Bovine Erythrocytic, Granulocytic and Macrophage Colony Formation in Culture. A Preliminary Report

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ABSTRACT

Bovine erythrocytic colonies containing up to 300 cells each were produced by using a plasma clot technique with five percent CO_2 at 37°C. with high humidity and 2.5 units of sheep step III erythropoietin per milliliter. Erythropoietin was essential for colony formation. The number of colonies ranged from 24 to 823 per 10^5 nucleated marrow cells plated, in different animals. Some of the erythroid colonies were mixed with granulocytes.

Granulocyte/macrophage colonies were produced in methyl cellulose cultures. Colonies contained up to 1000 cells and the number of colonies ranged from 13 to 981 per 10^5 nucleated marrow cells plated, from different animals. Glass adherent cells appeared to produce colony stimulating activity in culture.

In both culture systems, there was a direct linear relationship between the number of nucleated marrow cells plated and the number of colonies produced.

RÉSUMÉ

Les auteurs ont réussi à obtenir des colonies de cellules érythrocytaires bovines qui comptaient chacune jusqu'à 300 cellules, en utilisant la technique d'un caillot de plasma et les facteurs suivants: 5% de CO_2, 37°C, une humidité élevée et 2.5 unités d'érythropoïétine ovine/ml. L'érythropoïétine s'avéra essentielle à la formation de colonies. Le nombre de colonies varia de 24 à 823 pour 10^5 cellules nucléées de moelle osseuse ensemencées, selon les sujets. Certaines colonies de cellules érythrocytaires contenaient des granulocytes.

Ils réussirent aussi à obtenir des colonies de granulocytes et de macrophages, en utilisant des milieux de culture au méthylcellulose. Ces colonies comptaient jusqu'à 1000 cellules et le nombre de colonies varia de 13 à 981 pour 10^5 cellules nucléées de moelle osseuse ensemencées, selon les sujets. Les cellules qui adhéraient aux lamelles semblèrent produire une activité stimulatrice de colonies, dans les cultures.

Chacune des deux méthodes de culture donna une relation linéaire directe entre le nombre de cellules nucléées de moelle osseuse ensemencées et le nombre de colonies obtenues.

INTRODUCTION

Culture *in vitro* of bone marrow granulocytic progenitors as developed for mice (2, 13, 14) and humans (15) has proved to be a very useful tool for the study of the kinetics and regulation of granulopoiesis. Similar techniques have been developed for the production of erythroid colonies in culture of mouse (17), human (18), rat (16) and dog (3). These assays for granulocytic and erythroid precursors can be used to quantitate the hemopoietic capacity of marrow samples.

Bovine granulocytic colonies have been successfully grown *in vitro* (19) but growth of bovine erythroid colonies *in vitro* has not yet been reported. The purpose of our study was to develop an *in vitro* assay of bovine erythroid precursors. We then intend to use erythroid and myeloid assays for *in vitro* quantitation of hemopoiesis and bioassay of colony stimulating activity (CSA) in body fluids of cattle suffering from diseases affecting the hemopoietic system, such as African trypanosomiasis.

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MATERIALS AND METHODS

MARROW COLLECTION

Bone marrow for granulocyte/macrophage culture was collected from six calves and for erythroid culture from four calves. The calves were Holstein-Friesian females ranging in age from one to six months. The sternal regions were shaved, washed and thoroughly disinfected. Bone marrow was then aspirated from sternebrae using a 16 gauge biopsy needle and a 20 ml disposable syringe. Ten ml were aspirated and mixed with 100 units of heparin in a disposable test tube (17 x 10 mm).

ERYTHROID COLONY CULTURE

The erythroid colonies were cultured in plasma clots as described by Tepperman *et al* (18). Human and bovine marrow samples were also cultured in methyl cellulose as described by Iscove *et al* (9). The plasma clot colonies were stained using 3, 3′-dimethoxybenzidine as described by McLeod *et al* (11). Benzidine positive cells (brown to orange) were identified as erythroid. Aggregates of eight or more erythroid cells were scored as colonies.

GRANULOCYTE/MACROPHAGE COLONY CULTURE

The granulocytes and macrophages were cultured in methyl cellulose using endotoxin serum for stimulation of colony formation as described by Walker *et al* (19). A Holstein-Friesian cow was injected with 25 μg endotoxin[1]/kg body weight into the jugular vein. Three hours postinjection, blood was collected from the contralateral jugular vein into a 500 ml vacuum bottle, the serum was separated, filtered through 0.45 μ millipore filter[2] and stored in 10 ml aliquots at -20°C until just prior to use. Human leukocyte conditioned medium (LCM)[3] was also tested for its activity in bovine marrow cultures. In some cases, the glass adherent cells were removed from marrow cell suspensions using the method of Messner *et al* (12).

The granulocyte and macrophage colonies were counted directly in the Petri dishes using an inverted microscope. Some of the colonies were aspirated using a Pasteur pipette and smears were made and stained with a peroxidase technique (20). Peroxidase positive cells were identified as macrophage or granulocytic. Colonies were scored at 20 cells and above.

STATISTICAL ANALYSIS OF THE RESULTS

In order to obtain homogeneity of variance all colony data were transformed to common logarithms. Linear regression analysis by the least squares method was conducted on the transformed colony data.

RESULTS

Erythroid colonies of up to 300 cells in size were produced in seven days in plasma clots (Fig. 1). The number of colonies produced ranged from 24 to 823 per 10^5 nucleated marrow cells plated, in different animals. Some of the colonies consisted entirely of either erythroid or granulocytic cells, while about one-third were mixed (Fig. 1).

Erythropoietin was found to be essential for erythroid colony formation. Bovine marrow samples cultured in methyl cellulose repeatedly failed to produce erythroid colonies, while human marrow produced many erythroid colonies.

GRANULOCYTE/MACROPHAGE COLONIES

The granulocyte and macrophage colonies reached maximum size by the seventh day and contained up to 1000 cells per colony. Colony formation was observed both in cultures with and without endotoxin serum. However, the addition of endotoxin serum produced a marked increase in the number and size of colonies. This difference was particularly conspicuous in cultures from

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[1]Piromen, a trypsinized whole cell culture of Pseudomonas aeruginosa, Baxter Laboratories, Malton, Ontario.

[2]Millex, Millipore Corporation, Bedford, Massachusetts.

[3]The Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario.

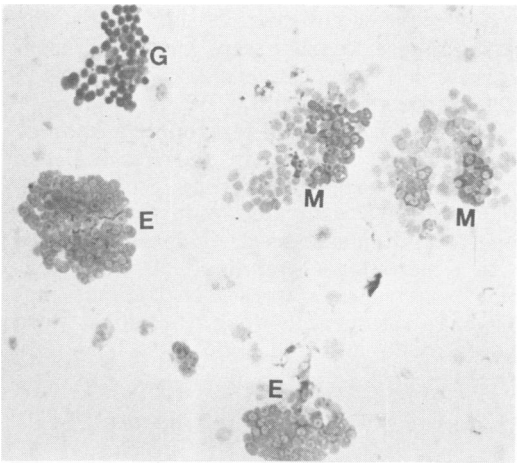

Fig. 1. Seven day old colonies in a plasma clot stained with benzidine technique and counterstained with hematoxylin. Present are erythroid colonies (E), a granulocyte colony (G) and mixed colonies (M). Magnification x95.

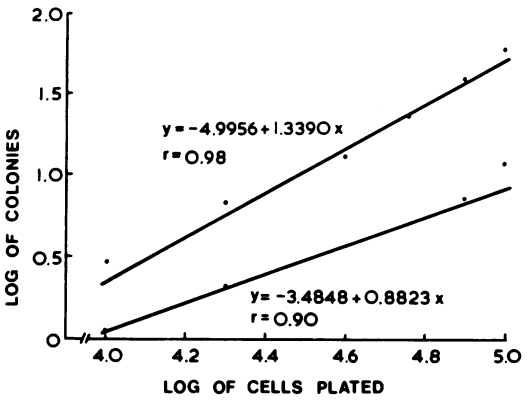

Fig. 2. Differences in stimulatory activities between the endotoxin (top line) and control sera (bottom line) on bovine granulocyte/macrophage cultures in the absence of glass adherent cells. Each point represents the mean of the colony counts of four dishes. The least squares linear regression lines are plotted.

which the glass adherent cells were removed prior to plating (Fig. 2). Cultures in which adherent cells were present showed a small difference in number of colonies produced between the endotoxin and control serum cultures. This difference decreased with increasing cell inocula until no effect of endotoxin serum was found (Fig. 3). Human LCM failed to stimulate colony formation in bovine cultures.

In both the erythroid and granulocytic assays, there was a direct linear relationship between the number of nucleated marrow cells plated and the number of colonies produced ($P<0.001$, Figs. 4 and 5).

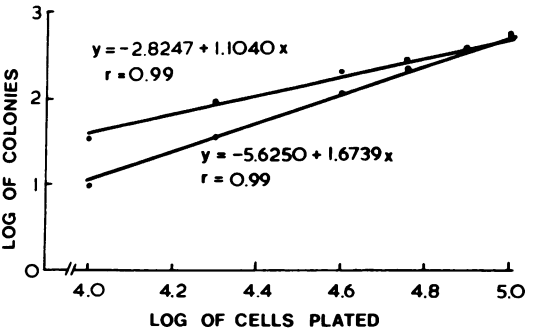

Fig. 3. Differences in stimulatory activities between the endotoxin (top line) and control sera (bottom line) on bovine granulocyte/macrophage cultures in the presence of glass adherent cells. Each point represents the mean of the colony counts of four dishes. The least squares linear regression lines are plotted.

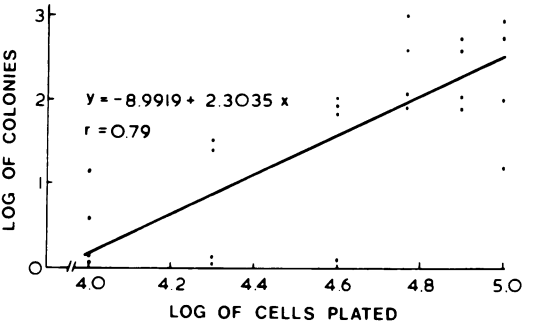

Fig. 4. Direct linear relationship between the number of nucleated marrow cells plated and the number of erythroid colonies produced from marrow aspirates from four calves ($P<0.001$). Each point represents the mean of the colony counts of four dishes. The least squares linear regression line is plotted.

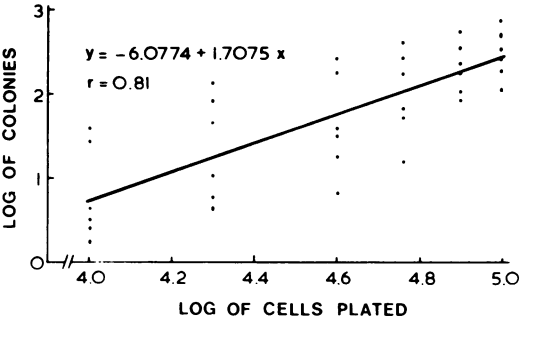

Fig. 5. Direct linear relationship between the number of nucleated marrow cells plated and the number of granulocyte/macrophage colonies produced from marrow aspirates from six calves ($P<0.001$). Each point represents the mean of the colony counts of four dishes. The least squares linear regression line is plotted.

DISCUSSION

Erythroid colonies have been grown in plasma clot cultures in mice (1, 4, 5, 7, 11, 17), man (8, 18) and in the dog (3). Erythroid colonies have also been successfully grown in methyl cellulose cultures in mice (6, 9, 10) and in man (9). The present paper reports culture of bovine erythroid colonies using plasma clot technique. Our repeated failure to culture bovine erythroid colonies in methyl cellulose suggests that the technique requires modification in order to be suitable for culture of bovine erythroid colonies. Erythropoietin has been found to be essential for erythroid colony growth in mice (5), in man (9) and in the dog (3). Our results have shown that none or very few bovine erythroid colonies are formed in the absence of erythropoietin.

A direct linear relationship between the number of nucleated marrow cells plated and the number of erythroid colonies produced has been demonstrated in mice (4, 5, 10), man (18), rat (16) and in the dog (3). In bovine erythroid colony culture, we have also demonstrated a direct linear relationship between the number of nucleated marrow cells plated and the number of erythroid colonies produced.

About one-third of our erythroid colonies were mixed with granulocytes. Tepperman *et al* (18) observed that in plasma clot cultures of human marrow 5% to 10% of the erythroid colonies were mixed with granulocytes. This mixture is likely to result from granulocytic and erythrocytic cells originating from pluripotential stem cells.

Walker *et al* (19) observed that the mean number of granulocyte/macrophage colonies per 10^5 nucleated marrow cells plated from four calves ranged from 24 to 59. From our six calves, which were comparable to those of Walker *et al* (19), the mean number of colonies per 10^5 nucleated marrow cells plated ranged from 86 to 732, indicating improved efficiency under our conditions. The source of the variation between animals is not known but may be related to variable degrees of dilution of marrow aspirate with blood.

Endotoxin serum had little stimulatory effect on cultures in which glass adherent cells were present. Messner *et al* (12) observed that in human marrow cultures colony numbers did not increase following addition of CSA to the cultures in the presence of adherent cells. They were able to demonstrate that the glass and plastic adherent cells produced CSA, and that the nonadherent cells formed few or no colonies in the absence of the adherent cells or human LCM. We also observed moderate colony formation in cultures containing adherent cells without addition of CSA (endotoxin serum). However, unlike Messner *et al* (12), we observed numerous colonies in bovine marrow cultures from which adherent cells were removed and without addition of CSA. This finding implies that our pre-endotoxin serum used in the cultures had CSA, unlike alpha medium used for human control cultures or the control serum of Walker *et al* (19).

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