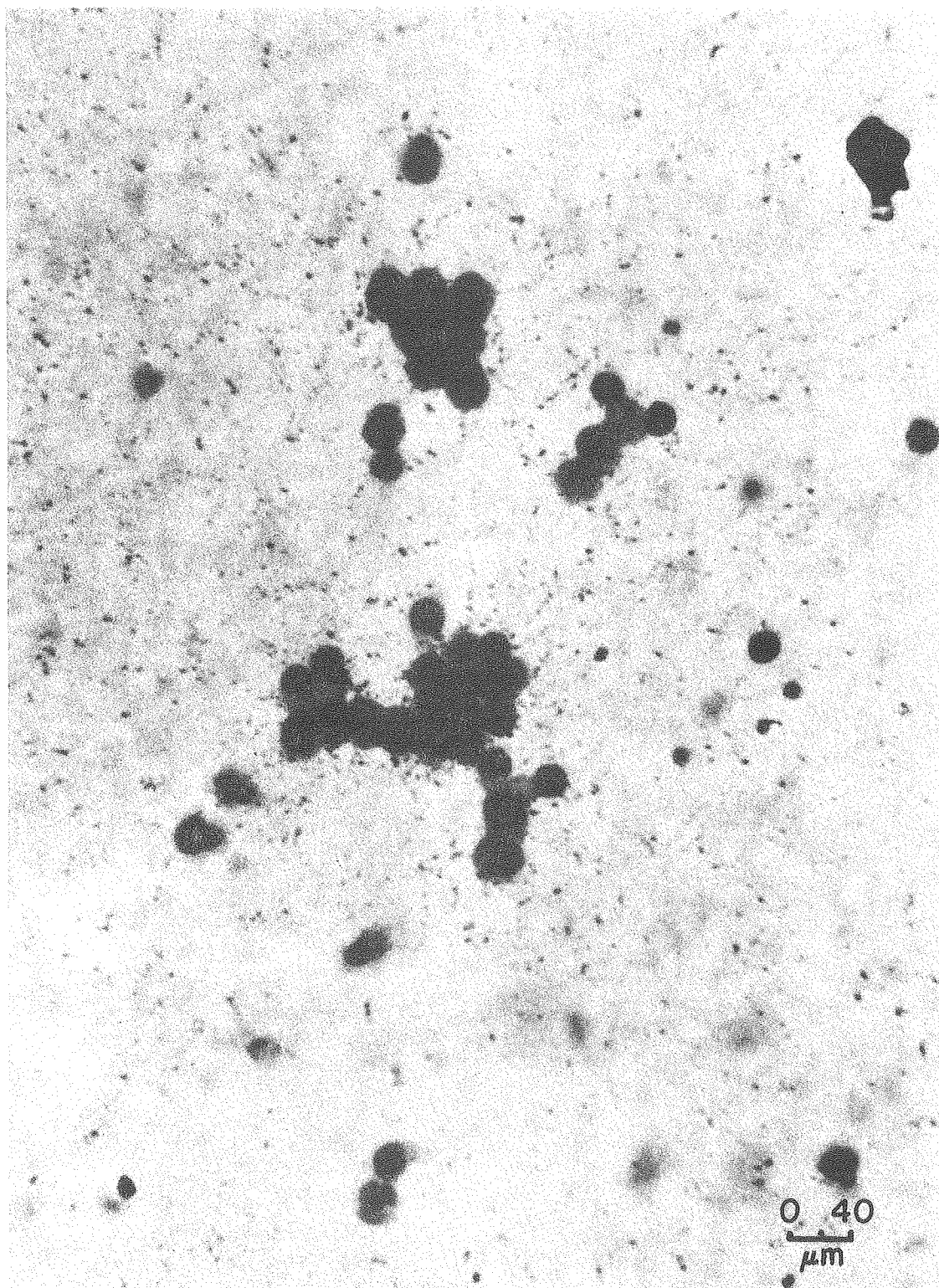
Fig. 1: Summary of present knowledge of the structure, kinetics, and regulation of differentiation leading to the mature red cell. Maturation involves a gradual decrease in proliferative potential (left of figure), and an increase in the number of cells present in S phase. (CFU-S = spleen colony forming unit; BFU-E = erythrocytic burst-forming unit; CFU-E = erythrocytic colony-forming unit; Ep = erythropoietin;  $^3\text{HTdR}$  = tritiated thymidine).

## EXPERIMENTAL DESIGN



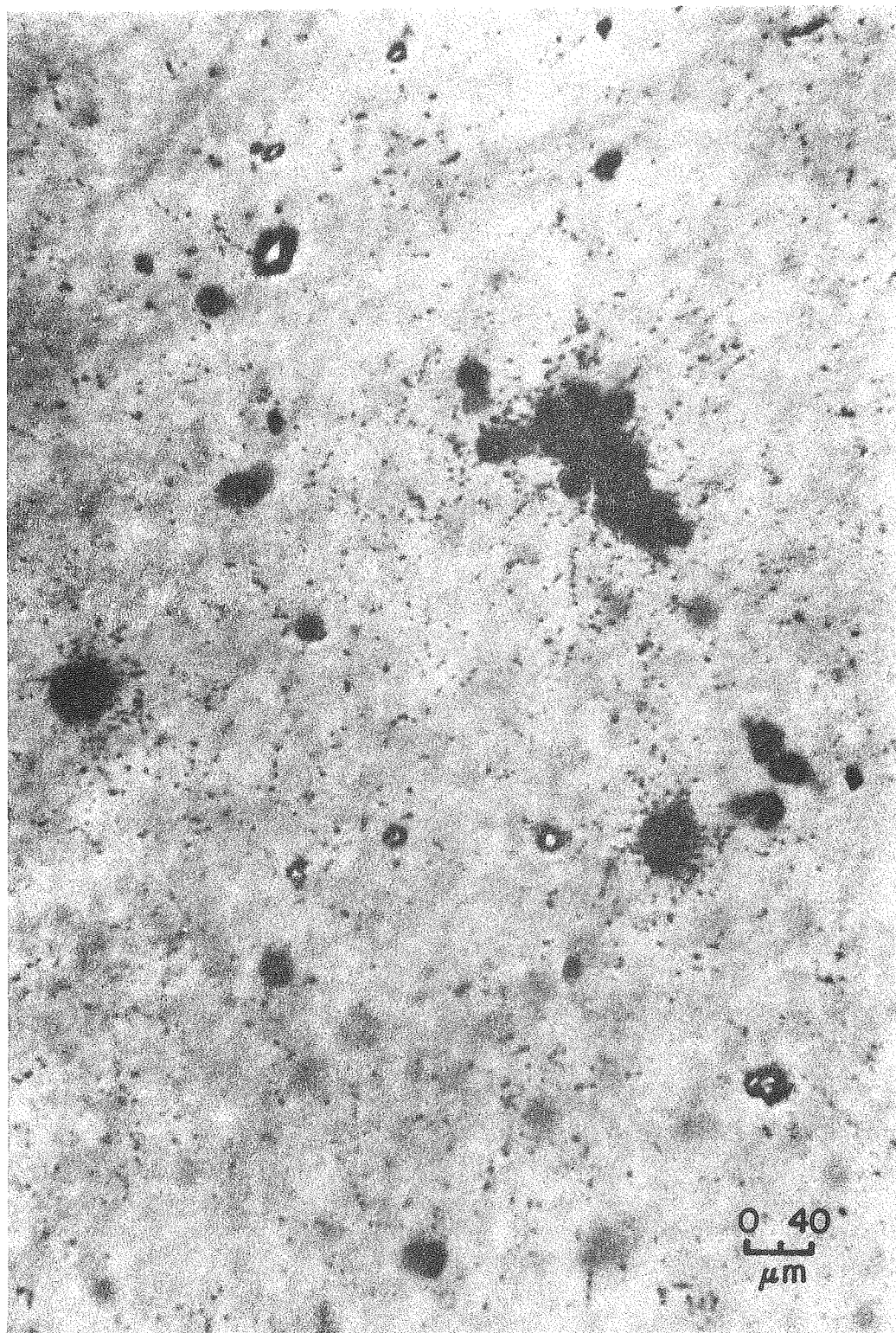
XBL7810-3595

Fig. 2: The basic design of the experiment showing times of addition of  $E_p$ , anti- $E_p$ , and  $^{59}\text{Fe}$ . The temperature of the cellular environment at each stage is included.



XBB-770-13015

Fig. 3:  $^{59}\text{Fe}$ -autoradiograph of erythroid colonies obtained from normal mouse marrow after 48 hours culture in plasma clot containing 0.25 U Ep per ml. Initial inocula  $6.8 \times 10^4$  cells per 0.15 ml clot.  $^{59}\text{Fe}$ -labelled cells are clearly above background, even in the small 4-cell erythroid cluster. Non-erythroid cells are not labelled. Giemsa stain, original magnification 400x.



XBB-770-13019

Fig. 4:  $^{59}\text{Fe}$ -autoradiograph of a colony which is slightly more mature than that of Figure 3. Culture conditions were identical to those in Figure 3. Giemsa stain, original magnification 400x.

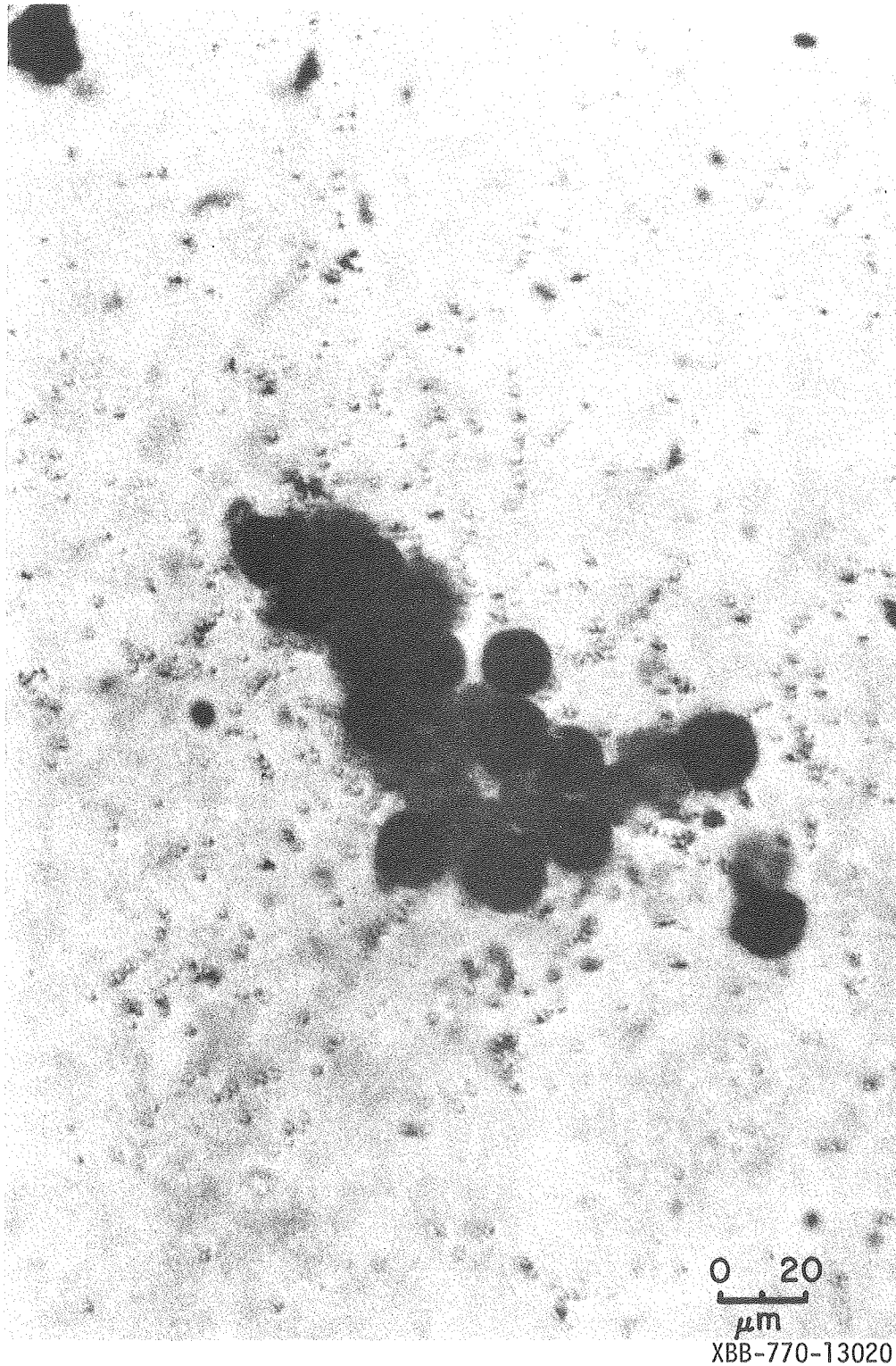
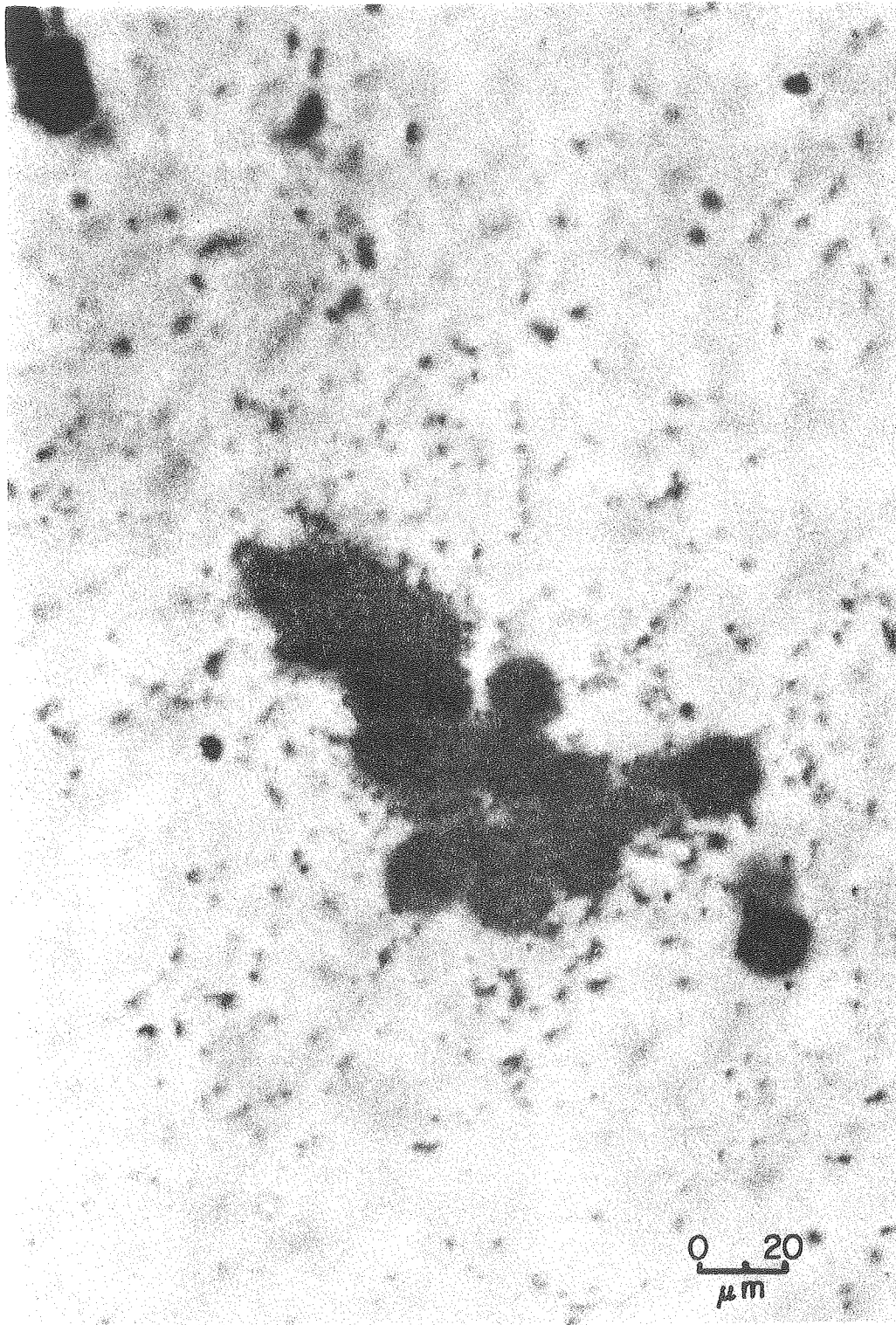
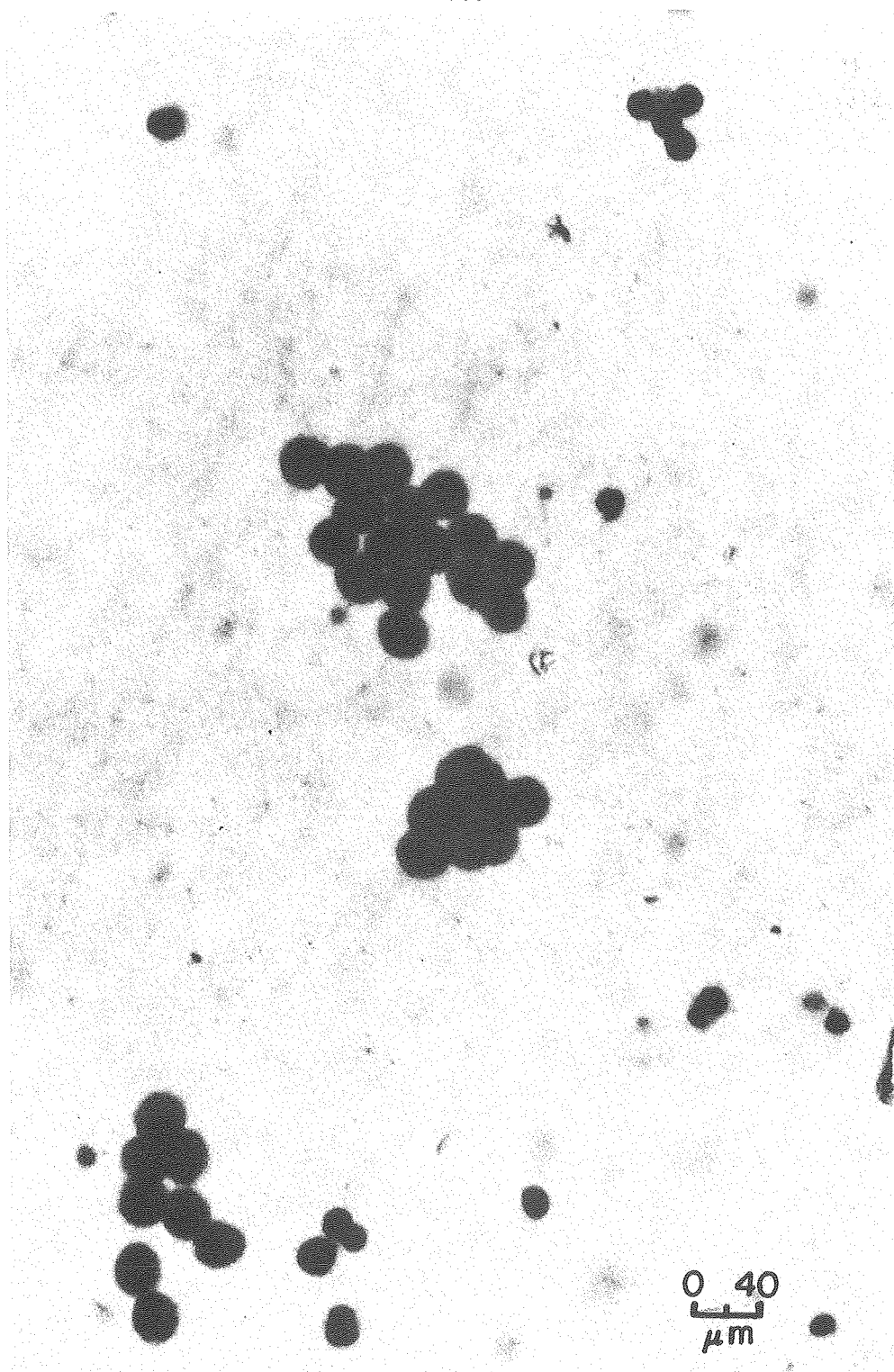


Fig. 5: Higher magnification photograph of the erythroid colony seen in Figure 4. Focus is on the cells. The variation in maturity among the cells is minimal. Giemsa stain, original magnification 1000x.



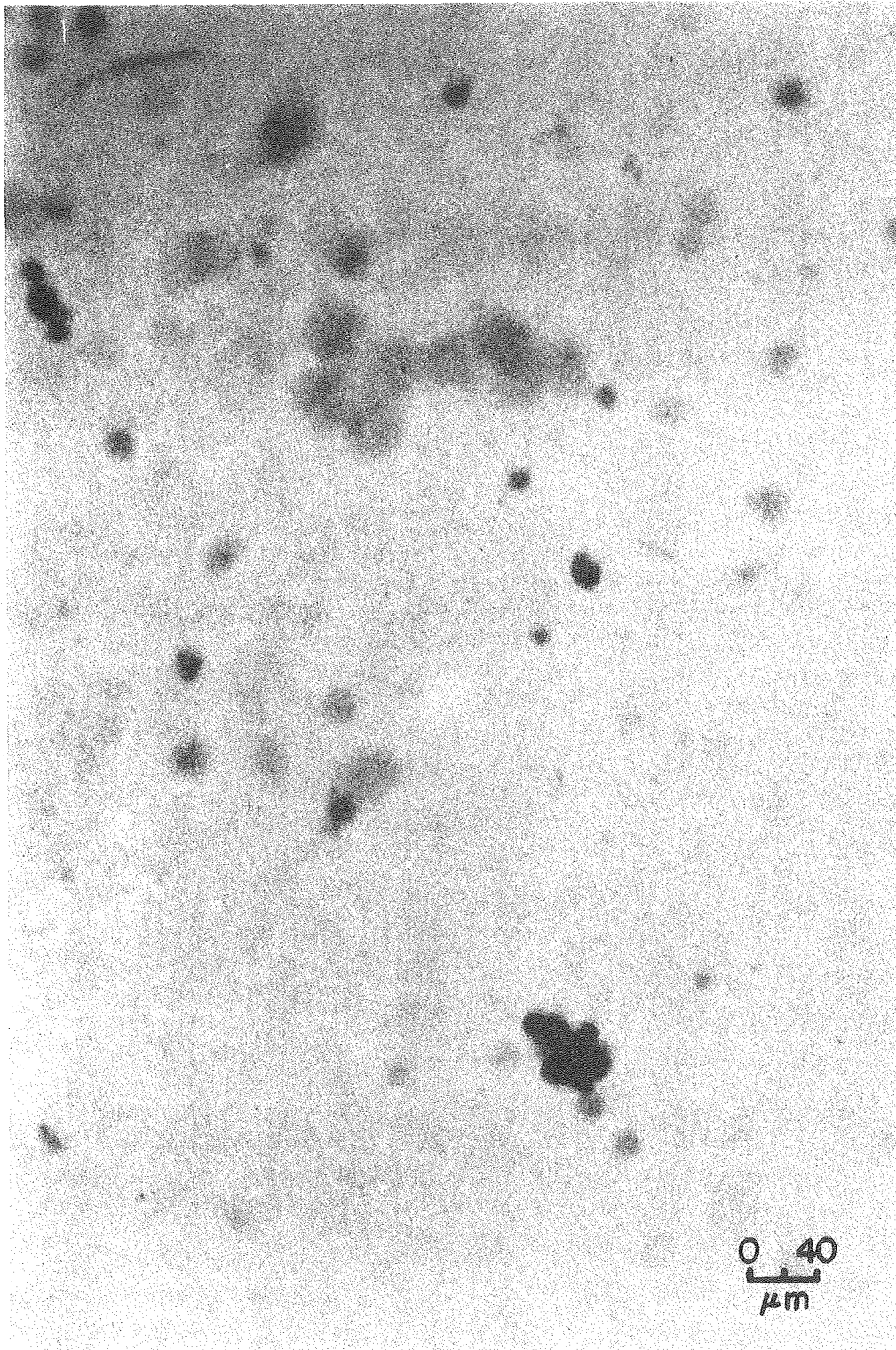
XBB-770-13021

Fig. 6: Same colony as Figure 5, but with focus on grains of the emulsion. The grains cover the colony uniformly. Giemsa stain, original magnification 1000x.



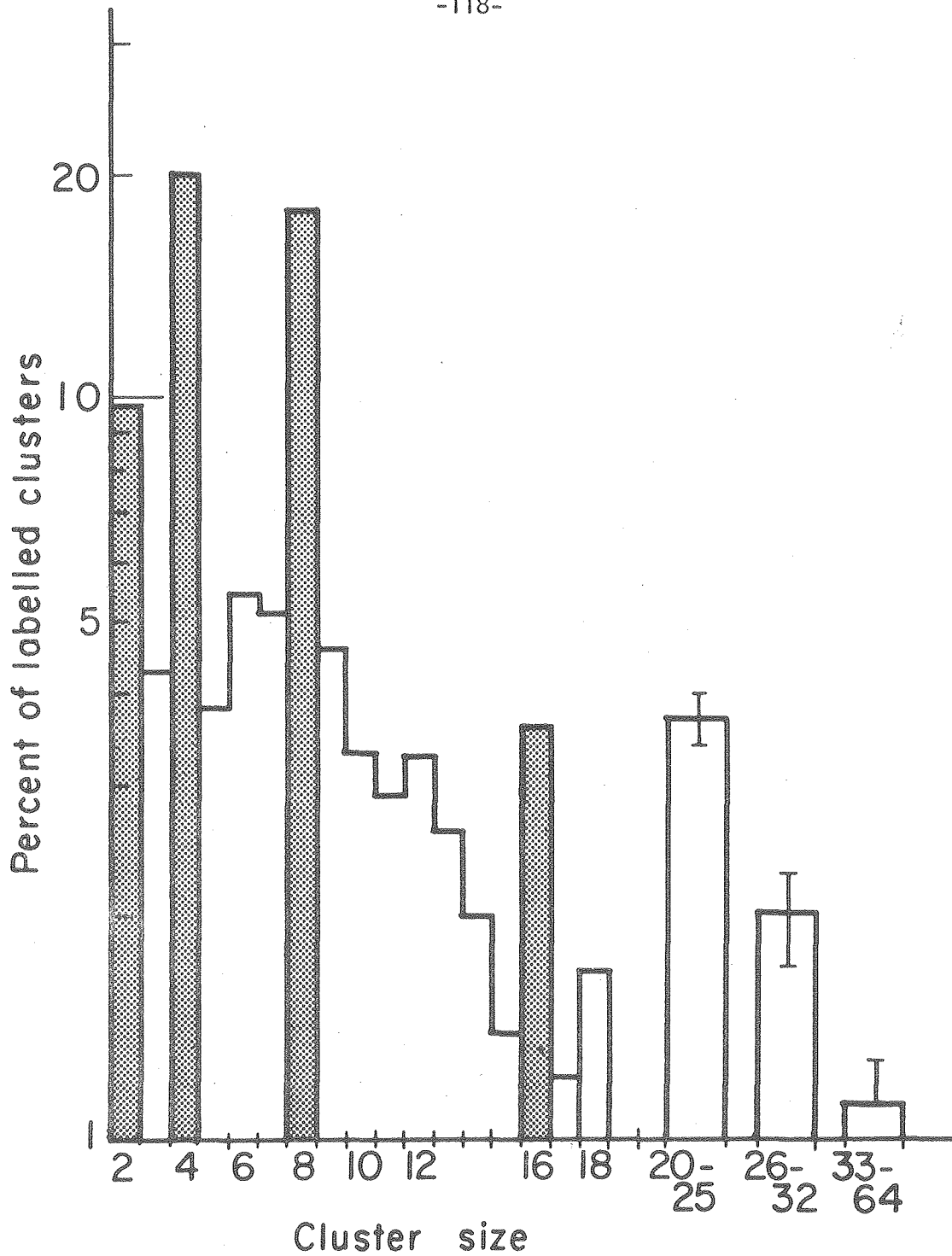
XBB-770-13017

Fig. 7:  $^{59}\text{Fe}$ -autoradiograph of clusters containing polymorphonuclear cells. Morphology and low  $^{59}\text{Fe}$  labelling identifies them as granulocytic clusters. Giemsa stain, original magnification 400x.



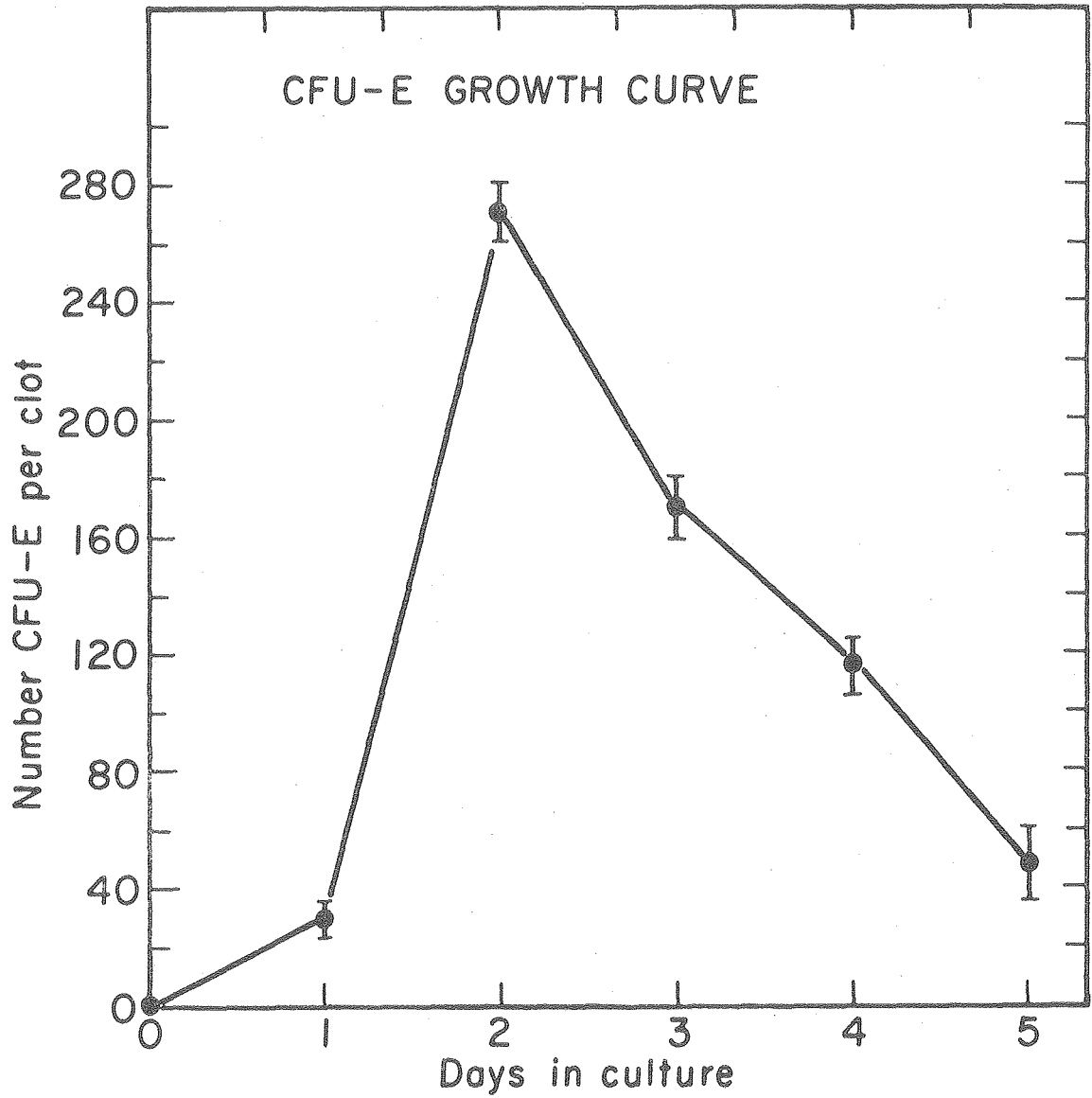
XBB-770-13025

Fig. 8: Typical erythroid (dark stain) and myeloid (light stain) clusters from normal marrow which was cultured 48 hours with 0.25 U Ep per ml.  $^{59}\text{Fe}$  was not added, but benzidine-hematoxylin stain was used. Original magnification 400x.



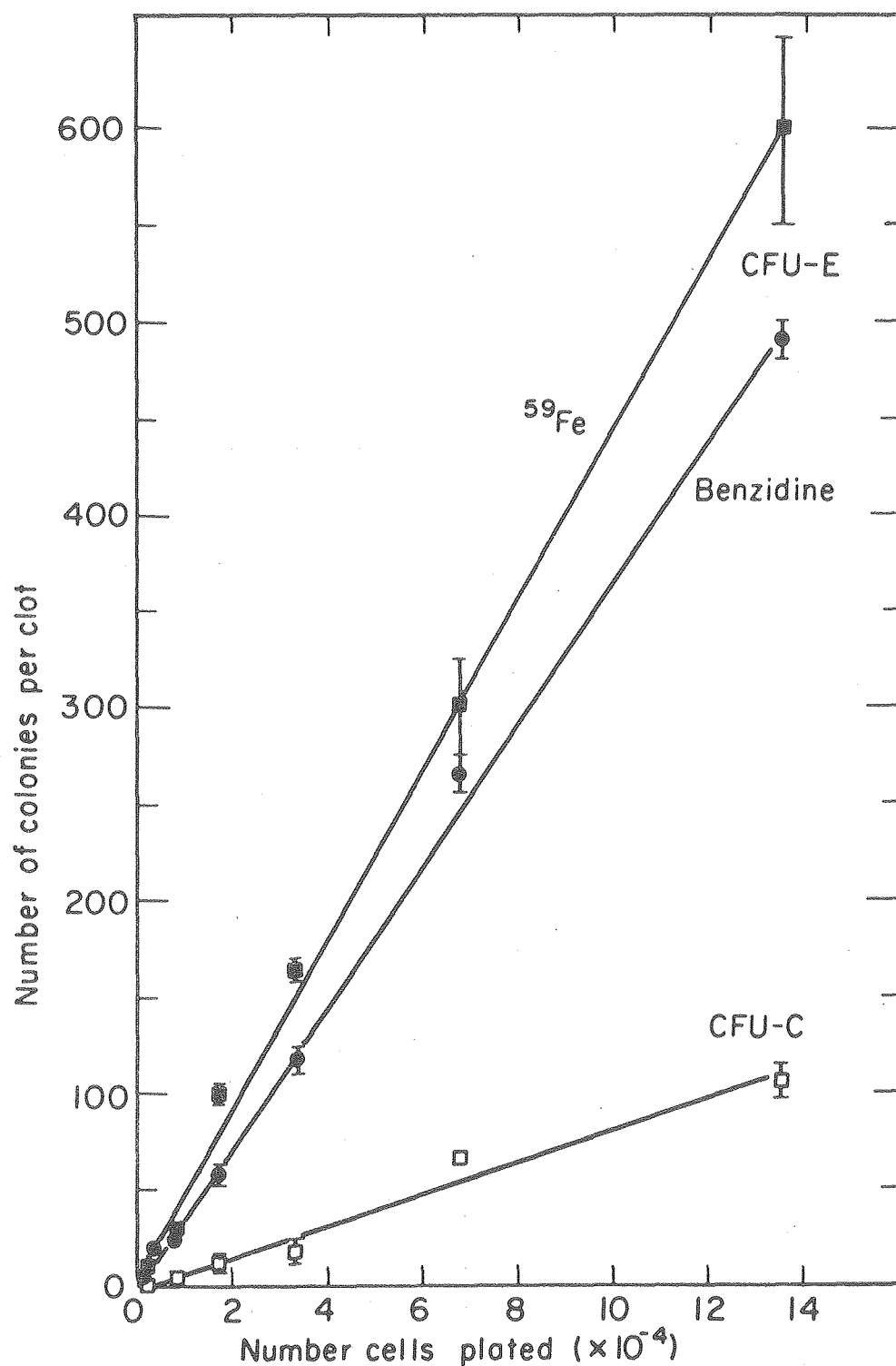
XBL7812-12384

Fig. 9: Frequency distribution of erythroid cluster sizes after 48 hour culture with 0.25 U Ep per ml. Groups of 2 to 64 cells are observed in a single clot, with higher frequencies at the binary cluster sizes (shaded). Average of four experiments (3 clots per experiment) with normal mouse marrow. Two hundred to 400 clusters were counted per clot. Error bars indicate standard error for larger cluster sizes.



XBL7812-12386

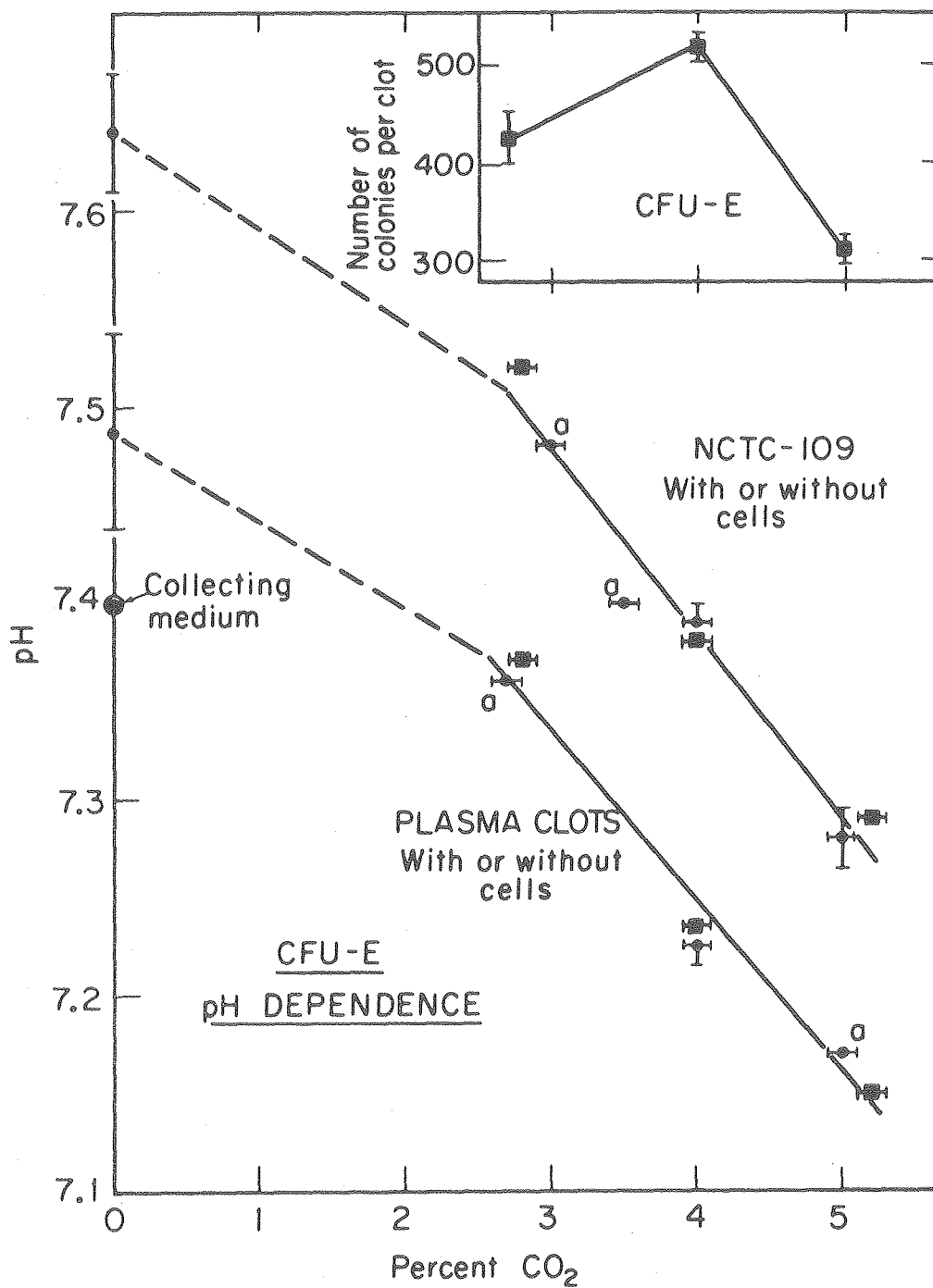
Fig. 10: Number of erythroid colonies observed as a function of time in culture. 0.25 U Ep per ml,  $4.5 \times 10^5$  cells per ml. Four  $^{59}\text{Fe}$ -autoradiographs per point  $\pm$  SEM.



XBL7812-12387

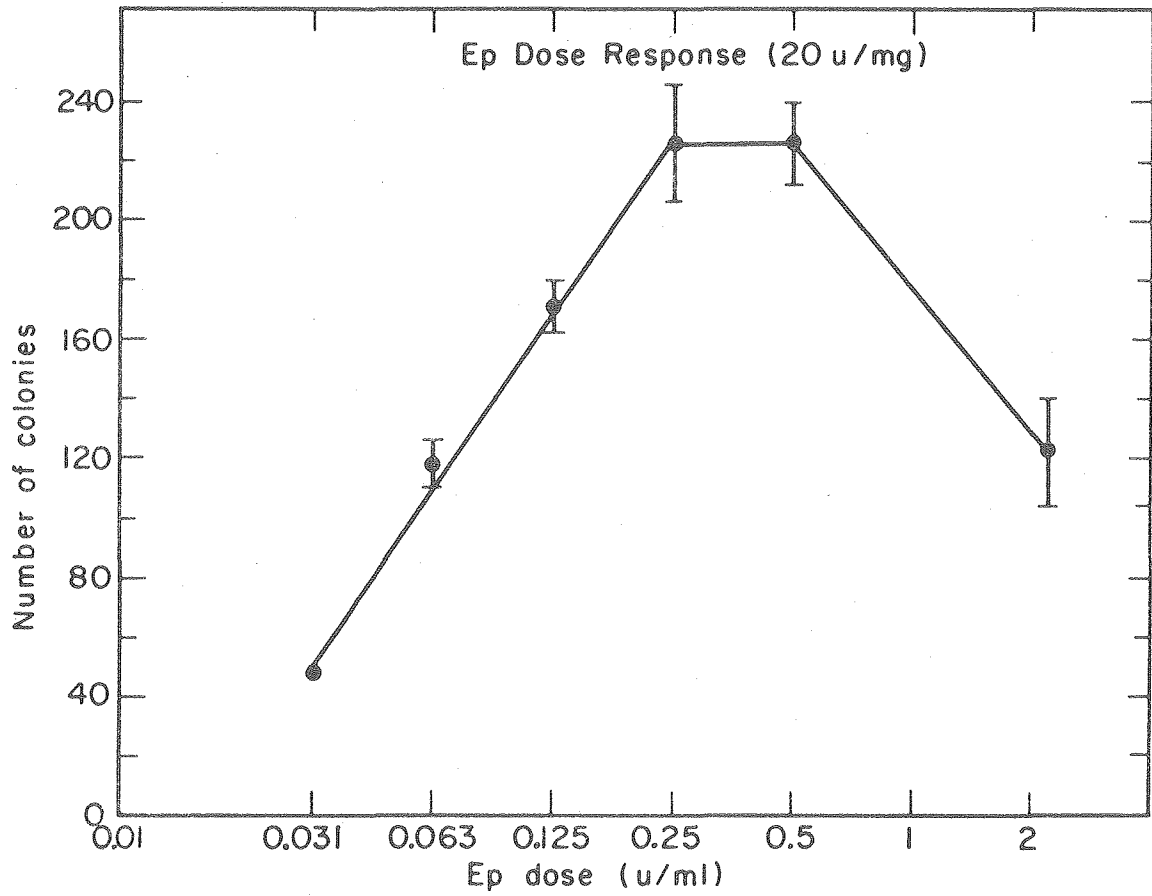
Fig. 11: Linearity of erythroid colony response (identified by either " $^{59}\text{Fe}$ " autoradiographs or "benzidine" stain) and myeloid cluster (CFU-C) responses as a function of cell inoculum. The same clots gave data for both the " $^{59}\text{Fe}$ " and "CFU-C" curves. 48 hour cultures of normal marrow with 0.25 U Ep per ml. Points represent mean  $\pm$  SEM of three clots. Lines drawn by least squares regression analysis.

Fig. 12: Erythroid colony production as a function of  $\text{CO}_2$  concentration. Lower curves relate the pH of plasma clots or NCTC-109 medium alone to  $\text{CO}_2$  concentration in culture. The number of erythroid colonies (upper curve) peaks at 4%  $\text{CO}_2$ , which translates to a pH of approximately 7.25. The pH of collecting medium was tested at ambient air  $\text{CO}_2$  (●). Dots marked "a" represent single measurements. Squares (■) represent one experiment, with three measurements per point. Error bars give standard errors.



XBL7812-12385

Figure 12



XBL788-3462

Fig. 13: Erythroid colony response to different doses of low specific activity Ep extract. Normal marrow. Points represent mean  $\pm$  SEM of three clots seeded with  $6.7 \times 10^4$  cells per clot.

Fig. 14: Comparison of erythroid colony dose responses to both low (15 U/mg protein) and high (150 U/mg protein) specific activity Ep preparations. Data points were obtained from one experiment and each point represents the mean  $\pm$  SEM of three clots ( $6.7 \times 10^4$  cells per clot).

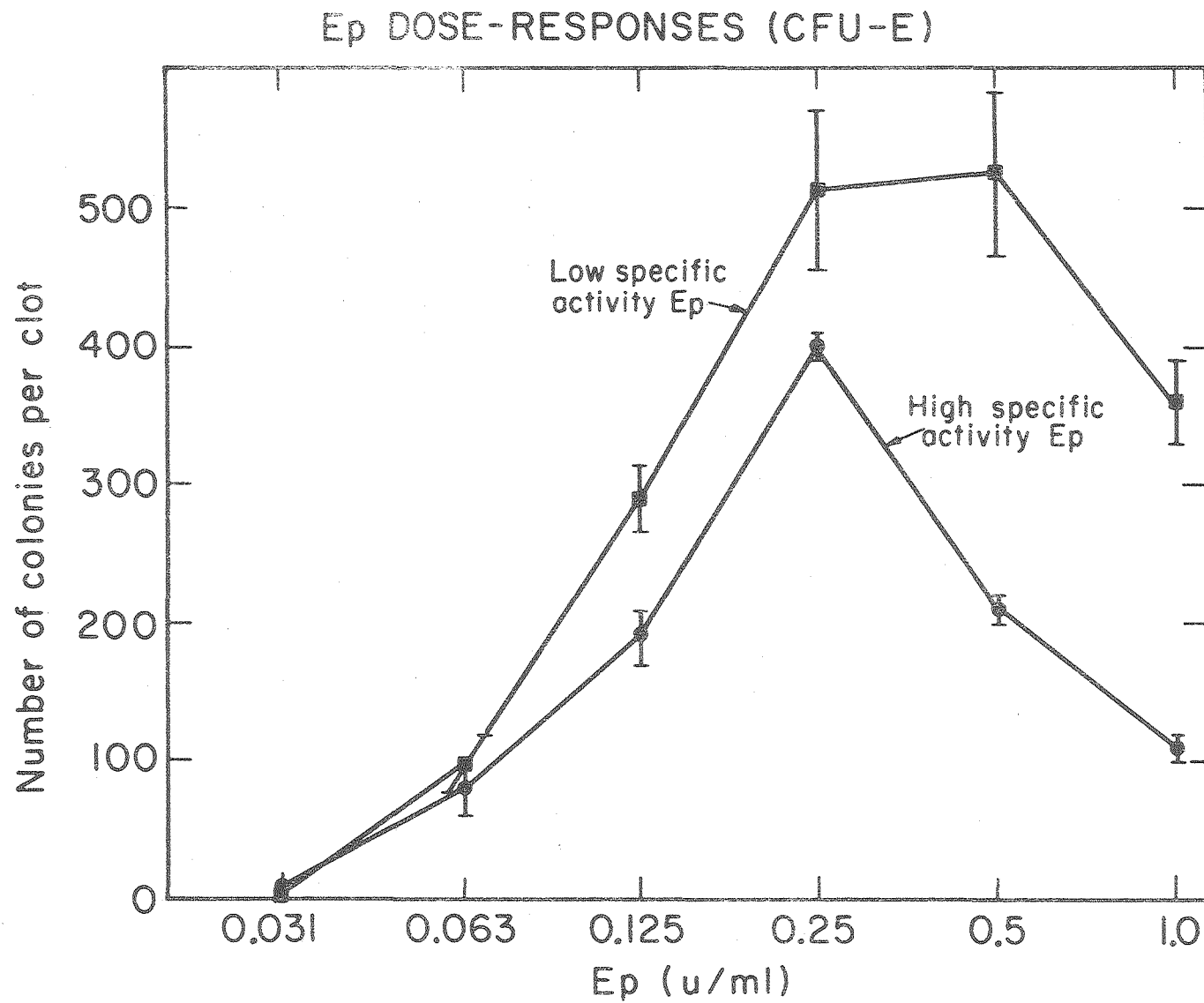
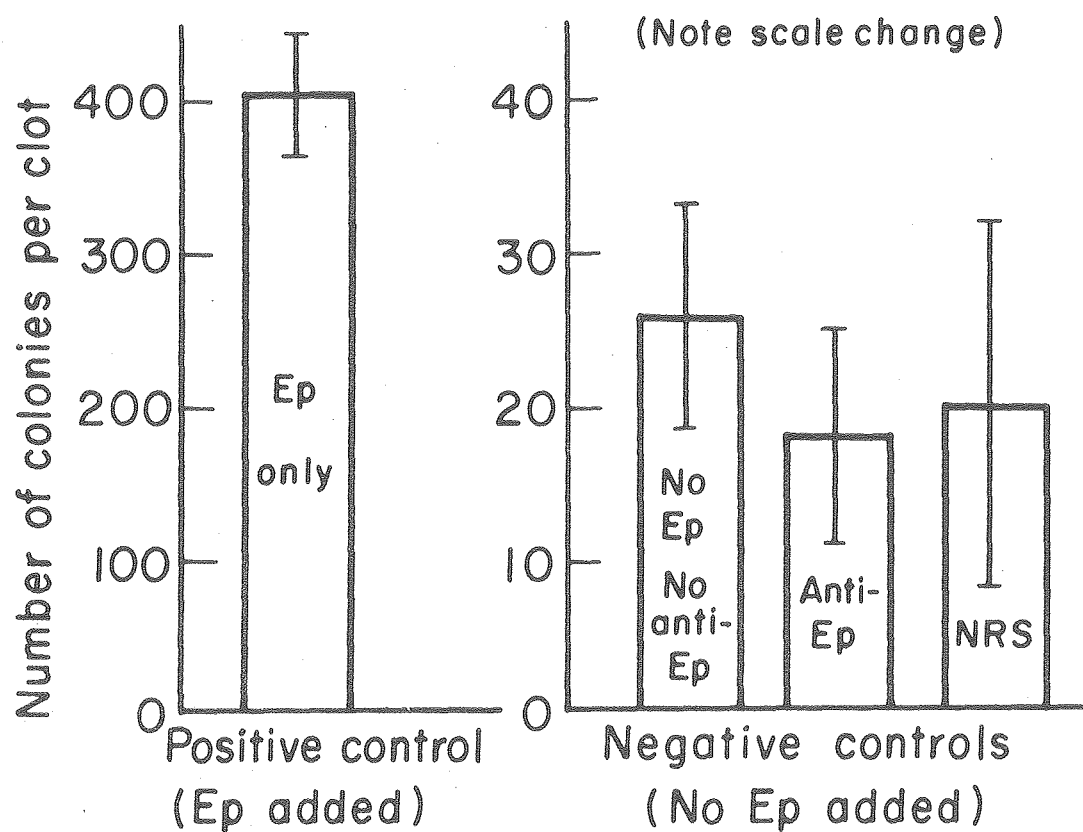


Figure 14

XBL792-3095

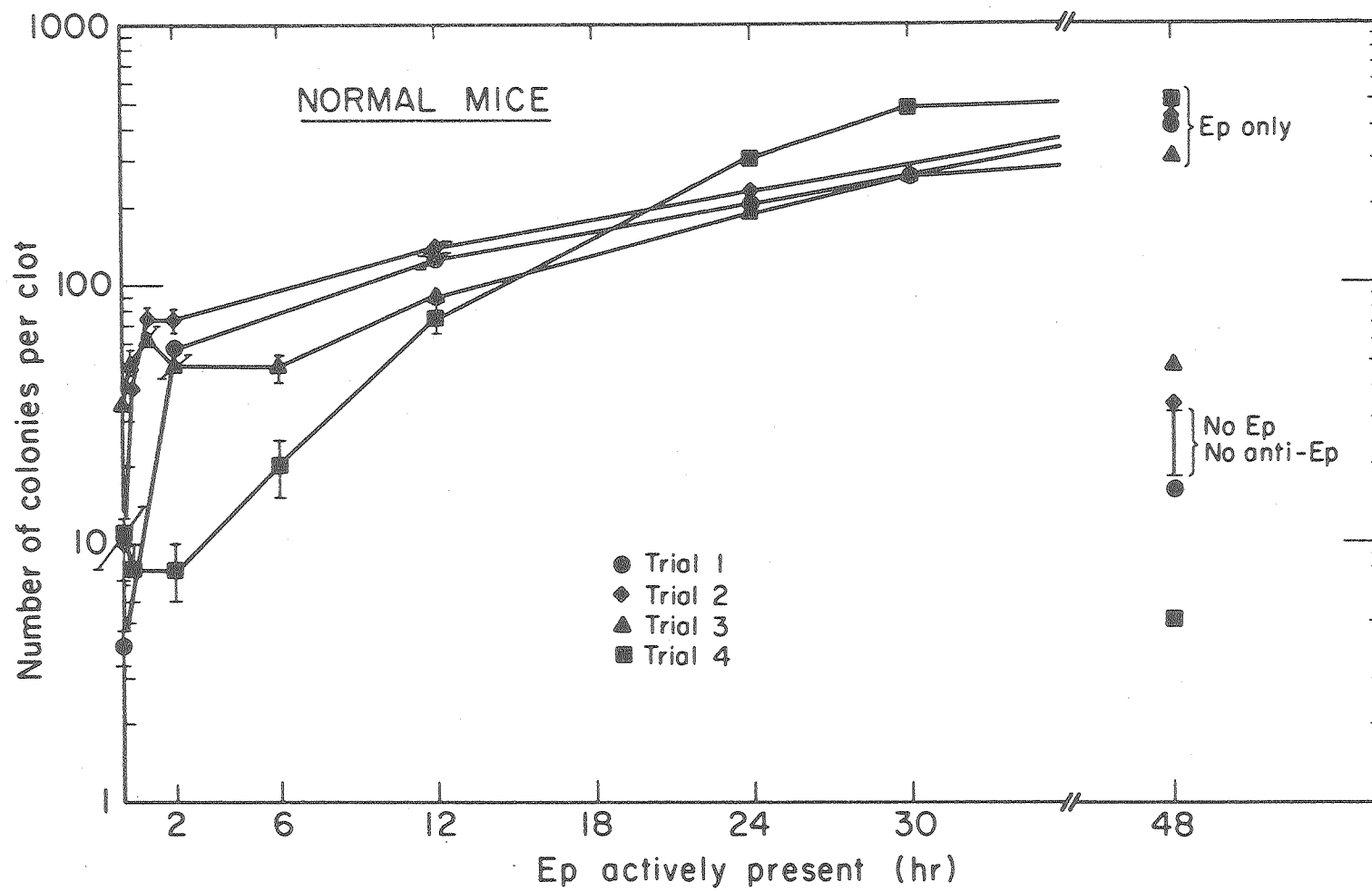
## NORMAL MICE



XBL794-3366

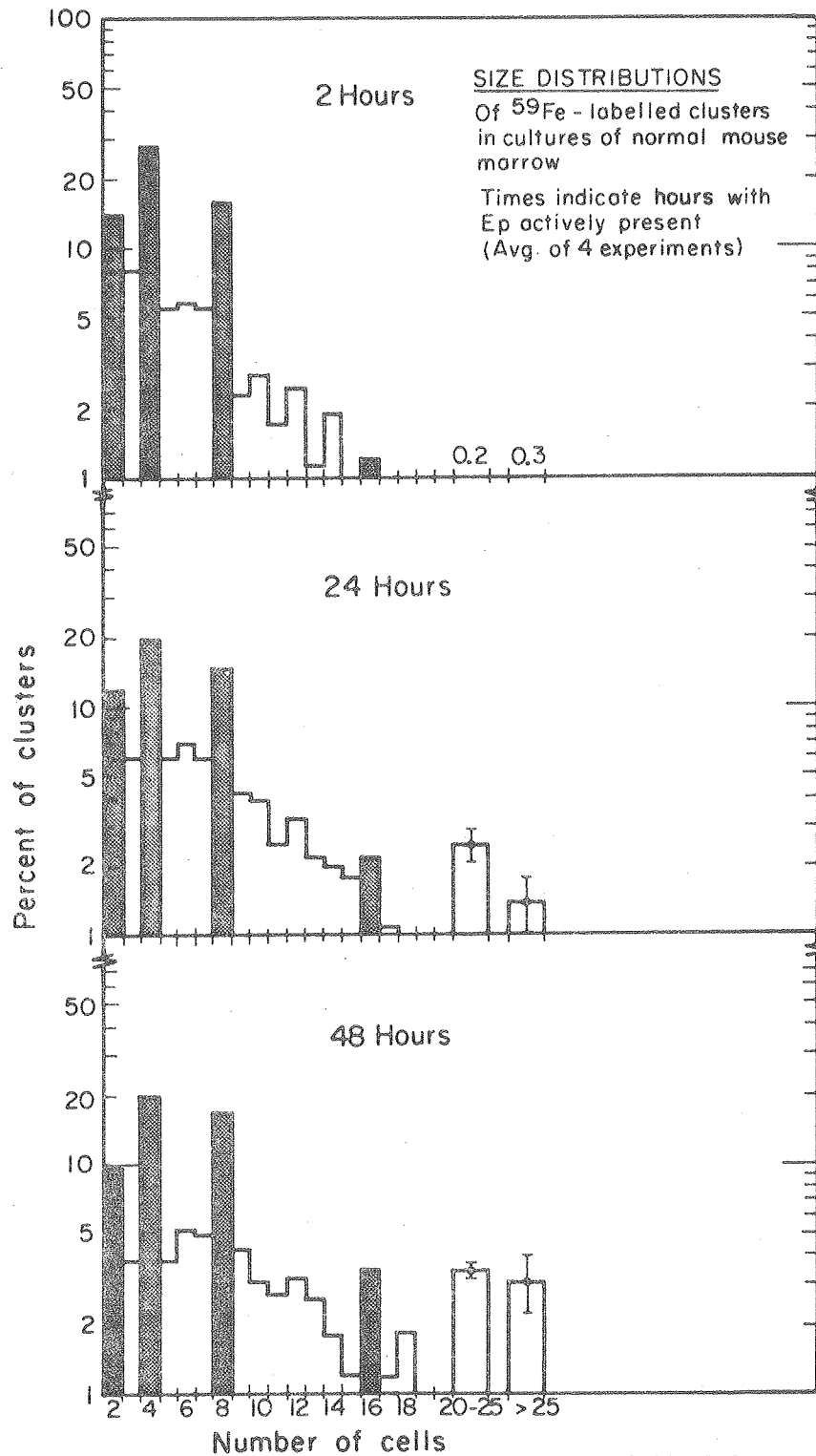
Fig. 15: Erythroid colony yield from control cultures of normal mouse marrow. Positive controls received 0.25 U Ep per ml at  $t=0$ . Additions of anti-Ep or NRS to negative controls were made close to  $t=0$ . All clots were 48 hour cultures of  $6.7 \times 10^4$  cells per clot. Points represent mean of four experiments  $\pm$  SEM.

Fig. 16: Erythroid colony production from normal marrow as a function of Ep exposure time. Abscissa indicates time anti-Ep was added after cultures were initiated with Ep at  $t=0$ . All cultures were terminated at 48 hours. Data indicates that two populations of colony-forming cells are present. Maximum erythroid colony response ("Ep only" control on right) requires presence of active Ep all of the culture period. Data points give mean colonies per 0.15 ml clot (3-4 clots per point)  $\pm$  SEM. Each clot contained  $6.7 \times 10^4$  nucleated cells. To all clots except "No Ep No anti-Ep" controls, 0.25 U Ep per ml was added.



XBL791-3044

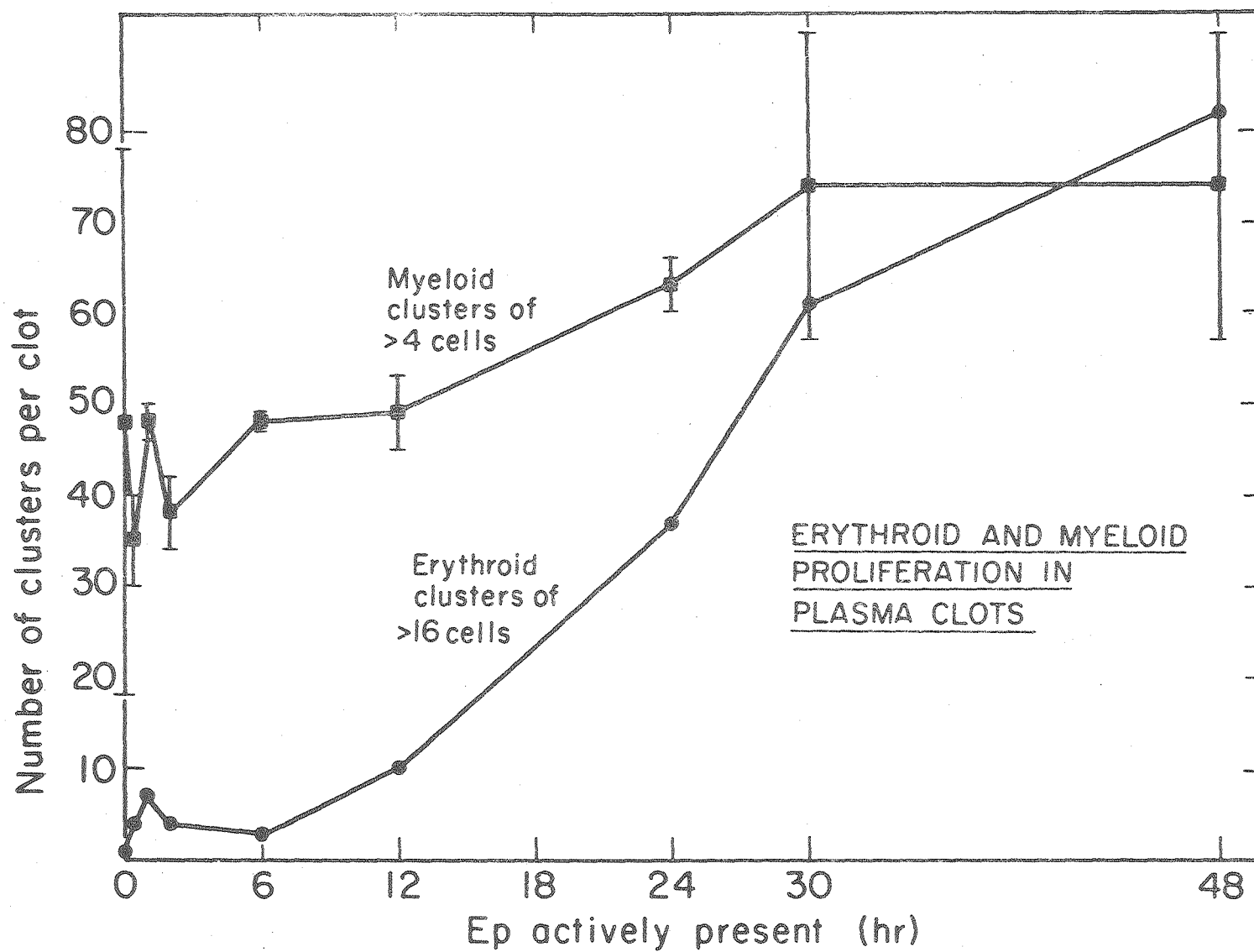
Figure 16



XBL 798-3464

Fig. 17: Frequency distribution of erythroid cluster sizes as a function of exposure time. As Ep exposure is prolonged, a shift toward larger cluster sizes becomes evident. Error bars indicate mean  $\pm$  SEM of four experiments. If available, 200 clusters per clot were grouped according to size.

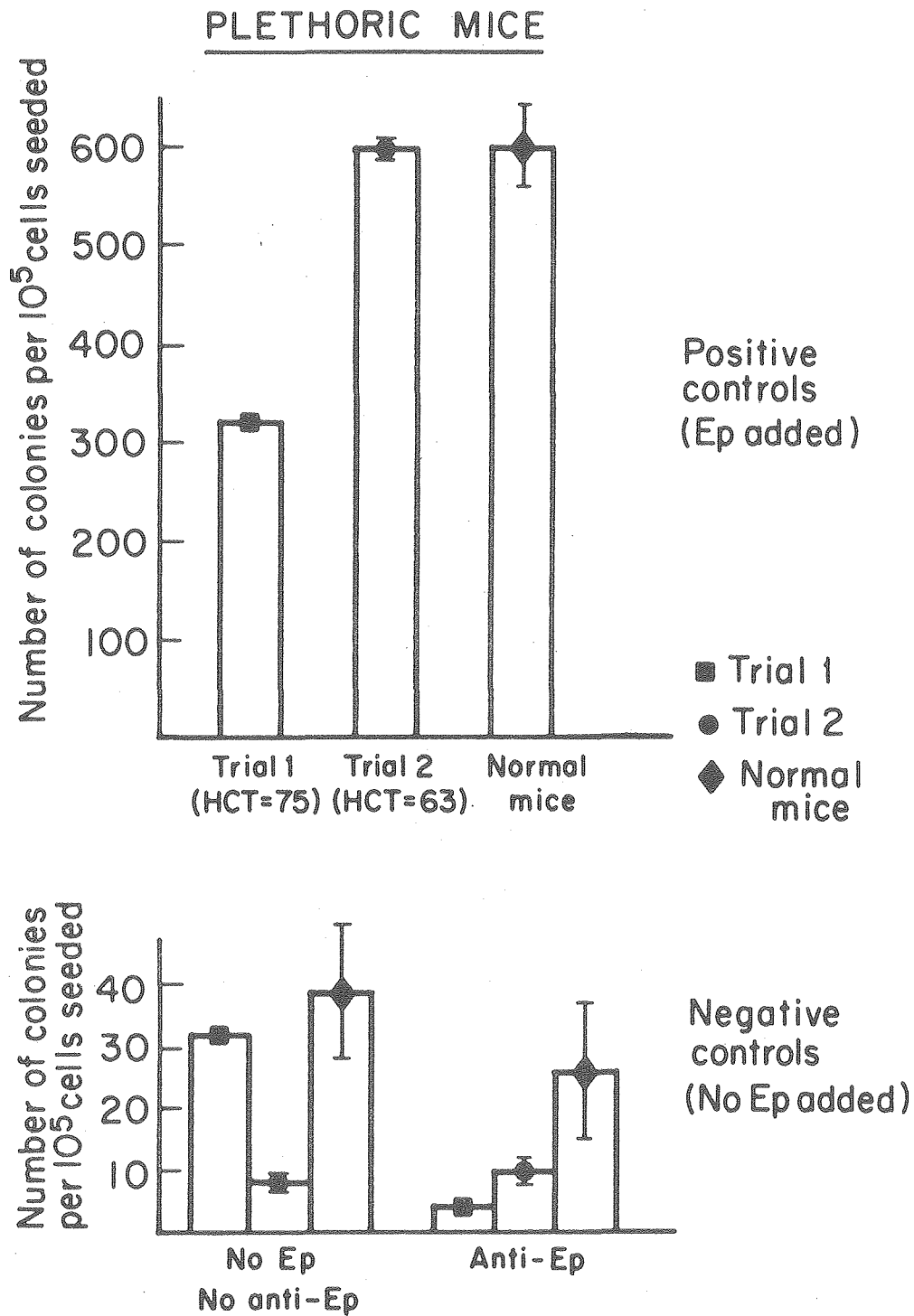
Fig. 18: Increase in number of larger erythroid and myeloid clusters as exposure to Ep is increased. The two types of cells respond at different rates to longer Ep exposures.



-131-

Figure 18

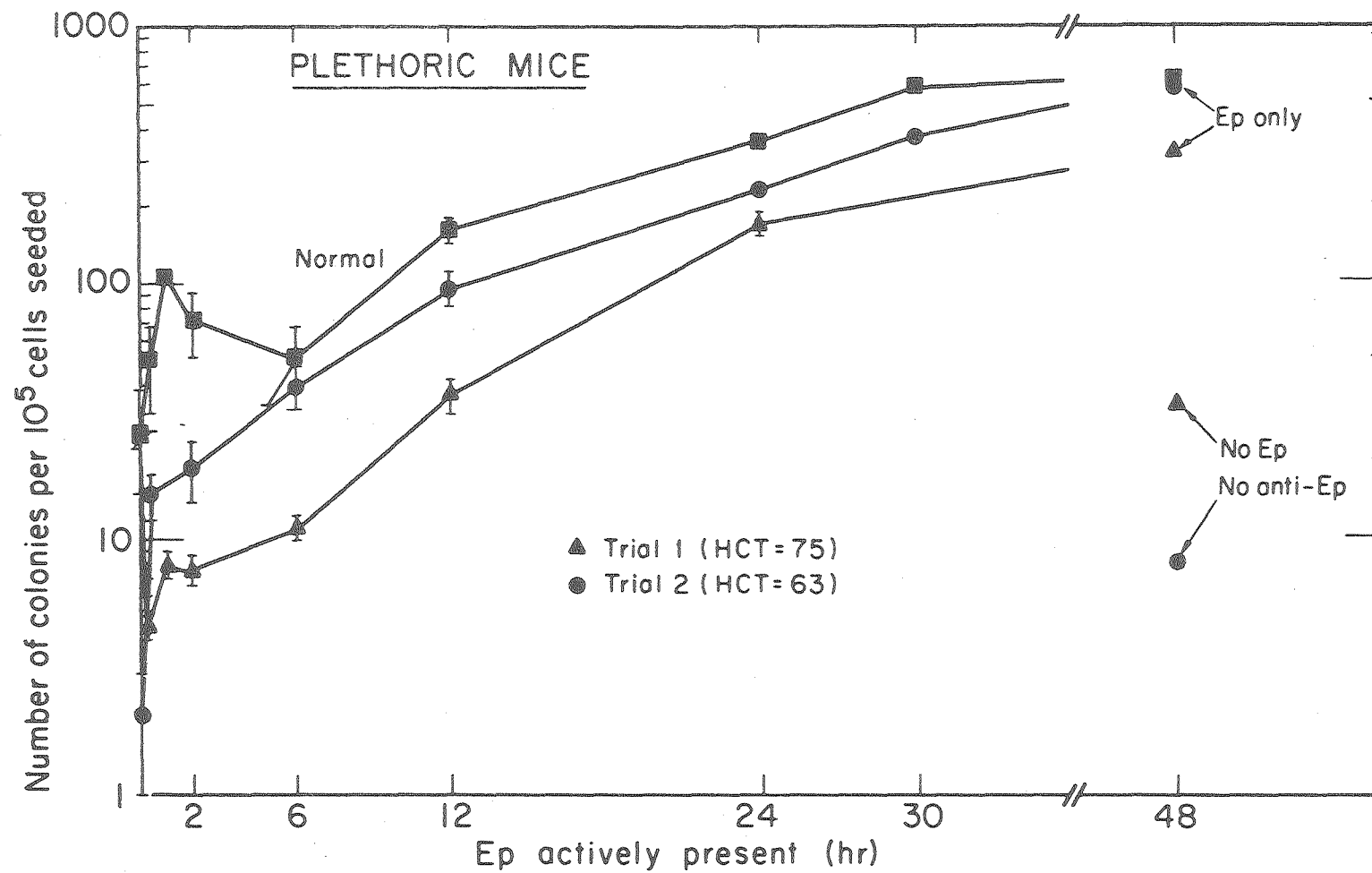
XBL792-3094



XBL791-3040

Fig. 19: Erythroid colony yield from control cultures of plethoric mouse marrow. Values obtained from four experiments with normal marrow are included for comparison. Culture conditions and treatments are the same as those described for Figure 15. Each data point from plethoric mice represents mean  $\pm$  SEM of three clots.

Fig. 20: Erythroid colony production from plethoric mouse marrow as a function of Ep exposure time. Data averaged from four experiments with normal marrow is shown for comparison. As with normal marrow (Figure 16), plethoric marrow appears to contain two populations of colony-forming cells that respond differently to Ep (0.25 U/ml). Three 0.15 ml clots per point were counted, each containing  $13.5 \times 10^4$  nucleated cells. Points represent colonies per  $10^5$  cells seeded  $\pm$  SEM.

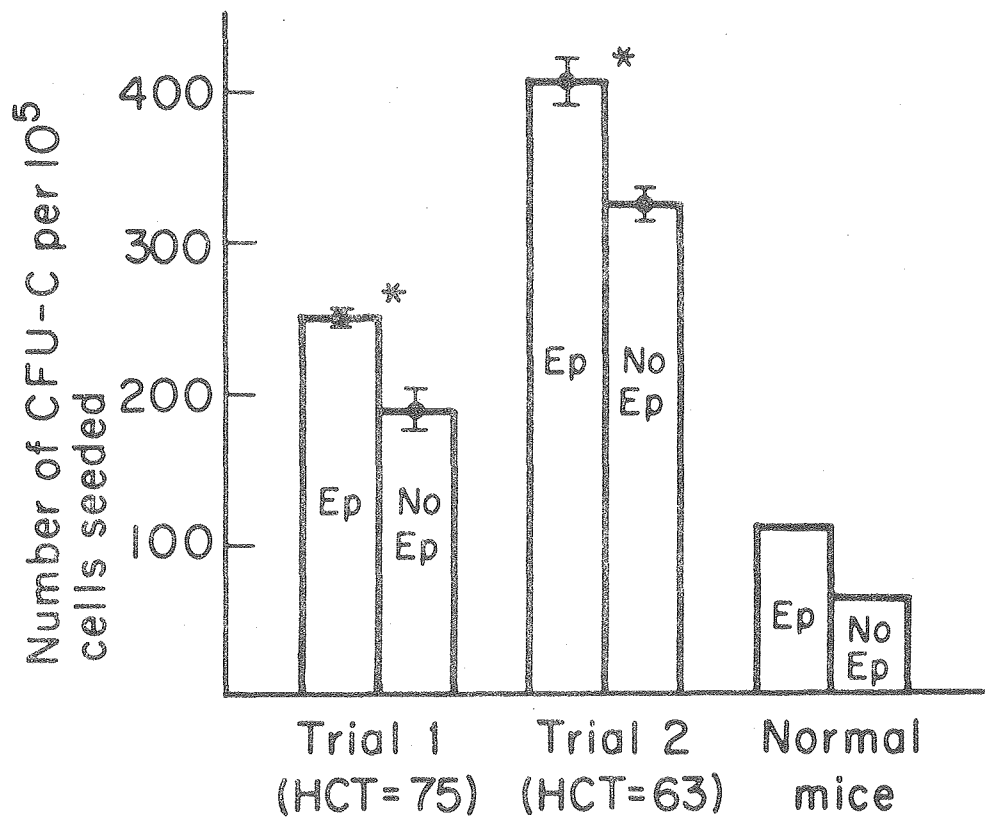


XBL791-3045

Figure 20

## PLETHORIC MICE

### Myeloid Cell Proliferation With or Without Ep

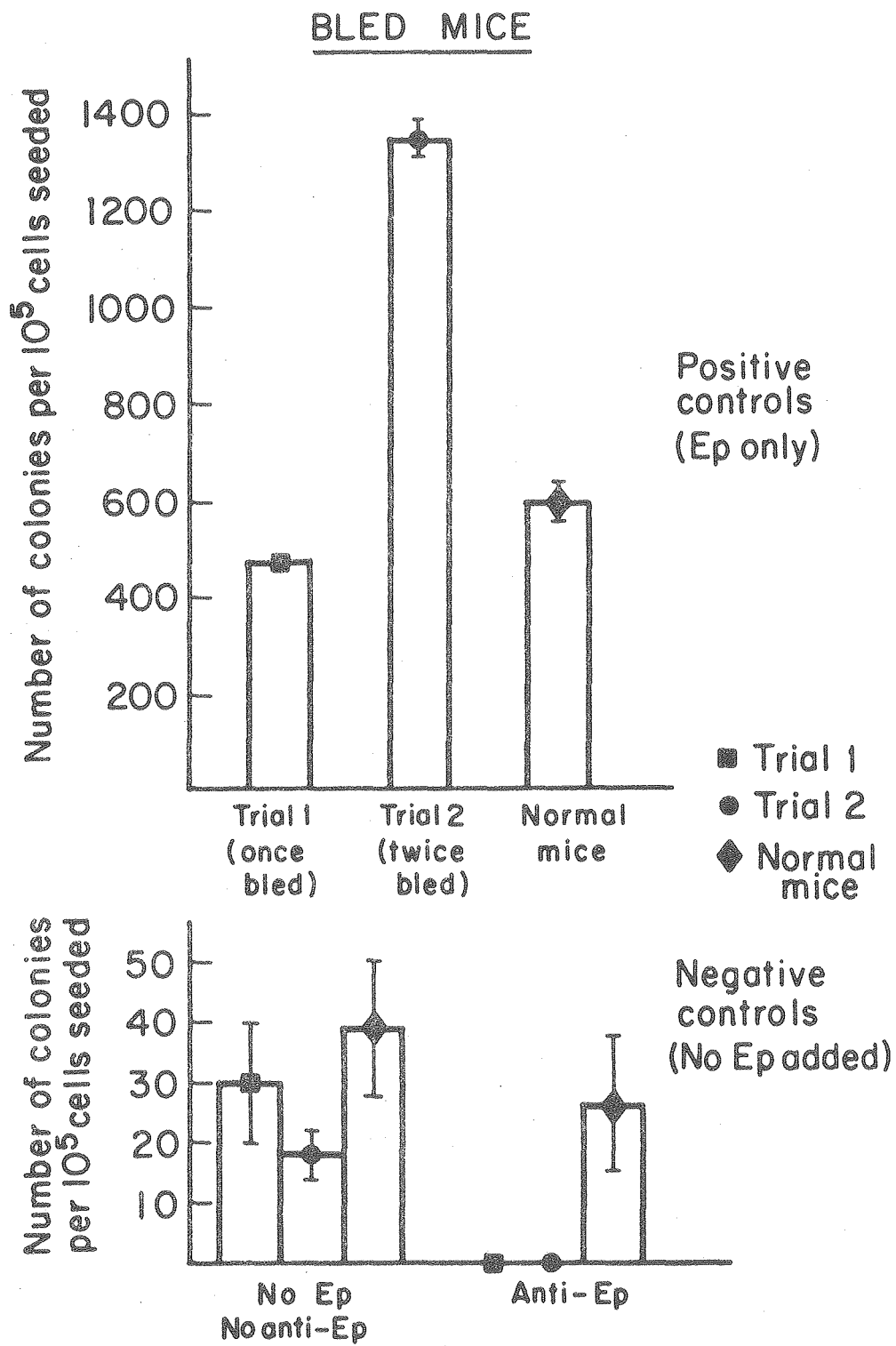


\* Difference between response with and without Ep is significant at  $p < 0.005$  level (by one-tailed  $t$ -test)

XBL791-3042

Fig. 21: Production of myeloid clusters in response to 48 hour exposures to Ep by marrow from plethoric mice. Values obtained from four experiments with normal marrow are presented for comparison. Myeloid precursors respond significantly to Ep extract. Data represent mean of three clots  $\pm$  SEM.

Fig. 22: Erythroid colony yield from control cultures of bled mouse marrow. Values obtained from four experiments with normal marrow are included for comparison. Culture conditions and treatments are the same as those described for Figure 15. Each data point from bled mice represents mean  $\pm$  SEM of three clots.



XBL791-3041

Figure 22

Fig. 23: Erythroid colony production from marrow taken from bled mice as a function of Ep exposure time. Data averaged from four experiments with normal marrow is shown for comparison. As with normal marrow (Figure 16), bled marrow appears to contain two populations of colony forming cells that respond differently to Ep (0.25 U Ep per ml). Three 0.15 ml clots per point were counted, each containing  $3.4 \times 10^4$  nucleated cells. Points represent colonies per  $10^5$  cells seeded  $\pm$  SEM.

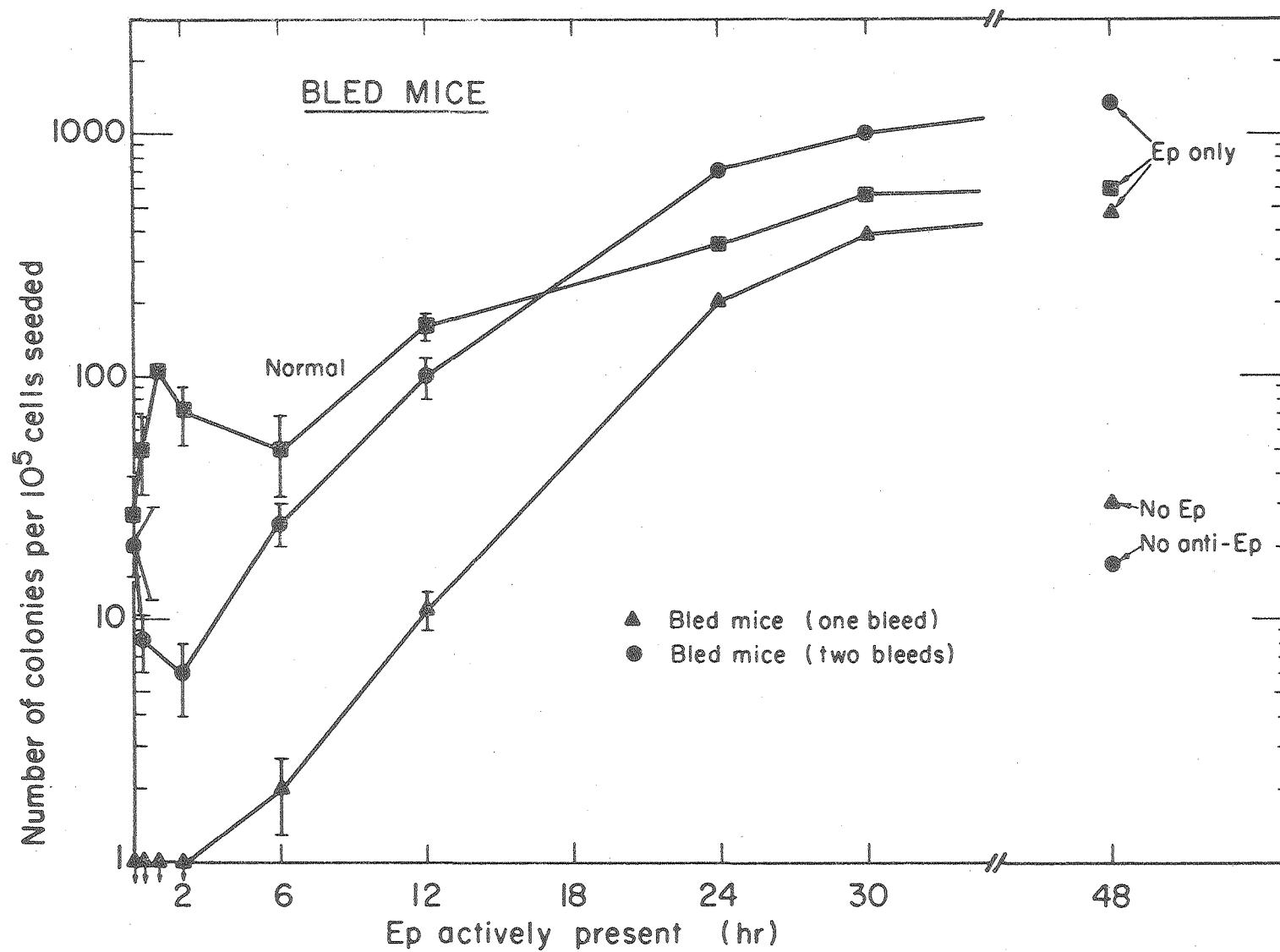
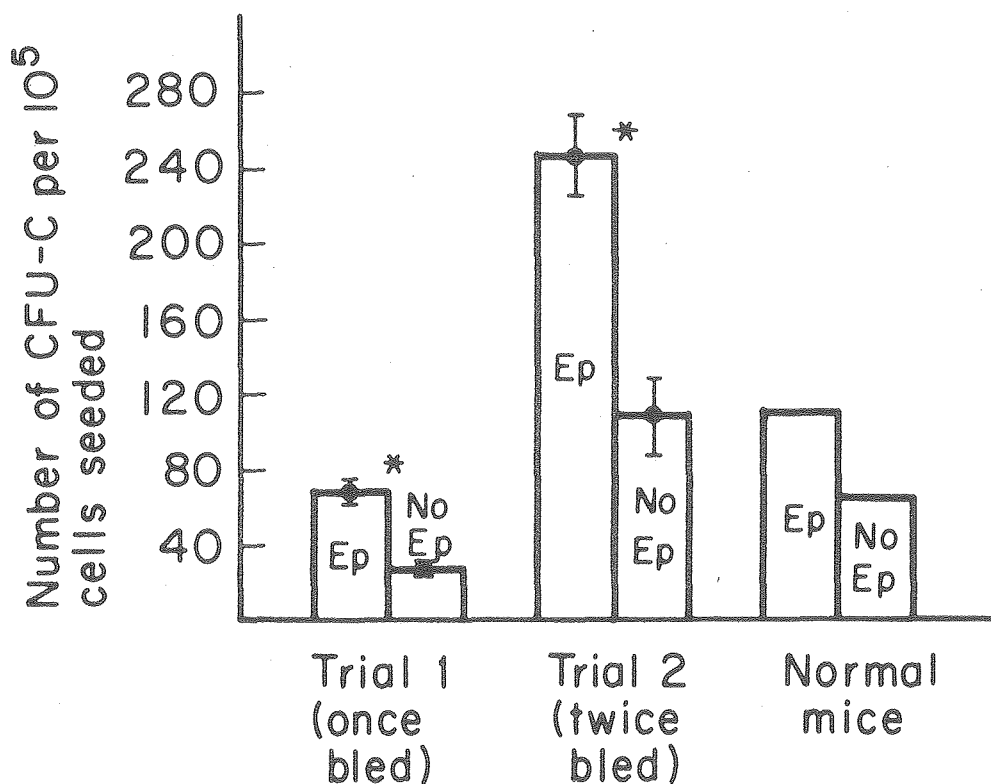


Figure 23

XBL791-3046

## BLED MICE

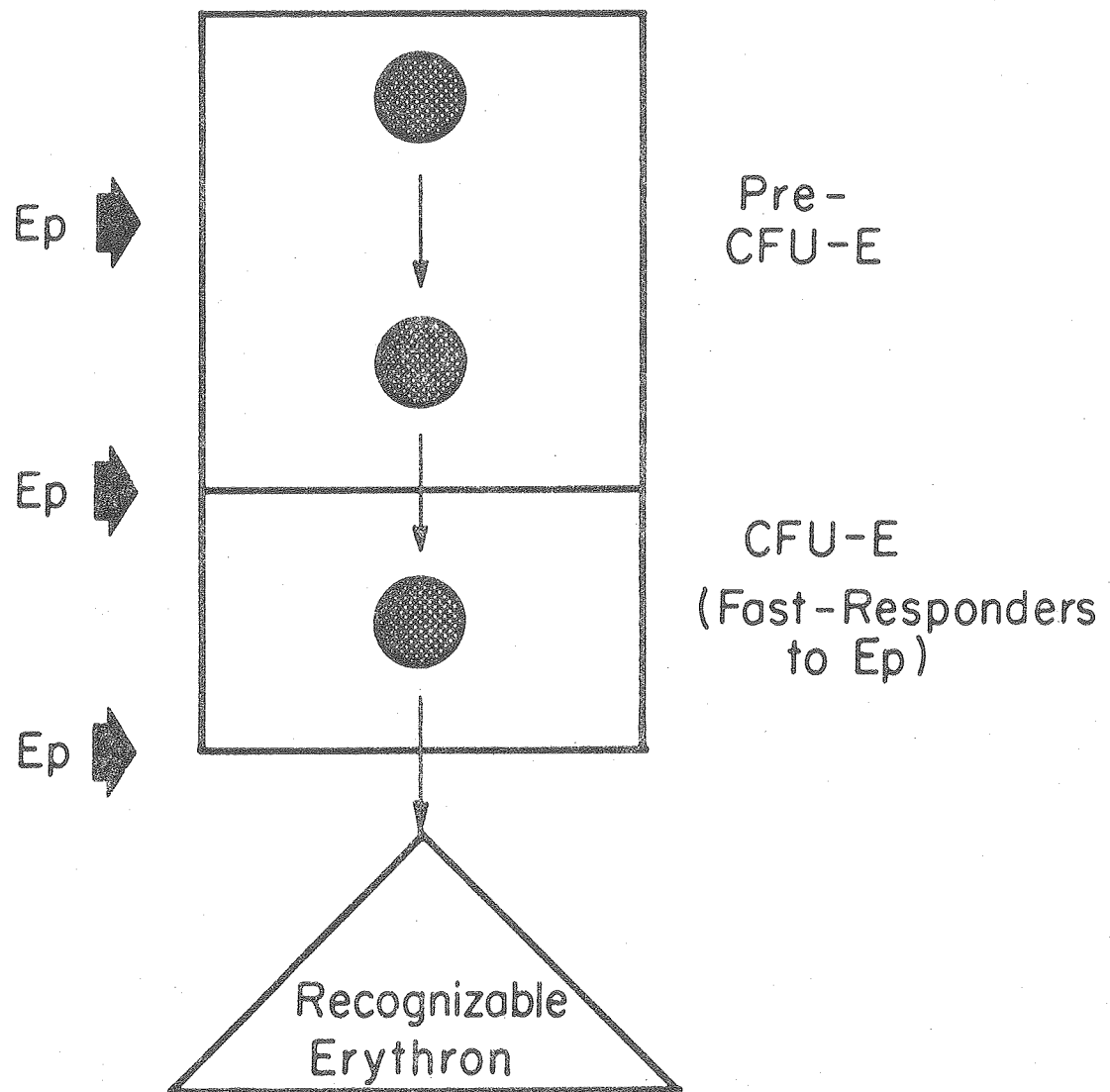
### Myeloid Cell Proliferation With or Without Ep



\*Difference between response with and without Ep is significant at  $p < 0.005$  level. (by one-tailed t-test)

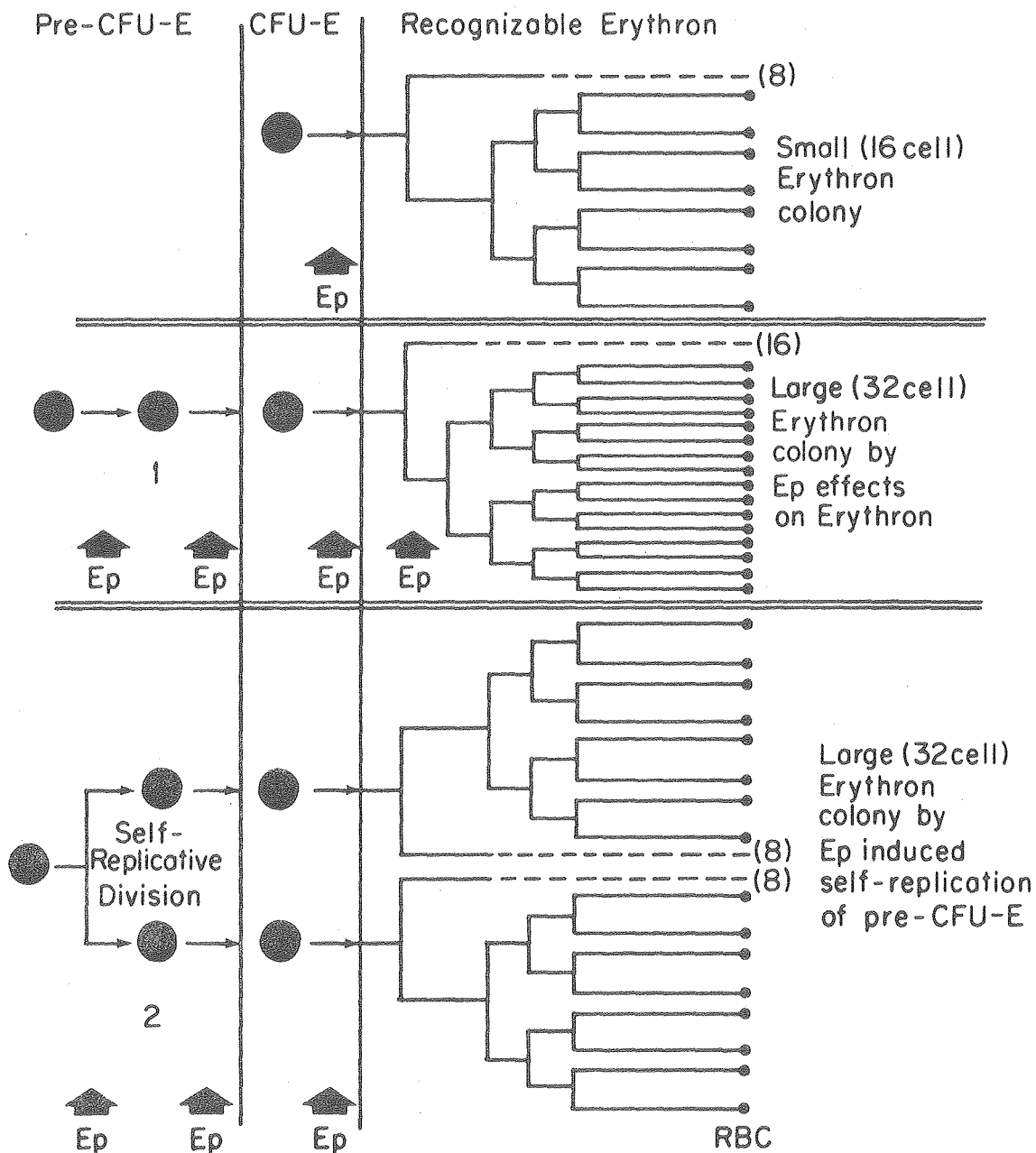
XBL791-3043

Fig. 24: Production of myeloid clusters in response to 48 hour exposures to Ep by marrow from bled mice. Values obtained from four experiments with normal marrow are presented for comparison. Myeloid precursors respond significantly to Ep. Data represent mean of three clots  $\pm$  SEM.



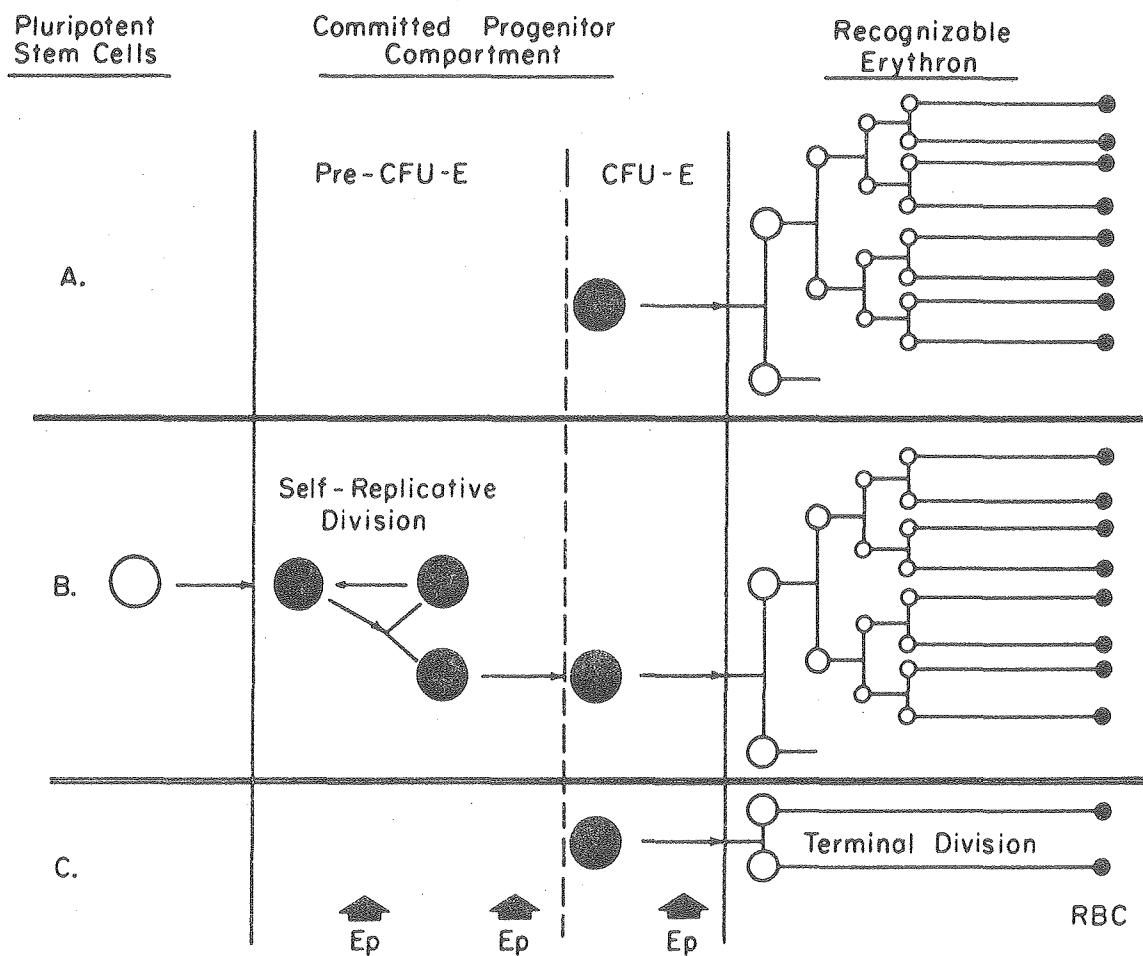
XBL794-3367

Fig. 25: Outline of possible structure in late erythroid colony-forming compartment. Data from the present study indicate the existence of two populations with maturation and entry into the recognizable erythron being regulated by Ep.



XBL794-3369

Fig. 26: Comparison of two possible ways Ep might induce larger erythroid colonies when present for longer times. Upper panel shows cell kinetics involved in making small colonies (16 cells or fewer). Case #1 shows production of a large colony if Ep acted to increase the number of divisions in the recognizable erythron. Case #2 shows how self-replication of pre-CFU-E would give rise to large colonies without Ep affecting the recognizable erythron. Available data supports case #2.



XBL794-3368

Fig. 27: A proposed structure and scheme for Ep regulation of the committed erythroid progenitor compartment. Regulation by Ep occurs throughout this portion of the committed progenitor pool.

