Bovine Granulocyte/Macrophage and Erythroid Colony Culture: Characteristics of the Colonies and the Assay Systems

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ABSTRACT

Bovine bone marrow granulocyte/macrophage colonies were cultured in vitro in methyl cellulose and in plasma clots using bovine endotoxin-stimulated serum as a source of colony stimulating activity. The endotoxin-stimulated serum was four times as potent as the control serum in the methyl cellulose cultures. No significant increase in the number of colony forming units was observed when bovine marrow cells were maintained in suspension cultures for various periods prior to plating in methyl cellulose. The percentage of glass/plastic adherent cells in bovine marrow cells was observed to be 43% ± 12 (SD). Benzidine positive erythroid colonies appeared in plasma clot cultures on day 4 and disappeared by day 9. No second population of erythroid colonies appeared either as a function of time or as a function of erythropoietin concentration. The optimum erythropoietin concentration for bovine erythroid cultures was found to be 1.0 unit/mL. A significant difference was observed between animals in their marrow capacity to produce erythroid colonies in culture but no significant difference was observed within individual animals over a period of three months.

RÉSUMÉ

Cette expérience visait à obtenir in vitro des colonies de granulocytes et de macrophages de la moelle osseuse de bovins, dans du méthylcellulose et dans des caillots de plasma enrichis de sérum bovin traité avec une endotoxine, comme source de stimulation de l'activité de ces colonies.

Le sérum traité avec une endotoxine se révéla quatre fois plus puissant qu'un sérum témoin, dans les cultures sur méthylcellulose. Le fait de maintenir les cellules de la moelle osseuse dans des cultures en suspension, pour diverses périodes, avant de les ensemencer sur du méthylcellulose, ne se traduisit pas par une augmentation appréciable du nombre d'unités formatrices de colonies. Le pourcentage des cellules de la moelle osseuse qui adhéraient au verre ou au plastic s'établissait à 43% ± 12 (SD). Quatre jours après l'ensemencement, des colonies de cellules érythroïdes, positives à la benzidine, apparurent dans les cultures sur caillots de plasma; elles disparurent cependant, cinq jours plus tard. Il ne se forma pas d'autres colonies de cellules érythroïdes, indépendamment du temps ou de la concentration d'érythropoïétine. La tenur optimale d'érythropoïétine, pour les cultures de cellules érythroïdes, s'avéra 1,0 unité/mL.

L'aptitude des cellules de la moelle osseuse à produire des colonies de cellules érythroïdes dans les cultures varia de façon appréciable, d'un bovin à l'autre; on ne nota cependant aucune différence appréciable, pour un même animal, sur une période de trois mois.

INTRODUCTION

In vitro granulocyte/macrophage colony culture was first reported in mice by Bradley and Metcalf (2) in Australia and by Pluznik and Sachs (23) in Israel. Thereafter, the *in vitro* granulocyte/macrophage culture technique was successfully applied in man (24), rat (3), cattle (29) and in the dog (18). *In vitro* erythroid colony culture was reported for the first time in mice by Stephenson *et al* (25) and has been successfully applied in man (27), dog (4) and cattle (17). Two different

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This research project was financed by International Development Research Center, Ottawa, Canada although opinions contained herein are not necessarily those of the Center.

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Submitted February 5, 1979.

classes of erythroid colonies have been reported in plasma clot cultures of mouse (1) and of human (27) marrow cells. The early population of erythroid colonies requires a lower erythropoietin concentration, shorter time, and the colonies are smaller in size as compared to the second population. The aim of our present research work was to study the characteristics of *in vitro* bovine marrow culture assay systems and to use the systems as tools for investigating the mechanisms of anemia and leukopenia in bovine trypanosomiasis. Such systems have already proved to be very useful in the study of human leukemias (15, 21, 24), human pure red cell aplasia and congenital hypoplastic anemia (12), and cyclic neutropenia in grey collie dogs (6).

Nomenclature

Committed granulocyte/macrophage progenitors capable of producing granulocyte/macrophage colonies *in vitro* are designated, "colony forming units in culture, or CFU-C" (2). Committed erythropoietic progenitors capable of producing erythroid colonies *in vitro* are designated "erythroid colony forming units or CFU-E" (25). Earlier erythropoietic progenitors capable of producing larger erythroid colonies *in vitro* and requiring more time and erythropoietin concentration are designated "erythroid burst-forming units or BFU-E" (1).Pluripotent stem cells capable of forming granulocytic, erythrocytic and megakaryocytic colonies in spleens of heavily irradiated mice are designated "colony forming units in spleen or CFU-S" (28).

MATERIALS AND METHODS

Experimental Animals

Twelve Boran (*Bos indicus*) steers ranging in age between one and two years were used as marrow cell donors. These steers were bought from a trypanosomiasis-free area of Kenya.

Marrow Aspiration

Animals were anesthetized by injecting a solution of 2% Rompun[1] into their jugular veins at a dosage of 1 mL/100 kg body weight and then their sternal regions were shaved and disinfected with 70% ethanol. Three mL of marrow were then aspirated from the third or fourth sternebra using a 16 gauge biopsy needle and 20 mL disposable syringe as described by Wilde (30). These 3 mL of marrow were put into 15 mL plastic disposable test tubes[2] containing 30 units of heparin[3] and mixed thoroughly.

Source of Colony Stimulating Activity (CSA)

Five hundred mL of blood were collected from a jugular vein of a Boran steer and serum was separated (preendotoxin serum). After collecting this first sample of blood, the steer was given an injection of a 1 mg/mL solution of bacterial endotoxin (Piromen)[4] at a dosage of 50 μg/kg body weight. At the nadir of peripheral leukocyte count (two hours later), 500 mL of blood were collected and serum separated (endotoxin-stimulated serum). Both the preendotoxin serum and the endotoxin-stimulated serum were put into 15 mL disposable test tubes and stored in 10 mL aliquots in a freezer at –20°C.

CFU-C Colony Culture

The CFU-C colonies were cultured in methyl cellulose using the method described by Walker *et al* (29). These colonies were also produced in plasma clots using the method described by McLeod *et al* (20).

Assay for Colony Stimulating Activity (CSA)

The preendotoxin serum and the endotoxin-stimulated serum were assayed for CSA by culturing a constant cell inoculum (10^5 cells) with various concentrations of these serum samples.

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[1]Bayer Laboratories, Nairobi, Kenya.

[2]Fisher Scientific Co., Ottawa, Canada.

[3]E.T. Monks, Nairobi, Kenya.

[4]Baxter Laboratories, Malton, Ontario, Canada.

Removal of Glass/Plastic Adherent Cells from Bovine Marrow Aspirates

The adherent cells were isolated from bovine marrow cell suspensions using the method described by Messner *et al* (21).

Linearity of the CFU-C Assay

The cell inoculum was varied from 1×10^4 to 10×10^4 in otherwise identical CFU-C cultures.

Suspension Cultures of CFU-C

Suspension cultures were established using optimum concentrations of CSA and a cell inoculum of 10^6 nucleated marrow cells per mL and incubated for different periods after which cells were recovered, washed in tissue culture medium and then cultured in methyl cellulose as described by Iscove *et al* (13).

The Erythroid Colony (CFU-E) Culture

The erythroid colonies were cultured in plasma clots using the method of McLeod *et al* (20).

Linearity of the CFU-E Assay

These cultures were established in the same way as the CFU-C linearity experiments, except that the cultures were in plasma clots.

Assay for Erythropoietin (Erythropoietic Stimulating Factor ESF)[5]

Several plasma clot cultures of erythroid colonies were established in identical manner except for the various concentrations of ESF.

Colony Staining

The peroxidase staining technique described by Yam *et al* (31) was used to identify the CFU-C colonies, while the benzidine staining technique described by McLeod *et al* (20) was used to stain the erythroid cells.

Culture of Bovine Erythroid "Bursts" (BFU-E Colonies)

In several attempts to culture two different populations of erythroid colonies, plasma clot cultures were established using a constant cell inoculum of 10^5 nucleated marrow cells and various ESF additions, as follows:

(a) 1.00 ESF units per mL followed by feeding some clots with 1.50 ESF units per clot for two, and some for four, consecutive days. Colonies were harvested daily for 15 days, stained and counted (N* = 128).

(b) 1.00 or 7.50 ESF units/mL. Cultures were harvested daily for 15 days and erythroid colonies counted (N = 128).

(c) 1.00 or 7.50 ESF units/mL and half of the cultures fed with 0.5 ESF unit per clot at 24 and 48 hours, followed by harvesting and counting colonies daily for 15 days (N = 128).

(d) 0.625, 1.25, 2.50, 5.00, 7.50 or 10.00 ESF units per mL followed by harvesting cultures daily for ten days (N = 264).

(e) 1.00, 5.00 or 10.00 ESF units per mL, colonies harvested and cells per colony counted from 100 colonies from each ESF concentration. The frequency distribution of various colony sizes was determined (N = 12).

Suspension Cultures of CFU-E

These cultures were established in the same manner as the suspension cultures of CFU-C, except that the plasma clot culture technique was used in this experiment and ESF was substituted for CSA.

Erythroid Colony Cultures in Methyl Cellulose

Attempts to culture bovine erythroid colonies in methyl cellulose using the meth-

[5]Sheep step III erythropoietin, Connaught Laboratories, Toronto, Canada.

*Number of plasma clots per experiment.

od described by Iscove and Sieber (14) were made. Since erythroid colonies repeatedly failed to grow, in subsequent experiments the cultures were supplemented with 1% beef embryo extract[6], 1% vitamin solution[7], 1% nonessential amino acids[8], 1% bovine serum albumin[9], β-mercaptoethanol (10^{-4} M)[10] and PHA-LCM[11].

An attempt to culture erythroid and granulocyte colonies from peripheral blood cells, in plasma clot cultures was made. 1×10^5 and 5×10^5 nucleated cells and 1.00 and 10.00 ESF units per mL were used.

Statistical Analysis

To determine levels of significance, the one way analysis of variance was used on log transformed data. Linear regression analysis by the least squares method was conducted on the log transformed colony data.

RESULTS

CFU-C Colonies

These colonies appeared in methyl cellulose cultures on day 4, reached maximum maturity on day 7, and thereafter disappeared gradually up to day 15 when colonies were not present. Colonies composed of large cells, often found attached to the bottom surface of the plastic Petri dishes, contained mostly macrophages, while compact colonies composed of small cells, often found suspended in methyl cellulose, contained predominantly granulocytes. Both types of colonies were peroxidase positive.

A direct linear relationship ($r = 0.88$) between the log of nucleated marrow cells plated and the log of CFU-C colonies was demonstrated between 1×10^4 and 10×10^4 cells plated (Fig. 1).

In eleven Boran steers, the percentage of glass/plastic adherent cells was found to be 43 ± 12 (SD) of total bovine marrow cells.

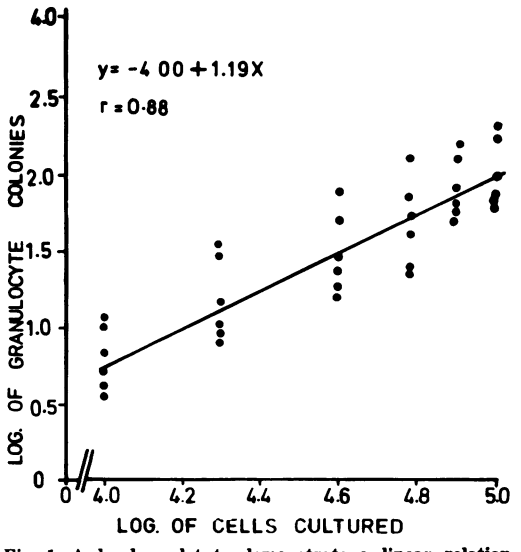

Fig. 1. A log-log plot to demonstrate a linear relationship between the log of bovine nucleated marrow cells cultured and the log of CFU-C colonies produced in six Boran steers. Each point represents mean colony count from four Petri dishes.

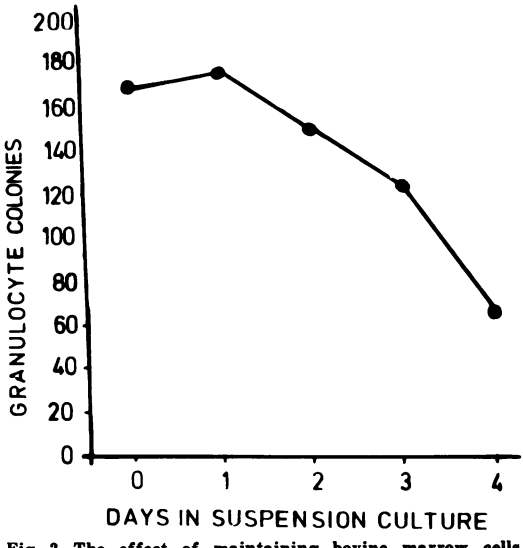

Fig. 2. The effect of maintaining bovine marrow cells in suspension cultures, prior to plating in methyl cellulose, on CFU-C colony production. Each point represents mean colony counts from four Petri dishes.

When bovine marrow cells were maintained in suspension cultures for various times in the presence of optimum levels of CSA prior to plating in methyl cellulose cultures, no significant increase in the number of granulocyte forming units was observed (Fig. 2).

The postendotoxin injection serum was four times as potent as the preendotoxin injection serum in stimulating CFU-C

[6,7,8,9]Grand Island Biological Co., New York, U.S.A.

[10]Eastman Kodak Co. New York, U.S.A.

[11]Phytohemagglutinin-stimulated human peripheral leukocyte conditioned medium, Ontario Cancer Research Institute, Toronto, Canada.

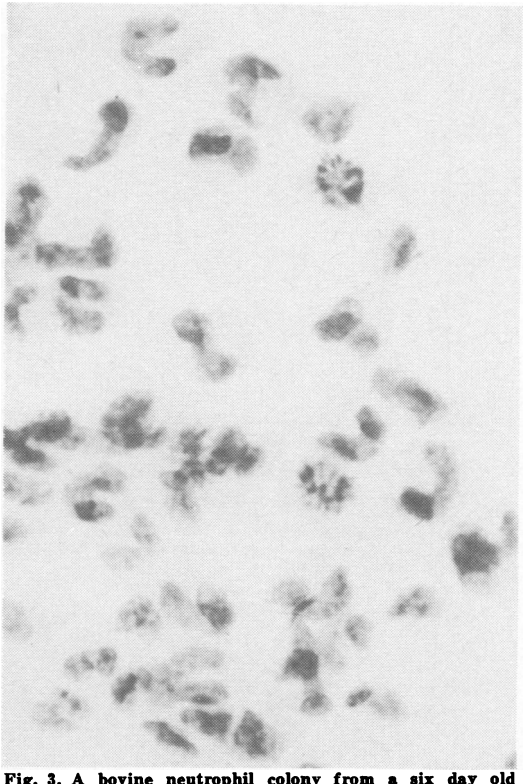

Fig. 3. A bovine neutrophil colony from a six day old plasma clot culture. Note the presence of metamyelocytes, bands and mitotic figures. X785.

colony growth in methyl cellulose cultures. The optimum concentration of postendotoxin injection serum required in the cultures was 4% (final concentration).

A significant difference ($P < 0.05$) between animals in the capacity of their marrow to form CFU-C colonies was observed.

Well differentiated granulocyte colonies were produced in plasma clot cultures by six days (Fig. 3). The optimum concentration of postendotoxin injection serum required in these cultures was found to be 10% (final concentration). However, this culture system was found to have very poor sensitivity to exogenous CSA, especially as far as colony size was concerned.

CFU-E Colonies

These colonies were cultured in plasma clots and colonies of up to 260 benzidine positive cells were produced. The colonies appeared in cultures on day 4 and disappeared by day 9. A direct linear relationship ($r = 0.87$) between the number of nucleated marrow cells cultured and the number of erythroid colonies formed was demonstrated (Fig. 4).

The optimum concentration of ESF required in these cultures was found to be 1.00 ESF unit per mL (Fig. 5). Culturing the colonies at 1.0 and 7.50 ESF units/mL followed by daily harvesting of some clots

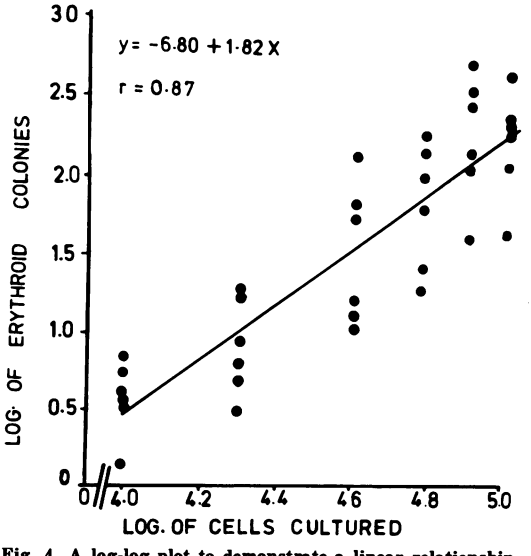

Fig. 4. A log-log plot to demonstrate a linear relationship between the log of bovine nucleated marrow cells cultured and the log of CFU-E colonies produced in six Boran steers. Each point represents mean colony counts from four Petri dishes.

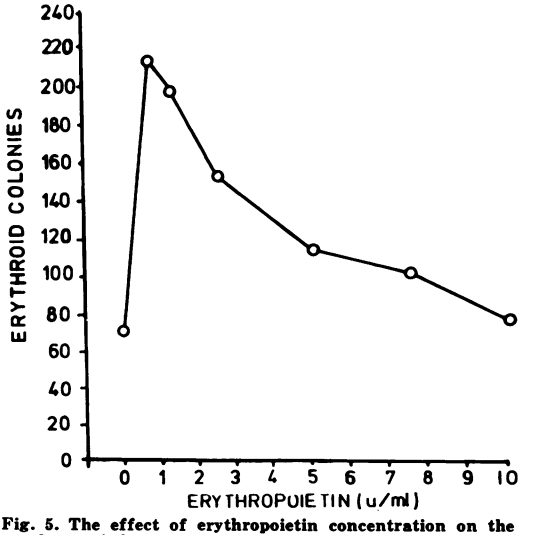

Fig. 5. The effect of erythropoietin concentration on the numbers of bovine CFU-E colonies formed in plasma clot cultures. The values represent the means of five experiments.

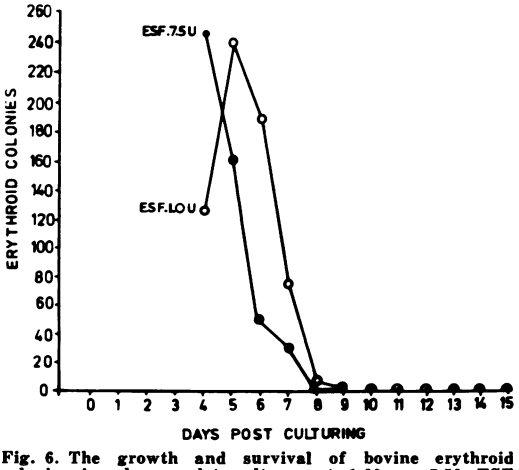

Fig. 6. The growth and survival of bovine erythroid colonies in plasma clot cultures at 1.00 or 7.50 ESF units per mL. Each point represents mean colony counts from our plasma clots.

for 15 days failed to reveal the presence of two different classes of erythroid colonies even when the clots were fed with extra ESF after 24 and 48 hours (Figs. 6 and 7). Frequency distribution charts of the number of erythroid colonies against the number of cells per colony revealed only one population of erythroid colonies even when different ESF concentrations were used (Fig. 8).

A significant difference ($P < 0.05$) was observed between animals in the capacity of their bone marrow to produce erythroid colonies. However, no significant difference was observed within an individual animal over a period of three months.

Maintenance of marrow cells in suspension cultures prior to plating in plasma clots resulted in a decrease in the number of erythropoietic progenitors (Fig. 9).

When bovine nucleated peripheral blood cells were cultured in plasma clots using 1×10^5 and 5×10^5 cells per clot and 1.00 and 10.00 ESF units per mL, neither erythroid nor granulocytic colonies developed. However, when the experiment was repeated using bovine nucleated marrow cells, several erythroid and granulocytic colonies developed.

DISCUSSION

Bovine CFU-C colonies were produced in methyl cellulose cultures *in vitro* using bovine endotoxin-stimulated serum as a source of CSA. The optimum period of incubation was observed to be seven days. This observation agrees with that of Walker *et al* (29). The endotoxin-stimulated bovine serum was observed to be four times as potent as the preendotoxin serum, suggesting that an injection into the steer of 50 μg endotoxin/kg body weight increased the serum concentration of CSA four times. Chervenick (5) observed that the levels of CSA in mice injected with 50 μg of endotoxin increased to 600% of control values.

Besides the increase in the number of CFU-C colonies observed in this experiment, the endotoxin-stimulated serum caused a marked increase in colony size. Messner *et al* (21) observed that in human marrow cultures, colony number increased markedly when CSA was added. Marrow cell suspensions consist of a population of glass/plastic adherent cells and a population of nonadherent cells. The glass/plastic adherent cells are the source of CSA and 20% to 50% of human marrow cells are of this type (21). In the present experiment, bovine marrow cells were found to consist of 20% to 66% ($\bar{x}$ 43%) plastic adherent cells. The proportions of adherent cells in man and in cattle are thus comparable.

A direct linear relationship between the number of nucleated marrow cells plated and the number of CFU-C colonies produced has been reported in mice (2), rat (3), man (15), dog (18) and cattle (29). In this experiment, a direct linear relationship between the number of bovine nucleated marrow cells plated and the number of CFU-C colonies produced was demonstrated. This kind of relationship suggests that the colonies originate from single cell progenitors.

CFU-C colonies were also cultured in plasma clots and well differentiated granulocyte colonies were produced within six days. However, this culture system was found to be less sensitive to exogenous CSA than the methyl cellulose culture system, especially in as far as colony size is concerned. This might be due to the presence of various ingredients of bovine origin (fetal calf serum, beef embryo extract and bovine plasma) which may contain a substantial amount of CSA in this culture system as compared to only fetal calf serum present in the methyl cellulose culture system.

Maintenance of bone marrow cells in

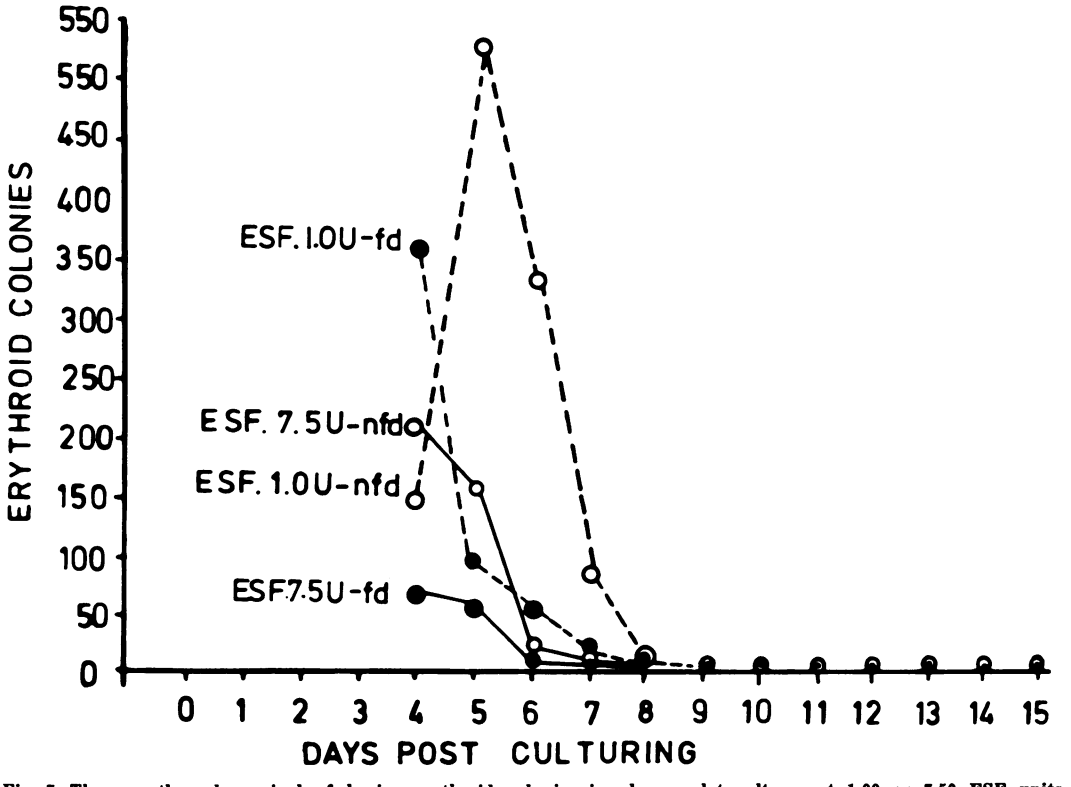

Fig. 7. The growth and survival of bovine erythroid colonies in plasma clot cultures at 1.00 or 7.50 ESF units per mL, followed by feeding the cultures with 0.50 ESF unit per clot at 24 and 48 hours. fd = fed, N-fd = non-fed. Each point represents mean colony counts from four plasma clots.

suspension cultures in the presence of CSA, prior to plating in methyl cellulose, results in a marked increase in the CFU-C content in mice (19, 26) and in man (13, 22). In cattle, however, only a slight increase occurs (29). The results of our experiment are consistent with the findings of Walker *et al* (29) that only a slight (nonsignificant) increase in the number of CFU-C occurs in bovine marrow cells maintained in suspension cultures in the presence of CSA. The increase in the number of CFU-C in marrow cells maintained in short-term suspension cultures is thought to result from recruitement of the CFU-C from the pluripotent stem cells (CFU-S) (13, 26). The failure of bovine CFU-C to increase when marrow cells are maintained in suspension cultures may imply that bovine marrow samples contain a very small proportion of pluripotent stem cells as compared to mouse and human marrow samples. This may explain why cattle respond very slowly to hematological stress as compared to other species of animals and to man.

Erythroid colonies have been successfully cultured in methyl cellulose in mice (10, 14) and in man (16). In cattle, however, several attempts to culture erythroid colonies proved unsuccessful even after various modifications of the culture system. Consequently, the plasma clot technique described by McLeod *et al* (20) was used and large bovine erythroid colonies of up to 260 cells were produced.

A direct linear relationship between the number of nucleated marrow cells plated and the number of erythroid colonies formed has been reported in mice (8, 9), man (27) and in cattle (17). In this experiment a direct linear relationship was also demonstrated between the number of nucleated marrow cells cultured and the number of erythoid colonies produced. This linear relationship suggests that the erythroid colonies originate from single cell precursors.

CFU-E colonies in plasma clots mature on day 2 in mice (11, 25) and on days 7 to 9 in man (27). In cattle, benzidine-positive colonies appeared on day 4 and peak

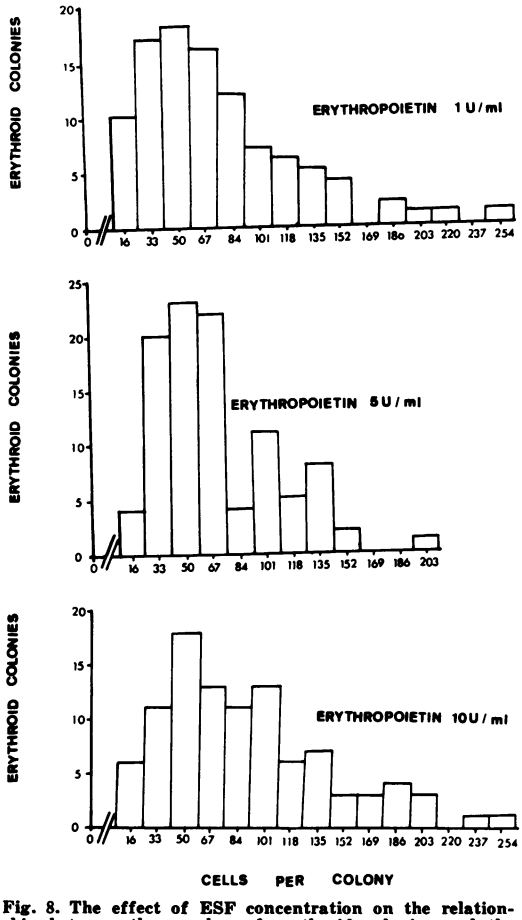

Fig. 8. The effect of ESF concentration on the relationship between the number of erythroid colonies and the number of cells per colony. Cells were counted from 100 colonies for each ESF concentration.

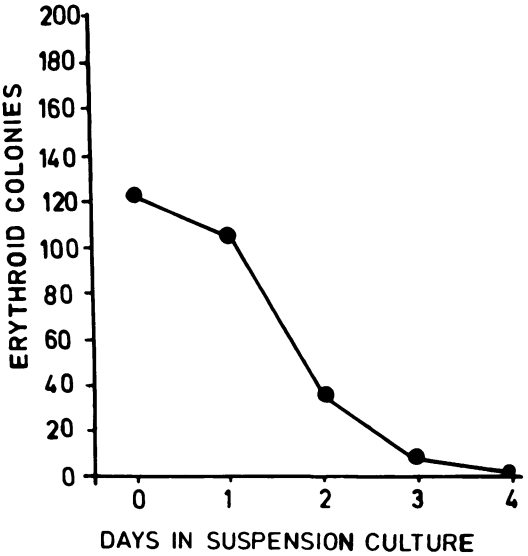

Fig. 9. The effect of maintaining bovine marrow cells in suspension cultures prior to culturing in plasma clots, on CFU-E colony production. Each point represents mean colony counts from four plasma clots.

maturity was reached on day 5. Thereafter, the colonies started disappearing until they had completely disappeared by day 9, leaving behind only the CFU-C colonies. The absence of day 2 benzidine-positive colonies in bovine marrow cultures as compared to mouse marrow cultures is an important indication of species difference.

About 5% of the bovine erythroid colonies were mixed with granulocyte/macrophage cells. In our previous publication (17) we reported the percentage of bovine mixed colonies to be 30%. The difference might have resulted from the fact that the plasma clot technique used in our previous experiment was a modification of Tepperman *et al* (27) while the technique used in the present experiment was a modification of McLeod *et al* (20).

The optimum ESF concentration in plasma clot erythroid cultures is about 0.25 unit/mL in mice (9, 21) and 2.0 to 5.0 units/mL in man (27). In the bovine erythroid cultures the optimum level of ESF was found to be 1.00 unit/mL, indicating another species difference.

Axelrad *et al* (1) and Heath *et al* (11) were able to produce a second population of mouse erythroid colonies (BFU-E colonies) by repeatedly feeding the clots with extra ESF. Tepperman *et al* (27) reported culture of human BFU-E colonies using 10.0 ESF units/mL without extra feeding with ESF. Our repeated failure to culture bovine BFU-E colonies using the methods mentioned above (1, 11, 27) may imply that bovine hemopoietic progenitors do not exist in a wide spectrum of maturation like human and mouse progenitors, thus providing another explanation to the slow hemopoietic recovery in the bovine species following hematolological stress.

Heath *et al* (11) observed that mouse CFU-E colonies from plasma clot cultures contained eight to 50 cells, while the BFU-E colonies contained up to 10^3 cells. Under similar culture conditions, Tepperman *et al* (27) observed that human CFU-E colonies contained eight to 32 cells while the BFU-E colonies contained up to 150 cells. In methyl cellulose cultures of mouse marrow cells Iscove and Sieber (14) observed that the CFU-E colonies contained eight to 60

cells while the BFU-E colonies contained up to 10^4 cells. In our experiments the bovine erythroid colonies ranged from eight to 260 cells. The upper size limit for the bovine CFU-E colonies is thus much higher than that of mouse and human CFU-E colonies.

Maintenance of bovine marrow cells in short-term suspension cultures prior to plating in plasma clots did not result in any increase in the CFU-E content of the marrow cell suspensions. This implies that under these experimental conditions CFU-E are not recruited from the more primitive progenitor cells.

A significant difference ($P < 0.05$) between different animals in their marrow capacity to produce erythroid colonies was observed. However, no significant difference ($P < 0.05$) was observed within one animal over a period of three months. This suggests that one can collect marrow samples from one animal for several days without having to worry about day-to-day variations, as long as the animal is kept under the same experimental condtions throughout.

CFU-C circulate in peripheral blood of normal human beings (7) and dogs (18). BFU-E have also been reported in peripheral blood of normal mice (10). Our attempts to culture CFU-C and CFU-E (or BFU-E) colonies from bovine peripheral blood leukocytes using 1×10^5 and 5×10^5 leukocytes and 1.00 and 10.00 ESF units per mL proved repeatedly unsuccessful. When the experiments were repeated using the same inocula of bovine marrow cells, several CFU-C and CFU-E colonies were produced. This suggests that the number of CFU-C and CFU-E circulating in normal bovine peripheral blood is less than 1 per 5×10^5 peripheral blood leukocytes. When this figure is compared with 15 CFU-C per 10^6 peripheral blood leukocytes in the dog (18) a marked species difference is noticed.

ACKNOWLEDGMENTS

We wish to thank Dr. W. Masiga, the Director of Veterinary Research Department, Kenya Agricultural Research Institute, Muguga, for providing us with research facilities.

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