The in vitro Growth of Erythroid Colonies from Dog Bone Marrow

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ABSTRACT

Enriched methyl cellulose media together with either human urinary erythropoietin or serum collected from phlebotomized dogs exposed to hypoxia was used in the study of the erythroid colony forming (CFU-E) capacity of dog marrow. The dog serum erythropoietin was found to be more efficient in stimulating CFU-E than comparable concentrations of human urinary erythropoietin. Numbers of CFU-E were directly related to the culture concentration of the stimulating serum and to the number of cells per plate. Sheep plasma erythropoietin was also found to be effective in stimulating CFU-E growth. The system described is chemically better defined and produced more consistent results than has been reported for the plasma clot method.

RÉSUMÉ

Cette expérience visait à vérifier la possibilité d'obtenir des cultures de cellules érythroïdes, à partir d'échantillons de moelle osseuse canine. Les auteurs utilisèrent à cette fin des milieux de méthylcellulose enrichis, auxquels ils ajoutèrent de l'érythropoïétine urinaire humaine ou du sérum prélevé par fonction veineuse, chez des chiens soumis à l'hypoxie. L'érythropoïétine sérique canine se révéla plus efficace pour stimuler la formation de colonies de cellules érythroïdes, qu'une quantité équivalente d'érythropoïétine urinaire humaine. Le nombre de colonies de cellules érythroïdes s'avéra directement proportionnel à la concentration de sérum stimulant, dans le milieu de culture, ainsi qu'au nombre de cellules ensemencées dans chacune des boîtes de Pétri. L'érythropoïétine sérique ovine se révéla aussi efficace pour stimuler la formation de colonies de cellules érythroïdes. Le système décrit dans cet article possède une meilleure définition chimique et permit d'obtenir des résultats plus constants qu'avec la méthode du caillot plasmatique.

INTRODUCTION

Although dogs are frequently used as experimental models of human hematologic diseases, reports describing *in vitro* culture systems for dog erythroid progenitor cells (CFU-E) have been sparse and limited to the plasma clot system (1,10). Erratic colony formation and clotting inconsistencies occurred repeatedly during our numerous attempts to grow dog CFU-E in plasma clots. Since canine marrow is capable of forming

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leukocyte colonies *in vitro* (CFU-C) (9) in a manner quite similar to man and rodents, we reasoned that CFU-E should also form consistently when optimal culture conditions were approached. This report describes a reproducible method of growing CFU-E using a methyl cellulose medium and employing dog erythropoietin as a growth stimulus.

MATERIALS AND METHODS

Twelve normal female dogs and one male dog were used as marrow donors. These donors varied in age from six months to one year. Dogs 149-B7 and 143-B7 were American foxhounds while all others were mongrels. The body weights among all dogs ranged from 15 to 30 kilograms. At the time of marrow collection, each dog had been immunized against distemper-hepatitis, hematocrits were within the normal range and all dogs were apparently in good health.

The sera used to stimulate CFU-E were obtained from a six month old male (dog #635) and a seven month old female (dog #599) by first removing 17 mL per kg of body weight of whole blood from the dog, placing the dog in a hypobaric chamber (0.45 atm) for 16-18 hrs and then collecting serum. Dogs subjected to hypoxia have been shown previously to produce increased quantities of serum erythroid stimulating factor (ESF) (8). The serum was assayed for ESF in the mouse bioassay system (6) and the *in vitro* fetal mouse liver cell technique (3,4). Human urinary ESF, obtained from the National Institutes of Health, had a potency of 15 or 18 units of ESF per mg of protein.[1] Step III sheep plasma erythropoietin was obtained from a commercial source.[2]

Xylazine was administered as an analgesic to all dogs prior to marrow aspiration. A 10 cc plastic syringe and an Illinois sternal needle were used to aspirate

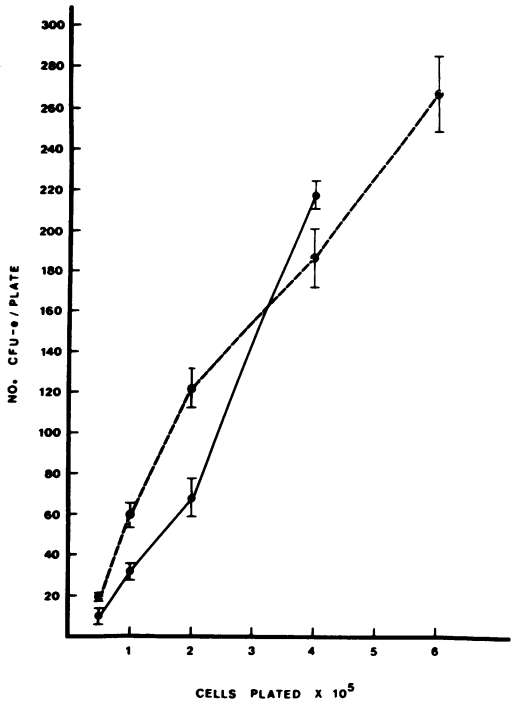

Fig. 1. Marrow cell dose response of dog 523 (● ——— ●) and dog 521 (● - - - - ●) to 0.1 mL serum per mL of culture. The serum was collected from dog 635 following phlebotomy and 18 hours of hypoxia. Four plates were counted for each point with the vertical bars showing standard error of the mean.

marrow from the head of the humerus or the iliac crest. The aspirated marrow was immediately placed in 5 mL of ice-cold alpha minimal essential medium[3] (MEM) containing glutamine but without ribosides and deoxyribosides. One hundred units of preservative-free heparin were added to each 5 mL of collecting medium. Nucleated cells were separated on Ficoll-Hypaque and washed twice with alpha MEM.

The culture procedure was modified from that of Rich and Kubanek (11). A 2% methyl cellulose solution was prepared as follows: methyl cellulose was mixed in the proportion of 4 g per 100 mL of hot distilled water, shaken vigorously and autoclaved for 15 min at 15 psi. Upon reaching room temperature an equal volume of cold, sterile 2x alpha MEM was added and the mixture was shaken and refrigerated over night to improve consistency. A 12 mL sterile plastic syringe was used to dispense the

[1]Procured from the Department of Physiology, University of the Northeast, Corrientes, Argentina; processed by the Hematology Research Laboratories, Children's Hospital of Los Angeles; distributed by the National Heart, Lung and Blood Institute under grant #HL-10880.

[2]Connaught Laboratories, Swiftwater, Pennsylvania.

[3]Grand Island Biological Co., Grand Island, New York.

TABLE I. Erythroid Stimulating Factors, Units/mL, [95% Confidence Limits] of Serum from Two Phlebotomized Dogs Exposed to an Hypoxic Environment for 16 to 18 Hours

Dog #	*In vitro* Fetal Mouse Liver Cell Assay	*In vivo* Ex-hypoxic Polycythemic Mouse Assay
635	1.7 [1.3 — 2.1]	0.9 [0.6 — 1.5]
599	7.0 [5.1 — 9.4]	2.7 [2.1 — 3.5]

TABLE II. Number of CFU-E Formed by Different Marrow Cell and Serum Combinations

Dog # of Marrow Donor	Dog # of Serum Donor	# CFU-E per 2 x 10^5 Cells			
		0.0 serum (S.E.)	0.025 mL (S.E.)	0.05 mL (S.E.)	0.1 mL (S.E.)
605	599	0	23 ± 2.1	31 ± 3.5	48 ± 4.2
714	603[a]	10 ± 1.3	10 ± 2.8	11 ± 3.0	11 ± 1.9

[a]Serum donor had been neither phlebotomized nor placed in hypoxia
(S.E.) = Standard Error of the Mean with four plates used for each figure

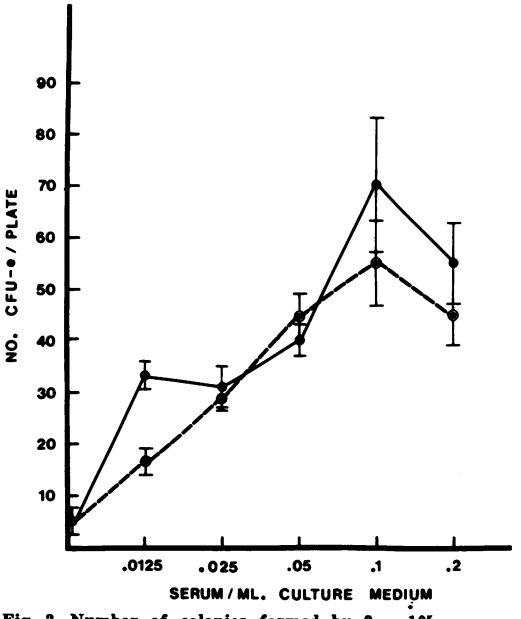

Fig. 2. Number of colonies formed by 2 x 10^5 marrow cells from dog 572 in response to increasing amounts of serum from dogs 599 (● ——— ●) and 635 (● - - - - ●). Both serum donors were phlebotomized and placed in hypoxia for 18 hours prior to serum collection. The vertical bars show the standard error of the mean of the four plates.

solution. To each tube containing 2.4 mL of 2% laboratory grade methyl cellulose (4000 centipoises) in alpha MEM was added: 1.2 x 10^6 viable cells, 1.8 mL fetal calf serum (not heat inactivated), and different amounts of hypoxic dog serum or urinary ESF. Step III sheep plasma erythropoietin at a level of 0.25 units per culture dish was used in certain experiments. In cell-dose response experiments, cell numbers were varied and serum or ESF was kept at a constant concentration. Alpha MEM (1x) was added to bring the volume of each tube to 6.0 mL. The tubes were mixed by inverting several times. Volumes of 1.2 mL were then dispensed into each of four plain plastic 35 mm Petri dishes with long 16 gauge needles and plastic syringes. Using aseptic techniques all dispensing and mixing was carried out under a tissue culture hood.

The plates were incubated at 36°C for 48 hrs in a humidified atmosphere containing 7.5% CO_2. Each plate was stained with 1.0 mL stain solution containing 2% benzidine dihydrochloride in 0.5% acetic acid (2). Fresh 30% H_2O_2 in the proportion of 0.2 mL per 50 mL of stain was added to each batch of stain just before use. Groups of six or more cells staining green within three to five minutes were enumerated as CFU-E. Colonies with similar morphology were picked from unstained culture dishes and stained with Wright's stain to confirm the erythroid cell composition.

RESULTS

The ESF activities of the phlebotomized hypoxic dog sera are shown in Table I. *In vitro* activities were higher

than the *in vivo* potencies, although this difference was not significant for dog #635.

When 0.1 mL serum from dog #635 was mixed with each mL of culture the number of colonies formed per plate varied directly with the number of nucleated cells plated (Fig. 1).

In Figure 2 is shown the number of colonies formed by 2 x 10^5 plated cells collected from dog #572 when different doses of sera #599 and #635 were used. Despite a threefold difference in the measured ESF activity (Table I), these two sera stimulated similar numbers of colonies of the same marrow preparation.

TABLE III. Number of CFU-E Formed by Various Marrow Cells (2 x 10^5) Cultured with 0.25 Units of Erythropoietin[a] and Three Different Sera[b]

Dog # of Marrow Donor	Dog # of Serum Donor	# CFU-E per 2 x 10^5 Cells	S.E.[d]
149-B7	635	86[c]	± 3.7
635	635	37	± 3.7
143-B7	739	40	± 0.0
143-B7	748	117	± 3.0
826	748	63	±14.7

[a]Step III sheep plasma erythropoietin
[b]0.1 mL. serum per culture
[c]Mean of eight experiments (32 cultures) with a range of 74-111
[d](S.E.) = Standard Error of the Mean with four plates used for each figure

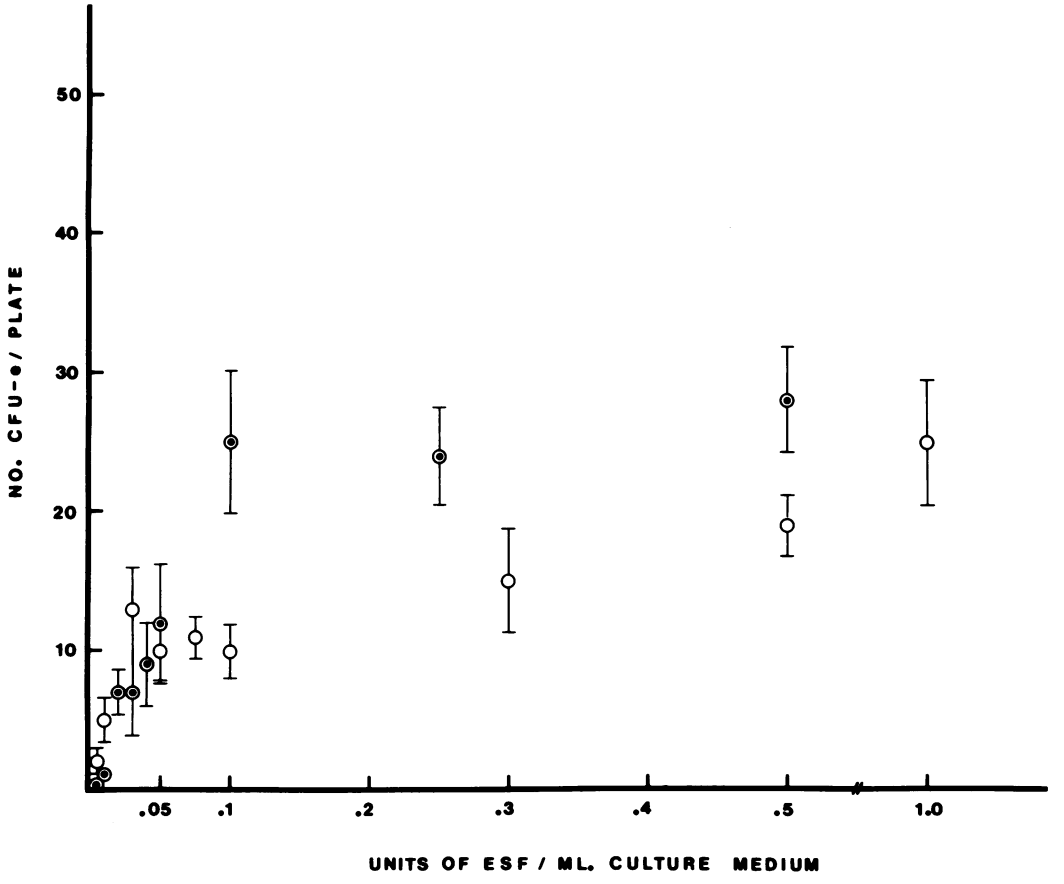

Fig. 3. Number of colonies formed by 2 x 10^5 marrow cells from dog 605 (●) and dog 600 (○) to increasing amounts of human urinary ESF. Vertical bars show standard error of the mean of four plates.

A second marrow preparation responded to different volumes of serum #599 as is shown in Table II. The number of colonies formed in response to serum from a normal dog that had been neither phlebotomized nor placed in hypoxia is also shown in Table II. The number of colonies formed by several different marrow-serum combinations together with Step III sheep plasma erythropoietin is shown in Table III. These cultures all contained 0.1 mL of dog serum and 2 x 10^5 marrow cells.

Human urinary ESF was incorporated into medium at seven dose levels (Fig. 3) with the number of cells plated held constant at 2 x 10^5 nucleated cells per mL. The 0.1 unit dose gave nearly maximal response with no statistically significant increase in colony numbers at higher doses.

In Figure 4 is shown the number of colonies formed by 0.1 to 4.0 x 10^5 cells per plate in response to either 0.25 units of human urinary ESF or 0.1 mL of hypoxic dog serum #635. As cell numbers increased, more colonies were formed in response to the hypoxic dog serum than to human urinary ESF, although, by bioassay, the serum provided less ESF than the human urinary preparation.

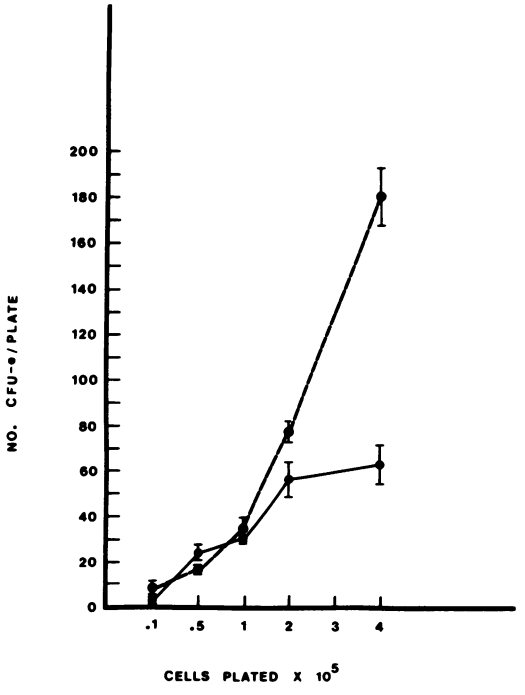

Fig. 4. Number of colonies formed by increasing numbers of dog marrow cells in response to the serum of dog 635 (● - - - - ●) [0.1 mL/mL culture medium] or human urinary ESF (● ——— ●) [0.25 μ/mL, 18 μ/mg protein]. Vertical bars indicate the standard error of mean of four plates.

DISCUSSION

The methyl cellulose culture system for canine marrow CFU-E fulfills the criteria for a quantitative assay in that colony numbers are relative to the number of nucleated cells plated over the range of 0.5 to 6 x 10^5 cells per culture (Figs. 1 and 4) and are directly proportional to the concentration of stimulating factors(s) (Fig. 2 and 3, Table II). The correlation between the CFU-E and bone marrow cell numbers plated (Fig. 1) were highly significant ($r = 0.959$, dog #521; $r = 0.975$, dog #523; $P < 0.001$ for both). However, the Y-axis intercept for zero cells plated was significantly different from 0 for both bone marrows (+ 14.5, dog #521; − 30.6, dog #523). The Y-intercept variation from less than 0 to greater than 0 provides little evidence that CFU-E number is not related to the number of bone marrow cells plated down to the limiting cell concentration.

Erythroid colony growth in methyl cellulose offers several advantages over the plasma clot system. Growth in the methyl cellulose system is more consistent than in plasma clots (1,10) and the reproducibility is comparable to other *in vitro* clonal assays (5,7,11). The methyl cellulose system has fewer inherent variables than the plasma clot technique and erratic gelling is not a problem. Also, scanning of colonies in the methyl cellulose cultures can be done directly with the aid of an inverted microscope and the benzidine stain (2), thus avoiding the tedious microscopic examination of plasma clots.

Dog sera containing elevated titers of ESF appear especially useful in culturing canine CFU-E. The dog sera were more efficient at generating erythroid colonies than were comparable concentrations of human urinary erythropoietin, particularly at higher bone marrow cell numbers (Fig. 4). The reasons for this are uncertain and are under investigation. It is possible that the addition of dog sera to dog cells offers some subtle growth advantages or that dog cells have

some degree of specificity for dog erythropoietin. However, cultures containing 0.1 mL of dog serum and Step III sheep plasma erythropoietin resulted in 37 to 117 CFU-E per 2 x 10^5 nucleated bone marrow cells, suggesting erythropoietin specificity is not critical to the growth of dog CFU-E. Furthermore, erythropoietin assays, either in mice *in vivo* or using fetal mouse liver cells *in vitro*, appear to be poor indicators of the growth-promoting activity of dog serum in methyl cellulose cultures. Both assays suggested a two- to threefold difference in potency between two sera (Table I) and yet both sera were equally effective at generating CFU-E from canine marrow.

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