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WEINBERG, Rona Singer, 1952-
REGULATION OF ERYTHROPOIESIS IN POLYCYTHEMIA
VERA AND FETAL SHEEP: COMPARATIVE IN VITRO
AND IN VIVO STUDIES.

City University of New York, Ph.D., 1977
Physiology

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1977

REGULATION OF ERYTHROPOIESIS IN POLYCYTHEMIA VERA
AND FETAL SHEEP: COMPARATIVE IN VITRO AND
IN VIVO STUDIES

by

Rona Singer Weinberg

A dissertation submitted to the Graduate
Faculty in Biomedical Sciences in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy, the City
University of New York.

1977

This manuscript has been read and accepted for the
Graduate Faculty in Biomedical Sciences in satisfaction
of the dissertation requirement for the degree of Doctor
of Philosophy.

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ABSTRACT

REGULATION OF ERYTHROPOIESIS IN POLYCYTHEMIA VERA AND FETAL SHEEP: COMPARATIVE IN VITRO AND IN VIVO STUDIES

by

Rona Singer Weinberg

Adviser: Dr. Esmail D. Zanjani

The in vitro plasma clot culture system was employed to investigate the mechanisms regulating the proliferation and differentiation of erythroid committed stem cells (CFU-E) in normal humans, patients with polycythemia vera, and fetal sheep. In this system, normal human bone marrow CFU-E responded to exogenous erythropoietin (Ep) by giving rise to large numbers of erythroid colonies. The number of colonies formed was directly proportional to the number of bone marrow cells cultured and the dose of Ep used. In all cases, maximal response (436 ± 23 erythroid colonies/ 6×10^5 bone marrow cells) was obtained with 2 IU of Ep. Neutralization of the exogenous Ep by the simultaneous addition of appropriate amounts of anti-Ep completely inhibited erythroid colony formation by normal human CFU-E, thus revealing the Ep dependent nature of the erythropoietic

activity of normal CFU-E. As with normal adult CFU-E, bone marrow cells from individuals with polycythemia vera (PV) and fetal sheep both responded to Ep by forming erythroid colonies. However, unlike normal adult CFU-E, both PV and fetal sheep CFU-E produced colonies (endogenous erythroid colonies, EEC) in the absence of Ep (122 ± 9 EEC/ 6×10^5 cells and 89 ± 8 EEC/ 6×10^5 cells respectively). Exposure of fetal calf serum and citrated bovine plasma to anti-Ep, prior to use in culture, resulted in near total inhibition of EEC formation by both PV and fetal sheep bone marrow (e.g., 183 ± 22 EEC/ 4×10^5 cells vs. 11 ± 5 EEC/ 4×10^5 cells and 123 ± 16 EEC/ 8×10^5 cells vs. 2 ± 1 EEC/ 4×10^5 cells, respectively). In addition, the production of colonies by PV bone marrow in response to exogenous Ep was inhibited by the presence of anti-Ep. Addition of as little as 0.001 IU of Ep to anti-Ep treated cultures of PV bone marrow resulted in the reappearance of EEC (28 ± 4 EEC/ 6×10^5 cells). These results suggest that the erythropoietic activity of CFU-E from normal humans, individuals with PV, and fetal sheep are all regulated by Ep. In PV and fetal sheep, however, there exists a subpopulation of CFU-E which is abnormally sensitive to small concentrations of Ep. The enhanced production of erythrocytes in patients with PV who generally exhibit lower than normal levels of

Ep may be attributed to the proliferative and differentiative activity of this population of highly Ep sensitive CFU-E. The possibility that the increased sensitivity of CFU-E in PV and fetal sheep may be related to the presence of a serum-borne erythrostimulatory factor, other than Ep, was also investigated. Mice were given daily injections of sera from normal humans, patients with PV, and fetal and adult sheep, and the erythropoietic status of these mice was observed in vivo and in vitro. Treatment of mice with PV sera for 5, 6, and 7 days or with fetal sheep sera for 10 to 14 days resulted in the formation of EEC by bone marrow cells from treated mice (48 ± 6 EEC/ 4×10^5 cells and 89 ± 11 EEC/ 4×10^5 cells, respectively). Bone marrow cells from untreated mice and from mice treated with normal human sera or adult sheep sera did not produce EEC. These results suggest that the myelostimulatory factor present in some patients with PV, which has previously been shown to affect the proliferation of hematopoietic stem cells, play a role in increasing the sensitivity of erythroid precursor cells to Ep.

ACKNOWLEDGMENTS

I would like to thank the many people who have taught and guided me throughout my graduate school career. My special thanks go to:

Dr. Esmail Zanjani, my thesis advisor, for stimulating my interest in erythropoiesis and for allowing me to pursue the study of this area in his laboratory. He always gave freely of his time, and his expert guidance during my research and the preparation of this thesis will never be forgotten.

Dr. Zia Penefsky and Dr. Michael Greenberg, for the time they spent reading this thesis and for their many thoughtful and helpful suggestions.

Dr. Joyce Shriver, my pre-thesis advisor, for her dedication to good graduate education and for being there to share the bad times as well as the good times.

Dr. Irving Schwartz, Dean of the Graduate School, for his help and encouragement over the years.

Dr. Terry Krulwich, Executive Officer of the Graduate School, for her advice and assistance.

Dr. John Lutton, Ms. Gertrude Lurinsky, and Dr. Ronald Hoffman, their technical knowledge and assistance made my laboratory work a little easier.

All my professors at Mount Sinai, especially Dr. Tibor Barka and the members of the Department of Anatomy.

Dr. Eileen Chusid, my friend, for always being ready, willing, and able to help.

My mother, father, and sister for teaching me to strive for the highest goals, and for their love and encouragement.

My husband, Artie, his constant love, understanding, and encouragement made my career possible and meaningful.

DEDICATION

This thesis is dedicated to

my father

my mother

and especially to my husband,

their love and encouragement

made it all worthwhile

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INTRODUCTION

Erythropoiesis is a complex process which can be influenced by many physiological factors. Perhaps the two most important requirements for normal adult mammalian erythropoiesis to occur are the presence of stem cells which can give rise to erythroid cells and the presence of the hormone erythropoietin (Ep).

The hormone erythropoietin acts in the adult to regulate erythropoiesis (Reissmann, 1950; Erslev, 1953; Krantz and Jacobson, 1970; Gordon, 1973). Carnot and Deflandre (1906) were the first to postulate the existence of this hormone and the role it plays in enhancing erythropoiesis in man and animals following exposure to hypoxia, when they observed that administration of serum from anemic rabbits stimulated erythropoiesis in normal rabbits. Although attempts to confirm this work were for the most part unsuccessful during the next forty years, the theory of humoral regulation of erythropoiesis was still considered a viable working hypothesis by many investigators (Bonsdorff and Jalavisto, 1948; Gordon, 1959).

In 1950, Reissmann demonstrated that, if one member of a pair of parabiotic rats was exposed to hypoxia, the bone marrow erythroid hyperplasia occurred in both rats.

It was postulated that this increase in erythropoiesis was caused by a humoral substance produced by the hypoxic rat which was able to exert its effect on the bone marrow cells of both partners because of the vascular connections between these two animals.

While this work supported the theory of humoral regulation of erythropoiesis, it was not fully accepted until Erslev in 1953 successfully demonstrated that plasma from bled rabbits could in fact stimulate erythropoiesis in normal recipient rabbits. In these studies it was shown that repeated injections of this plasma resulted in significant reticulocytosis, as well as a definite increase in percent nucleated red cells in the bone marrow and increased red blood cell count and hematocrit in the recipient animal. Furthermore, Stohlman et al. (1954) described a patient with regional hypoxia and polycythemia secondary to a patent ductus arteriosus. This patent connection between the pulmonary artery and the aorta caused the oxygen saturation and P_{O_2} levels of blood reaching the sternal bone marrow to be normal, while the oxygen saturation and P_{O_2} levels of blood reaching the iliac crest bone marrow were greatly reduced. Since hypoxia had been known for many years to be a fundamental erythropoietic stimulus, this difference in bone marrow oxygenation presented a unique opportunity to evaluate the influence of hypoxia on red blood cell formation. The observation of normoblastic

hyperplasia in normally oxygenated areas of bone marrow such as the sternum, as well as in poorly oxygenated areas of bone marrow such as the iliac crest, provided further evidence for the involvement of a humoral agent in the regulation of erythropoiesis in response to hypoxia.

These data are in accord with the hypothesis that the relation between tissue oxygen supply and demand of the animal determines the quantity of Ep produced (Fried et al., 1957). It is now generally accepted that hypoxia, the fundamental erythropoietic stimulus, exerts its effect by increasing the production of Ep, thereby leading to an increase in circulating Ep levels and finally to an increase in red blood cell production (Krantz and Jacobson, 1970; Gordon, 1973). Thus circulating Ep levels are increased in response to hemorrhagic or hemolytic anemia, hypoxic hypoxia, or cobaltous chloride (Gordon, 1959). Conversely, Ep production decreases when oxygen supply to the responsive tissue exceeds the demand. In this regard, transfusion induced polycythemic, hyperoxic, hypophysectomized, thyroidectomized, or fasted animals have a suppressed rate of erythropoiesis accompanied by decreased production of Ep (Fried, 1957; Reissman, 1964; Gordon et al., 1966).

Ep, the prime regulator of erythropoiesis, has not been

totally purified and characterized biochemically due to the unavailability of large supplies of the hormone and the difficulty in working with glycoproteins. However, biochemical separation and purification studies have shown that Ep is a glycoprotein with a molecular weight of approximately 46,000 (Goldwasser and Kung, 1972). At present the best sources of Ep are plasma from sheep rendered polycythemic by phenylhydrazine (White et al., 1960; Goldwasser et al., 1962; Goldwasser and Kung, 1968, 1972a), and urine from anemic human subjects (Espada and Gutinsky, 1970a,b). The most purified preparations (9-12,000 IU/mg. protein) have been shown to contain hexoses, hexosamines, and sialic acid in addition to several other amino acids (Goldwasser and Kung, 1972). It has been demonstrated that the removal of sialic acid by neuroaminidase (Lowy et al., 1960; Schooley and Garcia, 1971) causes inactivation of erythropoietin activity in vivo as determined by bioassay in the polycythemic mouse. In addition, injection of bacterial neuraminidase in vivo destroys circulating erythropoietin (Schooley and Mahlmann, 1971). However, the desialated hormone retains its ability to stimulate heme synthesis in isolated bone marrow cells in vitro (Goldwasser and Kung, 1968). It has been suggested that desialated erythropoietin does not have sufficient time to act on

hematopoietic tissues in vivo since it is removed more rapidly from circulation than the native form of the hormone (Goldwasser, 1966; Goldwasser et al., 1973). This theory is supported by the observations of Morell et al. (1968) that desialated glycoproteins in general are removed from the circulation by the liver more rapidly than sialated forms of the same glycoproteins. In addition, it has been shown that administration of desialated ceruloplasmin blocks the clearance of desialated Ep by binding to receptor sites in the liver and that by preinjecting desialated orsomucoid subsequent administration of desialated erythropoietin is effective in stimulating erythropoiesis (Gordon, 1973). In vitro, in the absence of such a rapid disappearance, however, desialated erythropoietin is capable of stimulating red blood cell production with no apparent loss of activity.

In order to determine the site of production of erythropoietin, Jacobson et al. (1957) removed various organs from rats and rabbits and then attempted to stimulate Ep production in these animals by phlebotomy or treatment with cobaltous chloride. Several hours later, plasma obtained from these animals was assayed in hypophysectomized rats to determine the presence of Ep. It was found that removal of the hypophysis, thyroid gland, spleen, adrenal glands,

gonads, liver, pancreas, stomach, or intestines did not diminish the animal's ability to produce Ep in response to hemorrhage or cobaltous chloride treatment. Following bilateral nephrectomy, however, neither rats nor rabbits retained the capacity to produce Ep in response to hemorrhage or cobaltous chloride treatment. It was also shown that the inability of nephrectomized animals to produce Ep was not due to uremia, but rather to the absence of renal tissue. Subsequent studies by these and other investigators (Jacobson et al., 1957b; Goldwasser et al., 1958) confirmed the role of the kidneys in the formation of Ep in many animal species including man (Gurney et al., 1957; Gallagher et al., 1959; Nathan et al., 1964; Mirand, 1968).

Although the kidney is recognized as the major site of Ep production, early attempts to extract the hormone from this organ were not successful (Naets, 1960; Rambach et al., 1961; Zangheri et al., 1962; Goldfarb and Tobian, 1963). Kuratowska et al. (1964) provided the first insight into the mechanism by which the kidney participates in Ep formation. They observed that, although perfusion of rabbit kidneys with normal saline solution produced no detectable Ep in the perfusate, mixture of this perfusate with plasma, serum, or an alpha globulin fraction of plasma did produce

active Ep. They, therefore, hypothesized that the kidney produces an inactive form of the hormone that requires a plasma alpha globulin for activation or stabilization. Kuratowska and Kopec (1969) and Kuratowska (1968) localized the inactive hormone in the acid nuclear protein fraction of the kidney and it was named renal erythropoietic factor (REF).

This work was confirmed and elaborated upon by Gordon and his co-workers (Gordon et al., 1967; Gordon, 1968; Gordon et al., 1968). They extracted a similar factor from kidneys of hypoxic rats and found it produced Ep when incubated with normal serum. This renal erythropoietic factor has subsequently been renamed erythroenin (Eg). Zanjani et al. (1967b) have shown that erythroenin is equally distributed in the cortical and medullary tissue of the kidney. Subcellular fractionation procedures have shown that only light mitochondrial and microsomal extracts of kidney tissue were capable of producing Ep when incubated with serum (Gordon, 1968). That Ep is in fact produced by the interaction of erythroenin and normal serum was confirmed by the ability of anti-Ep to neutralize the product of the interaction (Zanjani et al., 1968). Furthermore, it appears that the interaction of erythroenin and normal serum to produce Ep may be enzymatic in nature (Zanjani

et al., 1967a; Smith and Fisher, 1973). In support of this theory, Zanjani et al. (1971) have shown that during hypoxic stress circulating levels of the substrate fluctuate significantly and that these changes precede similar changes in serum Ep concentrations. While the erythroenin-Ep mechanism is reminiscent of the renin-angiotensin system, renin and erythroenin have different chemical and physiological properties (Contrera and Gordon, 1966; Zanjani et al., 1967b; Zanjani et al., 1967a; Varkarakis et al., 1976) and therefore cannot be assumed to be the same substances.

Although this hypothesis is supported by a considerable amount of experimental data, other theories have also been proposed which offer an alternative to the erythroenin-substrate mechanism of Ep production. One of these suggests that Ep is in fact present in the kidney but that it is bound to an inhibitor which renders it inactive. In this regard, Erslev and Kazal (1968) confirmed earlier reports that kidney extracts contain a factor capable of inactivating human urinary Ep in vitro. It was also observed that such a factor could be extracted in minute quantities from livers and spleens as well. Further studies by Erslev et al. (1971, 1972) indicated that this factor, isolated from kidneys, is a lipid. This renal lipid inhibitor was shown to inactivate Ep, and it was proposed that Ep is stored in

the kidney bound to a lipid substance. Furthermore, recovery of active Ep from this complex can be observed in the presence of plasma protein(s) of as yet undefined nature. It was suggested that during periods of hypoxic stress an increase in the synthesis and/or release of the plasma proteins leads to the enhancement of Ep release from the lipid-Ep complex. These two theories are somewhat modified and incorporated into a third theory proposed by Peschle et al. (1975). They suggested that the REF-Ep system is similar to the pro-insulin-insulin system in that the biogenesis of renal Ep involves the activation of an inactive, kidney produced, proerythropoietin molecule by a serum factor.

In addition to these theories, there is increasing evidence that the kidney may also produce and release at least some quantity of active hormone. Busuttil et al. (1971) treated anemic and normal human kidney tissue with antibody against human urinary Ep and fluorescent labeled antiglobulin in order to determine the site of production of Ep in the kidney. Intense fluorescent staining of visceral epithelial cells of glomerular tufts was observed in anemic human kidney tissue. Similar but less intense staining was observed in normal human kidney tissue. The tubules, collecting ducts, interstitium, juxtaglomerular

apparatus, parietal layers of Bowman's capsule, and the peritubular capillaries did not show any fluorescent labeling. The specific staining observed suggests that Ep is either produced or stored in the glomerulus. In addition, Burlington et al. (1972) have reported Ep activity in the culture medium of long-term tissue cultures of goat glomeruli. Although these authors provided evidence against the involvement of activator agents in their system, serum was used in the culture media, thus raising the possibility of an REF-serum substrate mechanism. Perhaps the most convincing evidence that the kidney produces the active form of Ep was reported by Erslev (1974). He subjected rabbits to hypoxia for three hours and then removed their kidneys and perfused the organs in vitro. These so-called "programmed" kidneys, first described by Zanjani et al. (1971), produced Ep even when the perfusion fluid contained no plasma proteins. Furthermore, since puromycin blocked the formation of Ep, it was proposed that the Ep released by these programmed kidneys was not stored and released, but rather produced by the kidneys in response to hypoxia.

While in some species the kidney appears to be the sole site of Ep production (e.g., the dog; Naets, 1960a,b), extra-renal sources of Ep production appear to be present in other species. In this regard, erythropoiesis continues

in anephric man (Nathan et al., 1964), nephrectomized mice respond to phenylhydrazine-induced anemia by Ep production (Jacobson et al., 1959), and bilateral nephrectomy in the rat does not completely suppress the rise in plasma Ep levels in response to hypoxic hypoxia (Mirand and Prentice, 1957; Goldwasser et al., 1958; Fried, 1972). The production of extra-renal Ep is related to the intensity of the hypoxic stimulus in the rat (Fried et al., 1969). Recent studies indicate that the liver is a major source of extra-renal Ep. Reissmann and Nomura (1962) perfused hypoxic rabbit livers in vitro and found that the perfusate contained very high erythropoietic activity. This work was supported by Fried (1972) who reported that nephrectomized rats with 80% of their livers removed prior to being made hypoxic had barely detectable Ep levels. Most recently, Schooley and Mahlmann (1974) have shown that, if nephrectomized rats are injected with lead, which causes severe anemia and renal tubular dysfunction in the intact animal, the production of extra-renal Ep by the liver is increased after hypoxic exposure. It appears that the reticuloendothelial system or Kupfer cells may be the site of production of Ep in the liver (Peschle et al., 1975). In addition to the liver, the spleen has also been suggested to be a site of extra-renal Ep production (de Franciscus et al., 1965; Kaplan

et al., 1973). Although extra-renal Ep production under stressful conditions in anephric animals is a well-documented phenomena, it has not yet been determined whether or not extra-renal Ep is produced by the intact, unstressed animal.

Erythrocytes are derived from a population of cells within the bone marrow, which is capable of extensive proliferation. As a result of this capacity, these cells are able to replenish their own numbers when they are depleted by injury or physiological conditions and can also lead to the production of mature cells with specific functional characteristics. These cells can also give rise to granulocytes and megakaryocytes and are, therefore, called pluripotent stem cells (McCulloch, 1970).

Although the pluripotent stem cell has not been morphologically characterized, it is believed to be morphologically similar to those of mature lymphocytes (Hillman and Finch, 1974). The existence of these cells can be confirmed by intravenously injecting previously irradiated mice (Till and McCulloch, 1961) or genetically anemic mice of genotype W/W^V (McCulloch et al., 1964) with a suitable number of normal mouse bone marrow cells and observing, nine to fourteen days later, the formation of macroscopic colonies of hematopoietic tissue on the spleens of the recipient mice. These colonies of cells are called spleen

colonies, and, therefore, the pluripotent stem cell which gives rise to them is often called the colony-forming unit (CFU or CFU-s).

Becker et al. (1973) used ionizing radiation to cause chromosomal damage and hence the formation of chromosomal markers in donor bone marrow cells. When spleen colonies formed by these cells were examined, a number of them contained radiation induced chromosomal markers. In each colony in which such markers were found, they proved to be unique and were present in all metaphase cells within the colony. This confirmed the hypothesis, derived indirectly from growth curve studies, that spleen colonies are clonal in nature, and, therefore, each individual colony is derived from one stem cell.

Several methods have been used to demonstrate that the CFU-s is pluripotent in nature. Firstly, histological sections of spleen colonies have shown that early in colony growth a single class of differentiated cells is recognizable in most colonies; however, at later times all three classes of differentiated cells can be identified in many spleen colonies (Curry and Trentin, 1967; Lewis and Trobaugh, 1964; Bleiberg et al., 1967). Secondly, when single cell suspensions were made of individual colonies, it was shown through peroxidase staining and radioactive iron incorporation studies that

both granulocytes and erythrocytes, respectively, are often present in the same colony (Fowler et al., 1967). Final conclusive evidence for the pluripotent nature of these cells was provided by Wu et al. (1967) through the use of radiation induced chromosomal markers. In these studies, a number of colonies each exhibited a different and unique chromosomal marker which could be observed in granulocytic, megakaryocytic, and erythrocytic cells within the same colony. In addition, these pluripotent cells can also give rise to cells capable of producing new spleen colonies when injected into appropriate recipient mice. They, therefore, are said to be capable of self-renewal (McCulloch and Till, 1964) and fulfill the important requirement for classification as a stem cell.

Bruce and McCulloch (1964) observed that hypoxia increased red cell production in the mouse spleen, but at the same time decreased the size of the splenic stem cell compartment, as evidenced by decreased transplantation potential of the CFU-s. It was suggested that this decrease in repopulating ability by the spleen was due to the existence of an intermediate precursor compartment being repopulated by the CFU-s compartment which in turn reduced the number of cells available for transplantation. In addition, they suggested the existence of an Ep-sensitive precursor cell

separate and distinct from the colony forming cell. The existence of such intermediate stem cell compartments (one each for erythroid, myeloid, and megakaryocytic cell lines) has been confirmed by methods which inhibit the replication of one or more, but not all of the stem cell compartments such as vincristine (Stohlman et al., 1968) and thymidine suicide (Lajtha et al., 1969). As a result of these studies, a model for the control of proliferation and differentiation of hemopoietic precursor cells was proposed by McCulloch (1972).

According to this model, the CFU-s are normally in a steady state with the majority of cells in a non-proliferative G_0 state. These cells can be triggered into either a proliferative cycle leading to cell division, or into "differentiation" into a committed stem cell line. The nature of proliferation control of the CFU-s is not known; however, the existence of the W/W^v and $S1S1^d$ (McCulloch et al., 1964, 1965) genetic mutants carrying "cell-defect" and "milieu-defect," respectively, implies the existence of some form of genetic regulation of this step in normal hematopoiesis.

Ep has been shown to regulate erythropoiesis by acting on the cells of the erythroid committed compartment called Ep-responsive cells (ERC) (Schooley, 1969). Its action appears to be threefold in that: (1) it can stimulate the

proliferation of the ERC (Reissmann and Udupa, 1972),

(2) it can enhance the differentiation of ERC into morphologically recognizable erythroid cells (Fisher, 1962; Hodgson and Eskuche, 1962; Gallagher et al., 1963), and

(3) it may also be responsible for the early release of reticulocytes from the bone marrow into circulation (Stohlman, 1959; Gallagher and Lange, 1962; Gordon et al., 1962). It also appears likely that the ERC are derived as the result of a partial differentiation of the CFU-s into a unipotential erythroid stem cell, which is at least partially self-maintaining in nature, and that these ERC can be induced by Ep to differentiate into proerythroblasts (Lajtha et al., 1969). Obviously, during accelerated erythropoiesis, a greater number of ERC is forced into differentiation, and, therefore, if the ERC population is to be sustained, the greater cell outflow must be balanced either by an increased inflow of cells from the CFU's compartment or by an increased self-replication of the ERC.

The exact mechanism and regulation of this important step in erythropoiesis is not as yet certain; however, the alkylating agent, busulfan (Myleran), has been used to selectively inhibit the proliferation and differentiation of the CFU-s compartment. These studies (Reissmann and Samorapoompichit, 1970; Reissmann and Udupa, 1972) have

shown that, following treatment with Myleran, ERC regeneration can be accelerated and maintained by administration of repeated injections of Ep. The inability of the CFU-s to proliferate and/or differentiate under the conditions of these studies indicates that the regeneration of the erythroid compartment is due to increased proliferation of a population of ERC in response to Ep.

Recently it has been shown in the in vitro plasma clot culture system (Axelrad et al., 1974) that the ERC compartment is made up of at least two separate populations of Ep-responsive cells which are capable of giving rise to erythroid colonies in culture. The early developing but small (25-50 cells) colonies presumably originate from existing pronormoblasts, and the later developing "bursts" of (100-200 cells) erythroid colonies presumably originate from cells which are more immature at the time of initiation of the culture. The cells giving rise to the early-formed colonies are called CFU-e, while those giving rise to the "burst" colonies are called the BFU-e. That the BFU-e and CFU-e are in fact two separate cells has been confirmed by the ability to separate them from one another and other bone marrow constituents by the velocity sedimentation method of cell separation (Heath et al., 1976).

The mechanism through which Ep exerts its effect is at present the subject of much investigation. It is generally accepted that Ep acts on the Ep-responsive cell to stimulate synthesis of RNA which in turn leads to increased protein synthesis in these cells. Exposure to Ep also leads to increased DNA synthesis and mitosis (Marks and Rifkind, 1972; Bank et al., 1970; Rifkind et al., 1971; Cantor et al., 1972; Necheles et al., 1968), as evidenced by an increase in the mitotic index of erythroid cells (Matoth and Kaufman, 1962; Paul et al., 1973).

The biochemical events occurring in Ep-responsive cells following exposure to Ep have been studied in vitro in rat bone marrow cells (Goldwasser and Kung, 1972). These cells have been shown to contain small amounts of 150S RNA within five minutes after exposure to Ep. This RNA is found in the nucleus, has a very short half-life of about six minutes, and is not present in control cells. Shortly after the formation of the 150S RNA, another species of RNA (60-70S) is seen with a half-life of several hours. The role of this RNA in differentiation is uncertain. Within fifteen minutes after exposure to Ep, r-RNA, t-RNA, an "iron effector" which allows iron to affect cellular function, and a 9S RNA (probably a globin messenger) are found within the cell (Gross and Goldwasser, 1970). Fetal liver cells

in vitro also exhibit increased RNA synthesis following incubation with Ep (Nicol et al., 1972). The RNA synthesis occurs predominantly in the proerythroblast compartment (Paul et al., 1973).

Erythropoietic tissue responds to Ep prior to cell replication. DNA synthesis in adult bone marrow cells does not increase until about two hours following exposure to Ep. Studies by Maniatis and Marks (1974) seem to indicate that DNA synthesis is not necessary for RNA synthesis but may be required for the stimulation of globin synthesis by Ep. In this regard, Datta and Dukes (1975) have shown that inhibition of DNA synthesis does not prevent differentiation of precursor cells into mature erythrocytes, although it does decrease the number of cells which do so. They have suggested that the mitosis stimulated by Ep allows the genes of these cells to be "reprogrammed" to initiate globin m-RNA synthesis and subsequent globin synthesis.

By four hours, new proteins are being synthesized. These proteins appear to influence the uptake of iron from the medium. The uptake of glucosamine and the formation of glycolipid (Dukes, 1968) and stroma (Dukes et al., 1964) do not occur until five hours after exposure to Ep. The induced cells begin to show increased hemoglobin synthesis within the next hour (Gross and Goldwasser, 1969). It

should be noted that, in polycythemic animals, hemoglobin synthesis is not seen until ten to twelve hours after exposure to Ep. Since the polycythemic bone marrow is devoid of any differentiated cells, it must be assumed that, in this instance, Ep is acting on a less differentiated cell. This is supported by the observation that treatment of fetal liver cells with Ep causes a flow of cells from the proerythroblast compartment to the basophilic erythroblast compartment (Paul et al., 1973).

Recent evidence (Spivak, 1976) indicates that Ep stimulates incorporation of uridine into RNA species with the sedimentation coefficients of 150, 55-65, 45, 28, 18, 9, 6, and 4, with the 150S RNA being formed first. These changes are due to the sequential activation by Ep of multiple forms of nuclear RNA polymerase (Piantadosi et al., 1976). In addition, Ep stimulates the synthesis of histone and non-histone proteins in the spleen of exhypoxic polycythemic mice prior to the appearance of morphologically identifiable erythroblasts in the spleen (Spivak, 1976).

In addition, Ep appears to play a role in the induction of enzymes required for the synthesis of heme. Hemoglobin is synthesized through most of the maturation process of

the red blood cell, about 95% being made before the nucleus is extruded and 5% in the reticulocyte stage (Labardini et al., 1973). Normal hemoglobin production is dependent on an adequate iron supply, as well as the synthesis of protoporphyrin and globin. Porphyrin synthesis begins in the mitochondria with the formation of delta-aminolevulinic acid (ALA) and continues in the cytoplasm with the combination of two molecules of ALA to form porphobilinogen (PBG). Four molecules of PBG are converted into uroporphyrinogen and coproporphinogen. The final steps, carried out in the mitochondria, involve the formation of protoporphyrin and the incorporation of iron to form heme. The most important rate limiting step in this pathway is the conversion of succinate and glycine to delta-aminolevulinic acid, a reaction catalyzed by ALA synthetase (Wintrobe, 1972).

This step in the formation of hemoglobin was shown by Bottomley and Smithee (1969) to be regulated by Ep by demonstrating that rabbit bone marrow cells in vitro synthesized increased amounts of delta-aminolevulinic acid synthetase (ALAS) when exposed to Ep. Furthermore, actinomycin D and puromycin prevented increased enzyme activity in response to Ep indicating that DNA dependent m-RNA

synthesis and subsequent protein synthesis were necessary for enzyme activity. The mechanism of early stimulation of RNA synthesis by Ep is unknown; however, there is some evidence that cyclic AMP may be involved. In this regard, Bottomley et al. (1971) demonstrated that c-AMP stimulated ALAS activity of rabbit bone marrow cells in vitro. Furthermore, the effects of Ep and cyclic AMP were synergistic and could be enhanced by theophylline. Cyclic AMP was also shown to exert an erythropoietic effect in vivo in polycythemic mice, and administration of c-AMP and Ep to polycythemic mice resulted in an additive effect on erythropoiesis.

Our present knowledge about Ep, the hormonal regulator of erythropoiesis, has come a long way since the days of Carnot and Deflandre (1906), but there are still many unanswered questions about this hormone and other regulators of erythropoiesis. It is now becoming apparent that factors other than Ep may also influence erythropoiesis. Examples of this can be seen in the pathological condition of polycythemia vera and in the normal erythropoiesis of the mammalian fetus.

Polycythemic states in man may be related to erythropoietin production. In those cases of secondary polycythemia where an increased red cell mass is appropriate to a physiologic (i.e., hypoxic) stimulus, basal urinary Ep levels

are either normal or increased, and phlebotomy results in markedly increased Ep excretion (Adamson, 1968). Examples of this include high altitude residence (Van Dyke et al., 1966), pulmonary disease with hypoxia (Shaw and Simpson, 1961), abnormal hemoglobin with increased oxygen affinity (Adamson and Stamatoyannopoulos, 1966), and cyanotic heart disease, most often congenital (Stroebel and Fowler, 1956).

An enigmatic category of erythrocytosis, termed paraneoplastic (Thorling, 1972), relates to those associated with certain tumors and cysts in man. These include renal adenocarcinomas and cysts, cerebellar hemangioblastomas, hepatomas, adrenal cortical adenomas, and uterine leiomyomas. In these states, elevated quantities of Ep are detected, on occasion not only in the tumor extract and/or cyst fluid, but in the plasma as well (Krantz and Jacobson, 1970; Thorling, 1972; Wrigley et al., 1971; Ossias et al., 1973). Although it is possible that these tumors are extracting Ep from the plasma and are merely storing this hormone, it would seem more likely that they are producing it, especially since removal of the neoplasm or cystic mass is often followed by regression of the polycythemia and the disappearance of Ep from the plasma. That it is actually Ep which is being produced by the tumors receives credence

from the finding that the erythropoiesis stimulating activity is completely neutralized by anti-Ep serum (Ossias et al., 1973).

The mechanism by which these neoplasms engage in inappropriate Ep production remains undetermined. One possibility is that, in the process of neoplastic transformation, the tumor cells, by depression of a genome, commence the production of a glycoprotein or some active biological core which resembles Ep chemically and possesses erythropoiesis stimulating activity (Thorling, 1972). A marked drop in hematocrit (from 64% to 39%) in a patient with erythrocytosis associated with a cerebellar hemangioblastoma did not result in any alteration in the amount of Ep produced, as judged by the urinary output, suggesting that autonomous production of Ep occurs in these neoplastic states (Adamson, 1968). Of interest are recent observations (Zanjani et al., 1972) that plasma from patients with tumor-associated erythrocytosis is considerably more active in releasing exogenous Ep bound to a lipid inhibitor than are plasmas obtained from normal humans. This finding raises the question as to whether the heightened plasma Ep levels noted in these paraneoplastic states arise not only from hyperproduction by the tumor but also from release of Ep from storage sites.

In contrast to the secondary polycythemia, primary polycythemia or polycythemia rubra vera (PV), a panmyelopathy, is not associated with increased levels of Ep. Polycythemia vera is one of the group of diseases of the blood forming tissue characterized by an increased proliferation of hematic and stromal cell lines derived from the multipotent primitive mesenchymal cell. The natural evolution of the disease ranges from a "benign neoplastic" orderly proliferation of cells (a panmyelosis) with preservation of cellular differentiation and maturation through the "spent phase" of myeloid metaplasia and myelofibrosis resembling, yet biologically distinct from, chronic myelocytic leukemia (Glasser and Walker, 1969) to a rapidly progressive stage of marrow decompensation with or without the appearance of a leukemoid syndrome that resembles acute leukemia (Wasserman, 1954; Lawrence et al., 1969). Increased erythroid activity is normally found in the early stages of the disease, as represented by an elevated red cell mass, accelerated but otherwise normal ferrokinetics, normal red cell survival, normal skeletal distribution of the active marrow compartment, and normal red blood cell, white cell, and platelet morphology in the peripheral blood. Varying degrees of megakaryocytic and myeloid hyperplasia are reflected in modest elevations of the platelets and

granulocytes. With time, varied and changing patterns of cytopathology occur in both intra- and extra-medullary sites. Increasing heteroplastic blood formation in the spleen and liver is associated with disturbed red cell kinetics (Pollycove et al., 1966), shortening of red cell survival and morphologic characterization of the increasing extra-medullary blood formation by the presence of oval, tear-drop, and bizarre-shaped erythrocytes, a leuko-erythroblastic blood picture, and an anemia. An accelerated malignant phase may supervene, blast forms may begin to proliferate in increasing numbers resulting in a rising white blood cell count, a progressive anemia and thrombopenia. The enlarging spleen can become the site of frequent infarction with fever and infection; and the terminal picture, occurring in 10-15% of cases, is indistinguishable clinically and hematologically from acute or subacute myeloblastic leukemia (Wasserman, 1954; Lawrence et al., 1969).

The failure to demonstrate a direct association between increased Ep titres and augmented erythropoiesis in polycythemia vera has led to a search for alternative explanations. Suggestions that the elevated numbers of circulating red cells in polycythemia vera result from the differentiation

of a clone of hematopoietic precursor cells which are not under the usual regulatory influences of Ep are based on the following reported observations: (1) unlike the secondary polycythemia, where increased production of Ep has been shown to accompany and be responsible for the elevated red cell mass (Ossias et al., 1973; Adamson and Finch, 1968; Gurney, 1973), increased levels of this hormone cannot be demonstrated in the plasma or urine of patients with polycythemia vera (Adamson and Finch, 1968; Adamson, 1968; Adamson, 1970). In most cases, urinary excretion of Ep is below normal (Adamson and Finch, 1968; Adamson, 1968; Adamson, 1970). This can be attributed to feedback inhibition by the increased circulating red cell mass (Adamson, 1968; Gordon et al., 1966), since the ability to produce Ep is not impaired in polycythemia vera (Adamson, 1970), but a reduction in red cell mass to normal levels is required for its demonstration (Adamson, 1970); and (2) bone marrow cells obtained from donors with polycythemia vera during relapse, when cultured in suspension, failed to respond to Ep to the same degree as normal marrow cells or cells derived from patients with secondary erythrocytosis (Krantz, 1968; Zucker et al., 1972).

Other evidence has been cited indicating that a population of normal cells (i.e., erythropoietin-responsive)

is also present in polycythemia vera (Krantz, 1968; Golde and Cline, 1973; Prchal and Axelrad, 1974). The existence of normal hematopoietic precursor cells with normal responses to Ep in polycythemia vera was shown by Krantz (1968). He found that suspension cultures of bone marrow cells obtained from patients with polycythemia vera during a busulfan-induced remission phase responded normally to erythropoietin. More recently, studies employing the plasma clot culture procedure which is more conducive to growth and differentiation of erythroid cells have shown that these cells are also present during the active phase of the disease.

The plasma clot culture system originally described by Stephenson et al. (1971) and modified by McLeod et al. (1974) permits the growth and differentiation of erythroid precursor cells in vitro. In this system bone marrow cells from normal animals (McLeod et al., 1974) and man (Tepperman et al., 1974), as well as Friend leukemia cells (Goldstein et al., 1974) cultured with Ep, form discrete colonies of hemoglobin-synthesizing cells. A direct relationship between the number of erythroid colonies formed and Ep concentrations has been established (McLeod et al., 1974). As was the case with the development of the in vitro culture technique for the study of the regulation of white blood cell formation (Iscoe et al., 1971), the technique for

the colony growth of erythroid cells in vitro now permits an accurate and meaningful assessment of the changes in the rate of formation of red blood cells and/or nature of these changes under a variety of clinical conditions.

When bone marrow cells from polycythemia vera patients in relapse were cultured in the plasma clot culture system, a significant response to Ep was noted (Prchal and Axelrad, 1974). The number of Ep-dependent colonies formed was directly proportional to the number of marrow cells cultured. Experiments by Golde and Cline (1973) using a liquid culture method yielded similar results. It should be noted, however, that, whereas normal bone marrow cells are totally Ep-dependent in these culture systems (Golde et al., 1974; Iscove et al., 1974), polycythemia vera bone marrow cells are not and continue to exhibit some erythroid activity in the absence of added Ep (Golde and Cline, 1973; Prchal and Axelrad, 1974). Addition of Ep to the latter increases the number of erythroid colonies. If, as has been suggested, each erythroid colony arises from a single precursor cell (McLeod et al., 1974; Iscove et al., 1974; Gregory et al., 1973), then these results suggest that the number of precursor cells with a "normal" dependence upon Ep exceeds those with altered characteristics, assuming equal plating efficiency. In the study by Prchal and Axelrad (1974),

the ratio of endogenous erythroid colonies to colonies formed in the presence of added Ep varied from patient to patient but did not exceed 37%.

The existence of precursor cells responsive to Ep in polycythemia vera is further shown by in vivo studies. Patients with polycythemia vera responded to anemia (Stohlman, 1966) and hypoxia (Bomchil et al., 1967) by increased red cell production. This increase occurred only in those patients in whom a rise in Ep production was detected (Adamson, 1970). The extent to which these normal (i.e., Ep-responsive) precursor cells contribute to the increased red cell mass in polycythemia vera remains a perplexing problem. In secondary polycythemias, where only normal precursors are thought to be present, the development of plethora is accompanied by, and dependent upon, elevated levels of Ep (Thorling, 1972). In polycythemia vera, on the other hand, high concentrations of Ep are not required for red cell formation (Gurney, 1973), which raises the question as to whether red cell production in polycythemia vera depends solely on the activity of the altered clone of cells. The presence of a relatively large population of Ep-responsive hematopoietic precursor cells in polycythemia vera (Golde and Cline, 1973; Prchal

and Axelrad, 1974) seems to rule out such a possibility. It is possible that the low concentrations of Ep present in polycythemia vera (Adamson and Finch, 1968) may be adequate for stimulating red blood cell production in this disorder. Moreover, there is still the possibility that factor(s) other than normal Ep may exist in polycythemia vera that can affect erythrocyte formation.

Recently, several investigators have reported that such a stimulatory factor capable of influencing red cell production may be present in the serum of patients with polycythemia vera (Reisner, 1967; Reisner, 1970; Robinson and Ward, 1972; Ward and Robinson, 1972; Ward et al., 1971; Zanjani et al., 1971; Zanjani et al., 1972). Reisner (1967, 1970), using serum from these patients, was able to show stimulation of heme synthesis in cells present in explants of normal human bone marrow. This factor also stimulated in vitro granulopoiesis to a lesser degree, and was not affected by treatment with anti-Ep, storage, or heat (Reisner, 1967; Reisner, 1970). The factor in polycythemia vera serum was also species restricted in the exhibition of its effect. Thus, no stimulation of heme synthesis occurred when polycythemia vera serum was added to cultures of canine bone marrow cells (Reisner, 1967; Reisner, 1970). This is in accord with the observations

of Krantz (1968) who was unable to demonstrate a direct stimulatory effect of polycythemia vera serum on murine bone marrow cells in vitro. Reisner (1970) attributed the inability to demonstrate erythropoiesis-stimulatory activity of polycythemia vera serum in polycythemic mice to species variations. However, evidence suggests that the failure to demonstrate such activity may relate more to the erythropoietic state (with regard to Ep levels) of the recipient animal than to the existence of species specificity. In this regard, Ep production, and hence erythropoiesis, is almost totally suppressed in polycythemic mice (Filmanowicz and Gurney, 1961). The absence of significant Ep production in mice rendered polycythemic by transfusion with red cells or exposure to hypoxia is the main feature for use in the Ep assay (Filmanowicz and Gurney, 1961). In earlier studies with polycythemia vera serum, employing assay animals in which Ep production and erythropoiesis were not markedly suppressed, several investigators observed increased erythropoietic activity in these sera (Contopoulos et al., 1957; Korst et al., 1959). More recently, a factor capable of stimulating erythropoiesis in both normal and polycythemic mice was demonstrated to be present in sera of some patients with polycythemia vera (Ward et al., 1971; Zanjani et al., 1972).

When separate groups of normal mice were given daily injections of sera from different individuals with polycythemia vera, over the course of several weeks, red cell production increased in groups of mice injected with serum from some patients. By the fifth week of treatment the animals had become plethoric. Continued administration of polycythemia vera serum was necessary, however, for the maintenance of the plethoric state, and the hematocrit returned to normal following the discontinuation of the serum injections. No significant effect on erythropoiesis was seen with a similar number of injections of normal human serum (Zanjani, 1976). Unlike the suppression of erythropoiesis that occurs in hypoxia-induced polycythemic mice within three to five days of removal from hypoxia, approximately eight days were required for feedback inhibition of erythropoiesis after cessation of polycythemia vera serum injections (Zanjani, 1976). Whether this is related to longer (than Ep) persistence of the factor from polycythemia serum in these mice remains unclear. Not all polycythemia vera sera exhibited erythrostimulatory activity in these studies, but nearly half of the sera from patients with polycythemia vera did contain the factor.

The factor in polycythemia vera serum does not appear to be antigenically similar to Ep, but data suggest that

Ep is required for the manifestation of its effect in vivo (Zanjani, 1976). Thus, the development of plethora in normal mice treated with active polycythemia vera serum was prevented by the simultaneous administration of anti-Ep to these animals. Mice receiving injections of immune serum (prepared in rabbits against human urinary Ep), in addition to polycythemia vera serum for two weeks, failed to demonstrate elevated hematocrits as compared to mice receiving normal rabbit serum and polycythemia vera serum (Zanjani, 1976). Anti-erythropoietin is known to inhibit erythropoiesis by its ability to neutralize circulating Ep (Schooley et al., 1968; Zanjani et al., 1974). The dependence of the factor in polycythemia vera serum on Ep is further indicated when the effect of this factor on erythropoiesis in polycythemic mice was examined (Ward et al., 1971). Thus, the stimulatory nature of polycythemia vera serum becomes apparent only when exogenous Ep is also administered to these mice. This enhancing effect does not appear to be mediated through an increase in the production of Ep in the host animal, a mechanism by which some androgenic hormones are known to influence red blood cell production (Mirand et al., 1965; Schooley, 1966). There was no increase in plasma Ep levels in polycythemia vera serum treated mice (Zanjani et al., 1971).

There are indications that the factor in serum of patients with polycythemia vera may operate by altering the proliferative characteristics and numbers of the hematopoietic precursor cells. Femoral bone marrow cells obtained from polycythemia vera serum treated mice contained significantly greater numbers of pluripotent stem cells (CFU-s) and cells capable of giving rise to leukocyte colonies in soft agar (CFU-c) than cells from mice treated with normal human serum. These cells also exhibited a greater response to Ep in vitro than bone marrow cells obtained from normal serum treated animals. The effect of serum from donors with polycythemia vera and myeloid metaplasia on granulocyte colony formation by mouse bone marrow cells was also investigated by Robinson and Ward (1972). They reported that, when compared with injections of normal human serum or saline given to mice for four days, polycythemia vera serum increased the numbers of bone marrow cells capable of giving rise to granulocytic colonies, although the production of colony stimulating activity in these mice was unaffected. Similarly, an enhancement of the in vitro response to Ep by these bone marrow cells was noted following treatment of mice with polycythemia vera serum. These results suggest that the factor in polycythemia vera serum is capable of expanding the erythroid (CFU-e)

and myeloid (CFU-c) precursor cell compartments.

By affecting all hematopoietic precursor cell compartments in mice, the factor in polycythemia vera serum is, in effect, influencing the production of not only red blood cells but also white blood cells. Studies in the mouse, however, suggest that, for the increase in red cell production to occur, Ep is also required (Zanjani, 1976). It is not unreasonable, however, to assume that the effect of the factor in polycythemia vera serum on the production of these white blood cells may require the presence of a physiological regulator, such as leukopoietin.

That the myelostimulatory factor in polycythemia vera serum acts by increasing the numbers and/or sensitivity of the hematopoietic precursor cells is further supported by the observation that it fails to influence hemoglobin synthesis in short-term cultures of human and murine bone marrow cells (Krantz, 1968). An effect on the precursor cell compartments would presumably require more time than is allowed in the suspension culture system. This is especially true if the polycythemia vera serum effect is through a common stem cell as has been suggested by Robinson and Ward (1972). Moreover, the culture system must permit cellular proliferation and differentiation over

a relatively long period of time. The plasma clot culture system (McLeod et al., 1974; Iscove et al., 1974; Schooley et al., 1968) is more suited to this type of approach than the Krantz (1968) cell suspension procedure.

Most studies concerned with the regulation of blood cell production in polycythemia vera have focused on the erythroid compartment. However, any hypothesis emerging from these studies must also include an explanation for the increased production of white blood cells and platelets in this disorder, since it is unlikely that the basic defect is different for the three cell types. There is little doubt that polycythemia vera is a disorder of proliferation (Wasserman and Gilbert, 1966), but the pathogenesis of this disorder is obscure. A change in the pluripotent stem cell could conceivably account for the observed deviation from normal hematopoiesis, if we were to assume that blood cell production in polycythemia vera is independent of physiological regulators. However, such an alteration would require not only increased differentiation of the common stem cell into the committed pools, but also autonomous differentiation of these pools into the recognizable hematopoietic cell lines. The demonstration of a response to Ep both in vivo (Adamson, 1970;

Stohlman, 1966; Bomchil et al., 1967) and in vitro (Golde and Cline, 1973; Prchal and Axelrad, 1974; Zanjani et al., 1975; Zanjani, 1976) makes it unlikely that the proliferation and differentiation of these cells occurs without the participation of the known physiologic regulators such as Ep or that a residual "normal" population exists. It is now an established fact that the production of Ep proceeds at a reduced level in polycythemia vera (Adamson and Finch, 1968). However, high levels of the hormone may not be necessary for red cell production in this disease.

In the absence of a suitable animal model, it is unlikely that such information will become available in the near future. However, recent studies concerned with the regulation of hemopoiesis in the mammalian fetus (Zanjani et al., 1974; Zanjani, 1971; Zanjani and Gordon, 1971; Zanjani et al., 1973; Zanjani et al., 1974) indicate that cells obtained from hemopoietic tissues of fetal sheep and cultured in vitro exhibit characteristics similar to bone marrow cells from patients with polycythemia vera.

As in the mammalian adult, Ep plays a major role in the regulation of erythropoiesis in the mammalian fetus. Kaiser et al. (1958) reported that exposure of pregnant ewes to high altitude for prolonged periods of time resulted in the delivery of polycythemic lambs. Shortly thereafter,

Finne (1964, 1967) reported the presence of erythropoietic stimulating activity in cord blood, amniotic fluid, and urine of normal and premature infants. Since this erythropoietic stimulating activity was found to be greater in cord blood and amniotic fluid from anemic human fetuses than normal fetuses, it was suggested that Ep or an Ep-like substance is responsible for this effect. Reports that heme synthesis by fetal hematopoietic tissue in vitro could be enhanced and maintained by Ep (Gallien-Lartigue, 1966; Cole and Paul, 1966) provided further support for the theory that Ep regulates fetal as well as adult erythropoiesis.

Unfortunately, the small size of fetuses of laboratory animals such as rats or mice made direct access to these fetuses in vivo difficult. Therefore, early studies on the mechanism(s) regulating fetal erythropoiesis centered around the manipulation of the erythropoietic status of the pregnant animal followed by observations of the effects these procedures had on the fetus.

In this regard, Schooley et al. (1968) treated pregnant rats with antibody prepared in rabbits against Ep and observed that this treatment resulted in the suppression of erythropoiesis in not only the mothers but in some of the fetuses as well. It was, therefore, concluded that

Ep was involved in the regulation of fetal erythropoiesis, although it was not certain whether the suppressive effect of anti-Ep was due to its influence on maternally produced Ep, presumably also available to the fetus, or Ep derived from sources within the fetus.

Matoth and Zaizov (1971) subjected pregnant rats to hypoxia induced by decreased atmospheric pressure and phlebotomy. These procedures resulted in increased production of Ep by maternal kidneys and were accompanied by significant increases in both maternal and fetal erythropoiesis. It was suggested that the rise in fetal erythropoiesis resulted from entry of maternally produced Ep into fetal circulation in these animals (Matoth and Zaizov, 1971). However, studies designed to determine whether Ep can traverse the placental barrier in rats contradicted this conclusion. Thus, Matoth and Zaizov (1971) demonstrated that large doses of exogenous Ep administered to pregnant rats failed to affect fetal erythropoiesis. It has also been shown (Zanjani and Gordon, 1971; Zanjani et al., 1974a) that the induction of high Ep levels in fetuses does not produce an increase in maternal Ep levels. These studies, therefore, indicate that the increase in fetal erythropoiesis in response to maternal hypoxia occurred independently of maternal Ep formation.

Jacobson et al. (1959) were the first to document the ability of the fetus to independently regulate its own erythropoiesis. These investigators observed that hypertransfusion of pregnant mice with red blood cells resulted in the inhibition of maternal but not fetal erythropoiesis. However, until the advent of surgical procedures which allowed the direct manipulation of the fetal erythropoietic status, it was impossible to confirm these findings. Recently, surgical techniques have been developed in large animals such as sheep and goats, which enable the direct manipulation of fetal erythropoiesis by means of catheters inserted into the femoral arteries of the fetus. That this procedure does not interfere with the normal development of the fetuses in utero is evidenced by the ability to maintain these fetuses until partuition at which time normal live animals have been delivered.

Using this surgical technique, Zanjani et al. (1969) induced anemia in fetal sheep by administering repeated doses of phenylhydrazine hydrochloride directly to the fetus for three weeks. Following treatment, the erythropoietic activity of maternal and fetal plasma was assayed in polycythemic mice. The results of this study indicate that, following the institution of phenylhydrazine treatment in fetal lambs, anemia develops in the fetus, and Ep levels

in fetal but not maternal plasma begin to rise. The possibility that the erythropoietic activity of plasma from phenylhydrazine treated fetuses was due to residual amounts of the chemical still present in the assayed samples was examined by Gordon et al. (1972) by treating plasma samples with anti-Ep prior to assay in polycythemic mice. Since treatment with anti-Ep neutralized the erythropoietic activity of the assayed plasma, it was concluded that the fetal lambs had, in fact, produced Ep in response to treatment with phenylhydrazine hydrochloride. This data provided the first direct evidence that the mammalian fetus is capable of producing Ep.

Similar studies using goat fetuses have shown that hypoxia produced in fetuses by bleeding (Zanjani et al., 1973) is also a stimulus for fetal but not maternal Ep production. Furthermore, following the appearance of increased Ep activity in plasma from these fetuses, increased erythropoiesis was observed, as evidenced by an increased rate of red blood cell radioiron uptake and increased reticulocytosis in anemic fetuses as compared to normal fetuses.

Zanjani et al. (1974a, 1975) have also shown that the rise in red blood cell production in the fetus in response to maternal hypoxia can be attributed to an increase in

Ep production from sources within the fetus. In these studies pregnant goats were made hypoxic by phlebotomy. Just prior to and at regular intervals following phlebotomy plasma samples were obtained from mothers and fetuses to determine plasma Ep levels. The induced anemia produced a significant increase in fetal plasma Ep levels within 15 minutes after the completion of maternal bleeding; however, maternal Ep levels exhibited no change for the first 10 hours and did not increase significantly until 15 hours after the completion of bleeding. Since increased plasma Ep levels in the fetus preceded increased plasma Ep levels in the mother by several hours, it was concluded that the fetus can produce Ep in response to maternal hypoxia and that the fetal system is more sensitive to reductions in maternal red blood cell levels than the mother. It was postulated that this increased sensitivity to hypoxia by the fetus was due to the presence of high affinity hemoglobin in fetal red blood cells which caused a greater hypoxic stress in the fetus than the mother as a result of maternal bleeding.

These techniques have also been used to study inhibitors of fetal erythropoiesis. In this regard, it has been shown that in fetal goats Ep production and hence erythropoiesis

are inhibited by rendering the fetuses polycythemic through hypertransfusion with red blood cells (Zanjani et al., 1973).

The role of Ep in the day to day physiological regulation of erythropoiesis in the fetus has been confirmed, as in the adult, by the use of antibodies produced in rabbits against Ep (Zanjani et al., 1974a, 1975). When anti-Ep is administered to fetal sheep, a significant depression of erythropoiesis, as evidenced by decreased radioiron incorporation by fetal red blood cells, fetal spleens, and fetal bone marrow cells, is observed. There is also a significant decrease in the numbers of circulating reticulocytes and of nucleated erythroid cells in the bone marrows of these fetuses. These effects are not seen in control fetuses treated with normal rabbit serum (Zanjani et al., 1974a, 1975).

In summary, it can be seen that studies involving the direct manipulation of the fetal erythropoietic state indicate that, as in the adult, fetal erythropoiesis is regulated by Ep. Furthermore, the fetus as well as the adult can produce Ep in response to hypoxia. In addition, the fetus has the unique ability to respond to maternal hypoxia through increased Ep production and hence increased erythropoiesis. Also, as in the adult, polycythemia

induced by hypertransfusion inhibits fetal erythropoiesis by inhibiting the production of Ep.

Experiments designed to determine the site of production of Ep in the fetus are similar to those which were employed for the same purpose in the adult, in that they involve the extirpation of organs, followed by induction of anemia, followed by determination of plasma Ep levels. One of the first organs to be tested for its ability to produce Ep was the kidney.

Bilateral nephrectomy of fetal goats failed to inhibit the production of Ep by these fetuses in response to phlebotomy (Zanjani et al., 1974a). The possibility that in the absence of the fetal mechanism to produce Ep the maternal system takes over has been ruled out by bilaterally nephrectomizing mothers as well as fetuses (Zanjani et al., 1974a). These studies have shown that maternal nephrectomy has no effect on Ep levels in the fetus. This indicates that Ep production observed in the nephrectomized fetus does not originate from the mother.

Splenectomy and hepatectomy of fetal goats and sheep have also been studied (Zanjani et al., 1974a, 1975) to determine their role in fetal Ep production. Although splenectomy did not cause any change in the ability of the

fetus to produce Ep in response to bleeding, hepatectomy resulted in a significant reduction of Ep production by the fetus. This inhibition of Ep production by hepatectomy in the fetus was observed regardless of the presence or absence of the kidney. In addition, significant amounts of Ep were found to be present in the perfusates of perfused anemic fetal livers (Zanjani et al., 1975).

The finding that the liver, rather than the kidney, is the primary source of Ep in the fetus is compatible with the structural and functional aspects of fetal circulation, since oxygenated blood returning from the maternal side of the placenta passes through the ductus venosus and partially bypasses the liver (Guyton, 1971). Therefore, the blood which is most fully oxygenated does not appear to go to the liver but rather flows directly to the heart, which pumps it to the liver and the rest of the fetus.

In the mammalian fetus, the production of red blood cells occurs in four distinct phases, the yolk sac stage, the hepatic stage, the splenic stage, and the myeloid stage. In the yolk sac hematopoiesis is generally confined to the blood islands of the yolk sac mesoderm. The cells from the yolk sac which give rise to blood cells are called hemangioblasts (Guyton, 1971).

Although they are mesodermal in origin, they will only develop in the presence of a favorable environment consisting of both endoderm and mesoderm. Evidence for this comes from the fact that erythroblastic differentiation is prevented in cultures where endoderm is not present. If, on the other hand, endoderm is present but separated from mesoderm by a cell impermeable filter, hematopoiesis will occur (Miura and Wilt, 1969).

Two theories have been proposed to explain the progression of hematopoiesis from the yolk sac, to the liver, to the spleen, and finally to the bone marrow. One theory, called the fixed theory, postulates that blood cells are derived from the cells of the tissues in which they are found, implying that each tissue in succession gives rise to a new population of cells which is capable of differentiating into hemopoietic cells. The second theory or cellular migration theory postulates that hemopoietic precursor cells migrate from one organ to another in succession and these cells differentiate when they reach their new environment (Wintrobe, 1974). The second theory seems to be more correct in light of recent observations. In this regard, if hematopoiesis of the fetus is studied in vitro, and the yolk sac is either destroyed or separated from the embryo by a filter which prevents the passage of cells, hepatic erythropoiesis fails to occur.

Although this evidence is not conclusive, the progression of hematopoiesis from the yolk sac to the liver does appear to be dependent upon the migration of cells from the former to the latter organ (Moore and Metcalf, 1970). In addition, once circulation is established, all stages of developing cells that are seen in the yolk sac can be seen in the blood, thus indicating the ability of these cells to migrate (Metcalf and Moore, 1971).

As in the adult, the morphological characteristics of hemopoietic stem cells in the fetus are not known; however, Lucarelli et al. (1968) have suggested that primitive cells with condensed nuclear chromatin and basophilic cytoplasm seen in fetal livers during hepatic erythropoiesis might be erythroid precursors. It is believed that, as in the adult, the recognizable precursors of the various hemopoietic cell lines are derived from pluripotent stem cells. Furthermore, throughout the gestational period, the total population of CFU-s per 10^5 nucleated cells in the hemopoietic organs has been shown to be ten times greater than in the adult (Moore and Owen, 1967a; Moore and Metcalf, 1970). The yolk sac appears to be the source of these pluripotent stem cells (CFU), since cells derived from murine yolk sac, when injected into irradiated mice, have the capacity to repopulate lymphoid organs (Moore and Owen, 1967;

Moore and Metcalf, 1970) and have also been shown to give rise to erythroid, granulocytic, and megakaryocytic colonies in vitro (Moore and Metcalf, 1970).

That Ep-responsive cells exist in the fetus is implied by the in vivo studies discussed earlier; however, the time at which they first appear is controversial. Cole and Paul (1966) reported that Ep is ineffective in the fetus during yolk sac erythropoiesis, whereas Bateman and Cole (1971) were able to detect a response by yolk sac cells to Ep in vitro. There is little disagreement, however, that, as soon as hepatic erythropoiesis commences in the fetus, a response to Ep can be demonstrated (Zanjani et al., in press). Stephenson and Axelrad (1971) have shown that, as in the adult, fetal liver cells which are responsive to Ep (ERC) are different from the pluripotent stem cell and can be separated from the CFU by velocity sedimentation.

In the fetal mouse, hepatic erythropoiesis begins between days ten and eleven of gestation, and hemoglobin containing cells increase in number exponentially, indicating rapid growth of the erythroid compartment, until day fifteen of gestation at which time the population doubling time of these cells increases from eight hours to two days (Paul et al., 1969). The rate of hepatic erythropoiesis continues

to decline until birth; only a few erythroid producing cells are still present in the liver (Silini et al., 1967). With the decline of hepatic erythropoiesis, splenic erythropoiesis begins to be seen. In the fetal mouse, the spleen can be detected by day thirteen of gestation, and active erythropoiesis can be seen in the spleen by day fifteen. By day seventeen, granulopoiesis has almost completely replaced erythropoiesis in the spleen (Cole et al., 1968). In fetal mice the bone marrow cavity forms at 17 days of gestation and then becomes populated by granulopoietic cells (Metcalf and Moore, 1971); however, erythropoiesis is not seen in mouse bone marrow until after birth (Petrakis et al., 1969).

Although fetal erythropoietic tissues have been shown to be responsive to Ep, there is some evidence that these tissues may not be regulated by Ep at all times during fetal life. In this regard, Cole and Paul (1966) and Cole et al. (1968) observed that addition of Ep to cultures of mouse fetal hepatic tissue at less than 15 days of gestation significantly increased hemoglobin synthesis. Although these tissues are still actively synthesizing hemoglobin in culture at 15 days of gestation, treatment of these tissues with Ep no longer effectively stimulates hemoglobin synthesis when added to hepatic tissue from fetuses 15

days of gestation or older in vitro. As mentioned earlier, recent studies have shown that cells obtained from hemopoietic tissues of fetal sheep and cultured in the plasma clot exhibited characteristics similar to bone marrow cells from patients with polycythemia vera. Thus, as with polycythemia vera marrow, cells derived from hepatic tissue at days 30-40 of gestation (normal gestation is 145 days), when liver is the major site of hematopoiesis in fetal sheep, formed erythroid colonies in the plasma clot in the absence of added Ep. Addition of Ep resulted in the formation of greater numbers of such colonies. At 60 days of gestation, splenic cells, and at 125 days bone marrow cells, behaved in a similar manner. An interesting feature of these studies was that cells with these characteristics were present in these tissues for a limited period of time, usually 10 days (liver and marrow), being replaced or altered to normal adult type cell thereafter. For example, bone marrow cells obtained from fetal sheep at 2 day intervals formed erythroid colonies in the absence of Ep from 115-130 days of gestation. Bone marrow cells obtained before day 115 and after day 135 of gestation did not form erythroid colonies in the absence of Ep. Normal adult and pregnant sheep bone marrow cells also failed to form Ep-independent colonies (Poster, 1975).

Determination of the mechanism(s) underlying this change

from what appears to be a relative independence from normal physiologic regulation to dependence on Ep for normal proliferative activity offers exciting prospects for the understanding of factors regulating normal and abnormal cellular proliferation. The similarities of growth and differentiation of bone marrow cells from normal fetuses and of bone marrow cells from patients with polycythemia vera, in the plasma clot culture system, raises many interesting questions. A study of these mechanisms underlying the proliferation and differentiation of endogenous colony forming precursor cells is no doubt important with regard to the possible treatment of individuals with polycythemia vera in the future. It also provides a unique opportunity to study and thereby provide a better understanding of the processes involved in the regulation of mammalian cell differentiation as manifested by hemoglobin synthesis. Specifically, the studies presented here were designed to investigate the following questions:

1a) What regulates endogenous erythroid colony (EEC) formation by bone marrow cells from patients with polycythemia vera in the in vitro plasma clot culture system?

1b) Are cells which form EEC responsive to erythropoietin?

2a) What regulates EEC formation by bone marrow cells from fetal sheep in the in vitro plasma clot culture system?

2b) Are cells which form EEC responsive to erythropoietin?

3a) Is there a factor (as has been previously suggested), in the serum of patients with polycythemia vera, other than erythropoietin, which acts as a myelostimulatory factor?

3b) Can in vivo administration of this factor to mice induce mouse bone marrow cells to produce endogenous erythroid colonies in the plasma clot culture system?

4a) Is there a myelostimulatory factor, other than erythropoietin, present in serum of fetal sheep?

4b) Can in vivo administration of this factor to mice induce mouse bone marrow cells to produce EEC in the in vivo plasma clot culture system?

MATERIALS AND METHODS

I. General

1. Human Bone Marrow Cells. Bone marrow cells were obtained from human donors by a physician by aspiration at the posterior iliac crest. In all instances, informed consent was obtained from both patients and normal donors.

2. Experimental Animals. Pregnant sheep (Dorset-Merino strain) with confirmed dates of pregnancy were purchased from the animal research facilities of Yale University, New Haven, Connecticut. All animals were maintained on a regular diet and given water ad libitum.

Mice (Swiss-Webster strain, 22-25 grams) were obtained from Taconic Farms, New York.

3. Collection and Preparation of Bone Marrow Cells Prior to Culture.

a. Human Bone Marrow Cells. Three ml. of the aspirated bone marrow was immediately suspended in 5 ml. Medium I containing 2% fetal calf serum. Medium I was prepared by adding the following components to 100 ml. of Supplemental Eagle's essential medium with Hank's balanced salt solution (supplemented MEM) (Grand Island Biological Co., Grand Island, New York): 1% nonessential amino acids (100mM), 1% l-glutamine (200mM), 1% Na pyruvate (100mM), 1.25% of 5% NaHCO₃, 0.1 ml. of 10,000 units/ml. heparin

(giving a final concentration of 10 units of heparin/ml.), and 1% penicillin-streptomycin mixture (giving final concentrations of 100 units/ml. and 100 µg./ml respectively) (all of the above were obtained from Grand Island Biological Co., Grand Island, New York). The penicillin-streptomycin solution was prepared by adding 20 ml. of bacteriostatic water to the lyophilized material to a final concentration of 10,000 units/ml. and 10,000 µg./ml. respectively. Medium I was prepared in advance and kept refrigerated until use. Just prior to collection of cells, 0.2 ml. of previously heat inactivated fetal calf serum (Grand Island Biological Co., Grand Island, New York) was added to 9.8 ml. of this mixture. The bone marrow cell suspensions were spun twice in a Sorval desk top centrifuge at 1,000 RPM for 10 minutes at room temperature and the cells resuspended in 5 ml. of a medium designated Medium II. The only difference between Medium I and Medium II is that Medium II contains no heparin. After centrifugation at 1,000 RMP for 10 minutes, the wash fluid was discarded, and the buffy coat was carefully removed and resuspended in 1-2 ml. of fresh Medium II. Single cell suspensions were obtained by passing the cells through a 25 gauge needle. A nucleated cell count was made for each bone marrow, and desired cell concentrations were made by diluting each stock of cells with Medium II. All

cells were then stored on ice until used. All cell suspensions were used within two hours after collection.

b. Sheep Bone Marrow Cells. Bone marrow specimens from maternal sheep were obtained by aspiration and immediately suspended in Medium I. Bone marrow specimens from fetal sheep were obtained by aspiration when possible and immediately suspended in Medium I. In some fetuses in which aspiration was not feasible the bones (usually femurs) were cleared of muscle tissues, cracked, and the marrow cavities were scraped into Medium I to obtain cells. Single cell suspensions were obtained from these specimens following the procedure outlined for human bone marrow.

c. Mouse Bone Marrow Cells. Following sacrifice, one femur and one tibia were quickly removed from each mouse and placed in a 35 x 10 mm. plastic Petri dish (Falcon Plastics, Oxnard, California) containing Hank's balanced salt solution (Grand Island Biological Co., Grand Island, New York). The distal end of each bone was cut off with a scissor, and a sterile 25 gauge, 5/8 inch long needle was used to puncture the proximal end of the bone in order to gain access to the marrow cavity. Another sterile 25 gauge, 5/8 inch long needle attached to a 1 cc. syringe containing Medium II was then inserted into the opening made at the proximal end of the bone. The cells

of the bone marrow were obtained by flushing the medium through the bone marrow cavity and collecting the medium containing the cells from the opening made at the distal end of the bone into a 10 ml. sterile plastic test tube (Falcon Plastics, Oxnard, California). Bone marrow cells from mice belonging to the same experimental group were pooled, and a nucleated cell count was determined for each pooled cell suspension. The desired cell concentrations were made by diluting each pooled cell suspension with Medium II. All cells were stored on ice until used. All cell suspensions were used within two hours after collection.

4. Culture Media. Lyophilized beef embryo extract (BEE) (Grand Island Biological Co., Grand Island, New York) that was 50% by volume when reconstituted with supplemented medium was used. This was kept frozen until used, at which time 1 ml. of the reconstituted extract was diluted with 5 ml. of medium NCTC-109 (Grand Island Biological Co., Grand Island, New York). L-asparagine (l-asp) (Calbiochem, San Diego, California) was prepared in NCTC-109 to a concentration of 2 mg./ml., sterilized through a Millipore filter apparatus containing a 0.45u Millipore filter and frozen until use. Prior to use 0.5 ml. of l-asparagine was diluted with 4.5 ml. NCTC-109. A 10% bovine serum

albumin (BSA) solution was prepared as follows. Fifty grams of BSA powder (Bovine serum albumin, fraction V, Sigma Chemical Co., St. Louis, Missouri) was added to 91 ml. of sterile distilled water and allowed to dissolve overnight at 4°C. The flask was rotated gently several times to insure that the powder on the sides would be dissolved. The next day 5 gms. of resin was added, and the mixture was allowed to stand overnight at 4°C. The BSA was then filtered through a sterile 4 x 4 piece of gauze (to remove the resin) into a sterile graduated cylinder. The solution was made isotonic by adding 1.1 ml. of 1M Dulbecco phosphate buffered saline (minus Ca^{++} and Mg^{++} ions) to each 15 ml. of BSA solution. For the final 10% BSA solution, the above was diluted with 0.1 M phosphate buffered saline (minus Ca^{++} and Mg^{++} ions). The solution was gently mixed well and then sterilized with a Millipore filtering apparatus containing a 0.45u Millipore filter. The solution was kept frozen until use. Prior to use, 0.5 ml. of 7% NaHCO_3 was added to the 10% BSA. NCTC-109 (Microbiological Associates, Bethesda, Maryland) was used as a component of the culture medium and also as a diluent for other components of the culture medium. Stock solutions of NCTC-109 were prepared by adding penicillin and streptomycin to a concentration of 100 units/ml. and 50ug./ml. respectively

and stored at 4°C. This stock solution was used as a component of the culture medium and also as a diluent for other components of the culture medium (except BEE which was diluted with NCTC-109 containing no added penicillin-streptomycin). Stock solutions of 0.1 M α -thioglycerol (α -TG) were prepared by pipetting 0.1 ml. of 10M α -TG (Aldrich, Milwaukee, Wisconsin) into 10 ml. NCTC-109. Prior to use 0.1 ml. of this stock solution was diluted with 10 ml. NCTC-109 to produce a 10^{-3} M solution. Fetal calf serum (FCS), which was previously heat inactivated (Grand Island Biological Co., Grand Island, New York), and citrated bovine plasma (CBP) (Grand Island Biological Co., Grand Island, New York) were kept frozen until used. Erythropoietin Step III from anemic sheep (Connaught Medical Research Laboratories, Ontario, Canada) containing 2.8 to 4.7 IU/mg. protein and human urinary erythropoietin containing 74 IU/mg. protein or 8,000-10,000 IU/mg. protein were diluted with NCTC-109 to desired concentrations and stored frozen until used. Human urinary Ep was obtained from the Blood Resources Division of the National Heart, Lung, and Blood Institutes, National Institutes of Health, Bethesda, Maryland.

5. Culture Method. Bone marrow cells were cultured in the plasma clot culture system. The procedures used

were a modification of the procedure originally described by Stephenson et al. (1971) and improved by McLeod et al. (1974). It has been shown that erythropoietin in these cultures will stimulate the differentiation of erythropoietin sensitive cells (ERC) into recognizable erythroid cells. In addition, a linear relationship exists between the number of cells plated and the number of colonies scored, indicating that each colony is derived from an individual ERC.

a. Human Bone Marrow Cells. One tenth ml. of human bone marrow cell suspension containing a predetermined number of cells was added to the following mixture: 0.3 ml. FCS, 0.1 ml. BEE, 0.1 ml. BSA, 0.1 ml. l-asparagine, 0.1 ml. NCTC-109, 0.1 ml. α -TG, and 0.1 ml. human urinary erythropoietin or an equivalent amount of NCTC-109 in cultures not containing erythropoietin.

b. Sheep Bone Marrow Cells. One tenth ml. of maternal or fetal sheep bone marrow cell suspension containing a predetermined number of cells was added to the following mixture: 0.3 ml. FCS, 0.1 ml. BEE, 0.1 ml. BSA, 0.1 ml. l-asparagine, 0.1 ml. NCTC-109, 0.1 ml. α -TG, and 0.1 ml. sheep Step III erythropoietin or an equivalent amount of NCTC-109 in cultures not containing erythropoietin.

c. Mouse Bone Marrow Cells. One tenth ml. of mouse bone marrow cell suspension containing a predetermined number of cells was added to the following mixture: 0.2 ml. FCS, 0.1 ml. BEE, 0.1 ml. BSA, 0.1 ml. l-asparagine, 0.1 ml. NCTC-109, 0.1 ml. α -TG, and 0.1 ml. sheep Step III erythropoietin or an equivalent amount of NCTC-109 in cultures not containing erythropoietin.

In all of the above cases, the resulting suspensions were then mixed well with 0.1 ml. citrated bovine plasma (CBP) that had been brought to a temperature of 37°C immediately prior to use. After thorough mixing was achieved by repeated drawing of the materials into a one ml. syringe fitted with a 20 gauge needle, one tenth ml. was placed in each of 8 wells of a microtiter plate (Disposo Trays, Linbro Chemical Co., New Haven, Connecticut). Groups of 4 wells each were placed in a 35 x 10 mm. Petri dish, and two such dishes were placed in an Optilux Tissue Culture Dish (100 x 20 mm.) (Falcon Plastics, Oxnard, California), along with another Petri dish containing distilled water. All but the water dish were covered. After allowing the mixtures to clot, they were examined under an inverted microscope to insure that there were no clumps of cells and also to check that the cells were

evenly dispersed. The dishes were then placed in an incubator at 37°C in a humidified atmosphere consisting of 5% carbon dioxide in air. Human bone marrow cells were incubated for 5-7 days; sheep bone marrow cells were incubated for 2-4 days; mouse bone marrow cells were incubated for 2-3 days. The clots were then removed from the wells and placed on a glass slide (4 clots/slide). The excess fluid around each clot was removed with a Pasteur pipette, and then a piece of filter paper (Whatman #1) was placed on each slide to absorb the remaining fluid. The clots were then gently flattened with the side of a Pasteur pipette and fixed in a gluteraldehydephosphate buffer mixture. The filter paper was then removed, the clots were allowed to dry, and then were dehydrated in absolute methanol for 30 minutes.

6. Staining Procedure. The following solutions were used:

a. 3,3' Dimethylbenzidine (Practical, Eastman Kodak Co., Rochester, New York). A 1% solution was made in absolute methyl alcohol. The stain was aged for one week before it was used and was stored in a dark bottle.

b. Hydrogen peroxide 30% (Fisher Scientific Co., Fairlawn, New Jersey). One part hydrogen peroxide was mixed with 11 parts 70% ethanol to give a final concentration of 3% hydrogen peroxide. This solution was aged

for one week before it was used and was stored in a dark bottle.

c. Hematoxylin Stain Solution (Harris-Lillie, Fisher Scientific Co., Fairlawn, New Jersey). The slides were placed in benzidine for 6-8 minutes, hydrogen peroxide for 1½ minutes, distilled water for 1 minute, hematoxylin for 5-10 minutes, and blued in running tap water for 10 minutes. They were then allowed to air dry. When dry, cover slips were mounted on the slides with Permount (Fisher Scientific Co., Fairlawn, New Jersey).

7. Scoring Colonies. Each clot was examined under 100X magnification. If 8 or more nucleated cells within a group were benzidine positive, i.e., exhibited a yellow brown to red cytoplasm, the group of cells was considered an erythroid colony.

8. Preparation of Anti-Erythropoietin Serum and Anti-Erythropoietin Serum IgG. Six white New Zealand rabbits weighing 2½ kg. each were immunized against human urinary erythropoietin. The Ep was prepared by carbowax concentration of urine from a patient with aplastic anemia. The Ep was boiled for half an hour in a water bath to denature the proteins. The potency of the original preparation was 10 IU/mg. protein. After boiling, distilled water was added to bring the solution back to its original

volume. The denatured Ep was then mixed with complete Freund's adjuvant in a 1:1 ratio. After complete mixing, 0.25 ml. of the solution was injected into the foot pad of the rabbits on each side, i.e., a total of 1 ml. with 10 IU Ep was injected. Ten to twelve days later Ep prepared in the same way was injected into each rabbit. This time each rabbit was injected IM with 0.25 ml. at 4 different sites.

Ten days later serum was taken from each rabbit (via the ear vein) and examined for anti-Ep activity by its ability to neutralize standard Ep as measured in polycythemic mice. Two rabbits had high levels, and they were then used for the preparation of anti-Ep serum. The rabbits were given booster shots 2 weeks after the last immunization with a preparation of heat-denatured Ep which had a potency of 60 IU/mg. protein. After the booster shots were given high potency anti-Ep titers were obtained. One ml. of anti-Ep serum neutralized 22 IU of sheep Step III Ep (Connaught Laboratories, Toronto, Canada) and 90 IU human urinary Ep.

The IgG fraction of the immune serum was separated from other serum components by DEAE-sephadex chromatography. In studies using the IgG fraction, a similarly prepared gamma globulin fraction from normal rabbit serum was used

as a control. One mg. of the IgG from the immune serum was found to neutralize approximately 6 IU of human urinary Ep, as determined by its effect on human urinary Ep assayed in polycythemic mice.

9. Pretreatment of Fetal Calf Serum and Citrated Bovine Plasma with Rabbit Serum IgG and Anti-Rabbit Gamma Globulin (GARGG). Fetal calf serum and citrated bovine plasma were pretreated with IgG from normal rabbit serum or IgG from immune rabbit serum (i.e., serum from rabbits immunized against human urinary Ep), in the following manner. To 50 ml. of each was added either 3 mg. of normal rabbit serum IgG or immune rabbit serum IgG. This mixture was incubated at 37°C for 2 hours with constant shaking and then allowed to stand at 4°C for 22 hours. Six mg. of IgG separated from commercially prepared goat anti-rabbit gamma globulin serum (GARGG) (Antibodies, Inc., Davis, California) was then added to each mixture, mixed thoroughly, and allowed to stand for 2 hours at 4°C. The mixtures were spun at 10,000X g for 20 minutes, the precipitates discarded, and the supernatants were passed through a Millipore filter and used in the plasma clot culture system. In order to insure uniformity, control preparations of untreated fetal calf serum and citrated bovine plasma

were subjected to the same procedure but without the addition of serum IgG.

10. Sheep Sera. Serum was obtained by Dr. Zanjani from maternal and fetal sheep by means of surgical procedures described elsewhere (Zanjani et al., 1975). Serum samples were collected one year prior to use and were stored frozen in sterile 10 cc. plastic test tubes (Falcon Plastics, Oxnard, California). Samples were thawed slowly by standing at room temperature just prior to use.

11. Human Sera. Serum was obtained by a physician from human donors by venous puncture. In all instances informed consent was obtained. Serum samples were stored frozen in sterile 10 cc. plastic test tubes (Falcon Plastics, Oxnard, California). Samples were thawed slowly by standing at room temperature just prior to use.

12. Determination of Percent ^{59}Fe Incorporation. The in vivo erythropoietic effect of sera from maternal and fetal sheep and from normal humans and individuals with polycythemia vera was determined in normal mice. In these studies, mice received varying dosages of serum followed by treatment with radioactive iron. Radioactive iron (0.5 microcurie/mouse) was administered intraperitoneally 24-48 hours following the final injection of test serum.

The mice were killed by exsanguination 56-72 hours later. At the time of sacrifice, the spleen, the liver, and one femur from each mouse were also removed. The percent ^{59}Fe incorporation in these organs and the blood was calculated as follows:

% ^{59}Fe incorporation/100 mg. spleen =

$$\frac{\text{counts/minute spleen}}{\text{spleen weight (mg.)}} \times \frac{10,000}{\text{counts/min. injected dose}}$$

% ^{59}Fe incorporation/100 mg. liver =

$$\frac{\text{counts/minute liver}}{\text{liver weight (mg.)}} \times \frac{10,000}{\text{counts/min. injected dose}}$$

% ^{59}Fe incorporation/femur

$$\frac{\text{counts/minute femur}}{\text{counts/minute injected dose}} \times 100$$

% ^{59}Fe incorporation by blood =

$$\frac{\text{blood volume} \times \text{counts/minute/ml. blood}}{\text{counts/minute injected dose}} \times 100$$

Blood volume was assumed to be 5% of body weight.

II. Experimental

A. Experiments Using Human Bone Marrow Cells

1. Erythroid Colony Formation by Normal Human Bone Marrow Cells In Vitro.

Bone marrow cells from 5 hematologically normal human donors were obtained and cultured separately in the plasma clot culture system. Each bone marrow specimen was cultured at concentrations of 2, 4, 6, or 8×10^5 cells/1.1 ml. of culture medium with no Ep or with 2.0 IU human urinary Ep. These cultures were incubated for 5 to 7 days.

2. Effect of Anti-Erythropoietin (Ep) on the Formation of Erythroid Colonies by Normal Human Bone Marrow Cells In Vitro.

Bone marrow cells obtained in Section II.A.1. were cultured in the plasma clot culture system at concentrations of 2, 4, 6, or 8×10^5 cells/1.1 ml. of culture medium with 2.0 IU human urinary Ep. One mg. IgG from immune rabbits, capable of neutralizing 6 IU human urinary Ep, was added directly to the culture medium just prior to incubation. Control cultures were incubated with 2.0 IU human urinary Ep and 1 mg. IgG from non-immunized rabbits. All cultures were maintained for 5 to 7 days.

3. Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera In Vitro.

Bone marrow cells were obtained from 22 patients with polycythemia vera. Each specimen was cultured separately at concentrations of 2, 4, 6, or 8×10^5 cells/1.1 ml. of culture medium. All cell concentrations were cultured with 2.0 IU human urinary Ep and without Ep. These cultures were incubated for 5 to 7 days.

4. Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera In Vitro, In The Absence of Erythropoietin.

Bone marrow cells were obtained from 7 donors with polycythemia vera. Bone marrow cells from 7 donors who were hematologically normal were used as controls. Cells from each donor were collected and cultured separately at a concentration of 6×10^5 cells/1.1 ml. of culture medium in the plasma clot culture system without Ep. These cultures were incubated for 5 to 7 days.

5. Effect of Erythropoietin (Ep) on Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera, In Vitro.

The following studies were performed employing cells from the same bone marrow specimens as in Section II.A.4. As in the previous studies, these bone marrow specimens

were cultured separately in the plasma clot culture system at a concentration of 6×10^5 cells/1.1 ml. of culture medium and normal human bone marrow cells served as controls. These bone marrow cells were cultured with 0.5 IU human urinary Ep, 1.0 IU human urinary Ep, 2.0 IU human urinary Ep, 3.0 IU human urinary Ep, 5.0 IU human urinary Ep, and 10.0 IU human urinary Ep for 5 to 7 days.

6. Effect of Immunoglobins (IgG) from Serum of Rabbits Immunized Against Human Urinary Erythropoietin (Ep) on Endogenous Erythroid Colony (EEC) Formation In Vitro by Bone Marrow Cells from Patients with Polycythemia Vera.

Bone marrow cells were obtained from 9 patients with polycythemia vera and cultured separately in the plasma clot culture system with IgG from rabbits immunized against human urinary Ep. As a control, these bone marrow cells were also cultured in the plasma clot culture system with IgG from serum of normal rabbits. For use in these studies, serum IgG from rabbits immunized against human urinary Ep (immune serum IgG) and serum IgG from normal rabbits (normal serum IgG) were both diluted to a concentration of 1 mg. IgG/0.1 ml. NCTC-109. One-tenth ml. NCTC-109 containing 1 mg. IgG was added to 0.1 ml. Medium II containing 4×10^5 cells along with the other constituents of the plasma

clot culture medium. Therefore, the final cell concentration in these studies was 4×10^5 cells/1.2 ml. culture medium. Each bone marrow specimen was cultured under each of the following six culture conditions: with 2.0 IU human urinary Ep, with 2.0 IU human urinary Ep and 1 mg. immune serum IgG, with 2.0 IU human urinary Ep and 1 mg. normal serum IgG, with no Ep, with no Ep and 1 mg. immune serum IgG, with no Ep and 1 mg. normal serum IgG. In order to maintain equal volumes in all cultures, an additional 0.1 ml. NCTC-109 was added to cultures not containing serum IgG. All cultures were maintained for 5 to 7 days.

7. Effect of Pretreatment of Fetal Calf Serum (FCS) and Citrated Bovine Plasma (CBP) with Anti-Erythropoietin (Anti-Ep) on the Formation of Endogenous Erythroid Colonies (EEC) by Bone Marrow Cells from Patients with Polycythemia Vera In Vitro.

Bone marrow cells were obtained from 7 patients with polycythemia vera and cultured separately in the plasma clot culture system with FCS and CBP pretreated with anti-Ep IgG and goat anti-rabbit gamma globulin (GARGG), as described in Section I.9. As a control, these bone marrow cells were also cultured with FCS and CBP pretreated with normal rabbit serum IgG and GARGG as described in Section I.9.

Untreated FCS and CBP used in this study were exposed to the same physical conditions as the pretreated FCS and CBP. Each bone marrow specimen was cultured in the plasma clot culture system at a concentration of 4×10^5 cells/1.1 ml. culture medium under each of the following six culture conditions: with no Ep and untreated FCS and untreated CBP, with no Ep and anti-Ep IgG treated FCS and anti-Ep IgG treated CBP, with no Ep and normal serum IgG treated FCS and normal serum IgG treated CBP, with 2.0 IU human urinary Ep and untreated FCS and untreated CBP, with 2.0 IU human urinary Ep and anti-Ep IgG treated FCS and anti-Ep IgG treated CBP, and with 2.0 IU human urinary Ep and normal serum IgG treated FCS and normal serum IgG treated CBP. All cultures were maintained for 5 to 7 days.

8. Effect of Small Doses of Highly Purified (Lot #H4H-35) and Larger Doses of Crude (Lot #H-12) Preparations of Erythropoietin (Ep) on Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera Cultured in Media Containing Fetal Calf Serum (FCS) and Citrated Bovine Plasma (CBP) Pretreated with Anti-Erythropoietin (Anti-Ep).

Bone marrow cells from 3 patients with polycythemia vera were collected and cultured separately in the plasma clot culture system at a concentration of 6×10^5 cells/1.1 ml.

culture medium. The culture medium contained FCS and CBP that were pretreated with anti-Ep and goat anti-rabbit gamma globulin (GARGG), as described in Section I.9. Control cultures contained FCS and CBP pretreated with normal rabbit serum IgG and GARGG, as described in Section I.9. Each bone marrow specimen was cultured without Ep and with the following doses of highly purified human urinary Ep (Lot #H4H-35, containing 8,000-10,000 IU of Ep/mg. protein): 0.001 IU, 0.01 IU, 0.04 IU, 0.08 IU, 0.10 IU, and the following doses of crude human urinary Ep (Lot #H-12, containing 75 IU Ep/mg. protein): 0.30 IU, 0.50 IU, 1.0 IU, 2.0 IU, 3.0 IU, 5.0 IU, and 10 IU. The cultures were maintained for 5 to 7 days.

B. Experiments Using Sheep Bone Marrow Cells.

1. Erythroid Colony Formation by Bone Marrow Cells from Fetal and Adult Sheep In Vitro, In the Absence of Erythropoietin (Ep).

Bone marrow cells were obtained from fetal sheep and their mothers at 115 to 125 days of gestation and cultured individually at concentrations of 6×10^5 cells/1.1 ml. culture medium in the plasma clot culture system without Ep. These cultures were incubated for 2 to 4 days.

2. Effect of Erythropoietin (Ep) on Erythroid

Colony Formation by Bone Marrow Cells from Fetal and Adult Sheep In Vitro.

The following studies were performed employing cells from the same bone marrow specimens as in Section II.B.1. As in the previous study, bone marrow cells from fetal sheep and their mothers were collected between 115 and 125 days of gestation and cultured at a concentration of 6×10^5 cells/1.1 ml. culture medium in the plasma clot culture system. Each bone marrow specimen was cultured with 0.2 IU and 0.4 IU of sheep Step III Ep. All cultures were incubated for 2 to 4 days.

3. Effect of Pretreatment of Fetal Calf Serum (FCS) and Citrated Bovine Plasma (CBP) with Serum IgG from Rabbits Immunized Against Erythropoietin (Anti-Ep) on the Formation of Endogenous Erythroid Colonies (EEC) by Bone Marrow Cells from Fetal Sheep In Vitro.

Bone marrow cells were obtained from fetuses between 115 and 125 days of gestation and cultured with FCS and CBP pretreated with anti-Ep and goat anti-rabbit gamma globulin (GARGG), as described in Section I.9. As a control, these bone marrow cells were also cultured with FCS and CBP pretreated with normal rabbit serum IgG and GARGG, as described in Section I.9. Untreated FCS and CBP used

in this study were exposed to the same physical conditions as the pretreated FCS and pretreated CBP. Each bone marrow specimen was cultured in the plasma clot culture system at a concentration of 6×10^5 cells/1.1 ml. culture medium under each of the following six culture conditions: with no erythropoietin (Ep) and untreated FCS and untreated CBP, with no Ep and anti-Ep treated FCS and anti-Ep treated CBP, with no Ep and normal rabbit serum IgG treated FCS and normal rabbit serum IgG treated CBP, with 0.5 IU sheep Step III Ep and untreated FCS and untreated CBP, with 0.5 IU sheep Step III Ep and anti-Ep treated FCS and anti-Ep treated CBP, with 0.5 IU sheep Step III Ep and normal rabbit serum IgG treated FCS and normal rabbit serum IgG treated CBP. All cultures were maintained for 2 to 4 days.

C. Experiments Using Mice.

1. Effect of In Vivo Administration of Serum from Patients with Polycythemia Vera (PV Serum) to Mice on Erythroid Colony Formation by Mouse Bone Marrow Cells In Vitro.

Mice were separated into groups consisting of 5 mice each. Each mouse received intraperitoneal injections of 0.5 ml. PV serum/day for 1 to 7 days. Mice belonging to the same group all received the same number of injections

of serum from the same donor. As a control, groups of 5 mice each received a daily dose of 0.5 ml. serum from a hematologically normal human donor (AB serum) for 1 to 7 days. A single uninjected control group was also used. The animals were killed by cervical dislocation 48 hours following the last serum injection. At the time of sacrifice, bone marrow cells were collected from each mouse. Bone marrow cells from mice belonging to the same group were pooled. Bone marrow specimens (4×10^5 cells) from each group of mice were cultured separately in the plasma clot culture system in the presence or absence of 0.4 IU Ep. These cultures were maintained for 2 to 3 days.

2. Effect of In Vivo Administration of Serum from Patients with Polycythemia Vera (PV Serum) on Erythropoiesis in Normal Mice.

Mice were separated into groups consisting of 5 mice each. Each mouse received a daily dose of 0.5 ml. PV serum by means of a single daily intraperitoneal injection for 1 to 7 consecutive days. Mice belonging to the same group all received the same number of injections of serum from the same donor. As a control, groups of 5 mice each received a daily dose of 0.5 ml. serum from a hematologically normal human donor (AB serum) for 1 to 7 days. A single

uninjected control group was also used. Twenty-four hours following the last serum injection each mouse received 0.5 μCi $^{59}\text{FeCl}_3$ in 0.2 ml. normal saline intravenously. Forty-eight hours later, each animal was weighed and sacrificed by exsanguination via cardiac puncture. At the time of sacrifice, 0.5 ml. blood from each mouse was placed in a separate glass test tube for determination of radioactivity. The peripheral hematocrit for each animal was determined by the micromethod using blood drawn from the heart. Following exsanguination, one femur, the entire liver, and the entire spleen were removed from each mouse, and placed separately in appropriate vials. The radioactivity of these tissues was determined in a well type gamma scintillation counter (Picker, Autowell II), the femurs discarded, and the spleens and livers were frozen until weighed. The percent incorporation of radioiron by red blood cells, liver, spleen, and bone marrow (represented by femurs) was calculated as described in Section I.12.

3. Effect of In Vivo Administration of Serum from Fetal Sheep on Erythroid Colony Formation by Mouse Bone Marrow Cells In Vitro.

Mice were separated into groups of 10 mice each. Sera for these studies were obtained from fetal sheep (fetal serum) between 115 and 125 days of gestation. Each

mouse received a single daily injection of 0.3 ml. of fetal serum by means of a single daily intraperitoneal injection for 1 to 14 consecutive days. Mice belonging to the same group all received the same number of injections of serum from the same fetus. As a control, groups of 10 mice each received a daily dose of 0.3 ml. serum from a maternal sheep by means of a single intraperitoneal injection for 1 to 14 days. Maternal serum used for this purpose was obtained at the same time as the fetal serum. The mice were killed by cervical dislocation 48 hours following the last serum injection. At the time of sacrifice, bone marrow cells were collected from each mouse. Bone marrow cells from mice belonging to the same group were pooled. Bone marrow cells from each group of mice were cultured separately in the plasma clot culture system in concentrations of 2, 4, and 6×10^5 cells/ml. culture medium in the presence or absence of 0.2 or 0.4 IU sheep Step III Ep. These cultures were maintained for 2 to 3 days.

RESULTS

A. Experiments Using Human Bone Marrow Cells.

1. Erythroid Colony Formation by Normal Human Bone Marrow Cells In Vitro.

In order to determine if normal human bone marrow cells are capable of erythroid growth and differentiation in vitro, bone marrow cells from hematologically normal human donors were cultured with and without Ep. The results of these studies are shown in Figure 1. No erythroid colonies were formed by these cells, at any cell concentration, in the absence of exogenous Ep. Addition of Ep to these cultures, however, resulted in the appearance of significant numbers of erythroid colonies at all cell concentrations examined. A linear relationship between the numbers of colonies formed and bone marrow cells cultured was seen.

2. Effect of Anti-Erythropoietin (Ep) on the Formation of Erythroid Colonies by Normal Human Bone Marrow Cells in Response to Erythropoietin (Ep) In Vitro.

In order to determine if neutralization of Ep with anti-Ep in the plasma clot culture system affects erythroid colony formation by normal human bone marrow cells, bone marrow cells from hematologically normal human donors were cultured with Ep and anti-Ep. The results of these studies

are shown in Figure 1. Addition of anti-Ep to these cultures resulted in the total inhibition of erythroid colony formation in response to Ep. Thus, no erythroid colonies were formed by these cells at any cell concentration when anti-Ep was added to the culture medium. Although anti-Ep inhibited erythroid colony formation, normal rabbit serum IgG did not exert a significant effect on erythroid colony formation by normal human bone marrow cells in vitro (Figure 1).

3. Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera.

In order to determine if bone marrow cells from individuals with polycythemia vera are capable of erythroid growth and differentiation in vitro, bone marrow cells obtained from donors with polycythemia vera were cultured in the plasma clot culture system with and without Ep. The results of these studies are shown in Figure 2. Unlike normal human bone marrow, cells from all 22 individuals with polycythemia vera formed erythroid colonies in the absence of exogenous Ep in the plasma clot culture system. The number of endogenous erythroid colonies (EEC) formed was correlated with the number of bone marrow cells cultured in a linear fashion. Addition of Ep to the culture medium resulted in the formation of significantly greater numbers

of erythroid colonies than when Ep was absent.

4. Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera, In Vitro, in the Absence of Erythropoietin (Ep).

In order to determine if bone marrow cells from patients with polycythemia vera contain a population of cells capable of erythroid growth and differentiation, in vitro, in the absence of Ep, bone marrow cells from patients with polycythemia vera were cultured in the plasma clot culture system without Ep. As can be seen in Table 1, bone marrow cells from patients with polycythemia vera produced 122 ± 9 erythroid colonies/ 6×10^5 bone marrow cells in the plasma clot culture system when no Ep was added to the culture medium, whereas bone marrow cells from hematologically normal human donors produced no erythroid colonies in vitro in the absence of Ep.

5. Effect of Erythropoietin (Ep) on Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera, In Vitro.

In order to determine if bone marrow from patients with polycythemia vera contains an Ep responsive cell (ERC) population, bone marrow cells from patients with polycythemia vera (PV bone marrow cells) were cultured in the plasma

clot culture system with the following doses of human urinary Ep: 0.5 IU, 1.0 IU, 2.0 IU, 3.0 IU, 5.0 IU, and 10.0 IU. As can be seen in Table 1, 0.5 IU human urinary Ep failed to increase erythroid colony formation by PV bone marrow cells significantly over erythroid colony formation by these cells in cultures not containing exogenous Ep. However, 1.0 IU human urinary Ep did produce a significant increase in the production of erythroid colonies by these cells. The number of erythroid colonies produced by these cells increased still further with the addition of 2.0 IU human urinary Ep to the culture medium; however, with doses of Ep greater than 2.0 IU, a plateau in erythroid colony formation by these cells was reached. In this regard, it was found that 3.0 IU, 5.0 IU, and 10.0 IU human urinary Ep failed to produce a significant change in erythroid colony formation when compared with erythroid colony formation in cultures containing 2.0 IU human urinary Ep. It can also be seen from Table 1 that 0.5 IU human urinary Ep barely stimulated erythroid colony formation by normal human bone marrow cells and that increasing doses of Ep resulted in the formation of increasing numbers of erythroid colonies to a certain point. As with PV bone marrow cells, a plateau in erythroid colony

formation was reached in cultures containing more than 2.0 IU human urinary Ep. It should also be noted from Table 1 that, comparatively, bone marrow cells from patients with polycythemia vera produced nearly twice as many erythroid colonies as normal human bone marrow cells at all Ep concentrations examined above 0.5 IU.

6. Effect of Immunoglobulin (IgG) from Serum of Rabbits Immunized Against Human Urinary Erythropoietin (Ep) on Endogenous Erythroid Colony (EEC) Formation In Vitro, by Bone Marrow Cells from Patients with Polycythemia Vera (PV Bone Marrow Cells).

In order to determine if small, otherwise undetectable, amounts of Ep present in the plasma clot culture medium are capable of inducing EEC formation by PV bone marrow cells, these cells were cultured in the plasma clot culture system with serum IgG from rabbits immunized against human urinary Ep (anti-Ep IgG). As can be seen from Table 2, all bone marrow specimens used in this study were capable of producing erythroid colonies in the plasma clot culture system in the absence of exogenous Ep. Furthermore, the formation of these so-called endogenous erythroid colonies (EEC) was either totally inhibited or at least significantly suppressed by the addition of anti-Ep IgG to the culture

medium. Normal rabbit serum IgG, however, did not significantly suppress EEC formation when added to the culture medium. It can also be seen that, as in previous studies, addition of Ep to the culture medium resulted in a significant increase in erythroid colony formation over cultures not containing Ep. The erythroid stimulatory effect of Ep in the culture system was not inhibited by the addition of normal rabbit serum IgG to the culture medium, although the erythroid stimulatory effect of Ep was inhibited by the addition of anti-Ep IgG to the culture medium. These results indicated that EEC formation was most probably dependent upon Ep.

7. Effect of Pretreatment of Fetal Calf Serum (FCS) and Citrated Bovine Plasma (CBP) with Anti-Erythropoietin (Anti-Ep) on the Formation of Endogenous Erythroid Colonies (EEC) by Bone Marrow Cells from Patients with Polycythemia Vera (PV Bone Marrow Cells) In Vitro.

Since the most likely sources of erythropoietin (Ep) in cultures not containing exogenous Ep are the FCS and CBP, PV bone marrow cells were cultured in the plasma clot culture system with FCS and CBP that were pretreated with anti-Ep and goat anti-rabbit gamma globulin (GARGG). Table 3 indicates that all PV bone marrow specimens used in these

studies were capable of producing erythroid colonies in the absence of exogenous Ep when untreated FCS and untreated CBP were used to prepare the plasma clot culture medium. Moreover, the use of FCS and CBP which were pretreated with anti-Ep in the culture medium resulted in a significant inhibition of EEC formation by all bone marrow cells studied. Use of FCS and CBP which were treated with normal rabbit serum IgG in the culture medium, however, failed to inhibit EEC formation by these bone marrow specimens. As in previous studies, the addition of Ep to the culture medium resulted in a significant increase in the number of erythroid colonies formed by PV bone marrow cells. This erythroid stimulatory effect of Ep was found to be the same in all cultures regardless of whether the FCS and CBP used in the culture medium were untreated, or pretreated with anti-Ep, or pretreated with normal rabbit serum IgG. These results suggested that the Ep present in FCS and CBP was sufficient to induce proliferation and differentiation of the EEC forming cells in the PV bone marrow and that the procedure employed to neutralize this Ep did not result in the presence of excess anti-Ep in the culture. This was apparent from the finding that the addition of exogenous Ep to these anti-Ep treated cultures resulted in the formation of the expected numbers of erythroid colonies. It should be pointed

out that the amounts of anti-Ep and GARGG and the efficacy of the procedure used in these studies had been previously determined in our laboratory by Dr. Zanjani and is demonstrated in Table 4.

8. Effect of Small Doses of Highly Purified (Lot #H4H-35) and Larger Doses of Crude (Lot #H-12) Preparation of Erythropoietin (Ep) on Erythroid Colony Formation by Bone Marrow Cells from Patients with Polycythemia Vera (PV Bone Marrow Cells).

In order to determine if small doses of highly purified Ep (comparable to the amount present in fetal calf serum and citrated bovine plasma combined) can stimulate erythroid colony formation by PV bone marrow cells, these cells were cultured in plasma clot culture medium containing fetal calf serum (FCS) and citrated bovine plasma (CBP) that were pretreated with anti-Ep and goat anti-rabbit gamma globulin (GARGG) in the presence or absence of 0.001 IU, 0.01 IU, 0.04 IU, 0.08 IU, and 0.10 IU of highly purified (Lot #H4H-35) human urinary Ep. The results of these studies can be seen in Table 5. As in previous studies, the use of FCS and CBP which were pretreated with anti-Ep in the culture inhibited the formation of endogenous erythroid colonies (EEC) by PV bone marrow cells, whereas the use of FCS and CBP which were pretreated with normal rabbit

serum IgG in the culture did not inhibit EEC formation by PV bone marrow cells. It can also be seen that the addition of 0.001 IU human urinary Ep caused the reappearance of EEC and that increasing doses of Ep up to 0.04 IU human urinary Ep continued to increase the number of erythroid colonies formed by these bone marrow cells. At doses of 0.04 IU human urinary Ep the number of erythroid colonies formed by the PV bone marrow cells in culture reached a plateau which remained constant up to 1.0 IU human urinary Ep. This plateau persisted with doses of 0.3 IU and 0.5 IU of more crudely prepared human urinary Ep. At doses of 1.0 IU human urinary Ep the number of erythroid colonies formed by PV bone marrow cells began to increase significantly. Increasing the dose of Ep continued to enhance the number of erythroid colonies formed up to doses of 2.0 IU, at which point a second plateau in erythroid colony formation by PV bone marrow cells was reached. In addition, the erythroid stimulatory effect of doses of Ep between 0.3 IU and 0.5 IU human urinary Ep was found to be the same in all cultures, regardless of whether the FCS and CBP used in the culture medium were pretreated with anti-Ep or pretreated with normal rabbit serum IgG.

B. Experiments Using Sheep Bone Marrow Cells.

1. Erythroid Colony Formation by Bone Marrow Cells

from Fetal and Adult Sheep, In Vitro, in the Absence of Erythropoietin (Ep).

In order to determine if bone marrow from fetal and/or adult sheep contains a population of cells capable of erythroid growth and differentiation in vitro in the absence of Ep, bone marrow cells were obtained from fetuses and their mothers at 115 to 125 days of gestation and were cultured in the plasma clot culture system without exogenous Ep. As can be seen in Table 6, these fetal bone marrow cells produced erythroid colonies in the plasma clot culture system in the absence of exogenous Ep. It can also be seen from Table 6 that, unlike the fetal bone marrow cells, all adult sheep bone marrow specimens failed to produce any erythroid colonies in the plasma clot culture system in the absence of exogenous Ep.

2. Effect of Erythropoietin (Ep) on Erythroid Colony Formation by Bone Marrow Cells from Fetal and Adult Sheep In Vitro.

In order to determine if bone marrow from fetal and adult sheep contains an Ep responsive cell (ERC) population, varying numbers of bone marrow cells (2 to 8×10^5 cells) from fetal sheep and their mothers, obtained at 115 to 125 days of gestation were cultured in the plasma clot

culture system with 0.4 IU sheep Step III Ep. As can be seen in Table 6, addition of Ep resulted in the production of significant numbers of erythroid colonies by bone marrow cells from adult as well as fetal sheep. It can also be seen from the data in Table 6 that a linear increase in the numbers of erythroid colonies was seen with increasing numbers of bone marrow cells cultured.

3. Effect of Pretreatment of Fetal Calf Serum (FCS) and Citrated Bovine Plasma (CBP) with Serum IgG from Rabbits Immunized Against Erythropoietin (Anti-Ep) on the Formation of Endogenous Erythroid Colonies (EEC) by Bone Marrow Cells from Fetal Sheep In Vitro.

In order to determine if small, otherwise undetectable amounts of erythropoietin (Ep) present in FCS and CBP in the plasma clot culture system are capable of inducing EEC formation by fetal sheep bone marrow cells, in vitro, bone marrow cells obtained from fetuses 115 to 125 days of gestation were cultured in the plasma clot culture system with FCS and CBP that were pretreated with anti-Ep and goat anti-rabbit gamma globulin (GARGG). It can be seen from Tables 6 and 7 that all fetal bone marrow specimens used in this study were capable of producing erythroid colonies in the absence of exogenous Ep (i.e., formed EEC) when

untreated FCS and untreated CBP were used to prepare the plasma clot culture medium. Furthermore, the use of FCS and CBP which were pretreated with anti-Ep in the culture medium resulted in a significant inhibition of EEC formation by all fetal bone marrow specimens studied. The use of FCS and CBP which were pretreated with normal rabbit serum IgG in the culture medium, on the other hand, did not inhibit EEC formation by fetal sheep bone marrow cells. As in the previous studies, the addition of Ep to the culture medium resulted in a significant increase in the number of erythroid colonies produced by each fetal bone marrow specimen. This effect of Ep was observed regardless of whether the FCS and CBP used in the culture medium were untreated, or pretreated with anti-Ep, or pretreated with normal rabbit serum IgG.

C. Experiments Using Mice.

1. Effect of In Vivo Administration of Serum from Patients with Polycythemia Vera (PV Serum) to Mice on Erythroid Colony Formation by Mouse Bone Marrow Cells In Vitro.

In order to determine if repeated administration of PV serum to mice alters erythroid colony formation by mouse bone marrow cells in vitro in the plasma clot culture system, bone marrow cells from mice treated with PV serum

for 1 to 7 days were cultured in the presence or absence of 0.4 IU sheep Step III erythropoietin (Ep). Table 8 shows that bone marrow cells from mice that were treated with PV serum for 5, 6, and 7 days were able to produce erythroid colonies in vitro in the absence of Ep. Bone marrow cells from mice which received fewer than 5 injections of PV serum, however, were unable to produce erythroid colonies in vitro in the absence of Ep. Furthermore, treatment of mice with serum from hematologically normal donors did not result in the formation of endogenous erythroid colonies by mouse bone marrow cells in vitro, regardless of the number of injections on serum these mice received. Table 9 shows the results of studies in which bone marrow cells from mice treated with PV serum for 7 consecutive days were cultured in the plasma clot culture system with 0.4 IU sheep Step III Ep. It can be seen from this table that bone marrow cells from mice which received 7 injections of PV serum produced significantly greater numbers of erythroid colonies than bone marrow cells from mice which received 7 injections of normal human serum.

2. Effect of In Vivo Administration of Serum from Patients with Polycythemia Vera (PV Serum) to Mice on Erythropoiesis in Normal Mice.

In order to determine if chronic administration of

PV serum to mice increases erythropoiesis by mice in vivo, mice were treated with PV serum for 1 to 7 consecutive days. The rate of erythropoiesis in these animals was measured by determining the percent radioactive iron incorporation by the red blood cells of each mouse 72 hours after the final serum injection. The results of these studies are shown in Table 10. It can be seen from this table that chronic treatment with PV serum resulted in a gradual increase in radioactive iron incorporation by murine red blood cells so that by day 4 of treatment the percent radioactive iron incorporation by red blood cells obtained from treated animals was significantly greater than that observed in mice not having received PV serum. Erythropoiesis remained elevated in these animals for the duration of the treatment. No significant increase was noted in mice which received similar numbers of injections of normal serum. The rate of erythropoiesis by the bone marrows of these animals was measured by determining the percent radioactive iron incorporation by one femur of each mouse 72 hours after the final serum injection. The results of these studies are shown in Table 11. It can be seen from this data that bone marrow radioactive iron incorporation was not significantly affected by 1, 2, or 3 injections of serum; however, 4 doses of PV serum resulted in a significant

decrease in radioactive iron incorporation by mouse bone marrow. Continued administration of these sera up to 7 doses resulted in the maintenance of these decreased levels of radioactive iron incorporation. Chronic treatment with normal serum did not significantly alter radioactive iron incorporation by mouse bone marrow. The percent radioactive iron incorporation by spleens obtained from these mice was also determined, and the results of these studies are shown in Table 12. It can be seen from this data that radioactive iron incorporation by mouse spleens was increased significantly by treatment with PV serum for at least 5 days. Continued treatment maintained this elevated level of radioactive iron incorporation for the duration of the study. However, similar increases were also observed with normal human serum. The percent radioactive iron incorporation by livers from these mice was determined, and the results of these experiments are shown in Table 13. Treatment of mice for 3 days with both PV and normal human sera resulted in a significant decrease in radioactive iron incorporation by mouse livers as compared with livers from mice which received no injections. However, as with the effect on splenic incorporation, greater numbers of injections of PV serum were required to bring about a decrease similar to that seen with 3 doses of normal serum.

3. Effect of In Vivo Administration of Serum from Fetal Sheep on Erythroid Colony Formation by Mouse Bone Marrow Cells In Vitro.

In order to determine if chronic administration of serum from fetal sheep (fetal serum) to mice can induce endogenous erythroid colony (EEC) formation by mouse bone marrow cells in vitro, bone marrow cells from mice treated with fetal and adult sheep sera for 1 to 14 consecutive days were cultured in the plasma clot culture system without erythropoietin (Ep). Table 14 shows that bone marrow cells from mice that were treated with serum obtained from the fetuses between 115 and 125 days of gestation were able to produce significant numbers of erythroid colonies in vitro in the absence of exogenous Ep. A minimum of 10 doses of fetal serum were required to induce EEC formation by mouse bone marrow cells. Chronic treatment with adult sheep serum failed to induce EEC formation by mouse bone marrow cells. Erythroid colony formation by the bone marrow cells obtained from these mice in the presence of Ep was also examined by culturing these cells with 0.4 IU sheep Step III Ep. Table 14 shows that bone marrow cells from mice treated for a minimum of 8 days with serum obtained from fetuses between 115 and 125 days

of gestation produced significantly greater numbers of erythroid colonies in vitro than did bone marrow cells obtained from mice treated with adult sheep serum. Chronic treatment with serum obtained from pregnant sheep did not affect erythroid colony formation by mouse bone marrow cells regardless of the number of injections given (Table 14).

DISCUSSION

It is now firmly established that erythropoiesis in normal animals and man is dependent upon and regulated by the hormone erythropoietin (Ep) (Reissmann, 1950; Erslev, 1953; Krantz and Jacobson, 1970; Gordon, 1973). Alterations in the rate of production of this hormone can lead to changes in the numbers of circulating red blood cells (Gurney, 1973). It is generally accepted that the increased production of erythrocytes in all cases of secondary polycythemia is associated with increased Ep formation (Ossias et al., 1973). Such an association cannot be demonstrated in polycythemia vera. In this regard, the decreased levels of Ep in polycythemia vera (Adamson, 1968) and the failure to elicit a normal response to Ep by bone marrow cells of these patients in suspension cultures (Krantz, 1968; Zucker et al., 1972) have led to the suggestion that the increased production of red blood cells in polycythemia vera results from the proliferation and differentiation of hemopoietic precursor cells not under the usual Ep control (Gurney, 1973). Studies in vivo (Bomchil et al., 1967; Stohlman, 1966; Adamson, 1970) and in vitro (Prchal and Axelrad, 1974; Golde and Cline, 1975; Zanjani, 1976) have provided evidence for the existence also of a population

of Ep-responsive erythroid precursor cells in polycythemia vera. The latter cell population, however, was suggested not to contribute significantly to the overall production of red blood cells during the active phase of the disease (Gurney, 1973). Recent studies utilizing culture procedures conducive to the proliferation and differentiation of erythroid precursor cells in vitro such as the plasma clot culture system (Tepperman et al., 1974) indicated that the latter cell population may represent a greater proportion of the total erythroid precursor cell pool in polycythemia vera than previously assumed (Prchal and Axelrad, 1974; Zanjani, 1976). These studies also provided further evidence for the existence of an erythroid stem cell population in polycythemia vera with altered proliferative characteristics (Prchal and Axelrad, 1974; Golde and Cline, 1975; Zanjani, 1976).

The development of the plasma clot culture system by Stephenson et al. (1971) and its later modification by McLeod et al. (1974) have revolutionized the study of the regulation of erythropoiesis by providing an in vitro culture method which allows for the growth and differentiation of animal and human erythroid precursor cells. The applicability of this culture system for the study of

erythroid cell differentiation is demonstrated by the results presented here. These results show the Ep-dependent nature of erythroid colony formation by normal human bone marrow cells in the plasma clot culture system. Thus, no erythroid colonies were formed when bone marrow cells of normal individuals were cultured in the absence of exogenous Ep. Moreover, addition of anti-Ep to cultures of normal marrow containing Ep resulted in the total inhibition of erythroid colony formation. These in vitro findings are consistent with the observations that neutralization of circulating Ep in normal animals by in vivo administration of anti-Ep resulted in suppression of erythropoiesis (Schooley et al., 1968) and, as such, provide further evidence for the suitability of the plasma clot culture system for the study of the regulation of erythropoiesis.

Unlike normal human bone marrow, cells from bone marrow of patients with polycythemia vera gave rise to significant numbers of erythroid colonies in the absence of exogenous Ep. These endogenous erythroid colonies (EEC) first appeared on day 5 of culture and reached their maximum number and size by day 7. The formation of EEC is most probably due to the presence of a population of erythroid precursor cells with altered proliferative characteristics in polycythemia vera. The existence of an

abnormal erythroid stem cell population contributing to the overall increase in circulating red cell mass in polycythemia vera was suggested by the finding that:

- 1) unlike secondary polycythemias, where increased production of Ep has been shown to accompany and be responsible for the elevated red cell mass (Gurney, 1973; Ossias et al., 1973), increased levels of this hormone cannot be demonstrated in plasma and urine of patients with polycythemia vera (Adamson, 1968; Adamson, 1970). In most cases urinary excretion of Ep is below normal (Adamson, 1968), which is attributable to feedback inhibition by the increased red cell mass on Ep formation (Gordon et al., 1968); and
- 2) bone marrow cells obtained from donors with polycythemia vera during relapse, when cultured in suspension, exhibited a significantly reduced response to Ep (Krantz, 1968).

More recently, Adamson et al. (1975) have provided direct evidence for the clonal origin of hemopoietic cell lines in polycythemia vera. The data presented here, however, do not support the view that the erythroid activity of the abnormal clone is independent of Ep, since the formation of EEC by polycythemia vera bone marrow was inhibited by the addition of anti-Ep to the cultures. The inhibitory influence exerted by this IgG on EEC formation can be

attributed to the neutralization of Ep present in fetal calf serum (FCS) and citrated bovine plasma (CBP), and not to a non-specific effect of the rabbit IgG. Support for this contention is derived from the findings that 1) addition of IgG from non-immunized rabbits to cultures of polycythemia vera bone marrow failed to affect colony formation, 2) treatment of the immune IgG with goat anti-rabbit gamma globulin (GARGG) prior to use in culture resulted in the inhibition of anti-Ep effect, and 3) small doses of highly purified Ep caused the reappearance of EEC in anti-Ep treated cultures. It is of interest to note that recent studies by Golde et al. (1977) have demonstrated that addition of anti-Ep to methylcellulose cultures suppresses EEC formation by bone marrow cells from patients with polycythemia vera; thereby indicating the Ep-dependent nature of EEC formation in the methylcellulose culture system as well.

The results presented here imply that sufficient amounts of Ep must have been present in FCS and CBP to initiate the proliferation and differentiation of these cells, despite the fact that repeated bioassays of the FCS and CBP used in these studies failed to demonstrate Ep activity. It is generally accepted that the polycythemic

mouse assay system is not sensitive to doses of Ep below 0.05 IU/ml. of test material (Camiscoli et al., 1968). There is little doubt that sera from normal adult animals and man (Weintraub et al., 1963) and normal mammalian fetuses (Zanjani et al., 1976) contain Ep. The concentration of Ep in normal serum has been reported to range from 0.0003 to 0.003 IU/ml. The amount may reach approximately 0.03 to 0.05 IU/ml. in fetal serum at the time of delivery (Zanjani et al., 1976). Since the combined volume of FCS and CBP used to culture a given concentration of marrow cells in these studies was about 0.4 ml., the total amount of Ep present in these cultures could not have exceeded 0.025 IU. This concentration of Ep is not an effective dose for erythroid colony formation by normal bone marrow cells, optimal colonies being produced with 2 to 3 IU of Ep. Therefore, these precursor elements in polycythemia vera bone marrow appear to be far more sensitive to Ep than normal erythroid precursors.

The antibody used in these studies was produced against a crude preparation of Ep, and, despite repeated absorption steps, we could not be certain that we were working with a pure anti-Ep preparation. Therefore, although the results of these studies appeared to be conclusive, the possibility existed that the suppression of EEC formation by anti-Ep was the result of neutralization

of proteins, other than Ep, in the culture medium. If this were the case, it would mean that EEC formation is influenced by a protein contaminate of the Ep preparation rather than by Ep. To examine this possibility, two approaches could have been used. One would require the use of an antibody preparation directed solely against Ep. Unfortunately, the antibody used in these studies was produced against crude preparations of Ep. The alternative approach, which was employed here, was to utilize the most pure preparation of Ep available. The ability of doses of pure Ep as low as 0.001 IU to induce erythroid colony formation by polycythemia vera bone marrow cells cultured in anti-Ep treated FCS and CBP indicates that the inhibition of EEC formation by anti-Ep is the result of the specific neutralization of Ep activity by this anti-sera. Admittedly, the most pure preparation of Ep available, and used here, cannot be considered absolutely free of contaminating proteins; however, it is unlikely that the very small amounts of non-Ep proteins that may be present in this preparation were sufficient to bring about the results observed in these studies.

These studies also provided further support for the contention that erythroid precursor cells in polycythemia vera are more sensitive to Ep than normal, since, following

the inhibition of EEC formation by neutralization of Ep present in FCS and CBP, addition of very small amounts of highly pure Ep to cultures of bone marrow cells from patients with polycythemia vera resulted in the reappearance of these colonies. Thus, a significant number of erythroid colonies were formed with as little as 0.001 IU Ep. A linear increase in colony formation was noted with increasing doses of this Ep up to 0.04 IU. No further increase occurred with concentrations of pure Ep greater than 0.04 IU. Similar concentrations of the highly purified Ep were without effect in cultures of normal human bone marrow cells. The limited ability of pure Ep prevented the examination of the effect of progressively larger doses of this Ep on erythroid colony formation in this system; however, using a more crude preparation of Ep, we found that this initial plateau persisted through 0.5 IU Ep. A further increase in erythroid colony formation occurred with 1.0 IU Ep with maximum numbers of erythroid colonies formed at 2 IU Ep/ 6×10^5 bone marrow cells. Larger doses of Ep (3, 5, and 10 IU) failed to induce a significant increase in erythroid colony formation beyond that produced by 2 IU Ep.

Interpretation of this data must be approached cautiously, since the possibility exists that the observed

plateau in erythroid colony formation is the result of intrinsic differences between the Ep preparations rather than the result of differences in dose response of different populations of Ep-responsive cells in polycythemia vera bone marrow; however, the results seem to indicate the presence of a population of cells in polycythemia vera with a normal Ep response. Addition of Ep to cultures of polycythemia vera bone marrow cells resulted in a significant increase in the numbers of erythroid colonies formed. If it is assumed that equal doses of both Ep preparations are capable of exerting the same erythroid stimulatory effect in the plasma clot culture system, several possibilities exist as to the nature of this increase in erythroid colony formation. One possibility is that the major portion of the erythroid precursor pool is composed of the highly sensitive type, but the amount of Ep present in FCS and CBP is not sufficient to trigger all these cells to undergo proliferation and differentiation. Therefore, the exogenous Ep provides the additional amounts needed. If this were indeed the case, it would be possible to achieve near maximal erythroid colony formation with relatively low doses of Ep. However, doses of Ep as high as 0.5 IU were not able to increase the number of erythroid colonies significantly above the EEC level.

A more likely explanation appears to be that normally responsive populations of erythroid precursor cells are also present in polycythemia vera which give rise to the erythroid colonies in the presence of exogenous Ep. Moreover, these results suggest that the number of normal precursor cells exceeds those with altered characteristics. Prchal and Axelrad (1974) have reported similar findings. The existence of these two precursor cell populations is further illustrated by the observation that two distinct areas of activity were seen when the effect of different concentrations of Ep on erythroid colony formation by polycythemia vera bone marrow was studied. The first region of erythroid activity was detected with small doses of Ep, whereas the second region required relatively large amounts of Ep. The two areas were clearly separated, as was demonstrated by the finding that a significant rise in erythroid colony formation above the first plateau (induced by 0.04 IU Ep) did not occur until 1.0 IU Ep was employed. Moreover, the numbers of erythroid colonies formed with larger Ep doses represented nearly 70% of the total numbers of colonies formed by polycythemia vera bone marrow.

These observations, combined with those previously published (Prchal and Axelrad, 1974; Golde and Cline, 1975;

Zanjani, 1976), lead to the conclusion that Ep plays an important role in the regulation of erythropoiesis not only in normal man but in individuals with polycythemia vera as well. A regulatory role of Ep in erythropoiesis in polycythemia vera has also been demonstrated by in vivo studies. Patients with polycythemia vera responded to anemia (Stohlman, 1966) and hypoxia (Bomchil et al., 1967) by increased red cell production. This increase occurred only in those patients in whom a rise in Ep production was detected (Adamson, 1970). The presence of a population of cells highly sensitive to Ep would be compatible with increased erythropoiesis in the face of low levels of circulating Ep in these patients. Polycythemia vera is a panmyelopathy, probably clonal in nature (Adamson et al., 1975), involving all three formed elements of the blood. The results presented here do not provide evidence for or against the clonal nature of this disease; however, evidence is provided to indicate that the final steps in the formation of red blood cells in polycythemia vera are subject to the usual Ep control. It is conceivable that a similar mechanism could be involved in the regulation of platelet and white cell formation.

It has not yet been determined what causes erythroid

precursor cells in bone marrow from individuals with polycythemia vera to be more sensitive to Ep than are the erythroid precursor cells in normal adult human bone marrow; however, several investigators (Reisner, 1967; Reisner, 1970; Ward and Robinson, 1972; Robinson and Ward, 1972; Ward et al., 1971; Zanjani et al., 1971; Zanjani et al., 1972) have reported the presence of a myelostimulatory factor in the serum of patients with polycythemia vera. The data presented here and by these other investigators indicate that this factor may be responsible for the unusual sensitivity to Ep exhibited by a population of erythroid precursor cells in polycythemia vera bone marrow. In this regard, Reisner (1967, 1970) demonstrated that addition of serum from patients with polycythemia vera to suspension cultures of normal human bone marrow cells significantly increased erythropoiesis by these cells when compared with suspension cultures of normal bone marrow cells containing serum from normal human donors. Serum from patients with polycythemia vera was found to stimulate granulopoiesis and megakaryocytopoiesis in these cultures as well (Reisner, 1970; Reisner, 1967). This factor has also been shown to increase erythropoiesis in mice in vivo (Zanjani et al., 1971), as evidenced by increased reticulocytosis, increased

hematocrit, and increased radioactive iron incorporation into red blood cells in chronically treated mice.

It has been suggested that this myelostimulatory factor exerts its effect(s) by increasing the number and/or sensitivity of hemopoietic precursor cells, perhaps through an effect on the pluripotent stem cell (Robinson and Ward, 1972). In support of this, it has been shown (Zanjani, 1976; Zanjani et al., 1977) that femoral bone marrow cells obtained from polycythemia vera serum treated mice contained significantly greater numbers of pluripotent stem cells (CFU-S) when compared with normal serum treated controls. Furthermore, treatment of mice with polycythemia vera serum increases granulocytic colony formation by mouse bone marrow cells in agar cultures in the presence of colony stimulating factor when compared with bone marrow cells from normal serum treated controls (Robinson and Ward, 1972; Ward et al., 1972; Zanjani et al., 1977), thereby indicating that the factor in polycythemia vera serum influences the production not only of red blood cells but of white blood cells as well. These results presented here confirm these findings and further demonstrate that the increased sensitivity of the erythroid committed cells to Ep can be manifested in the plasma clot culture system.

Bone marrow cells from mice treated with polcythemia

vera serum for 5, 6, and 7 days were capable of forming erythroid colonies in the plasma clot culture system in the absence of exogenous Ep much the same way as polycythemia vera bone marrow cells. Although it has not been determined whether or not these EEC will continue to be formed if bone marrow cells from polycythemia vera serum treated mice are cultured in medium containing FCS and CBP pretreated with anti-Ep and GARGG, it is likely that EEC formation by these cells is due to increased sensitivity to Ep so that the small quantity of Ep present in the culture medium is sufficient to stimulate the growth and differentiation of erythroid precursor cells. Support for this theory is twofold. Firstly, it has been shown that treatment of mice with polycythemia vera serum for three days significantly increases the erythropoietic response of mouse bone marrow cells to subsequent treatment with Ep in vivo (Ward et al., 1971; Zanjani et al., 1971). Secondly, similar studies have shown that treatment of mice with polycythemia vera serum results in significantly increased granulopoiesis by mouse bone marrow cells in agar cultures in response to colony stimulating factory (Robinson and Ward, 1972; Ward and Robinson, 1972; Zanjani et al., 1977). It is also of interest that, as in the case of bone marrow from patients

with polycythemia vera, the bone marrow cells obtained from polycythemia vera serum treated mice responded to exogenous Ep in vitro.

The hematocrit (Gordon et al., 1954) and the percent radioactive iron incorporation by mouse red blood cells, bone marrow cells, spleens (Plzak et al., 1955), and livers (Gordon, 1973) are commonly determined measurements which serve as indices of the level of erythropoiesis occurring in a given animal at a given time. It was, therefore, decided to also observe these parameters in mice treated with polycythemia vera serum. In these studies, treatment of mice with polycythemia vera serum failed to increase the hematocrit or bone marrow radioactive iron incorporation significantly over those of normal serum treated control animals. Although it has been previously reported that chronic treatment of mice with polycythemia vera serum results in increased hematocrits and increased radioactive iron incorporation by bone marrow cells, treatment for approximately five weeks was required in order to observe these effects (Zanjani, 1976). Since the animals in these studies were treated for only 7 days, it is not surprising that these effects were not observed in these studies. In spite of this, there were other indications

(in addition to endogenous erythroid colony formation by bone marrow cells from treated animals) that treatment with polycythemia vera sera increased erythropoiesis in treated animals. In this regard, increased radioactive iron incorporation into red blood cells and spleens of these mice was observed. The decreased radioactive iron incorporation by the livers of polycythemia vera serum treated mice suggests decreased storage and increased utilization of iron for erythropoiesis by these animals. These results indicate, although not conclusively, that the alterations in the proliferative activity of the hematopoietic stem cells in polycythemia vera may result from the activity of a humoral factor with growth promoting characteristics for several stem cell lines.

Considerable evidence indicates that, in polycythemia vera, erythropoiesis can occur in extramedullary sites, such as liver and spleen, in addition to the bone marrow (Wasserman and Gilbert, 1966). In man and most laboratory animals, splenic and hepatic erythropoiesis is seen only during fetal development, and hematopoiesis in the adult is generally confined to the bone marrow (Wintrobe, 1974). Therefore, the reversion to fetal type hematopoiesis observed in patients with polycythemia vera prompted the examination of the regulation of erythropoiesis by fetal

bone marrow cells in vitro. The results of these studies revealed that a great deal of similarity may exist between fetuses and patients with polycythemia vera with regard to the in vitro behavior of their erythroid committed stem cells and the in vivo effects of their sera. Thus, although it is generally accepted that Ep regulates erythropoiesis in mammalian fetuses (Zanjani et al., 1969; Matoth and Zaizov, 1971; Zanjani and Gordon, 1971; Gordon et al., 1971; Zanjani et al., 1974a; Zanjani et al., 1975), the data presented here indicate that bone marrow cells from fetal sheep (115 to 125 days of gestation) contain a population of cells capable of erythroid growth and differentiation in this culture system even when Ep is not added to the culture medium. Since the ability of these bone marrow cells to produce EEC paralleled the condition observed when polycythemia vera bone marrow cells are cultured in the plasma clot culture system, it became of interest to determine if, as in polycythemia vera, fetal hemopoietic tissue contains erythroid precursors with increased sensitivity to Ep.

The data presented here indicate that fetal bone marrow cells cultured in medium containing FCS and CBP that were pretreated with anti-Ep and GARGG did not produce EEC. This was due to the neutralization of Ep activity in the FCS and

CBP by anti-Ep since pretreatment of FCS and CBP with normal serum IgG did not inhibit EEC formation by fetal bone marrow cells. It can, therefore, be concluded, as in the case of bone marrow cells from individuals with polycythemia vera, that EEC are formed by a population of cells, present in fetal bone marrow, which is more sensitive to Ep than are normal adult bone marrow cells.

While the data demonstrate that erythroid precursor cells in fetal bone marrow can also respond to Ep in vitro by producing increased numbers of erythroid colonies, it cannot be determined from these studies whether there is a single population of cells present in fetal bone marrow which can grow and differentiate into erythroid cells in the presence of both large and extremely small doses of Ep or whether there are two separate cell populations present in these bone marrows, i.e., one which is more sensitive to small doses of Ep and one which responds to Ep in a manner similar to adult sheep bone marrow cells as is the case with polycythemia vera bone marrow.

The similarities between the growth and differentiation of bone marrow cells from patients with polycythemia vera and bone marrow cells from fetuses in the plasma clot culture system, i.e., the increased sensitivity of these cells to Ep, raised the question of whether there might not be some

factor present in the serum of fetuses, as in the serum of individuals with polycythemia vera, which renders some fetal bone marrow cells more sensitive to Ep. The studies presented here indicate that this may be the case, since chronic treatment of mice with serum from fetuses 115 to 125 days of gestation induced EEC formation by mouse bone marrow cells. Although inhibition of this EEC formation by pretreatment of the culture medium with anti-Ep and GARGG has not been attempted in these studies, the findings that sera from fetal sheep contain this stimulatory factor at the time during gestation when fetal bone marrow cells give rise to EEC and also that these colonies form as a result of increased sensitivity to Ep present in the culture medium have led to the hypothesis that EEC formation by mouse bone marrow cells following chronic treatment with fetal serum is also the result of increased sensitivity of erythroid precursor cells to Ep.

As in the case of polycythemia vera serum (Zanjani, 1976), this erythrostimulatory factor has been shown to be antigenically different from Ep. Preliminary studies show that treatment of fetal serum with anti-Ep and GARGG failed to affect the erythrostimulatory activity of fetal serum.

The data presented here, along with reported by other

investigators (Prchal and Axelrad, 1974; Golde and Cline, 1975; Zanjani, 1976), lead to the conclusion that Ep plays an important role in the regulation of erythropoiesis not only in normal adults but in individuals with polycythemia vera and in fetuses as well. A regulatory role of Ep in erythropoiesis in polycythemia vera has also been demonstrated by in vivo studies. Individuals with polycythemia vera responded to anemia (Stohlman, 1966) and hypoxia (Bomchil et al., 1967) by increased red blood cell production. This increase occurred only in those patients in whom a rise in Ep production was detected (Adamson, 1970). The presence of a population of cells highly sensitive to Ep is compatible with the observed increased erythropoiesis in the face of low levels of circulating Ep in these patients. Polycythemia vera is a panmyelopathy, probably clonal in nature (Adamson et al., 1975), involving all three formed elements of the blood. Thus, any consideration of the regulation of erythropoiesis in this disorder must also include an explanation for the increased production of white cells and platelets, since it is unlikely that the basic defect is different for the three cell types. The results presented here do not provide evidence for or against the clonal nature of this disease. Evidence is provided to indicate that the final steps in the formation

of red blood cells in polycythemia vera are subject to the usual Ep control, although it appears that there is a population of erythroid precursor cells in the bone marrow of individuals with polycythemia vera which is more sensitive to the hormone than are normal human bone marrow cells. It is conceivable that a similar mechanism could be involved in white cell and platelet formation.

The myelostimulatory factor present in the serum of some individuals with polycythemia vera may play a major role in the regulation of hematopoiesis in this disorder. Other investigators have shown that chronic treatment of mice with these sera results in: 1) an increase in the number of CFU-S (Zanjani, 1976; Zanjani et al., 1977), 2) increased response of polycythemia mice to Ep in vivo (Ward et al., 1971; Zanjani et al., 1971), and 3) increased granulopoiesis in vitro in the presence of colony stimulating factor (Robinson and Ward, 1972; Ward and Robinson, 1972), and it has been shown in the studies presented here that it causes murine erythroid precursor cells to become more sensitive to Ep so that they respond to smaller than normal doses of Ep in vitro.

The site and mechanism of production of the myelostimulatory factor in polycythemia vera is not known. The factor is non-dializable and is not recognized by anti-Ep.

Preliminary biochemical studies suggest that it is a glycoprotein with characteristics similar, but not identical, to Ep (Zanjani, 1976). Whether its absence in some polycythemia vera patients is indicative of a fundamental difference between these two groups of people or a manifestation of our inability to detect low concentrations of this factor is not known (Zanjani, 1976). Further studies on the nature of this myelostimulatory factor are no doubt needed.

It is quite apparent from these studies that at least some of the erythroid precursor cells in bone marrows from patients with polycythemia vera and fetuses (between 115 and 125 days of gestation) are more sensitive to Ep than are normal adult erythroid precursor cells; however, the relationship between fetal erythropoiesis and erythropoiesis in polycythemia vera is not very clear. The presence of a myelostimulatory factor in the serum of patients with polycythemia vera and normal fetuses (between 115 and 125 days of gestation) raises the question of whether or not polycythemia vera is the result of a reversion to fetal type hematopoiesis. Supporting evidence for this comes from the fact that in this disorder splenic and hepatic hematopoiesis occurs in addition to the bone marrow hematopoiesis (Wasserman and Gilbert, 1966), whereas under normal conditions splenic and hepatic hematopoiesis

occur only in the fetus and not in the adult. It could be that, having reached a stage of extramedullary hematopoiesis, a myeloproliferative factor the same as or similar to that found in the mammalian fetus can act on the very erythropoietic tissue to presensitize it to Ep. Alternatively, the abnormal situation observed in polycythemia vera could arise from or be preceded by an increase in the production of the fetal type serum factor which then stimulates accelerated, as well as extramedullary, hematopoiesis. Unfortunately, this question will remain unanswered until the myelostimulatory factor(s) present in polycythemia vera and fetal serum is (are) isolated and characterized biochemically. Nevertheless, the results presented here demonstrate that the final steps in the differentiation of the erythroid elements in polycythemia vera and fetuses are under the influence of a normal, physiological hormone (Ep).

Table 1. Comparison between the response of normal human and polycythemia vera (PV) bone marrow cells to different doses of erythropoietin (Ep) in vitro.

Ep Concentration (IU)	Number of Erythroid Colonies/ 6×10^5 cells ¹			
	Normal	P value ²	PV	P value ²
0	0		122 $\pm$ 9	
0.5	2 $\pm$ 0.6	N.S.	134 $\pm$ 6	N.S.
1.0	98 $\pm$ 8	<0.001	206 $\pm$ 11	<0.001
2.0	436 $\pm$ 23	<0.001	713 $\pm$ 24	<0.001
3.0	449 $\pm$ 36	N.S.	747 $\pm$ 30	N.S.
5.0	413 $\pm$ 19	N.S.	710 $\pm$ 12	N.S.
10.0	387 $\pm$ 29	N.S.	692 $\pm$ 44	N.S.

¹The erythroid colony counts represent the mean $\pm$ 1 standard error of the mean from 7 separate studies each involving a separate donor. The erythropoietin concentrations represent Ep concentration/1.1 ml. of culture.

²Determined by Student's t test; compared to colony numbers induced by the Ep dose immediately preceding.

Table 2. Effect of immunoglobulins from either normal rabbit serum (normal IgG) or serum from rabbits immunized against erythropoietin (Ep) (anti-Ep IgG) on erythroid colony formation by bone marrow cells from patients with polycythemia vera.

Number of Erythroid Colonies/ 4×10^5 cells ¹						
<u>Patient Number</u>	<u>No Ep</u>	<u>Normal IgG</u>	<u>Anti-Ep IgG</u>	<u>+ Ep</u>	<u>Ep + Normal IgG</u>	<u>Ep + Anti-Ep IgG</u>
1	154 $\pm$ 42	168 $\pm$ 36	0	598 $\pm$ 89	--	8 $\pm$ 6
2	109 $\pm$ 31	92 $\pm$ 9	10 $\pm$ 4	1132 $\pm$ 287	987 $\pm$ 169	0
3	183 $\pm$ 22	173 $\pm$ 43	11 $\pm$ 5	632 $\pm$ 71	959 $\pm$ 106	0
4	139 $\pm$ 25	158 $\pm$ 39	7 $\pm$ 3	446 $\pm$ 83	532 $\pm$ 43	0
5	132 $\pm$ 14	147 $\pm$ 29	0	597 $\pm$ 62	503 $\pm$ 79	12 $\pm$ 7
6	140 $\pm$ 56	146 $\pm$ 20	12 $\pm$ 2	875 $\pm$ 106	847 $\pm$ 88	0
7	44 $\pm$ 8	--	0	741 $\pm$ 66	603 $\pm$ 38	0
8	77 $\pm$ 11	63 $\pm$ 14	11 $\pm$ 4	395 $\pm$ 53	474 $\pm$ 16	0
9	32 $\pm$ 6	42 $\pm$ 18	0	449 $\pm$ 88	--	14 $\pm$ 2

¹The erythroid colony counts represent the mean $\pm$ 1 standard error of the mean of 8 separate clots.

Table 3. Effect of Pre-treatment of fetal calf serum (FCS) and citrated bovine plasma (CBP) with anti-erythropoietin (Ep) on the formation of erythroid colonies by bone marrow cells from patients with polycythemia vera in vitro.

Number of Erythroid Colonies/4 x 10 ⁵ Cells ¹						
<u>Patient Number</u>	<u>Untreated</u>	No Ep	Anti-Ep	<u>Untreated</u>	+ Ep	Anti-Ep
		Normal IgG- Treated	IgG- Treated		Normal IgG- Treated	IgG- Treated
1	175 ± 20	163 ± 23	23 ± 11	472 ± 56	512 ± 37	372 ± 73
2	108 ± 26	119 ± 42	28 ± 9	649 ± 75	493 ± 49	576 ± 67
3	174 ± 31	190 ± 51	9 ± 3	397 ± 45	432 ± 87	412 ± 57
4	186 ± 39	147 ± 48	23 ± 12	843 ± 69	789 ± 39	888 ± 90
5	97 ± 12	122 ± 30	4 ± 2	483 ± 59	563 ± 85	415 ± 62
6	108 ± 13	96 ± 27	12 ± 6	593 ± 70	502 ± 76	631 ± 54
7	86 ± 26	84 ± 10	0	731 ± 86	809 ± 84	780 ± 66

¹The erythroid colony counts represent the mean ± 1 standard error of the mean of 8 separate clots.

Table 4. Erythropoietin (Ep)-neutralizing activity of anti-Ep before and after treatment with goat anti-rabbit gamma globulin (GARGG).

<u>Material Assayed</u> ¹	<u>% RBC-⁵⁹Fe Incorporation = SEM</u> ²	<u>P Value</u> ³
Saline	0.56 ± 0.15	
1.0 IU Ep ⁴	24.62 ± 1.97	<0.001 ^a
1.0 IU Ep + 0.025 ml. normal rabbit serum	23.98 ± 1.32	N.S.
1.0 IU Ep + 0.05 ml. normal rabbit serum	25.14 ± 2.08	N.S.
1.0 IU Ep + 0.025 ml. anti-Ep	16.19 ± 1.32	<0.05 ^b
1.0 IU Ep + 0.05 ml. anti-Ep	0.47 ± 1.09	<0.001 ^b
1.0 IU Ep + 0.025 ml. GARGG	27.32 ± 3.18	N.S.
1.0 IU Ep + (0.05 ml. anti-Ep + 0.025 mg. GARGG) ⁵	23.12 ± 1.25	N.S.

¹All volumes were adjusted to 6 ml. with saline.

²Each value represents mean ± 1 standard error of results.

³Compared to a) saline, b) 1.0 IU Ep, determined by Student's t test.

⁴Step III, sheep plasma Ep (Connaught Laboratories, Toronto, Canada).

⁵Anti-Ep and GARGG were incubated together for 1 hour before addition of Ep.

Table 5. Effect of small doses of highly purified (Lot #H4H-35) and larger doses of crude (Lot #H-12) preparations of erythropoietin on erythroid colony formation by bone marrow cells from patients with polycythemia vera cultured in media containing either normal IgG or anti-Ep treated fetal calf serum (FCS) and citrated bovine plasma (CBP).

<u>Ep Preparations and dose (IU)</u>	<u>Number of Erythroid Colonies/6 x 10⁵ Cells¹</u>	
	<u>Type of FCS and CBP Used</u>	
	<u>Normal IgG Treated</u>	<u>Anti-Ep Treated</u>
<u>H4H-35</u>		
0	88 ± 13	0
0.001	--	28 ± 4
0.01	--	53 ± 8
0.04	--	73 ± 11
0.08	--	69 ± 9
0.10	--	62 ± 12
<u>H-12</u>		
0.30	99 ± 8	71 ± 7
0.50	85 ± 23	74 ± 10
1.0	179 ± 18	157 ± 21
2.0	359 ± 38	374 ± 32
3.0	--	429 ± 38
5.0	412 ± 19	398 ± 30
10.0	--	408 ± 22

¹The erythroid colony counts represent the mean ± 1 standard error of the mean of 3 separate studies, each involving a different bone marrow donor. The -- represents undetermined colony counts.

Table 6. Erythroid colony formation by bone marrow cells from adult and fetal sheep in vitro.

<u>Concentration of Cells</u>	<u>Number of Erythroid Colonies¹</u>			
	<u>No Ep</u>		<u>Ep</u>	
	<u>Adult</u>	<u>Fetus</u>	<u>Adult</u>	<u>Fetus</u>
2 x 10 ⁵	0	32 ± 4	379 ± 36	412 ± 47
4 x 10 ⁵	0	57 ± 11	642 ± 71	766 ± 22
6 x 10 ⁵	0	89 ± 8	814 ± 80	1094 ± 52
8 x 10 ⁵	0	123 ± 16	1106 ± 112	1469 ± 44

¹The erythroid colony counts represent the mean ± 1 standard error of the mean from 5 separate studies each involving a different fetal and maternal sheep.

Table 7. Effect of pretreatment of fetal calf serum (FCS) and citrated bovine plasma (CBP) with serum IgG from rabbits immunized against erythropoietin (anti-Ep) on the formation of endogenous erythroid colonies (EEC) by bone marrow cells from fetal sheep in vitro.

<u>Type of FCS and CBP Used</u>	<u>Number of Erythroid Colonies/8 x 10⁵ Cells¹</u>	
	<u>No Ep</u>	<u>With Ep</u>
Untreated	149 $\pm$ 12	1599 $\pm$ 105
Normal IgG-Treated	137 $\pm$ 18	1480 $\pm$ 126
Anti-Ep Treated	2 $\pm$ 1	1695 $\pm$ 87

¹The erythroid colony counts represent the mean $\pm$ 1 standard error of the mean from 5 separate studies, each involving a different fetal sheep.

Table 8. Effect of in vivo administration of serum from patients with polycythemia vera (PV serum) to mice on erythroid colony formation by mouse bone marrow cells in vitro, in the absence of erythropoietin.

<u>Number of Injections</u>	Number of Endogenous Erythroid Colonies/ <u>4 x 10⁵ cells¹</u>	
	<u>Normal Serum</u>	<u>PV Serum</u>
0	0	0
1	0	0
2	0	0
3	0	0
4	0	0
5	0	35 $\pm$ 4
6	0	48 $\pm$ 6
7	0	25 $\pm$ 2

¹The erythroid colony counts represent the mean $\pm$ 1 standard error of the mean from 3 separate studies each involving a different normal and polycythemia vera serum donor.

Table 9. Effect of chronic administration of serum from patients with polycythemia vera (PV serum) to mice on erythroid colony formation by mouse bone marrow cells in vitro.¹

<u>Cell Concentration</u>	<u>Number of Erythroid Colonies²</u>			
	<u>Normal Serum</u>		<u>PV Serum</u>	
	<u>No Ep</u>	<u>0.04 IU Ep</u>	<u>No Ep</u>	<u>0.04 IU Ep</u>
2 x 10 ⁵	0	142 ± 16	12 ± 2	246 ± 39
4 x 10 ⁵	0	292 ± 24	31 ± 3	497 ± 47
6 x 10 ⁵	0	408 ± 39	49 ± 7	714 ± 122

¹Each mouse received 0.5 ml. normal or PV serum/day for 7 days.

²The erythroid colony counts represent the mean ± 1 standard error of the mean from 3 separate studies, each involving a different normal and polycythemia vera serum donor.

Table 10. Effect of in vivo administration of serum from patients with polycythemia vera (PV serum) to mice on radioactive iron incorporation by murine red blood cells.

<u>Number of Injections</u>	<u>Mean % ⁵⁹Fe Incorporation by Blood¹</u>			
	<u>Normal Serum</u>	<u>p² Value</u>	<u>PV Serum</u>	<u>p² Values</u>
0	24.90 ± 3.3		24.90 ± 3.3	
1	26.79 ± 2.0	N.S.	25.31 ± 3.2	N.S.
2	—		31.51 ± 2.6	N.S.
3	32.12 ± 6.0	N.S.	28.80 ± 6.0	N.S.
4	—		34.73 ± 4.5	N.S.
5	24.83 ± 2.8	N.S.	42.86 ± 4.7	<0.05
6	—		39.44 ± 2.7	<0.05
7	12.29 ± 7.6	N.S.	39.88 ± 4.5	<0.05

¹Each value represents the mean ± 1 SEM from 3 separate studies, each involving separate serum donors and 5 mice per group.

²Compared to non-injected controls, determined by Student's t test.

When values were compared daily, no significant difference existed.

Table 11. Effect of in vivo administration of serum from patients with polycythemia vera (PV serum) to mice on radioactive iron incorporation by murine bone marrow.

<u>Number of Injections</u>	<u>Mean % ⁵⁹Fe Incorporation by Bone Marrow¹</u>			
	<u>Normal Serum</u>	<u>P Value²</u>	<u>PV Serum</u>	<u>P Value²</u>
0	0.78 ± 0.06		0.78 ± 0.06	
1	0.47 ± 0.02	P < .0025	0.72 ± 0.05	N.S.
2	--		0.83 ± 0.08	N.S.
3	0.46 ± 0.10	N.S.	0.67 ± 0.09	N.S.
4	--		0.50 ± 0.12	N.S.
5	0.47 ± 0.10	N.S.	0.54 ± 0.02	P < .005
6	--		0.57 ± 0.07	N.S.
7	0.34 ± 0.06	P < .0025	0.36 ± 0.06	P < .0025

¹Each value represents the mean ± 1 SEM from 3 separate studies, each involving separate serum donors and 5 mice per group.

²Compared to non-injected controls, determined by Student's t test.

Table 12. Effect of in vivo administration of serum from patients with polycythemia vera (PV serum) to mice on radioactive iron incorporation by murine spleens.

<u>Number of Injections</u>	<u>Mean % ⁵⁹Fe Uptake by Spleen/100 mg. Liver¹</u>	
	<u>Normal Serum</u>	<u>PV Serum</u>
0	0.19 ± 0.03	0.19 ± 0.03
1	0.86 ± 0.04	0.17 ± 0.02
2	--	0.14 ± 0.01
3	0.79 ± 0.16	0.22 ± 0.17
4	--	0.21 ± 0.22
5	0.77 ± 0.04	0.72 (1)
6	--	0.81 ± 0.09
7	0.78 ± 0.08	0.70 ± 0.07

¹Each value represents the mean ± 1 SEM from 3 separate studies, each involving separate serum donors and 5 mice per group.

Table 13. Effect of in vivo administration of serum from patients with polycythemia vera (PV serum) to mice on radioactive iron incorporation by murine livers.

<u>Numbers of Injections</u>	<u>Mean % ⁵⁹Fe Uptake of Liver¹</u>	
	<u>Normal Serum</u>	<u>PV Serum</u>
0	0.87 $\pm$ 0.14	0.87 $\pm$ 0.14
1	0.54 $\pm$ 0.01	0.84 $\pm$ 0.08
2	--	0.93 $\pm$ 0.09
3	0.39 $\pm$ 0.09	0.41 $\pm$ 0.15
4	--	0.52 $\pm$ 0.01
5	0.34 $\pm$ 0.05	0.51 $\pm$ 0.06
6	--	0.40 $\pm$ 0.05
7	0.43 $\pm$ 0.05	0.33 $\pm$ 0.05

¹Each value represents the mean $\pm$ 1 SEM from 3 separate studies, each involving separate serum donors and 5 mice per group.

Table 14. Effect of in vivo administration of serum from fetal sheep on erythroid colony formation by mouse bone marrow cells in vitro.

<u>Number of Injections</u>	Number of Erythroid Colonies/4 x 10 ⁵ Cells ¹			
	<u>No Ep</u>		<u>With Ep</u>	
	<u>Fetal Serum</u>	<u>Adult Serum</u>	<u>Fetal Serum</u>	<u>Adult Serum</u>
0	0	0	217 ± 18	247 ± 30
4	0	0	319 ± 25	--
8	0	0	382 ± 39	276 ± 19
10	57 ± 7	0	473 ± 52	290 ± 41
12	63 ± 4	0	--	272 ± 22
14	89 ± 11	0	443 ± 46	--

¹The erythroid colony counts represent the mean ± 1 standard error of the mean from 6 separate studies, each involving different fetal and maternal sera.

Legends to Figures:

Figure 1. Effect of anti-erythropoietin (Ep) on the formation of erythroid colonies by normal human bone marrow cells in response to Ep in vitro.

Each point represents the mean $\pm$ 1 standard error of the mean of 5 separate studies, each involving a different donor. Normal rabbit serum IgG was used as control, shown by the line designated Ep.

Figure 2. Erythroid colony formation by bone marrow cells from patients with polycythemia vera. Each point represents the mean $\pm$ 1 standard error of the mean of 22 separate studies, each involving a different donor.

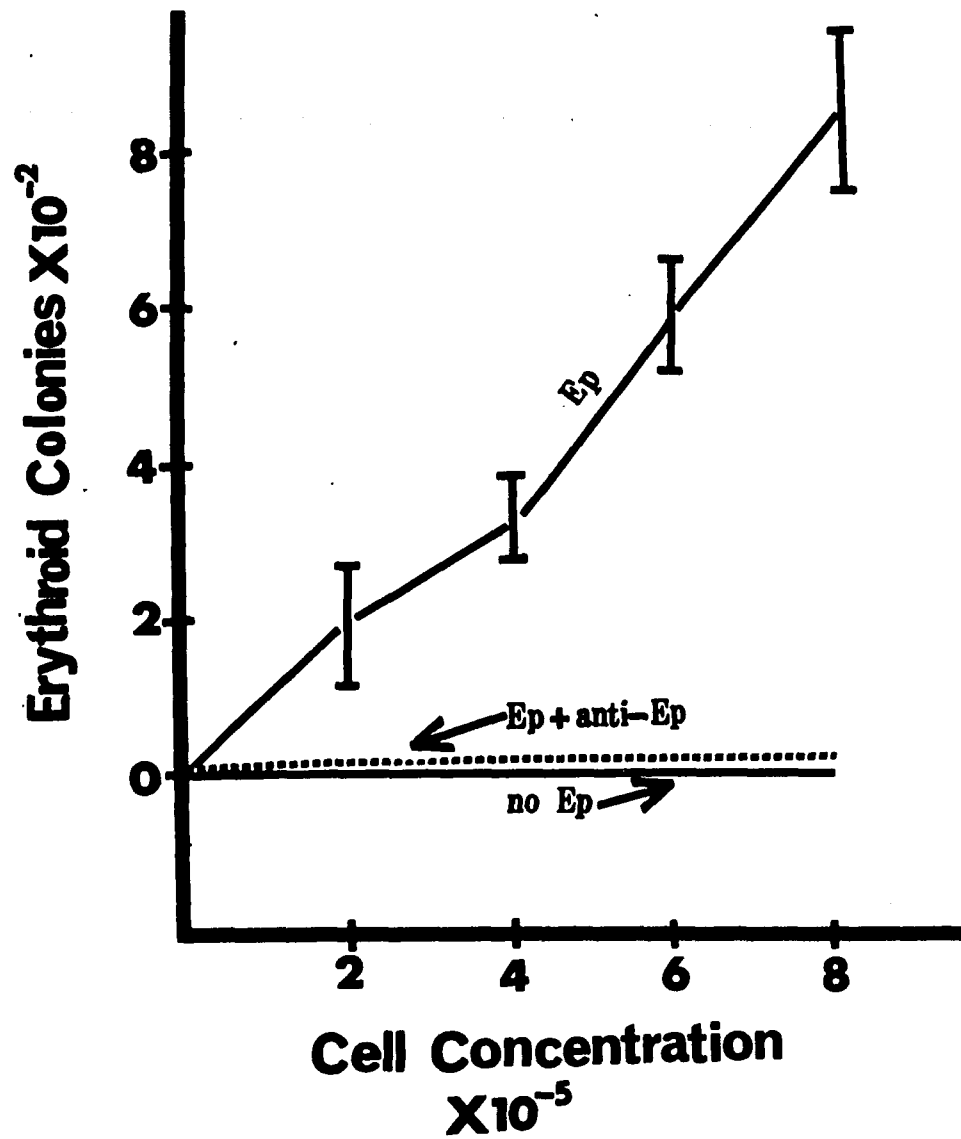


Figure 1

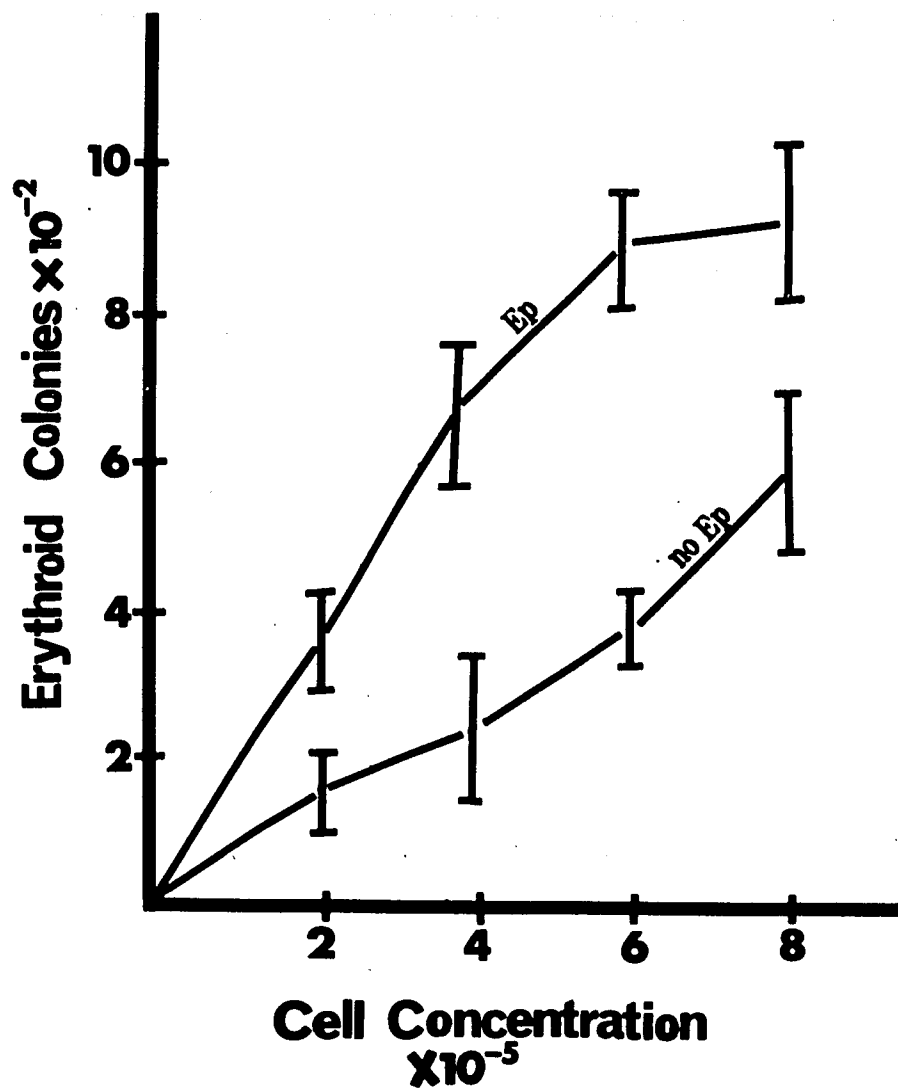


Figure 2

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