1980

Rabbit Erythropoiesis in vitro

K. G. M. Brockbank
Medical University of South Carolina

RABBIT ERYTHROPOIESIS IN VITRO

by

KGM Brockbank

A dissertation submitted to the Faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the School of Graduate Studies.

Department of Pathology

1980

Approved by:

Chairman, Advisory Committee

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ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Ogawa for giving me a stimulating apprenticeship in experimental hematology.

I am also indebted to Dr. M. Spector, Dr. S. Kurtz, Dr. R. Galbraith, Dr. P.N. Porter and Mrs. A. Leary for their advice and encouragement during the course of my studies

Chapter 1 of this thesis will be published in-part in an invited review:

Brockbank, K.G.M. and Spector M.: Experimental Heterotopic Hemopoiesis. Clin. Orthoped., in press, 1980.

Chapter 3 of this thesis has been accepted for publication:

Brockbank, K.G.M., Ogawa, M., and Spector, M.: Hemopoiesis in Demineralized Bone Allografts. Exp. Hemat., 8: 763, 1980.

Chapter 4 of this thesis has been submitted for publication:

Brockbank, K.G.M., Porter, P.N., and Ogawa, M.: Burst-Promoting Activity Derived from Rabbit Bone Marrow Conditioned Media: A Quantitative Assay and Cellular Mechanisms of Action. 1980.

ABSTRACT

KGM Brockbank. Rabbit Erythropoiesis *In Vitro*.

The objective of the studies described was to analyze inductive and proliferative phenomena involved in hemopoiesis. In order to perform these studies in the rabbit it was necessary to vindicate the use of the methylcellulose assay for erythroid precursors in this species.

The erythropoietin (Ep) dose response of erythroid colony-forming units (CFU-e) and erythroid burst-forming units (BFU-e) from rabbit bone were similar to murine erythroid precursor Ep requirements. Bone marrow and peripheral blood contained a continuum of erythroid precursors at differing stages of maturation. The earliest BFU-e were assayed on day 10. CFU-e were observed in bone marrow, but not in peripheral blood. The most mature erythroid precursors observed in peripheral blood were intermediate BFU-e, assayed on day 5. A linear relationship exists between the number of nucleated bone marrow cells plated and the number of 3 day colonies and 10 day bursts observed. The 10 day bursts were composed of erythroid cells at all stages of maturation and these cells contained normal adult hemoglobins. The methylcellulose assay was then used

to characterize the hemopoietic tissue in ossicles induced by demineralized allogeneic bone matrix (DBM) and to investigate the mechanisms of action of burst-promoting activity (BPA).

DBM, implanted in muscle, induces the formation of an ossicle within which hemopoietic tissue develops. Analyses of ossicle marrow in vitro demonstrated the presence of committed hemopoietic precursors; colony-forming units in culture (CFU-c), CFU-e and BFU-e by 6 weeks postimplantation. The time courses of colony and burst formation by erythroid precursors in ossicle and femoral marrow were similar. Induction of hemolytic anemia by phenylhydrazine hydrochloride at six weeks post DBM implantation showed that the ossicle marrow was responsive to systemic erythropoietic stimuli. The DBM implant is a unique model for studying the development of hemopoietic microenvironments within bone.

Rabbit bone marrow conditioned media (BMCM) was found to contain potent erythroid BPA. In order to further characterize the mechanisms of action of BPA and to improve the quantitation of BPA, we studied the effects of BMCM on the number of bursts, cells per burst and ^{59}Fe incorporation into heme. Examination of erythroid precursors at different maturational stages revealed that the sensitivity of erythroid precursors to BPA decreases with maturity. Delayed addition of BMCM to cultures demonstrated a requirement

for BPA during the early stages of burst formation. BMCM did not enhance granulocyte/macrophage colony formation. The enhancement of heme synthesis by BMCM was routinely much greater (range, 7- to 109-fold) than the increase in burst number (range, 1- to 2-fold). The latter observation suggested that BPA might increase the size of bursts in addition to augmenting burst number. Simultaneous measurement of cell number and ^{59}Fe incorporation in individual bursts showed a strong correlation between these parameters. In this experiment, the total enhancement of cell numer and ^{59}Fe incorporation was 6.6- and 8.0-fold, respectively. These results suggest that a major effect of BMCM BPA is to promote cell division during the early phase of burst formation.

Chapter 1.

GENERAL INTRODUCTION: REGULATION OF ERYTHROPOIESIS

INTRODUCTION

The purpose of this dissertation was to study inductive and proliferative phenomena in hemopoiesis. The first objective was to study the induction of bone marrow in association with bone (1) in heterotopically placed demineralized bone matrix (DBM) implants (2). The second objective was to examine factors involved in erythroid proliferation. The rabbit was utilized for these studies and culture methods were devised which could be used for analysis of erythroid precursor cells. In particular the in vivo methylcellulose assay, originally described by Iscove et al. (3) for murine erythroid precursors, was employed after the appropriate adaptation. The initial part of my thesis involves the characterization of this assay for rabbit erythroid precursors.

The DBM model has been utilized for the study of bone formation (2). The implantation of DBM induces a cascade of biological events which culminate in the formation of an ossicle with central marrow. This model has potential for the study of early events in bone marrow formation.

During the characterization of the methylcellulose

assay it became apparent that the rabbit might be an excellent model for the study of factors, known as burst promoting activity, which are involved in the regulation of the early stages in erythropoiesis. Previous studies by Wagemaker (4), Ploemacher et al. (5) and Kurland et al. (6) have implicated the macrophage as a source of burst promoting activity. Morphologic evidence based on the observation that differentiating erythroblasts are frequently associated with macrophage in erythroblastic islands (7) has for many years been thought to imply that macrophage have a role in the formation of erythroid hemopoietic microenvironments. In order to put my work into perspective I will present an overview of erythropoietic regulation and introduce the assays and models available for its study in this chapter.

THE ERYTHROID MICROENVIRONMENT

Murine pluripotent hemopoietic stem cells may be assayed by transplantation of bone marrow cell suspensions into lethally irradiated mice (8). The transplanted stem cells generate macroscopic colonies of erythroid, granulocytic or megakaryocytic cells in the spleen. The stem cells assayed by this technique have been designated spleen colony forming units (CFU-S). There is a considerable amount of evidence supporting a clonal origin for spleen colonies. The curve relating the number of nucleated marrow cells that are transplanted to the number of colonies that develop is linear (8,9). More direct evidence has been obtained by inducing chromosome abnormalities in CFU-S prior to implantation. Recognizable chromosome abnormalities were observed in 10% of the spleen colonies and in each case at least 95% of the cells possessed the specific abnormality (10). The CFU-S gives rise to precursor cells capable of differentiation in only one hemopoietic line of development via a process known as commitment. In the spleen colony assay the colonies usually consist of cells following one line of development during the first 8 to 10 days (10-14). Later as the colonies get

larger they develop secondary lines of development. Trentin (13,14) proposed that this phenomena is due to colonies encroaching upon areas of hemopoietic stroma which induce uncommitted spleen colony cells to become committed to other hemopoietic development lines. These areas of hemopoietic stroma which induce commitment to specific lines of development are known as hemopoietic microenvironments. In the murine spleen erythroid microenvironments predominate (11), while in the bone marrow granulocytic microenvironments are more frequent (15). Wolf and Trentin (15) demonstrated quite clearly that the microenvironment distribution in spleen and bone marrow is a function of endogenous factors. Both tissues when implanted ectopically retained their original ratio of erythroid and granulocytic spleen colonies. In marrow implants in spleen the colonies growing across the junction of the two tissues showed abrupt transitions in cell type, erythroid cells on the spleen side and granulocytic cells on the bone marrow side. Further evidence for specific erythroid microenvironments was obtained via the spleen colony assay in hypertransfused mice (11,16). Hypertransfusion suppressed the formation of erythroid colonies. In place of macroscopic erythroid colonies microscopic nests of undifferentiated erythropoietin sensitive cells were observed. These undifferentiated colonies did not become granuloid even

though normal granulocytic colony formation was occuring in adjacent areas.

McCuskey et al. (17) have dissected the hemopoietic microenvironment morphologically into three compartments: a microvascular compartment consisting of arterioles, capillaries, sinusoids and venules; a connective tissue compartment composed of fibers substance and cells; and neural elements associated with both the blood vessels and stroma. The study of McCuskey et al. (17) demonstrated specific alterations in blood flow and glycosaminoglycan type in response to erythropoietic stimulation and repression. During erythropoietic repression the blood flow decreased, only sulphated acid glycoaminoglycans were observed, granulopoiesis predominated, and small undifferentiated colonies were frequent. During erythropoietic stimulation the blood flow was elevated, neutral glycosaminoglycans were observed in addition to sulphated acid glycosaminoglycans, and erythropoiesis was enhanced. Whether or not these changes in vivo during erythropoietic modulation directly effect erythropoiesis can not be determined from these studies. However, Ploemacher et al. (18) have shown an in vitro effect of acid glycosaminoglycans on erythroid proliferation and differentiation. So it is likely that the ground substance components of the hemopoietic microenvironment effect erythropoiesis.

EXPERIMENTAL HETEROTOPIC BONE MARROW MODELS

There are a number of models which might be useful for the study of the development of hemopoietic microenvironments in adult mammals. Heterotopic hemopoiesis can be established by implantation of medullary marrow (19) or demineralized bone matrix (2), and via a number of techniques in association with epithelial cells (20). In all these models bone formation preceeds the development of hemopoietic tissue and the general impression is that the histogenesis of the bone marrow recapitulates its ontogeny.

Implantation of medullary marrow into extramedullary sites leads to the formation of an ossicle with a central marrow in rats (19,22), mice (22), and rabbits (23,24). Tavassoli and Crosby (19) have described the sequence of events culminating in ossicle formation. Within 24 hours of marrow implantation capillaries from the surrounding host tissue penetrate the implant. These vessels rapidly establish an extensive capillary bed. By day 3-4, the implant consists of proliferating fibroblasts interspersed between large numbers of capillaries. Some isolated concentrations of osteoid may be seen and very few of the original implanted hemopoietic cells remain. By day 6-7 most of the implant is occupied by bone; between the areas of bone is a loose connective tissue stroma and highly branched intercommunicating

vascular sinuses. The first hemopoietic elements may be seen after day 10. During the ensuing weeks the bone resorbs and the marrow extends. The product of these simultaneous processes is an ossicle with a thin shell of bone and a large medullary cavity four weeks after implantation. The ossicles remain for at least six months (24). The ossicle marrow demonstrated enhanced erythroid proliferation in response to phenylhydrazine induced anemia indicating that the marrow is functionally normal (163). Friedenstein et al. (25) have refined this model by demonstrating that isolated marrow stromal cells produce ossicles by the same developmental sequence. Chromosome marker studies indicate that the bone cells (22) and the marrow stroma precursor (26) are of donor origin, while the hemopoietic cells are of recipient origin (22). Therefore the implantation of marrow is basically the transfer of hemopoietic microenvironment. This is an excellent model for the study of hemopoietic microenvironment development. The only reservation being that the earliest stages of hemopoietic tissue development are obscured by the presence of many stromal cells, which make it difficult to tell whether their distribution is coordinated in any particular manner.

Bone and hemopoietic tissue may be induced by the interaction of connective tissue cells with several sources of epithelial cells. The best studied epi-

thelial inductor is the transitional epithelium (TE) lining the urinary tract and bladder. The inductive properties of TE have been examined in a variety of mammals (27-37). The hemopoietic tissue requires further study before judgement is made with regard to its suitability as a model for bone marrow development. A number of epithelial cell lines (FL, WISH, Hep-2, Hela, KB, CLV-X and CLV-4) have been shown to induce bone and bone marrow in cortisone treated mice (38-42). This model is not suitable for the study of hemopoietic microenvironment formation because only 50% of the grafts induce hemopoietic tissue and the induced tissues are rapidly resorbed.

The last model I would like to discuss involves the implantation of demineralized bone matrix (DBM). DBM implantation in a variety of heterotopic sites (43) produces an ossicle with a central marrow (2). The most common site of implantation is in a muscle pouch (2,44,45). Urist (2) has reported successful ossicle induction by DBM in mice, rats, rabbits guinea pigs and dogs. The events during development of DBM-induced ossicles have been described by Urist (2), his observations have been corroborated and the hemopoietic tissue described in more detail by Reddi and Huggins (44), Bombi et al. (45), and Friedenstein et al. (46). At approximately 5-10 days after implantation the DBM is

invaded by mesenchymal cells from the surrounding connective tissue. At 10 days mesenchymal cells may be observed in synchrony with the appearance of matrix resorbing multi-nucleated giant cells and capillary ingrowth. By 15-20 days newly differentiated osteoblasts start to elaborate calcifiable osteoid. After 20 days, bone formation, bone remodeling, marrow stroma and sinusoid development and the initiation and expansion of hemopoiesis occur in a coordinated manner. A fully formed ossicle with a bone cortex and hemopoietic medulla is present at two weeks and may still be present after 300 days (44). Ultrastructurally the hemopoietic cells are normal (44,45) and ^{59}Fe incorporation into heme was used to detect hemoglobin synthesis in the ossicles (44). This model would appear to be the best of the models discussed for the study of hemopoietic microenvironment formation. The DBM ossicles are discrete which permits easy location and removal. Multiple implants can be made in each experimental animal, which would permit sequential studies or multiple analyses to be made. The ossicles have a long life span. Finally, the DBM possesses no viable cells at implantation, so the earliest events in hemopoietic microenvironment formation may be observed. However, it should be noted that prior to using this model for the study of hemopoietic microenvironment formation it

is necessary to demonstrate that the hemopoietic tissue is normal both in hemopoietic precursor cell content and their functional responses to proliferative stimuli.

HUMORAL REGULATORS OF ERYTHROPOIESIS

Erythropoietin (Ep):

Ep is considered the primary regulator of vertebrate erythropoiesis. Ep is a glycoprotein produced principally by the kidney in response to tissue hypoxia (47,51). Ep has been purified from sheep plasma (52) and from anemic human urine (53). The international standard unit of Ep is equivalent to 1.48mg. of a crude human Ep assayed by the method of Kajal and Erslev (54) in either hypertransfused or exhypoxic mice.

Ep initiates red cell differentiation in a morphologically uncharacterized cell known as the erythropoietin responsive cell (ERC) (55,56). The ERC is thought to be a committed erythroid precursor and there is a lot of evidence which differentiates the ERC from the CFU-S. Ep administration does not influence CFU-S numbers (57). When CFU-S are depleted the ERC can still be detected (58) and vice versa, when the ERC are depleted by tritiated thymidine the CFU-S are scarcely effected (59). Furthermore, the ERC can be separated from the CFU-S by unit gravity sedimentation (60).

Ep promotes proliferation and differentiation of the ERC. Within a short period of time (3 days) after Ep administration, the incorporation of labelled thymidine and uridine (61), RNA synthesis (62), DNA synthesis

(63), mitotic index (63), and hemoglobin synthesis (64) are increased. The speed with which Ep acts to promote erythropoiesis is thought to imply that the ERC is a relatively mature erythroid precursor not far removed from the early erythroblast. Marks and Rifkind (65) showed that Ep increased the number of cells synthesizing hemoglobin and that the amount of hemoglobin synthesized (measured by tritiated leucine incorporation) per cell did not change. Thus Ep would appear to promote proliferation and differentiation of the ERC.

Further characterization of the ERC was difficult because of the lack of morphologic characterization. The development of two in vitro assays for erythroid precursor cells, the plasma clot (66) and methylcellulose (67) techniques in the early 1970's were major breakthroughs. In these assays two committed erythroid precursor cells could be detected. Stephenson et al. (66) described the formation of small (8-32 cells) erythroid colonies from erythroid precursors, dubbed erythroid colony-forming units (CFU-e), in murine bone marrow after two days of culture. Axelrad et al. (68) demonstrated the presence of a second class of committed erythroid precursor cells, known as erythroid burst-forming units (BFU-e), which produce bursts of small colonies after longer periods in culture. The CFU-e require approximately physiological concentrations of

Ep (0.01-0.25 units) to form colonies *in vitro* and are thought to be relatively mature erythroid precursor cells placed just before the pronormoblast in the erythroid series. The BFU-e require high levels of Ep (0.1-2.5 units) for burst formation and are believed to be an early erythroid precursor cell separated from the CFU-S by committment to erythroid development (69,70,71). Both the CFU-e and BFU-e derived colonies have been shown to be clonal in origin (72,73,74). Further study has demonstrated the presence of a continuum of intermediate precursor cells between the BFU-e and CFU-e (71,75,76,67,77). BFU-e, but not CFU-e, have been observed in the blood of mice (78,79) and humans (80,81). The BFU-e in blood are found in the null-cell leukocyte fraction (81). Bone marrow BFU-e and CFU-e have been differentiated by separation at unit gravity and by sensitivity to tritiated thymidine (82,75,83,84).

The response of erythroid precursors to ambient Ep levels *in vivo* can be determined *in vitro*. Elevation of Ep levels can be produced by bleeding, induction of hemolytic anemia with phenylhydrazine hydrochloride (PHZ), Ep injection and intermittent hypobaric hypoxia. Depression of Ep levels can be produced by hypertransfusion with packed erythrocytes or by prolonged subjection to a hypobaric atmosphere followed

by a few days at normal atmospheric pressure (exhypoxic). Murine CFU-e increase following in vivo elevation of Ep (84,78,79,83,85) and decrease when Ep is depressed (68,84,79,83). Murine BFU-e migration from bone marrow to spleen may be enhanced by elevation of Ep (78,83), but no significant changes in marrow BFU-e number occur following either increase or decrease of Ep (71,84,86,78,83,85). The proliferative state of BFU-e and CFU-e, assayed by sensitivity to tritiated thymidine, is not changed during erythropoietic stimulation and suppression (83,84). These observations indicate that Ep stimulates proliferation of intermediate erythroid precursors to produce increased CFU-e numbers and that Ep has little effect on BFU-e.

Burst-Promoting Activity

In the section on Ep it was noted that BFU-e require higher than physiological concentrations of Ep in order to produce bursts. In addition BFU-e did not appear to be influenced by *in vivo* Ep fluctuations. These observations suggest that factors other than Ep may be involved in the production of BFU-e from CFU-S and for regulation of BFU-e differentiation to an Ep responsive stage.

During the past few years investigators have observed the enhancement of burst number by addition of media conditioned by human peripheral blood leukocytes (87,88), lectin stimulated murine spleen (89), bone marrow (90), T-cells (91,92), monocytes (93), macrophage (94,6) and non-adherent (non T-cell) peripheral blood mononuclear cells (95). The factor(s) responsible for increasing burst number has been designated burst-promoting activity (BPA). BPA has also been detected in urine (96) and in serum (97) of anemic humans. In addition to enhancing burst number conditioned media with BPA have also been reported to induce the formation of mixed colonies (colonies with cells of more than one hemopoietic line) (87,89,98), increase hemoglobin synthesis (90), and enhance human fetal hemoglobin synthesis (99) in culture. The increase in burst number may be due to stimulation of a

BFU-e population with high BPA requirements or uncommitted pluripotent stem cells. The latter would explain the observation of mixed colonies. A requirement for BPA during early proliferation in bursts prior to the development of Ep responsiveness has been shown, but Ep is still required for terminal differentiation to recognizable erythroid cells (86,90,100). The mechanism(s) of action of BPA are still uncertain.

A very active area of BPA research has been the identification of cell types which produce BPA. Isolated studies have shown that radioresistant, non-adherent, non T-cell peripheral blood mononuclear cells produce BPA (95) and that marrow stromal cell addition to the erythroid precursor cell assay enhanced BFU-e and CFU-e (101). Most evidence, however, implicates T-cells and monocyte-macrophages as sources of BPA.

A role for lymphocytes in erythropoiesis was indicated by studies in the early 1970's which showed that thymocytes could promote erythrocyte formation (102,103). More recently data has specifically implicated T-cells. Removal of T-cells from mononuclear leukocyte preparations led to a decrease in burst number which was corrected by replacing the T-cells (104). Phytohaemagluttinin (PHA) stimulated marrow cells or thymocytes produce an increase in erythroid colonies in bone marrow suspensions placed intraperi-

toneally in diffusion chambers. Pretreatment of the PHA stimulated cells with antitheta serum and complement eliminated the colony increment (105). The genitically anemic W/W^V mouse could not be cured by T-cell depleted marrow transplants (106). Nathan et al. (91) reported that the addition of T-cells or media conditioned by tetanus toxoid stimulated T-cells enhances burst formation. The most convincing evidence for T-cells as a source of BPA comes from the production of BPA by a human T-lymphocyte cell line (Mo) (92).

A role for the macrophage in erythropoiesis has been suggested by the observation in morphological studies of an intimate physical relationship between macrophage and developing erythroid cells in erythroblastic islands (7). In vitro studies have generated much stronger evidence. Rinehart et al. (107) showed that 20% monocytes in the BFU-e assay completely suppressed burst formation and that low concentrations of monocytes (1%) favour erythropoiesis. Wagemaker (108) has observed BPA by addition of irradiated bone marrow cells to the BFU-e assay. The cells responsible for this BPA, which he termed burst feeder activity, have a bouyant density of 1.083 g/cm^3 and a modal sedimentation rate of 4.7mm/hr. (4). Intravenous administration of inert polystyrene latex particles, which were mainly phagocytosed by macrophages, induced changes

in the femoral BFU-e content following an increase in burst feeder activity (5). These observations indicate that macrophage may be responsible for burst feeder activity. Murphy and Urabe (109) cultured mouse non-adherent bone marrow cells in the presence of varying concentrations of peritoneal macrophage and observed enhanced burst and colony formation. Kurland et al. (6) have carried out similar studies and also showed that macrophage conditioned media could enhance erythroid colony formation. Interestingly the sedimentation velocity characteristics of the stimulatory peritoneal macrophage resemble those of the cells responsibile for burst feeder activity in the study of Wagemaker et al. (4). Two recent studies, one with a murine macrophage cell line (WEHI-3) and the other with a human monocyte cell line (GCT), are the most convincing evidence for a role of monocyte-macrophage in the production of BPA (94,93).

It is likely that the BPA produced by these cell types is not the same, and it is also possible that some of these BPAs do not effect BFU-e directly. The T-cell BPA may modulate monocyte-macrophage BPA production or vice versa. The other cell types, mentioned briefly. which have been associated with BPA, may also be involved. Complex lymphocyte-macrophage interactions have been well documented in other cell systems (110).

Other Hormones

Although erythropoietin is the major regulator of erythropoiesis other hormones may influence red cell production. Considerable information has been obtained from *in vivo* experiments. Androgenic steroids can stimulate erythropoiesis (111) *in vivo* by enhancing erythropoietin production (112). The *in vivo* evidence for glucocorticosteroids is contradictory (113-116), however, dexamethasone appears to stimulate erythropoiesis by an effect on erythropoietin production (117). Thyroid hormones have also been shown to increase erythropoiesis when administered to animals (118-120) and anemia is regularly observed in the hypothyroid man (121). It is not clear from *in vivo* studies whether thyroid hormones directly effect erythroid precursor cells or act primarily upon erythropoietin production (122,121,119,123,124). In general the *in vivo* approach to analysis of hormonal effects on erythroid precursors suffers from the problem of whether any effects observed are direct or indirect.

In vitro analysis of hormone interactions with erythroid precursors removes the possibility of indirect effects. Androgens (125) and phosphodiesterase inhibitors (126) enhance erythroid colony formation. Golde et al. (127) has reported that dexamethasone stimulates erythroid colony formation in mouse and human

bone marrow cultures. Most investigators, however, have found glucocorticosteriods to be inhibitory for rodent bone marrow erythroid precursors [cortisol (125), cortisone (128), and dexamethasone (129,128)]. Urabe et al. (130) compared the effects of dexamethasone on human and murine bone marrow and found dexamethasone inhibitory for mouse and stimulatory for human erythroid precursors. Other hormones which potentiate erythroid colony and burst formation in vitro are thyroid hormones (131,132), B-adrenergic agonists (133,134), dibutryl cyclic AMP (135,136,133,134), prostaglandins (135,137,138,134), and growth hormone (134).

MATURATION AND DELIVERY OF ERYTHROCYTES

Erythroblastic islands consisting of maturing erythroblasts and one or two central macrophage have been observed in the liver of mice following induction of hemolytic anemia with phenylhydrazine (139), and in normal bone marrow (140), spleen (141), and yolk sac (142). In the erythroblastic island, the erythroid cells are arranged in one or more corona with the cells of each corona being at approximately the same maturational stage, the most mature cells being on the outside (143, 144,141,145,139). A number of roles have been proposed for the central macrophage; (A) delivery of ferritin to the maturing erythroid cells (114), (B) detainment of differentiating erythroblasts, which would inhibit premature delivery into the circulation and (C) the ability to phagocytose expelled normoblast nuclei and deranged erythroid elements (146,147).

The vascular sinuses of the marrow are the site of transmural migration of blood cells. Their wall consists of an endothelium, a discontinuous adventitia and basement membrane (148). The components of the sinus wall may play an important role in erythrocyte delivery to the circulation. Several investigators have presented evidence which indicated that junctional structures might be involved in maintaining endothelial cell contact (149-151). More recent evidence based on freeze

fracture and ultrastructural analysis of lanthanum localization in intra-endothelial spaces indicates that there are no junctional structures present (152). These last observations indicate that transmural transport may be interendothelial rather than transendothelial as proposed previously (153,150,151). The absence of tight junctions may permit the endothelial cells to slide over one another resulting in sinus lumen diameter changes. This would explain the rhythmic sinusoid dilations observed by Branemark (154) during in vivo microscopic studies of bone marrow. These periodic fluctuations in sinus diameter could serve as a mechanism for deliver of hemopoietic cells into the circulation (152).

The adventitial covering of sinusoid consists of radio-resistant, long lived reticular cells (155). These cells, unlike endothelial cells, possess contractile cytoplasmic filaments (156-158). Tavassoli (159) observed a reduction in the quantity of sinus wall covered by adventitial cells after phlebotomy. This reduction may be promoted by erythropoietin (160,161). It is still not clear whether the effect of erythropoietin is direct or indirect. McCuskey and Meineke (162) suggested that erythropoietin may mediate the release of a vasoactive substance from erythropoietin responsive stem cells.

SUMMARY

Due to the development of a pluripotent stem cell assay in the mouse, during the 1960's, studies of murine hemopoiesis have dominated experimental hematology. Approximately ten years later *in vitro* assays for murine erythroid precursors were described. Studies using these assays, repeated in humans when possible, have granted us considerable insight into the regulatory mechanisms of erythropoiesis. The accumulated data suggests that the earlier stages of erythropoiesis, such as commitment of pluripotent hemopoietic stem cells to the erythroid line of development and functional differentiation of committed stem cells, are controlled at the cellular level by interaction with hemopoietic microenvironments. Potential models for the study of hemopoietic microenvironment development were discussed. The demineralized bone matrix system appeared to be the best model for such studies, but further characterization is required. Humoral factors, such as erythropoietin and a variety of nonspecific hormones, are involved in the intermediate stages of erythropoiesis during which proliferation and differentiation occur. Recent evidence for humoral factor(s) with burst-promoting activity which are required for early erythroid proliferation *in vitro* was presented. The mechanism(s) of action of burst-promoting activity are still speculative. The

final stages in erythropoiesis, maturation and delivery, appear to be a function of the hemopoietic organ structure.

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Chapter 2.

CHARACTERIZATION OF RABBIT ERYTHROPOIESIS IN CULTURE

INTRODUCTION

Rabbits are an excellent experimental animal for hematological studies because repeated blood and bone marrow sampling can be carried out. Rabbit erythroid colonies have been described previously in the methylcellulose assay (1) and in the plasma clot assay (2). Rabbit bursts have been described in the plasma clot assay (3). The purpose of this study was to characterize rabbit erythroid precursors in the methylcellulose colony assay.

MATERIALS AND METHODS

Cell Preparation:

Male New Zealand white rabbits weighing 2.5-3.5kg. were employed in the study. Bone marrow was aspirated from the femur and collected in 6 ml. falcon plastic tubes containing heparin without preservatives (Chromalloy Pharmaceuticals, Inc., St. Louis, MO). The buffy-coat cells were collected after centrifugation and resuspended in α- medium (Flow Laboratories, Inc., Rockville, MD). Peripheral blood was obtained by bleeding from the car veins and mononuclear cells were harvested using the Ficoll-Isopaque technique described by Boyum (4) with slight modifications (5).

Bone Marrow Conditioned Media (BMCM):

Marrow buffy-coat cells (10^6 cells/ml) were cultured in glass flasks in 10 ml. of α-medium containing 1% deionized bovine serum albumin (Calbiochem, San Diego, CA), 1% fetal calf serum (FCS) Flow Laboratories, Inc.) and 10^{-4}M mercaptoethanol (Fisher Scientific Co., Norcross, GA). After 1, 2, 3, 4, and 5 weeks of culture, one-half of the culture medium was removed and replaced with fresh medium. BMCM was centrifuged at 800g and the supernatant stored at -70°C.

Erythropoietic Cell Culture:

Aliquots of 10^5 marrow nucleated cells or 5×10^5 peripheral blood mononuclear cells were plated in Lux standard non-tissue culture dishes (#5221P, Flow Laboratories, Inc.) in 1 ml. of media per dish.

The media consisted of α-medium, 0.8% methylcellulose (Fisher Scientific Co.), 1% deionized bovine serum albumin, 30% FCS, 10^{-4}M mercaptoethanol and 1.0 U of Step III preparation of sheep plasma erythropoietin (Ep) with specific activity of 13.7 U/mg. protein (Connaught Labs., Ltd. Willowdale, Ontario, Canada). When BMCM (10% V/V) was added to experimental culture FCS content was reduced to 10%. Controls with no BMCM contained the same concentrations of all other media components as the experimental cultures with BMCM. The dishes were in-

cubated at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Red colonies and bursts were counted using an inverted microscope (65X) on days 3 and 10, respectively.

Incorporation of ^{59}Fe into Heme:

Cultures were labeled for 24 hours by carefully overlaying each dish with 0.3ml of α-medium containing 50% heat-inactivated rabbit serum (30 minutes at 56°C) and 0.5 μCi of ^{59}Fe-citrate (6) on day 10 of incubation. Heme was extracted by the cyclohexanone method (7).

Analysis of Hemoglobin:

Hemolysates of rabbit erythrocytes were prepared by the method of Drabkin (8) and were stabilized by the addition of 40μl of 0.1 M KCN to prevent oxidation (9). Burst hemoglobin was labelled for 24 hours by overlaying 2μCi of uniformly C^{14}-labelled amino acid mixture (NEC-445, New England Nuclear, Boston, Mass.) in 0.3ml. of phosphate buffered saline (PBS) on day 10 of culture. Hemolysates from C^{14}-labelled and non-labelled bursts was prepared by lifting each burst with a 10μl pipet and pooling the bursts of each dish in 200ul of PBS in a microcentrifuge tube. The cells were then washed twice in PBS by centrifugation and the cell pellets were frozen at -70°C. The frozen cell pellets were thawed at room temperature and lysed by addition of a solu-

tion containing 40ul of 0.01M KCN and 10ul of 10% nonidet P-40. The hemolysates were subjected to isoelectric focusing, which was carried out by using an LKB Multiphor apparatus and the conditions described by Allen et al. (10) and Drysale et al. (11). Ampholytes in the pH range 6-8 were used. After separation the gels were fixed in 15% trichloracetic acid, stained with dimethoxybenzidine P-509, and destained in 10% acetic acid. When C^{14}-labelled hemolysates were used, gels were stained with Coomassie Blue R-250 and dried. After drying, the labelled gels were placed in contact with Kodak RP "X_Omat" film (Eastman Kodak Co., Rochester, New York) that had been previously exposed to a brief flash of light using an electronic photographic flash unit (Vivitar 283, Vivitar Corp., Div. of Ponder and Best Inc., Santa Monica, Ca.). Pre-exposure of the film raised the level of background fog absorbance by 0.15 OD and established a linear relationship between radioactivity of the sample and density of the fluorogram band (12). Densitometric tracings of the bands were carried out on a Joyce Loebl 3CS microdensitometer (Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne, England).

Light Microscopy:

Bursts were individually lifted with a 10ul pipet and applied to slides via a cytospin centrifuge. The

cells were stained by Wright's stain or for red cell pseudoperoxidase activity by incubating with the substrate 3-3' diaminobenzidine (DAB). The latter stain utilized the technique of Graham and Karnovsky (13) with a slight modification (14). The substrate medium contained 9 mg. of DAB dissolved in 30ul of 0.05 M Tris-HCl buffer, pH 7.6 Six milliliters of 3% hydrogen peroxide solution were added to the DAB solution just prior to use.

Transmission Electron Microscopy:

One ml. of fixative containing 4% glutaraldehyde with 0.1 M cacodylate, buffered at pH 7.4, was added to culture dishes containing bursts and incubated at room temperature for 1 hr. The content of each dish was solidified by addition of 1 ml. of 2% Bacto-agar (14) and removed as an intact disc. The disc was rinsed overnight in 0.1M cacodylate buffer. Intact individual bursts were dissected out of the disc, post-fixed for 1 hour in 2% osmium tetroxide in 0.1M cacodylate buffer, dehydrated through graded ethanol to polypropylene oxide and embedded in Epon. Thin sections of bursts were examined after staining with uranyl acetate and lead citrate in a Hitachi 12A electron microscopy.

RESULTS

In preliminary studies of methyl-cellulose cultures containing 10^5 rabbit marrow nucleated cells and 1.0 U/ml of Ep, it was found that red cells could first be observed in colonies on day 3 after plating. These colonies consisted of 8-50 cells and rapidly disintegrated after hemoglobinization. A time course study of colony formation (Figure 3, Chapter 3) revealed that these colonies were most numerous on day 3 and rapidly decreased with longer periods of culture. On day 4 of culture colonies composed of 3-10 subcolonies were observed and with longer periods of culture larger colonies composed of many subunits were seen. These colonies composed of three or more subunits are known as bursts. Time course studies of burst formation in cultures containing rabbit bone marrow cells or rabbit peripheral blood cells are presented in Table 1. Peripheral blood cultures contained fewer small and intermediate sized bursts and no small 3 day colonies. Because very few bursts formed later than 10 days in culture it can be assumed that 10 day bursts are derived from early BFU-e. Day 10 was selected for burst quantitation.

Table 2 shows results obtained when colony and burst formation responses at different Ep concentrations were examined. CFU-e required approximately physiological concentrations of Ep in order to form the colonies.

The BFU-e required much higher than physiological concentrations of Ep for burst formation. Based on these studies 1.0 U per ml. of erythropoietin was selected for further studies of colony and burst formation.

Due to unknown and possibly variable plating efficiencies affecting the reliability of erythroid precursor quantitation it is necessary to have a well defined, preferably linear, relationship between the number of cells plated in culture and the number of colonies that form. Linearity of the culture system was tested by varying the cell concentration in the presence of 1.0U/ml of Ep. The number of 3 day colonies formed correlated well (r-0.9975) with the number of cells plated between 10^4 and 2.5 x 10^5 nucleated cells per dish (Fig. 1). Similarly the number of 10 day bursts correlated well (r-0.9792) with the number of cells plated between 10^4 and 1.5 x 10^5 nucleated cells per dish (Fig. 2). It was not possible to quantitate bursts at higher cell numbers because they tended to merge into one another.

It has been reported that human BMCM can promote human burst formation (6). The addition of rabbit BMCM to the rabbit erythroid precursor assay at low FCS concentrations resulted in enhanced burst number and heme ^{59}Fe incorporation similar to the results obtained in the previously reported human study (6) (Table 3). The bursts were qualitatively much larger in the presence

of BMCM than in its absence (Chapter 4, Fig. 1). The ability of BMCM to promote burst formation varied between batches and the first sample in each batch was usually of poor quality. Batches of BMCM were screened for their ability to enhance burst formation at low FCS. Table 3 compares the burst promotion by two batches of BMCM which were produced over a five week period, batch 2 is of excellent quality and batch 1 is of poor quality. The reason for such variation between BMCM batches is not known.

The factor(s) responsible for burst promotion by BMCM had a molecular weight greater than 10,000, based upon retention by ultrafiltration. Burst enhancement by BMCM was lost after heating at 100°C for twenty minutes.

Cytospin preparations of burst cells stained with Wright's stain revealed erythroid cells at all stages of maturation. The majority of burst cells examined stained positive for hemoglobin with DAB. Toluidine blue stained thick section of Epon embedded bursts also revealed erythroid cells at all stages of maturation. Ultrastructural studies of less mature erythroid cells were performed in order to confirm that they were early erythroblasts. Figure 3 shows a small cluster of such cells. These cells appear to be early erythroblasts, in that they possessed slightly irregular nuclei with condensed chromatin and one to three nucleoli, while

the cytoplasm was full of free ribosomes and polyribosomes, several mitochondria and pinocytotic vesicles. I failed to observe any non-erythroid cells or abnormal erythroid cells in bursts.

Burst hemolysate analysis by isoelectric focusing revealed two major protein bands with Coomassie blue. These bands corresponded to rabbit hemoglobins A1 and A2 when compared with erythrocyte hemolysates. Both of these bands stained positively for hemoglobin with benzidine. These hemoglobins were resolved most clearly by fluorography of ^{14}C-labelled burst hemolysates. Figure 5 shows a representative densitometric tracing of fluorographed burst hemoglobins.

DISCUSSION

The methylcellulose assay as described for murine erythroid precursor (16,17) was found to support the growth of rabbit erythroid precursors. Using the methyl-cellulose assay a spectrum of erythroid precursors was detected after different periods of culture. In nucleated bone marrow cell preparations the latest precursor detected was the CFU-e which produced small colonies on day 3 of culture and the earliest precursors were BFU-e which produced large bursts on day 10. In peripheral blood mononuclear cell preparations no CFU-e were observed, the latest precursors detected were inter-

mediate BFU-e, BFU-e which formed bursts on day 10 were the earliest precursors observed. These results are very similar to observations for murine erythroid precursors in bone marrow (19) and blood (20,6). The CFU-e Ep dose response was very similar to that reported by Moriyama and Fisher (1) for rabbit CFU-e in the methylcellulose assay. The Ep dose response of BFU-e resembles the murine BFU-e dose response (18,19). The linearity of colony and burst formation at different cell numbers is inconclusive evidence that they are of clonal origin.

Morphologically the contents of the bursts were erythroid and the majority of the erythroblasts examined stained positive for hemoglobin. Isoelectric focusing of burst hemolysates demonstrated the presence of normal adult rabbit hemoglobins. The addition of BMCM to low FCS cultures enhanced burst number and heme ^{59}Fe incorporation. Similar results were obtained by Porter el al. (6) with human BMCM, but the increase in heme ^{59}Fe incorporation observed was much greater. In addition BMCM appeared, qualitatively, to enhance the size of bursts. These studies indicate that rabbit BMCM may promote hemoglobin synthesis or erythroid proliferation. Further study is necessary to clarify this point.

In summary, these results demonstrate that the methylcellulose assay supports the growth of rabbit erythroid precursors. The bursts contained erythroid cells at all stages of maturity and normal adult rabbit hemo-

globins. Furthermore the results indicate that the rabbit is an excellent model for the study of the BMCM factor(s) which promote bursts.

TABLE I

Erythropoietin Dose Response of Erythroid Precursors

Ep Dose	3 Day Colonies*	10 Day Bursts
0.0	220 ± 64	3 ± 1
0.001	284 ± 20	(nd)
0.01	404 ± 12	13 ± 2.5
0.1	544 ± 20	27 ± 3
1.0	480 ± 40	28 ± 3.5
2.0	(nd)	30 ± 3
5.0	(nd)	39 ± 2

* All data expressed as mean ± standard error of duplicate dishes.

nd = not done.

TABLE 2

Time Course of Burst Formation by Bone Marrow and Peripheral Blood BFU-e

Day of Culture	Bone Marrow Burst #	Peripheral Blood Burst #
4	63 ± 1*	0
5	112 ± 17	8 ± 1
6	110 ± 19	15 ± 2
7	98 ± 9	22 ± 2
8	83 ± 7	29 ± 1
9	55 ± 0	29 ± 1
10	43 ± 3	29 ± 3
11	28 ± 2	20 ± 7
12	15 ± 2	21 ± 1
13	11 ± 3	19 ± 1

* All data expressed as the mean ± standard error of duplicate dishes.

TABLE 3

Production of BMCM by Bone Marrow Cultures

CM	Week	Bursts/Dish	^{59}FE CPM/Dish
1	1	43 ± 6*	479 ± 311*
1	2	54 ± 2	921 ± 80
1	3	59 ± 7	477 ± 329
1	4	47 ± 0	362 ± 139
1	5	43 ± 6	605 ± 29
2	1	43 ± 3	708 ± 80
2	2	52 ± 4	835 ± 50
2	3	71 ± 11	1909 ± 274
2	4	84 ± 4	2685 ± 620
2	5	90 ± 6	2690 ± 793
Control		43 ± 6	195 ± 33

Rabbit bone marrow nucleated cells (10^5/dish) were cultured in the presence of BMCM collected from bone marrow flask cultures at weekly intervals and in the absence of BMCM (control).

* Data expressed as mean ± standard error of duplicate dishes.

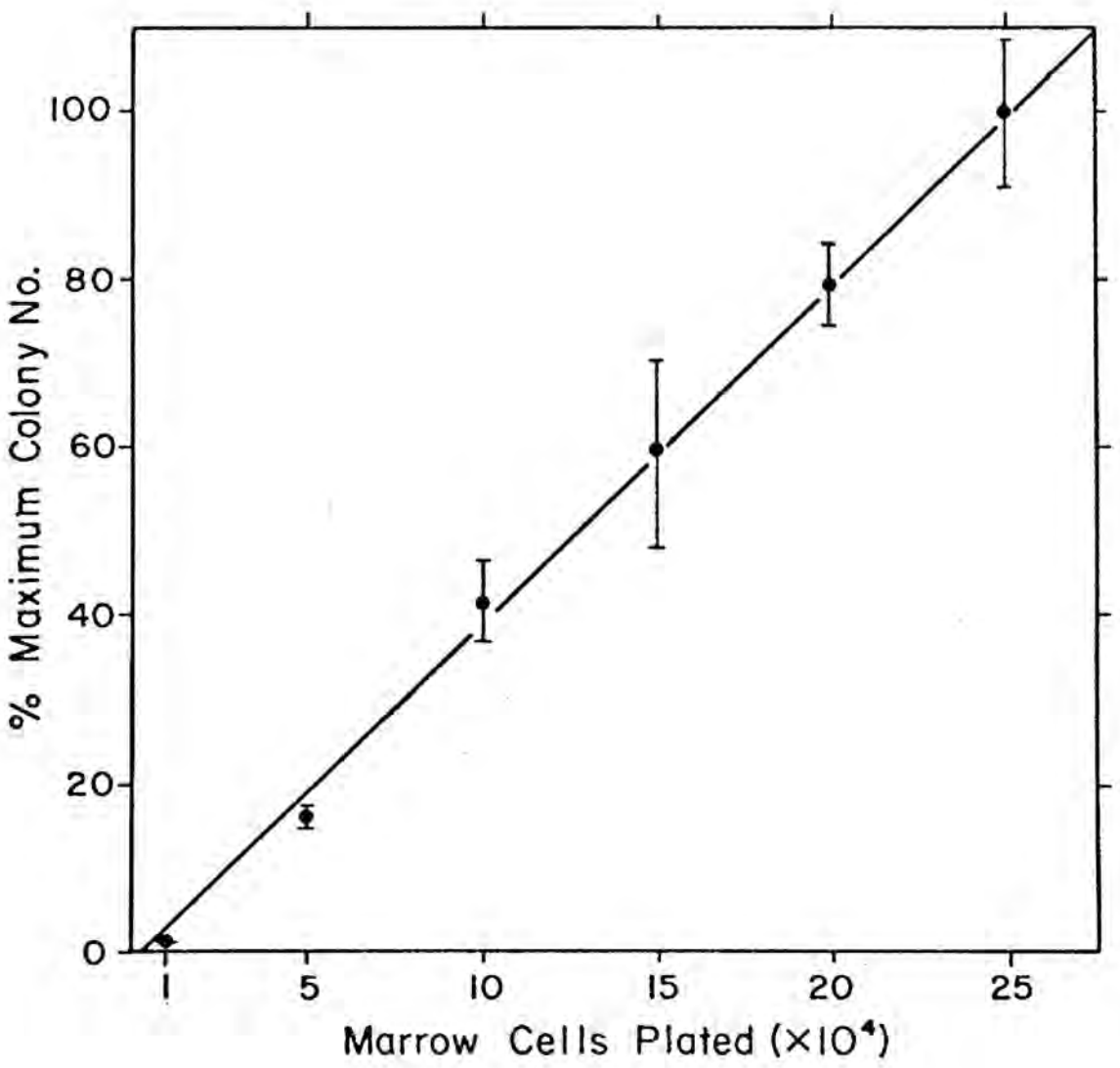

Fig. 1 Linear relationship (r-0.9975) between the number of bone marrow nucleated cells plated and the number of 3 day colonies observed. The data is expressed as the mean ± standard error of quadruplicate dishes.

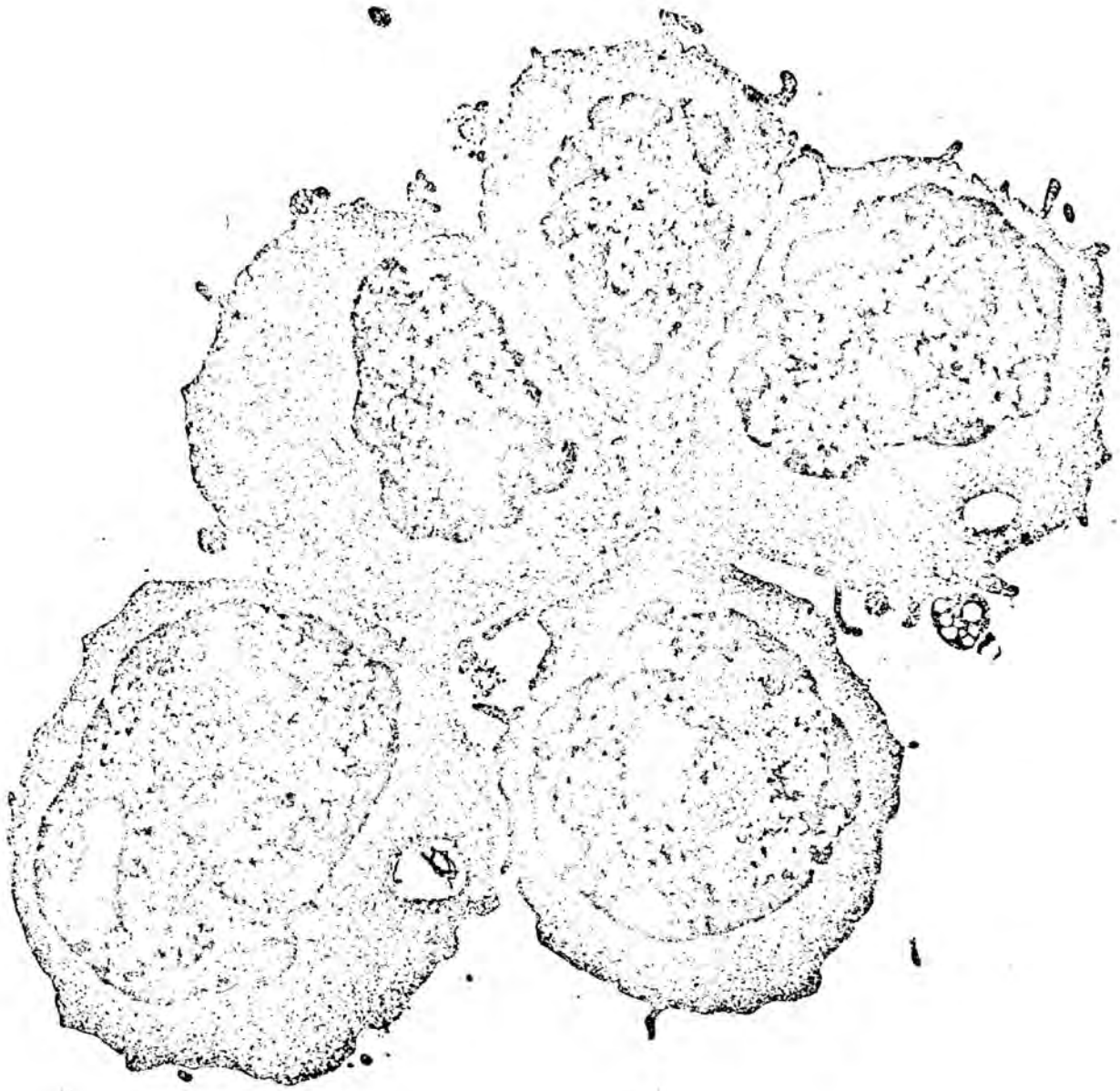

Fig. 3. A group of early erythroblasts form a burst. The cytoplasm of the cells contains many ribosomes and polyribosomes, several mitochondria and pinocytotic vesicles. The nuclei are slightly irregular with condensed chromatin and one to three nucleoli (x8000).

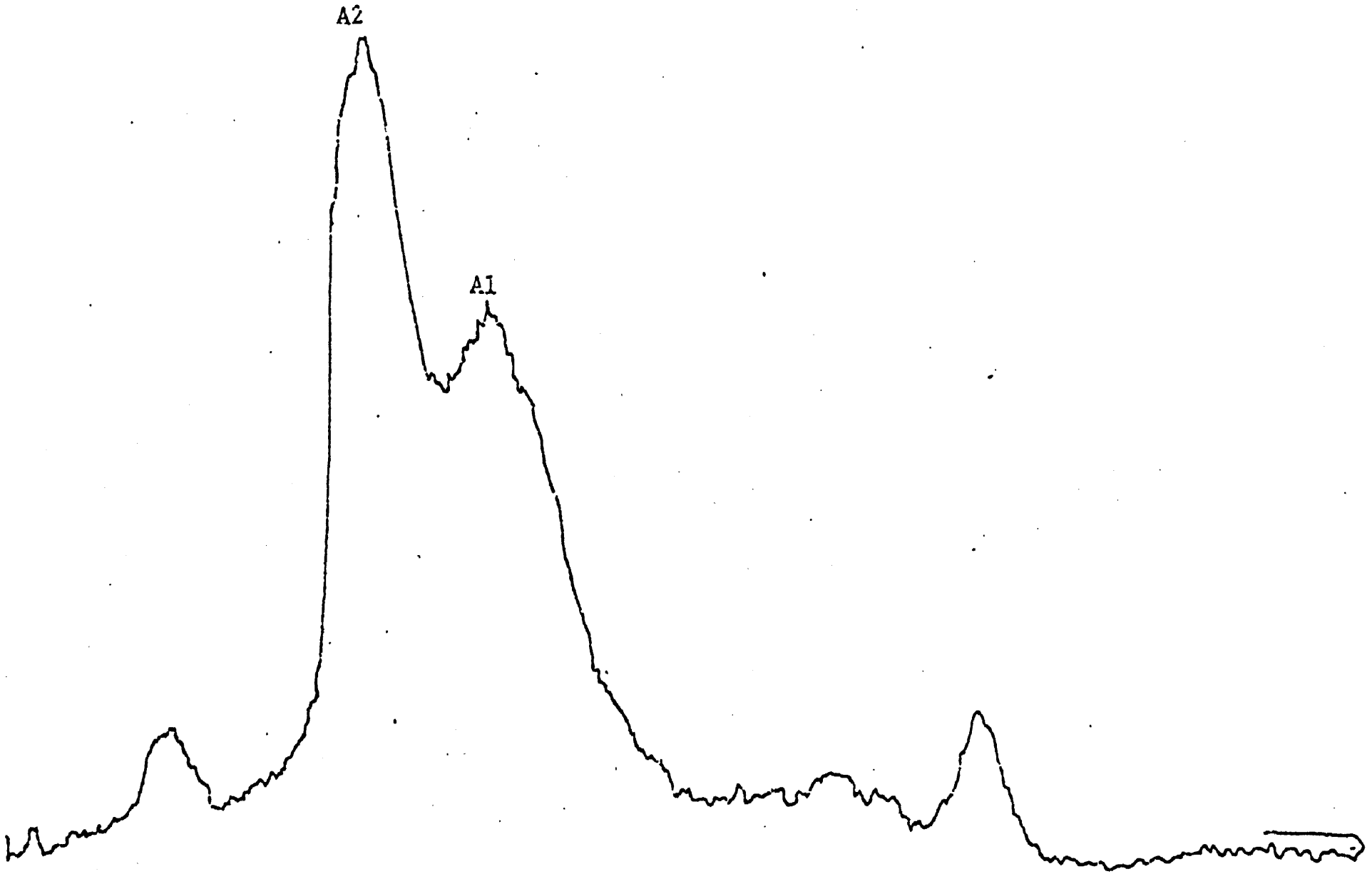

Fig. 3.4 Densitometric tracing of fluorographed burst hemolysate. A1 and A2 are normal adult rabbit hemoglobins.

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CHAPTER 3

HEMOPOIESIS IN DEMINERALIZED BONE ALLOGRAFTS

INTRODUCTION

Hemopoiesis in intimately, but not exclusively, associated with bone in mammals. Marrow has been observed in areas of ectopic ossification (1) and may be induced experimentally by implantation of demineralized allogeneic bone matrix (DBM) in a variety of tissues (2,3). The DBM implant has been extensively employed as a model for the study of bone development (3) and has been used clinically as a bone graft material. Implantation of DBM into a muscle bed produces an ossicle, with central marrow within four to six weeks, depending on the species of experimental animal (3,4). When DBM is implanted, fibroblastic mesenchymal cells in the connective tissues near the implant are induced to differentiate in a complex sequence into the cell types found in cartilage, bone and marrow stroma (5). The objective of this study was to determine if the DBM implant marrow possesses proliferative functions which are responsive to systemic hemopoietic control mechanisms. Results of these studies suggest that DBM implants are useful for investigation of bone marrow formation and hemopoiesis.

MATERIALS AND METHODS

Preparation, Implantation of DBM and Ossicle Retrieval:

Male New Zealand white rabbits weighing 2.5-3.5kg were used. Segments of femurs from sacrificed animals were demineralized using a modification of a reported method (6). Freshly excised cortical bone segments were demineralized in 0.6 N HCl (10gm/bone L) at 4°C for 24 hours. The DBM, prior to implantation, was rinsed in 70% ethanol, dissected into segments approximately 1x4x4 mm, and stored in 70% ethanol. Before implantation, the segments were rinsed in sterile saline.

Using sterile techniques, DBM segments were implanted in muscles, 4 in the quadricep and 8 in the abdominal muscles of eight rabbits. Two rabbits were sacrificed at 3, 6, and 10 weeks. At least three femoral and three abdominal ossicles from each rabbit were retrieved under sterile conditions for clonal cell culture; the remaining ossicles were processed for histological evaluation.

Ossicles from the left hind limbs of two rabbits were obtained by sterile biopsy after six weeks. Hemolytic anemia was subsequently induced by one subcutaneous injection of phenylhydrazine hydrochloride (20mg PHZ/kg). Hematocrits were measured before and three days after PHZ injection using blood obtained from peripheral ear

veins. The rabbits were sacrificed three days after PHZ injection, and the ossicles were processed as described above.

Methylcellulose Cell Culture:

Cell suspensions for clonal analyses were obtained from implants by dissection in α-medium (Flow laboratories, Inc., Rockville,MD). The cells from three implants were combined. Single-cell suspensions were prepared by flushing the cells through a #23 gauge needle, repeated pipetting with 1.0ml serological pipet and filtering through a #100 stainless steel mesh screen. Control marrow cells were obtained from the femur of anesthetized rabbits by aspiration, using a sterile procedure. The buffy coat cells, collected by centrifugation, were suspended in α-medium, counted and plated at a concentration of 10^5 nucleated cells per ml of tissue culture medium.

Hemopoietic precursors were cultured in methylcellulose (7). Duplicate or quadruplicate cultures were established in Lux standard non-tissue culture dishes (Flow Laboratories, Inc.) in 1 ml of media per dish. The medium consisted of α-medium, 0.8% methylcellulose (Fisher Scientific Co., Norcross, GA), 1% bovine serum albumin (Calbiochem, San Diego, CA), 30% fetal calf serum (Flow Laboratories, Inc.), 10^{-4} M mercaptoethanol (Fisher Scientific Co.) and 1.0 U of

Step III preparation of sheep plasma erythropoietin (EPO) (Connaught Labs, Ltd., Willowdale, Ontario, Canada). The dishes were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 in air.

The erythroid colonies were counted when the cells showed clear signs of hemoglobinization, either daily or on day 3 for erythroid colony-forming units (CFU-e) (8), and on day 7 for erythroid burst-forming units (BFU-e) (9). Granulocyte-macrophage colonies were counted on day 12. All colony counts were performed using an inverted microscope.

Histology:

Undecalcified specimens for histologic analysis were fixed in formalin and processed in methylmethacrylate. The sections, cut on a Jung model-K hard tissue microtome, were stained with hematoxylin and eosin.

RESULTS

Histology:

Histological evaluations of the 3-, 6- and 10-week ossicles from both the femoral and abdominal sites were consistent with previous findings (3,4,5,10). Hemopoietic elements were observed in the 6- and 10-week ossicles. The area occupied by marrow appeared to be closely related to the size of the ossicle. There was

some variation in the size of the ossicles in any one animal, despite the uniformity in size at implantation. The 6-week post-implantation ossicles revealed a highly vascular, developing marrow (Fig. 1a). Stromal cells were the predominant cell type, amongst which were scattered mature and immature erythroid and granulocytic cells. An occasional megakaryocyte was observed. The 10-week post-implantation ossicles had many more hemopoietic cells, especially granulocytic cells and megakaryocytes. The stromal cells were not the predominant cell type at this time. The marrow in 6-week ossicles, when examined three days after PHZ injection (Fig. 1b), was more cellular than the ossicles biopsied prior to PHZ administration. There was an increase in erythroid elements.

Cell Culture Analysis of Hemopoietic Precursors:

At 6-week post-implantation, the DBM implant contained both erythroid and granulocytic precursor cells. Erythroid colonies were seen at 3-6 days, bursts were seen after day 5 and granulocyte-macrophage colonies appeared after the 7th day. A representative burst (day 7) is shown in Fig. 2. The time courses of erythroid colony and burst formation (Fig. 3) from DBM ossicle and femoral marrow were approximately the same, but the plating efficiency of ossicle marrow was less than one-tenth that of femoral marrow. This difference

may be due to the disproportionately large number of stromal and bone-associated cells in the ossicles as compared to femoral marrow. Granulocyte-macrophage colonies measured on two occasions were 85 ± 10.5 and 31.5 ± 3 (mean ± SE) per 10^5 cells, respectively.

Erythropoietic Precursors in Ossicles Before and After PHZ Treatment:

Hematocrits, before and three days after PHZ treatment, were 44.1 ± 1.1% (mean ± SE) and 21.8 ± 1.3%, respectively. Anemia induced by PHZ was accompanied by an increase in the erythroid colony count and no change in the burst count of DBM ossicles (Fig. 4). These results indicate that the DBM induced ossicle marrow was responsive to systemic erythropoietic control mechanisms.

DISCUSSION

The results of this study show that the DBM-associated ossicle marrow possesses erythroid and granulocyte-macrophage precursors. The proportions of erythroid precursors at different maturational stages in the time course experiment were similar to those in the femoral marrow (Fig. 3). Furthermore, we observed that the erythroid elements in the ossicle marrows were responsive to erythropoietic stimuli present in PHZ-induced anemia as determined by both morphologic and functional assays. Our observations are in basic agreement with data on the changes in number of CFU-E and BFU-E in the marrow of mice following erythropoietic stimulation (11,12). These observations can probably be explained by the reported differences in erythroid precursor sensitivity to erythropoietin; the CFU-E being derived from EPO responsive precursor cells (11) and the BFU-E from precursor cells which are not EPO responsive (13). The proliferative potential of ossicle marrow cells appears to be similar to that of femoral marrow cells.

The ossicle that develops at the site of the DBM implant may provide a good model for the study of *de novo* development of marrow and hemopoietic functions *in vivo*. When compared with other models, the DBM implant system appears to have a number of potential ad-

vantages. Currently, four experimental models are available for in vivo study of cellular interactions in developing hemopoietic tissues; regenerating marrow (14), extramedullary marrow implants (14), intraperitoneal implants of cellulose acetate membrane (15) and transplants of urinary tract transitional epithelia (16). In the first model, the regeneration process following the evacuation of long bone marrow may be observed. The principle limitation is that there are only two comparable sites in any one animal. In the DBM model, multiple implants may be made at specific sites in each experimental animal. In the second model, a hemopoietic nodule surrounded by a shell of bone is formed at the site in which medullary marrow has been placed. In this model, the stromal cells are implanted whereas in the DBM implant model, cellular elements are absent at the time of implantation. In the third model, implanted cellulose acetate membranes provide an environment for predominantly granulocytic colony formation and only a few erythroid colonies are formed. This model is not suitable for the study of erythroid development and the cellulose acetate membranes begin to disintegrate after two weeks. The hemopoietic precursors in DBM-induced ossicles give rise to both granulocytic and erythroid colonies. Also, DBM-associated ossicles have a much longer lifespan and they have been observed to support marrow until 700 days post-implantation (10) in rats. The last model may be suit-

able for similar studies in the dog, cat or guinea pig, but it has a poor success rate in the rabbit (16). The DBM implant model may be of particular interest in the analysis of the development of marrow stromal cells, collectively called the hemopoietic inductive microenvironment (HIM) (17), and their interaction with functional hemopoietic precursors.

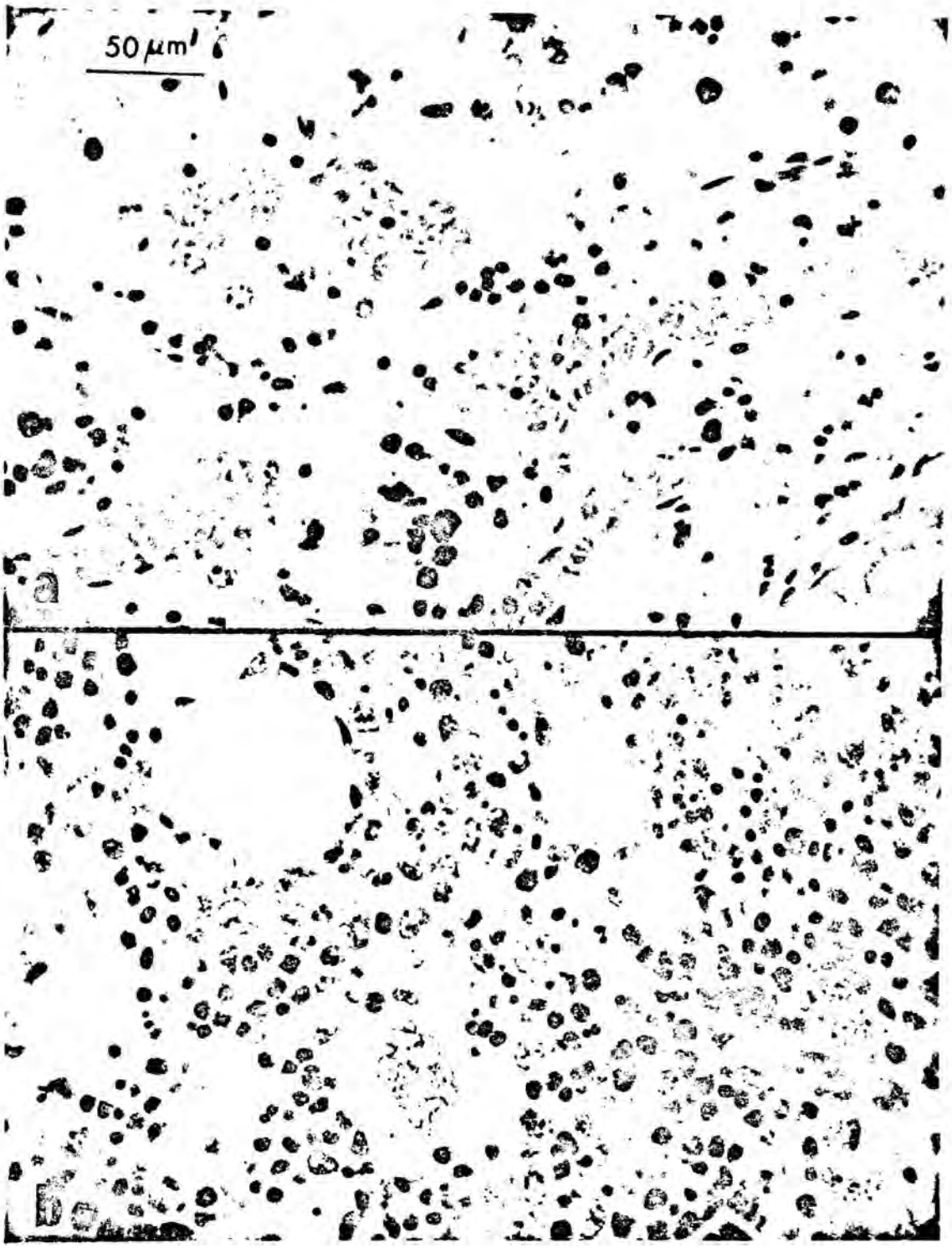

Fig. 1: Undecalcified ossicles were fixed in formalin, processed in methylmethacrylate, and stained with hematoxylin and eosin.
a) Bone marrow in an ossicle six weeks after implantation of DBM. The marrow contained many blood vessels amid a loose connective tissue stroma (330X).
b) Marrow in a six-week ossicle from a rabbit which received phenylhydrazine three days prior to sacrifice. The marrow was organized with fewer blood vessels and less stroma (330X).

Fig. 2: A large erythropoietic burst, observed on day seven of culture, from DBM-induced ossicle marrow (220X).

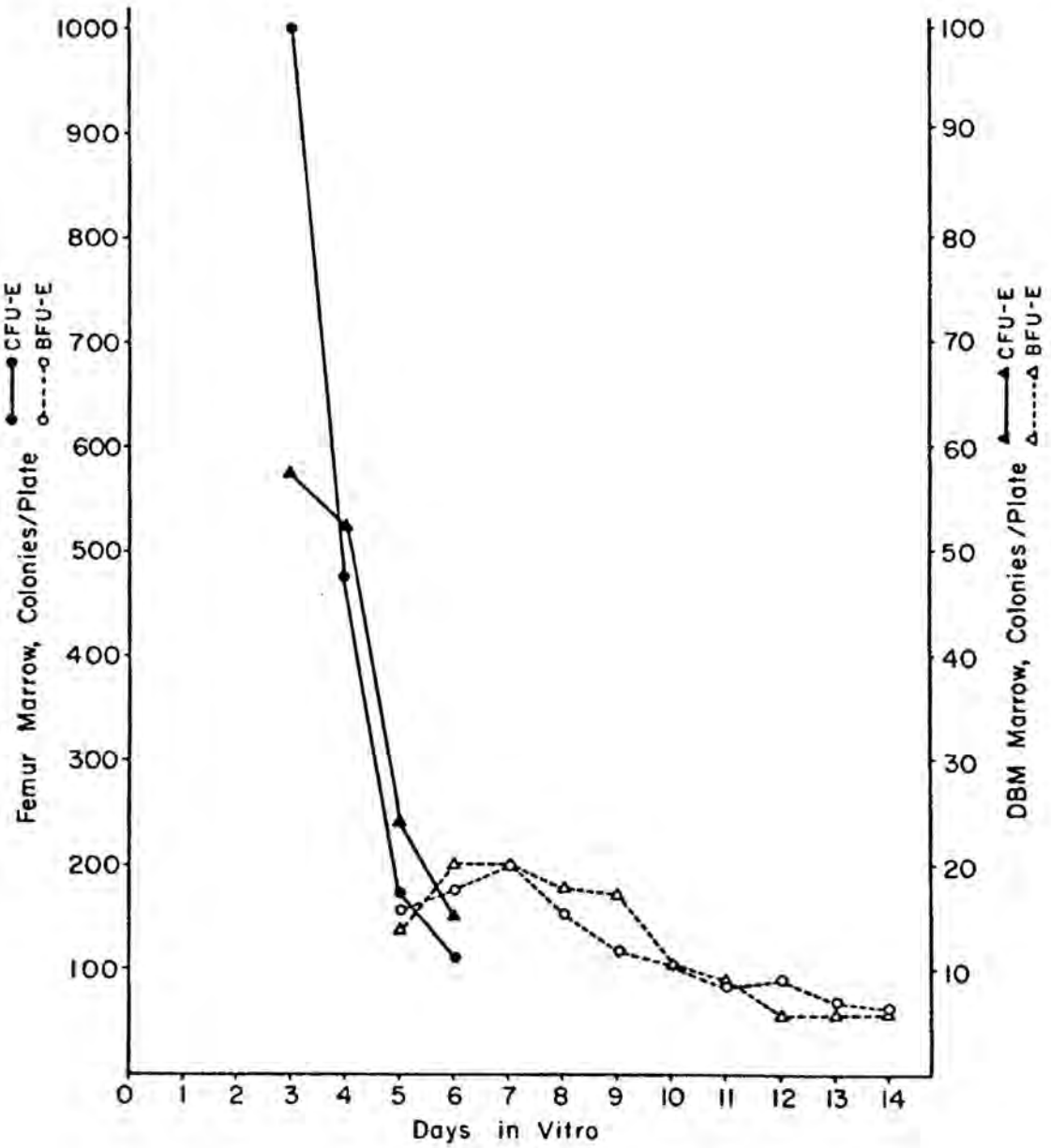

Fig. 3: Time course observation of erythroid colony and burst formation from six week post-implantation DBM-induced ossicle marrow (n=2) and from femoral (n=4). Only hemoglobinized colonies and bursts were scored.

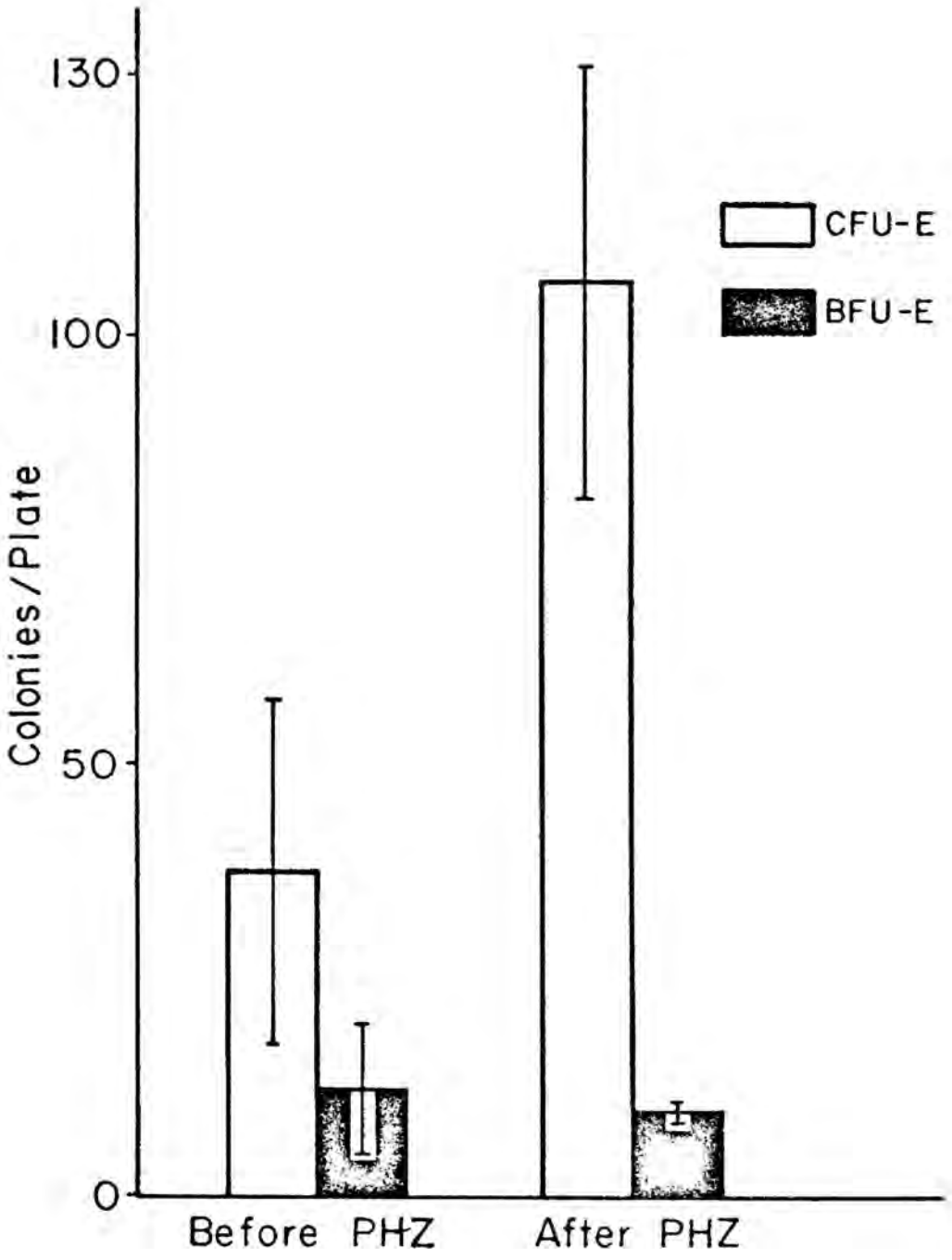

Fig. 4: Comparison of CFU-E and BFU-E from DBM-induced ossicle marrow before and after PHZ-induced hemolytic anemia. On the left, the height of the bars represents the mean of the two duplicate experiments. On the right, the ossicles were removed three days after PHZ administration, the height of the bars represents the mean of two quadruplicate experiments. The verticle lines represent the standard error of the mean.

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CHAPTER 4.

BURST-PROMOTING ACTIVITY DERIVED FROM BONE MARROW CONDITIONED MEDIA: A QUANTITATIVE ASSAY AND CELLULAR MECHANISMS OF ACTION.

INTRODUCTION

Using clonal cell culture assays, it is possible to study sequential stages of erythroid development and factors which influence erythroid maturation. Two distinct populations of erythroid precursors have been described between which occurs a continuum of intermediate stages (1-3). Late precursors, erythroid colony-forming units (CFU-e) (4), give rise to small colonies after short incubation periods. Early erythroid progenitors, erythroid burst-forming units (BFU-e) (5), give rise to large colonies (bursts) after longer incubation periods. Erythropoietin (Ep) is now recognized as the primary regulator of erythropoiesis and is known to act on precursors at relatively late stages of development. However, factors other than Ep, which are tentatively designated burst-promoting activity (BPA), may be important for early stages of erythropoiesis in culture. Aye (16) observed that the addition of human leukocyte conditioned medium to human bone marrow cultures produced an increase in burst number. Other investigators have since observed BPA in media conditioned by spleen cells (7,8), T-cells (9), a T-cell line (10), peripheral blood non-adherent, non-

rosetting mononuclear cells (11), and bone marrow (12). BPA has also been noted in the urine (13) and in the serum (14) of anemic patients. The BPA assays used in different laboratories differ in the means employed to reduce endogenous BPA. Endogenous BPA has been reduced by: (A) selective removal of adherent cells (6,2); (B) lowering the cell number plated to minimize burst feeder activity (15); and (C) lowering the serum concentrations used (8,12).

For precise characterization of BPA, a quantitative assay for BPA is necessary. The use of burst number has been used successfully for murine BPA (8), but this assay does not yield any information on the quality of the bursts. In our culture system, we consistently observed an increase in burst size, even in experiments where only a small increase in burst number is seen. Recently, we presented preliminary evidence that ^{59}Fe incorporation into heme may be a quantitative assay of BPA in human bone marrow conditioned media (BMCM) (12). We report here that rabbit BMCM also possesses potent BPA, which increases burst number and hemoglobin (Hb) synthesis. By analysis of cell numbers and ^{59}Fe incorporation into heme in individual bursts, we have further characterized the mechanism of action of rabbit BPA. The results described in this report indicate that the major effect of BPA in BMCM is on cell proliferation

during the early phase of burst formation and that the sensitivity of erythroid precursors to BPA decreases with maturity.

MATERIALS AND METHODS

Cell Preparation:

Male New Zealand white rabbits weighing 2.5-2.5 kg were employed in the study. Bone marrow was aspirated from the femur and collected in 6 ml Falcon plastic tubes containing heparin without preservatives (Chromalloy Pharmaceuticals, Inc., St. Louis, MO). The buffy-coat cells were collected after centrifugation and resuspended in α-medium (Flow Laboratories, Inc., Rockville, MD). Peripheral blood was obtained by bleeding from the ear veins and mononuclear cells were harvested using the Ficoll-Isopaque technique described by Boyum (16) with slight modifications (17). Human peripheral blood was obtained by venipuncture from antecubital veins and the mononuclear cells harvested as described above.

Bone Marrow Conditioned Media:

Marrow buffy-coat cells 10^6 cells/ml) were cultured in glass flasks in 10ml of α-medium containing 1% de-ionized bovine serum albumin (BSA) (Calbiochem, San Diego, CA), 1% fetal calf serum (FCS) (Flow Laboratories,

Inc.) and 10^{-4} M mercaptoethanol (Fisher Scientific Co., Norcross, GA). After 1, 2, 3, 4 and 5 weeks of culture, one-half of the culture medium was removed and replaced with fresh medium. The BMCM was centrifuged at 800 g and the supernatant stored at -70°C.

Erythropoietic Cell Culture:

Aliquots of 5×10^4 marrow nucleated cells or 5×10^5 peripheral blood mononuclear cells were plated in Lux standard non-tissue culture dishes (#5221R, Flow Laboratories, Inc.) in 1 ml of media per dish. Human peripheral blood mononuclear cells were plated at 2×10^5 cells per dish. We used 5×10^4 bone marrow nucleated cells per dish since we observed that at this cell concentration, the endogenous BPA due to burst feeder activity is low as has been reported by Wagemaker (15) in culture of mouse marrow cells.

The media consisted of α-medium, 0.8% methylcellulose (18) (Fisher Scientific Co.), 1% deionized BSA, 6.6% FCS, 10^{-4} M mercaptoethanol and 1.0 U of step III preparation of sheep plasma Ep with specific activity of 13.7 U/mg protein (Connaught Labs, Ltd., Willowdale, Ontario, Canada). This concentration of FCS was selected in order to minimize the endogenous BPA in our culture system. Human peripheral blood cells were cultured in the same media with two alterations; 10% FCS and no mercaptoethanol (12). BMCM was added to experimental

cultures at a concentration of 10% (v/v). The control medium contained the same concentrations of all components as the experimental controls, except BMCM. The dishes were incubated at 37°C in a humidified atmosphere consisting of 5% CO_2 in air. Red colonies, intermediate bursts and bursts were counted using an inverted microscope (65X) on days 3, 5 and 10, respectively. Granulocyte/macrophage colonies were counted on day 10.

Incorporation of ^{59}Fe into Heme:

Cultures were labeled for 24 hours by carefully overlaying each dish with 0.3 ml of α-medium containing 50% heat-inactivated rabbit serum (30 minutes at 56°C) and 0.5 uCi of ^{59}Fe-citrate (Porter et al. 1980). For the assay of individual bursts, 1.0 uCi of ^{59}Fe-citrate was used. Heme was extracted by the cyclohexanone method (19).

Individual Burst Analysis:

Individual bursts were lifted from the culture dishes with a 10 ul Eppendorf pipet and placed in a total volume of 200 ul of phosphate-buffered saline (PBS) containing 2% FCS. A 10 ul aliquot of the cell suspension was then counted on a hemocytometer. Incorporation of ^{59}Fe into heme was measured in individual bursts with or without simultaneous cell counts. When simultaneous cell counts were performed, the ^{59}Fe incorporation was estimated from

the remaining 190 ul of cell suspension. Twenty-five ul of rabbit packed red cells were added to each tube prior to preparation for heme extraction in order to prevent loss of cells during washing.

RESULTS

The effect of BMCM on the number and ^{59}Fe incorporation of bursts, intermediate bursts and colonies derived from bone marrow erythroid precursors and of bursts derived from peripheral blood erythroid precursors is shown in Table 1. No enhancement was observed for colonies. The intermediate bursts were moderately enhanced and bursts from both bone marrow and peripheral blood were more dramatically enhanced. In each experiment, the increase in ^{59}Fe incorporation was much greater than the increase in burst number. Although burst size qualitatively varied over a wide range, they were consistently larger in the presence of BMCM (Fig. 1). These results demonstrated that sensitivity to BPA is inversely parallel to the maturational stages of erythroid precursors and that the enhancement of heme synthesis by BPA is far greater than the increase in burst number. Comparison of granulocyte/macrophage colony and burst number at high (30%) and low (6.6%) FCS showed a decrease in both colony types in low FCS condition (Table 2). The addition of BMCM had no effect

on granulocyte/macrophage colony number, while BMCM significantly augmented burst formation. This observation indicated that colony stimulating factor (CSF) is undetectable in rabbit BMCM.

Walters et al. (20) demonstrated that immature leukemia leukocytes possess heme synthesizing capacity. In order to exclude the possibility that some of the heme synthesis we observed was from immature cells in the granulocyte/marcophage colonies, we estimate the ^{59}Fe incorporation in all the granulocyte/macrophage colonies (n=14) from two dishes. An equivalent number of erythroid bursts were lifted at random from the same cultures. The ^{59}Fe incorporation by granulocyte/macrophage colonies was undetectable, while the erythroid bursts revealed an incorporation of 3472 cpm. This result clearly negated the possibility that granulocyte/macrophage colonies might be contributing significantly to the observed ^{59}Fe counts.

In order to further delineate the target population of BPA, we carried out an experiment in which BMCM was added to cultures after varying intervals in incubation. We observed a gradual decline followed by a plateau in the burst number and in contrast, a sharp decline in ^{59}Fe incorporation (Fig. 2). This observation supported our conclusion from the previous experiment that the sensitivity to BPA inversely parallels the maturational stages of precursors. In addition, the plateauing of

burst number at control levels in the presence of a continuing decline of ^{59}Fe incorporation strongly indicated that a major effect of BPA is to sustain hemoglobin synthesis in individual bursts.

In order to examine two possible mechanisms of heme synthesis enhancement by BPA; namely enhancement of hemoglobinization of individual cells with no increase in the cell number per burst or augmentation of cell proliferation with resultant enhancement of hemoglobinization in individual bursts; we examined ^{59}Fe incorporation into heme and cell number of individual bursts in the presence and absence of BMCM. On day 10 of culture, all the bursts in an experiment and a control dish were picked and cell counts performed. At the same time, the duplicate dishes were labeled with ^{59}Fe for 24 hours for examination of individual bursts for heme ^{59}Fe incorporation. The size of bursts ranged from 4.5×10^2 to 5.9×10^4 cells per burst in the absence of BMCM and from 10^2 to 2.9×10^5 cells per burst in the presence of BMCM (Fig. 3). The amount of ^{59}Fe incorporated into heme ranged from 0-219 cpm/burst in the absence of BMCM and from 11/929 cpm/burst in the presence of BMCM (Fig. 4). For both cell number and ^{59}Fe incorporation, BPA produced a shift in the relative cumulative frequency distribution without changes in the shape of the distribution of the entire burst formation

(Figs. 3,4).

We then carried out simultaneous quantitation of cell number and ^{59}Fe incorporation into heme of individual bursts in order to examine the relationship between these parameters. Marrow cells were grown in the presence of BMCM and labeled with ^{59}Fe. All the bursts were lifted individually from the culture and simultaneous quantitation of cell number and ^{59}Fe incorporation performed. Linear regression analysis clearly demonstrated that there is a relationship between the number of cells and the heme ^{59}Fe incorporation in the bursts (correlation coefficient, 0.811; $p<0.001$ by Student t-test) (Fig. 5). The enhancement of cell number was 6.6-fold and the enhancement of heme synthesis was 8.0-fold. These results supported the notion that enhanced hemoglobinization by BPA is due primarily to augmented cell proliferation in the bursts.

To ascertain whether the BPA in rabbit and human BMCM are species-specific, we cultured cells from rabbit bone marrow and human peripheral blood in the presence of BMCM from each source (Table 2). Rabbit BMCM enhanced both burst number and ^{59}Fe incorporation in human bursts to the same degree as human BMCM. Human BMCM did not enhance rabbit bursts at all.

DISCUSSION

Our initial experiments were designated to further characterize the target cells of BPA. First, we compared the sensitivity of early, intermediate and late erythroid precursors to BPA. Rabbit BMCM mildly inhibited the growth of colonies, enhanced intermediate bursts moderately and dramatically enhanced bursts derived from very early precursors. Thus, the sensitivity of erythroid precursors to BPA decreases with maturity as has been observed by others for different sources of BPA (2,21,8). The delayed addition of BMCM experiment demonstrated a requirement for BPA during burst formation and is in agreement with the data of Iscove (8), Tsang and Aye (22) and Porter et al. (12). Since no CSF activity was detectable, the BPA in rabbit BMCM appears to be specific for erythroid precursors. We conclude from these experiments that the primary target of BPA is positioned very early in the committed erythroid sequence and that the presence of BPA is required for their survival in culture.

Analysis of our data shows that the use of ^{59}Fe incorporation into heme was a very sensitive assay for BPA. Dramatic increases in Hb synthesis were seen in the presence of BMCM. We considered two mechanisms which might be responsible for the enhancement of Hb synthesis by bursts in culture: 1) enhancement of hemoglobinization

in individual cells with no increase in cell number; and 2) augmented cell proliferation resulting in increased synthesis. It is possible that both are operative. In order to distinguish between these potential mechanisms, we tested the second hypothesis. Analysis of the number of cells in each burst demonstrated a consistent enhancement in the size of the bursts, with some containing as many as 2 x 10^5 cells. Similarly, analysis of the heme ^{59}Fe incorporation per burst demonstrated a consistent increase. Simultaneous analysis of these two parameters revealed a highly significant correlation between burst size and heme ^{59}Fe incorporation in individual bursts. In addition, the enhancement of cell numbers (6.6-fold) and heme ^{59}Fe incorporation (8.0-fold) was similar. These results supported the idea that the elevated heme ^{59}Fe incorporation is a reflection of enhanced proliferation. We thus concluded that the BPA in BMCM increases the number of cell divisions between BFU-e and hemoglobinization. The shift in the frequency distributions of burst size and burst heme ^{59}Fe incorporation induced by BPA (Figs. 3,4) suggest that almost all BFU-e respond to BMCM.

Our data, however, do not exclude the possibility that BPA may also recruit younger cell populations for proliferation in culture, since we consistently observed an increase in burst number with BMCM. These BFU-e would

probably be several cell divisions less mature than those present in low BPA conditions and may require the presence of high BPA concentrations in order to initiate burst formation. Alternatively, BPA, may induce burst formation from an uncommitted population. This possibility is supported by the observations that phytohemagglutinin-stimulated leukocyte conditioned media (23) and pokeweed mitogen-stimulated spleen cells (7) promote the growth of mixed colonies from pluripotent stem cells. Although our bursts appear erythroid in nature, we have not excluded the possibility that some of the bursts may contain non-erythroid cells. While these possibilities need further investigations, our observations in this report appear to suggest that a major effect of BMCM BPA is on cell proliferation during the early phase of burst formation and that the elevation of ^{59}Fe incorporation into heme induced by rabbit BMCM is a reflection of this enhanced proliferation.

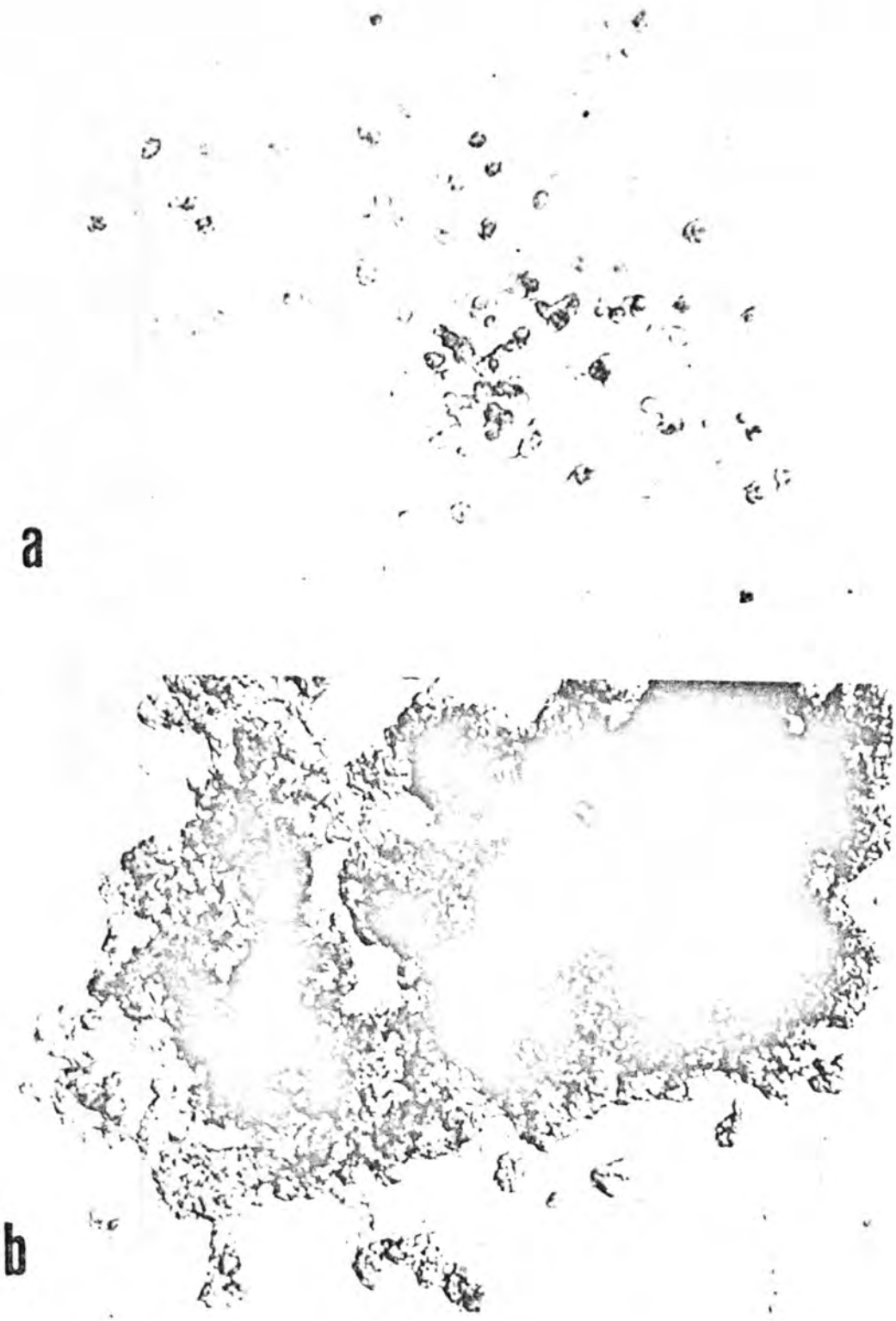

Fig. 1: The largest bursts observed in culture of peripheral blood mononuclear cells in (a) the absence of and (b) the presence of BMCM. Original magnification - 30X.

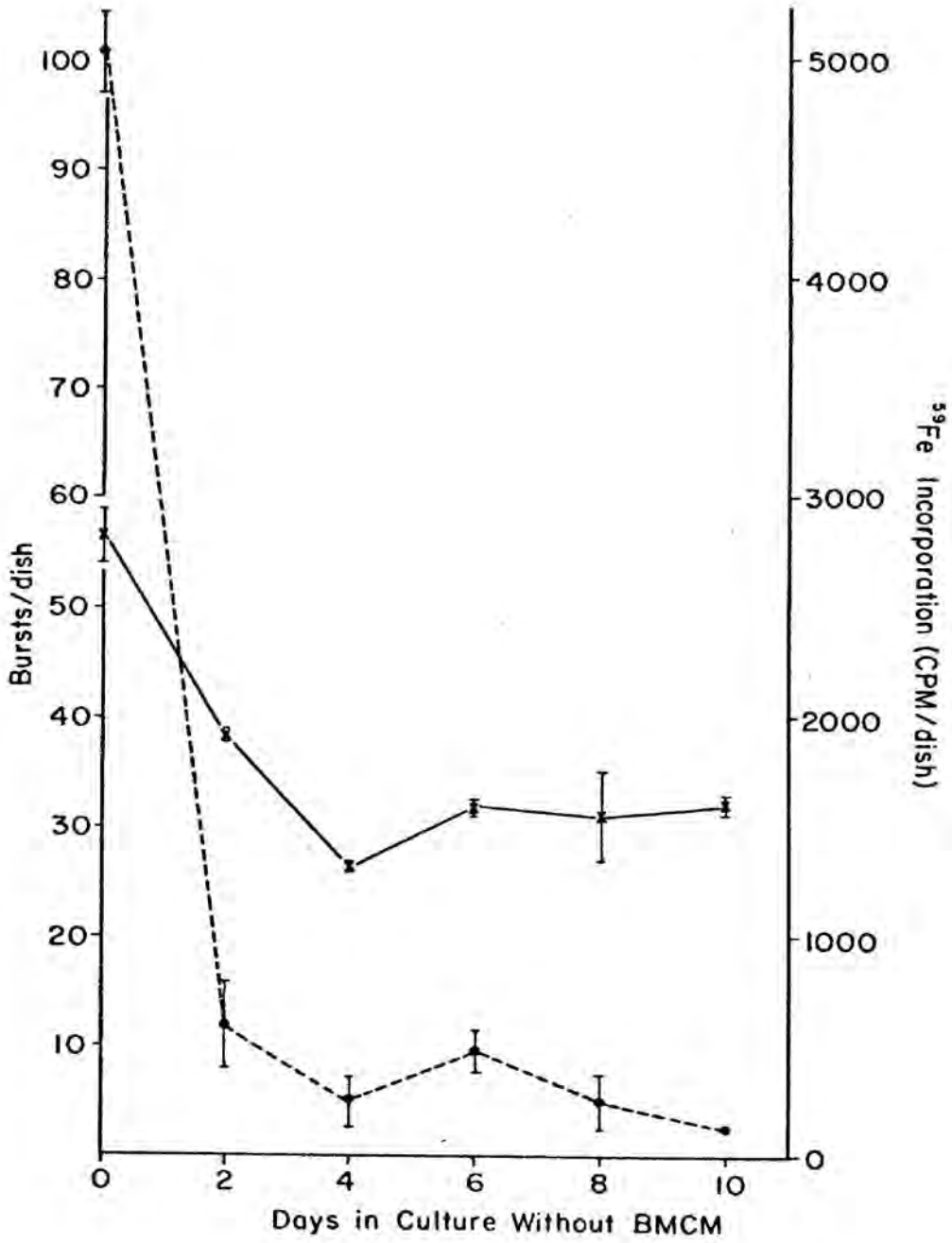

Fig. 2: Effects of delayed addition of BMCM on burst formation. BMCM was added at different times of culture. The data are expressed as mean ± SE of duplicate cultures. Burst number per dish (X——X) is expressed on the left-hand ordinate and ^{59}Fe incorporation per dish (0----0) on the right-hand ordinate.

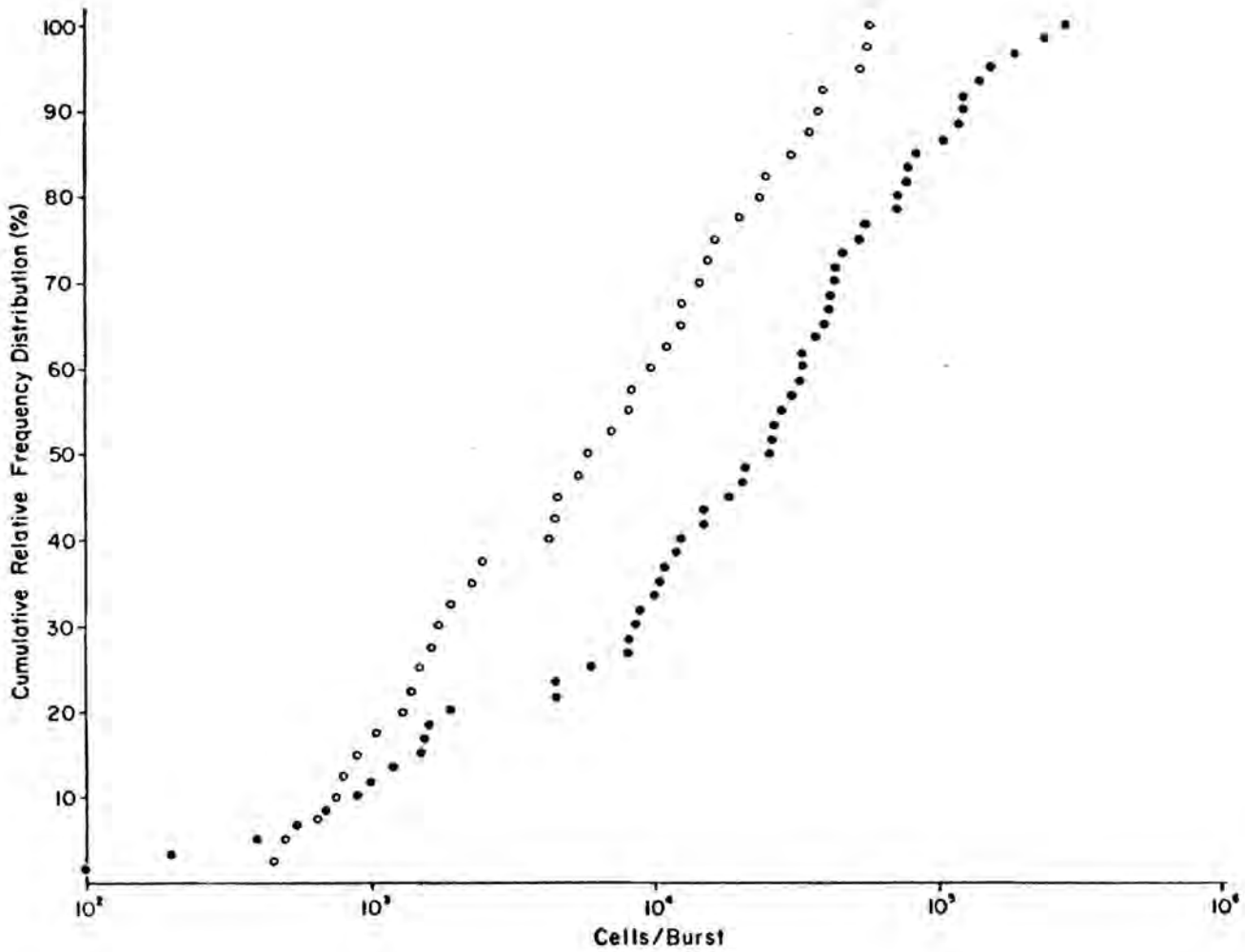

Fig. 3: Cumulative, relative frequency distribution of individual bursts varying in cell number. Bone marrow nucleated cells were cultured (a) in the absence of BMCM (0) or (b) in the presence of BMCM (o). The bursts were individually lifted and cell counts performed.

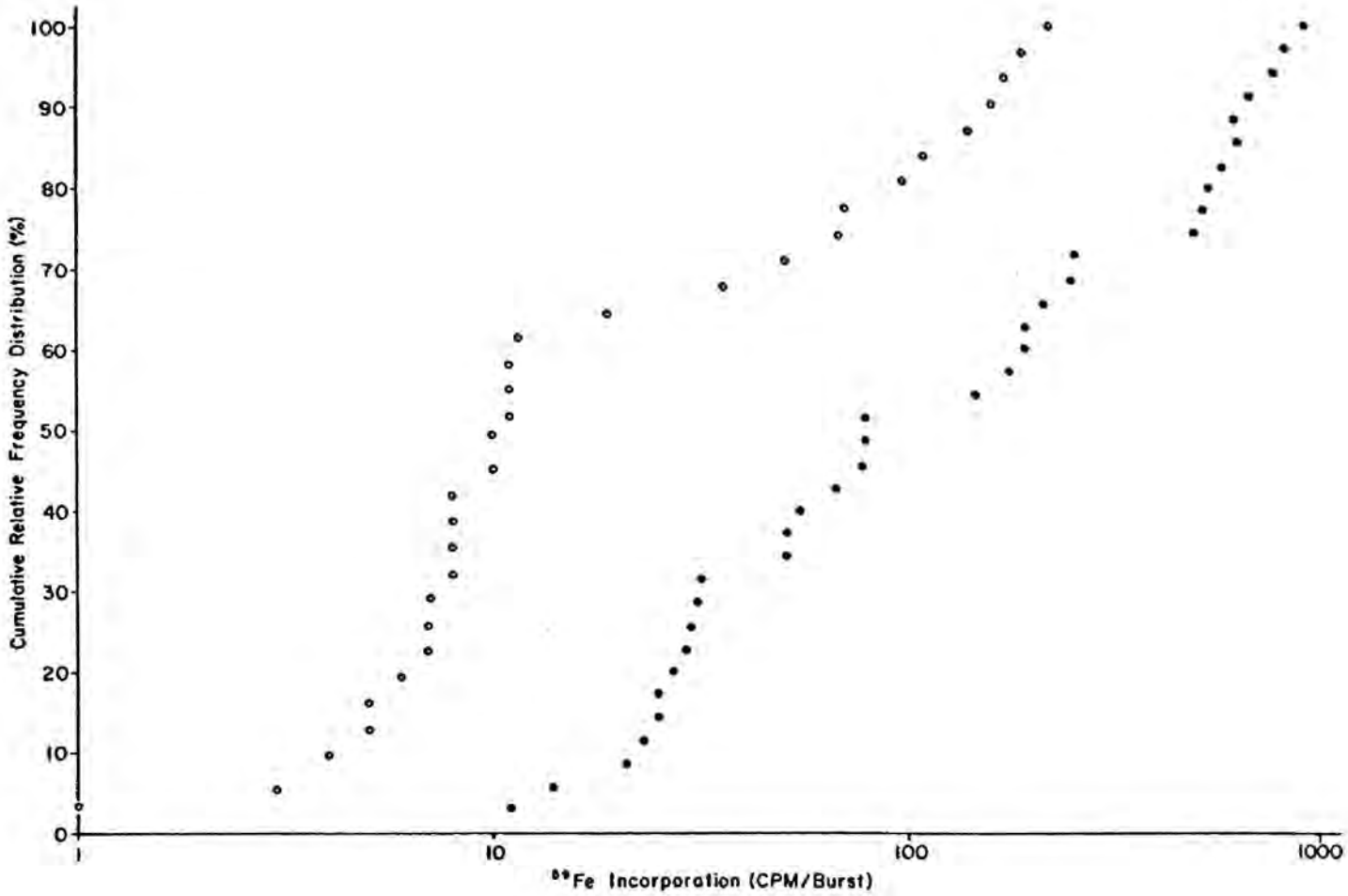

Fig. 4: Cumulative, relative frequency distribution of individual bursts differing in heme ^{59}Fe incorporation. Bone marrow nucleated cells were cultured (a) in the absence of BMCM (0) or (b) in the presence of BMCM (o).

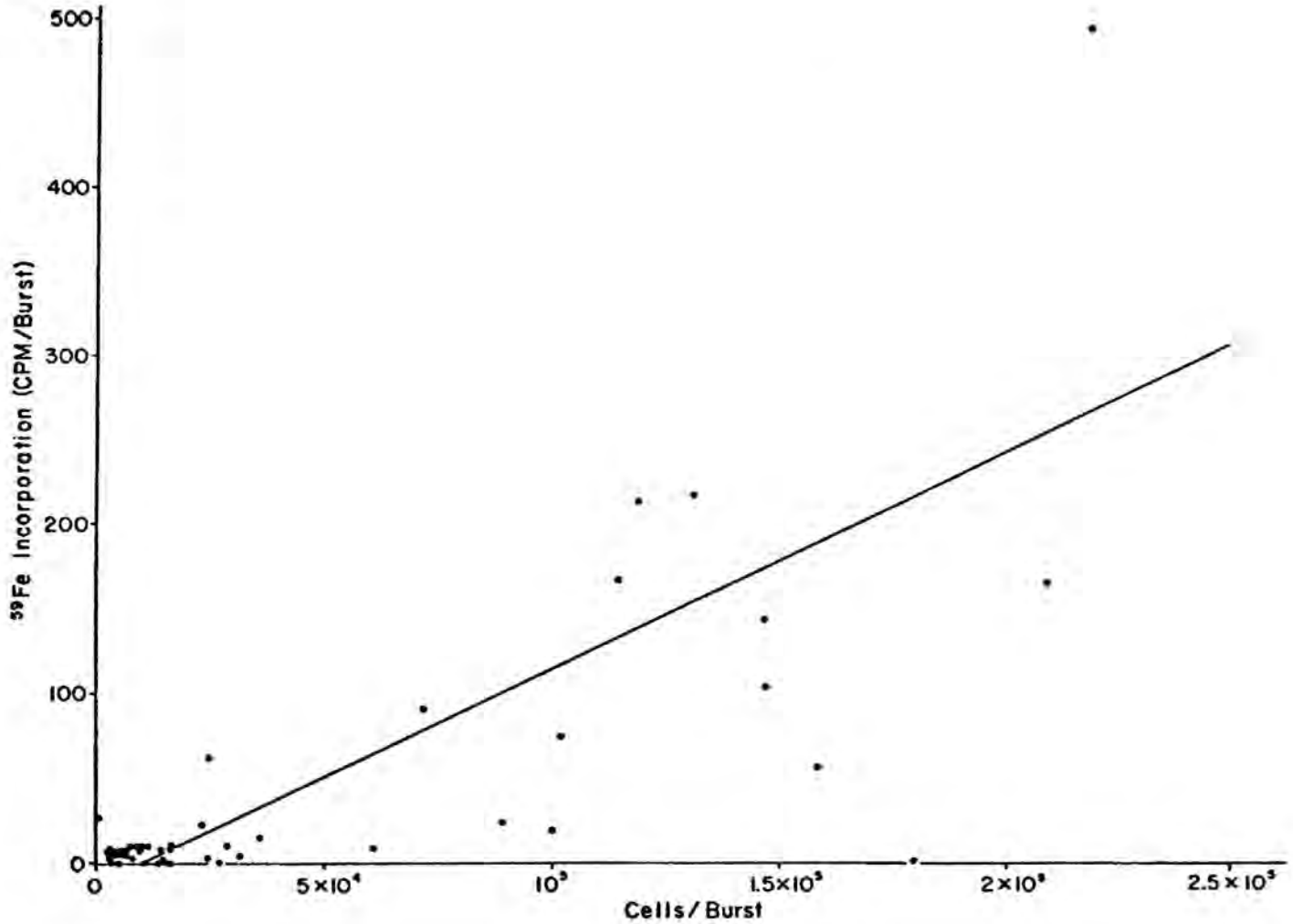

Fig. 5: Simultaneous examination of cell number and ^{59}Fe incorporation in individual bursts. Bone marrow nucleated cells were cultured in the presence of BMCM. The solid line represents a linear regression line for the data; the correlation coefficient = 0.81, $p<0.001$, (Student t-test).

TABLE 1

Effect of Rabbit BMCM on Colony Formation and Heme ^{59}Fe Incorporation By Rabbit Bone Marrow and Peripheral Blood Erythroid Progenitors

Colony Type	EXP #	Control Colony #	BMCM Colony #	Control ^{59}Fe CPM	BMCM ^{59}Fe CPM
3-day Colony	1	279 ± 5*	235 ± 12	168 ± 1	126 ± 9
	2	349 ± 10	305 ± 11	204 ± 6	158 ± 0
	3	140 ± 6	103 ± 9	107 ± 6	72 ± 4
5-day Intermediate Burst	1	36 ± 7	31 ± 3	516 ± 83	1075 ± 60
	2	33 ± 10	50 ± 1	431 ± 49	1693 ± 169
	3	21 ± 2	14 ± 1	85 ± 6	216 ± 64
10-day Burst	1	48 ± 5	53 ± 2	547 ± 115	3910 ± 1068
	2	41 ± 3	85 ± 4	32 ± 5	3475 ± 1008
	3	16 ± 1	29 ± 2	24 ± 2	1260 ± 64
Peripheral Blood 10-day Burst	4	10 ± 2	17 ± 2	72 ± 35	1708 ± 422
	5	7 ± 3	12 ± 5	51 ± 21	971 ± 308
	6	8 ± 1	12 ± 5	43 ± 38	1025 ± 685

*All data are expressed as mean ± standard error of duplicate dishes.

TABLE 2

BMCM Effect on Burst and Granulocyte/Macrophage Colony Number

Condition	Burst #	G/M Colony #
30% FCS	51 ± 5*	115 ± 5
6.6% FCS	31 ± 1	9.5 ± 0.5
6.6% FCS+BMCM	47.5 ± 6.5	11.5 ± 3.5

* All data are expressed as mean ± SE of duplicate dishes containing 5×10^4 nucleated bone marrow cells.

TABLE 3

Species Specificity of Rabbit and Human BMCM

	Rabbit BMCM		Human BMCM		No Conditioned Medium	
	Burst #	^{59}Fe Counts	Burst #	^{59}Fe Counts	Burst #	^{59}Fe Counts
Human Peripheral Blood	51 ± 7 *	1175 ± 231	54 ± 4	1297 ± 272	39 ± 5	354 ± 57
Rabbit Bone Marrow	34 ± 2	2605 ± 436	19 ± 2	57 ± 3	16 ± 1	78 ± 14

*All data are expressed as mean ± standard error of duplicate dishes.

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CHAPTER 5

DISCUSSION AND PROPOSAL

INTRODUCTION

In the preceeding chapters I have characterized two systems by which early events in erythropoiesis may be examined. First, I examined the demineralized bone matrix (DBM) ossicle hemopoietic tissue and found that it was normal. Second, I developed an assay for humoral factors known as burst-promoting activity (BPA), involved in the early development of erythroid cells. Rabbit bone marrow conditioned media (BMCM) was a potent source of BPA. The measurement of ^{59}Fe incorporation into heme was revealed as a much more sensitive assay for BPA than burst number. Analysis of individual bursts showed that heme ^{59}Fe incorporation and cell number were strongly correlated. Thus it appeared that heme ^{59}Fe incorporation was detecting increased hemoglobin synthesis due to cell proliferation. On the basis of these observations I proposed that a major mechanism of action of BMCM BPA is to promote cell proliferation during the early phase of burst formation. These studies were discussed at length in Chapter 3 and 4. In this chapter I will propose some avenues for future research.

Applications of the DBM Model

The DBM model has potential as a system for morphological study of events during hemopoietic microenvironment formation. The major events would involve the interaction of fibroblastic, vascular and hemopoietic cells. These interactions can be examined by standard techniques utilizing the light and electron microscope. The role of glycosaminoglycans in specific hemopoietic microenvironment formation (1) could be analyzed during ossicle development using histochemical techniques (2).

Another use for the DBM model is the investigation of the relationship between bone and bone marrow. A considerable amount of evidence points to such a relationship. Bone marrow forms in ectopic bone (3,4) under normal conditions. Hemopoiesis in non-bone sites is only observed when hemopoietic cells are undergoing compensatory hemopoietic proliferation or inappropriate hyperplasia (5). Bone and bone marrow have a common blood supply (6) which may facilitate the exchange of hypothetical biologically active substances between these two tissues (7). The cells of bone and bone marrow are derived from the same precursor cells. Friedenstein and his colleagues have shown that bone marrow stromal cells can give rise to bone cells (8) and marrow stromal cells (9). Osteoclasts are derived from pluripotent

stem cells via the monocyte differentiation pathway (10). The DBM model might be employed to examine the relationship between these two tissues by modulation of proliferation in one tissue and examining the other tissue for effects (11). Some parameters which can be quantitated are, the osteoclastic index, bone: bone marrow ratio, erythrocyte: granulocyte ratio and hemopoietic precuror cells.

Further Studies of Burst-Promoting Activity

Prior to further study of BMCM some degree of purification is required. The rabbit is an excellent source of BPA for purification because liter batches of BMCM may be produced from one animal. Preparation of large batches of human BMCM is not feasible because of the small volume of bone marrow obtained from aspiration biopsies. Rabbit BMCM contains BPA for both rabbits and human erythroid precursors. Thus purified rabbit BMCM could be used to further characterize the biological role of BPA in the development of human as well as rabbit erythroid precursors.

I have shown that early erythroid committed stem cells respond to BPA, but the possibility still remains that uncommitted pluripotent stem cells may also be responding to BPA (12,18,14). The culture system I have employed strongly favor erythroid colony formation.

An approach to aid in the detection of mixed colonies (colonies derived from pluripotent stem cells) would be to add colony stimulating factor (CSF) to the assay. Thus committed granulocyte/macrophage precursors in presumptive mixed colonies may be encouraged to differentiate.

A long-term bone marrow culture system has been described (15,16) in which hemopoietic stem cell maintenance and proliferation occurs. This system has been used to analyze factors involved in the control of proliferation and differentiation of early hemopoietic cells (16,17,18,15,19). Long-term bone marrow cultures could be used to detect the target cell population of BPA. Addition of BPA to the system followed by systematic analysis of the numbers of erythroid precursors at different maturation stages would indicate which cell populations are responsive to BPA. An increase in early BFU-e would indicate an effect on pluripotent cells and if the colonies are not mixed a role in committment might be proposed. Similarly an increase in intermediate BFU-e would indicate that earlier erythroid precursors are responsive.

The possibility of a BPA role in committment could be further examined in this system by looking for changes in the relative numbers of committed hemopoietic precursor cells for different cell lines in the presence and

absence of BPA. Development of a long term human BFU-e culture system has so far met with technical difficulties (Ogawa, personal communication). It is likely that such a culture system may be facilitated by the addition of rabbit BMCM to the media.

Another topic which requires further attention is the possibility that complex cell interactions may be involved in BPA production. In Chapter 1, I discussed at length the enhancement of burst formation by T-cells and monocyte-macrophage and proposed that BPA from one of these sources may stimulate BPA production from the other source. To examine this possibility experiments could be performed in which monocyte-macrophage BPA is incubated with T-cells, or vice versa, and the resulting conditioned media tested on bone marrow suspensions from which monocyte-macrophage and T-cells have been removed.

With a highly purified rabbit BPA preparation it should be possible to identify BPA sensitive human early erythroid precursors. This might be done by incubating suspensions of human hemopoietic cells with rabbit BPA, washing, and then a further incubation with labelled anti-rabbit antibodies prior to observation at the light or ultrastructural level depending on the type of label used.

Development of a Chemically Defined Medium

BMCM may facilitate the development of a serum free BFU-e assay similar to the assay described by Iscove (20) for CFU-e. The culture assays for erythroid precursors require serum in order to supply undefined needs. These requirements are probably multiple and it is difficult to distinguish between the effects of nonspecific nutritional factors and those of specific regulatory factors. The presence of serum makes it particularly difficult to investigate hormone effects because hormones may bind to serum proteins or there may already be hormones present in the serum. Serum is also a source of BPA (13). Thus it is important to replace serum with chemically defined media components. I have obtained burst formation in the presence of BMCM, selenite, transferrin, albumin, cholesterol and lecithin in serum free media. However, the bursts were very small and heme ^{59}Fe incorporation was not significant. This suggests that with further adjustments to the media a successful serum free BFU-e assay may be realized under serum free conditions. The development of a chemically defined medium will permit study of the regulation of early erythroid development and proliferation in much more detail than has been previously possible.

CONCLUSION

The future of the *in vivo* model and *in vitro* assay described appears promising because they enable one to study the early events in erythropoiesis. The DBM ossicle may be used for morphologic studies of hemopoietic microenvironment formation and to investigate the relationship between bone and bone marrow. Rabbit BMCM BPA may be used to further define the target cells of BPA, to investigate the possibility that complex cell interactions are involved in BPA production and in the development of a chemically defined media for the BFU-e assay.

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