CLONING OF CANINE HEMATOPOIETIC CELLS in vitro

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Culture of canine hematopoietic cells in agar by a method enabling the number of committed precursor cells of granulocytopoiesis and of humoral factors causing their proliferation to be determined, is described. Canine serum in a concentration of 20% was used as the source of colony-stimulating activity. A linear relationship was established between the number of explanted cells and the number of colonies developing within the range of $0.25 \cdot 10^5$ and $8 \cdot 10^5$ cells/ml.

KEY WORDS: precursors of granulocytes and macrophages; bone marrow; colony-stimulating factors.

With the creation of the method of culture of hematopoietic cells on semisolid nutrient media [1, 7] it became possible to study committed precursor cells of granulocytes and macrophages and the number of units forming colonies in culture (CFUc, which are considered to belong to the class of semistem cells, responsible for the quantitative regulation of hematophoiesis.

Cloning in vitro permits not only a quantitative estimation of this class of cells, but also a determination of the influence of various factors on them, in particular, of presumed humoral regulators of granulocytopoiesis.

This paper describes a modified method suitable for determining CFUc in canine bone marrow. This model is particularly interesting for the investigation of humoral mechanisms of regulation of granulocytopoiesis, for the kinetics of human and canine granulocytes in vitro is similar [2]; moreover, human serum is a factor which can facilitate the formation of colonies by canine bone marrow cells. This system can therefore be used to investigate serial samples of human blood for its content of colony-stimulating activity (leukopoietin ?).

EXPERIMENTAL METHOD

Adult mongrel dogs of both sexes weighing 8-12 kg were used. Bone marrow was aspirated aseptically from the head of the humerus or the iliac crest, mixed half and half with plasma of the same dog, and allowed to stand (to sediment the red cells) for 45-60 min at room temperature. The supernatant containing nucleated cells was removed and mixed with the basic medium in the required dilution.

The nutrient medium for culture was prepared by mixing the double-strength medium with a 0.75% aqueous solution of Bactoagar. The composition of the double-strength medium was: medium No. 199 in Earle's salt base (10 ×) 20%, 5% solution of sodium bicarbonate 8.8%, 100 mM solution of sodium pyruvate 1%, 200 mM solution of L-glutamine 2%, L-asparagine in a concentration of 20 mg/ml 0.2%, triple-distilled water 68%. Penicillin and streptomycin were added to the medium in a dose of 50 units/ml of each. The Bactoagar was made up in triple-distilled water. Immediately before use it was sterilized by boiling for 10-15 min and kept at 40°C on a water bath. To the mixture of double-strength medium and agar 20% of

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TABLE 1. Comparison of Different Sources of CSA

No. Expt.	Source of CSA	CFUc per 10^5 cells
1	Canine serum	22.8
	Conditioning medium from canine lung cultures	19.5
	Calf embryonic serum	25.8
2	Human serum	48.0
	Calf embryonic serum	53.0
	Canine serum	73.3
3	Calf embryonic serum	21.0
	Conditioning medium from cultures of PHA-treated lymphocytes	17.0
4	Canine serum	79.2
	Horse serum	13.8
5	Calf embryonic serum	28.0
	Calf serum	3.0
	Chicken serum	0
6	Feeder from peripheral blood leukocytes	83.0
	Calf embryonic serum	84.5

TABLE 2. Comparison of Action of Sera in Two-Layer System

Source of CSA	No. of CFUc to 10^5 cells	
	serum in top layer	serum in bottom layer
Canine serum	79,2	39,0
Calf embryonic serum	15,3	6,4
Horse serum	13,8	0
Without serum	0	0

TABLE 3. Effect of Serum Concentration in Medium on Number of Committed Precursor Cells of Granulocytes and Macrophages

Source of CSA	Concentration of serum in medium (%)				
	10	20	30	40	50
Human serum	27,0	47,0	41,0	—	—
Calf embryonic serum	8,3	25,8	12,8	2,6	5,7

the factor serving as the source of colony-stimulating activity (CSA) and the hematopoietic cells were added. The suspension was poured into Leighton's tubes in a volume of 1-2 ml. After 20 min, when the agar had solidified at room temperature, the tubes were blown through with a mixture of 10% CO_2 and 90% air, hermetically sealed, and incubated at 37°C for 6-14 days. The cultures were examined under an inverted microscope with a magnification of 38 ×. Collections of 40 or more cells were taken as colonies and groups of 3 to 40 cells as clusters. Each specimen was set up in two tubes. The scatter between them usually did not exceed 20%.

EXPERIMENTAL RESULTS

Colony formation took place only when sources of CSA were present in the medium. These sources are species-specific and, consequently, each species requires its own source of CSA [5]. The effect of various potential sources of CSA was therefore studied, notably conditioning media from lung cultures or from canine lymphocytes stimulated by phytohemagglutinin (PHA), human, canine, or calf serum, horse and chicken serum, and a feeder consisting of canine peripheral blood leukocytes.

As Table 1 shows, the cloning efficiency differed in the different experiments, as is also characteristic of other systems of agar cultivation [5]. Comparison of the results within the individual experiments showed that all sources studied except chicken and calf serum possessed CSA. Canine serum possessed the highest CSA, and under the conditions used it ensured maximal cloning efficiency. Feeder from peripheral blood leukocytes had no advantages as regards CSA over canine serum. This result is in agreement with those obtained by other workers [3], who found that canine CFUc do not require feeder, which can be replaced by CSA of the sera.

Consequently the system was suitable for determining substances influencing granulocytopoiesis.

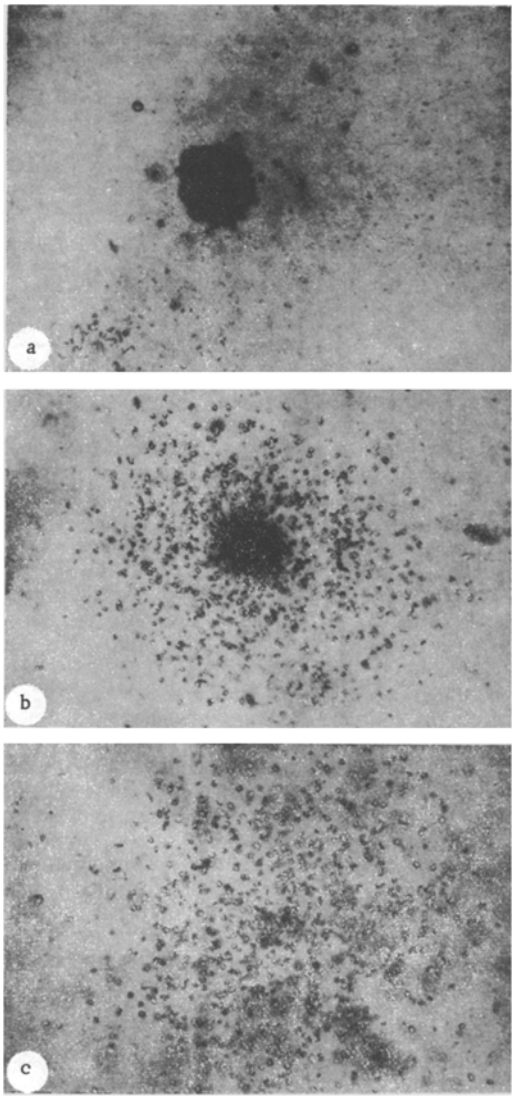

Fig. 1. Types of colonies developing in agar (200 ×): a) compact colony of type I; b) colony of type II with dense center and diffuse "halo"; c) diffuse type III colony.

Some other systems have been shown to contain inhibitors which reduce cloning efficiency [4] and appreciably distort the results of determination of the number of CFUc. The inhibitors have high molecular weight and diffuse with difficulty through the agar layer.

To detect potential inhibitors, tests were carried out in a two-layer system: The source of CSA was contained in the bottom layer (0.5% complete agar medium) and the cells in the top layer (0.3% complete agar medium).

As Table 2 shows no high-molecular-weight inhibitor was discovered in the system: Not only was the cloning efficiency not increased in the two-layer system, but it was actually reduced, evidently because of the lower concentration of CSA in the top layer.

The study of the effect of CSA concentration in the medium on cloning efficiency showed that the optimal concentration for both calf embryonic serum and human serum is 20% (Table 3).

Bone marrow cells of practically all animals except mice, if in a high concentration, can form colonies in agar spontaneously (without any exogenous source of CSA) [6]. Spontaneous colony formation also is observed in dogs with cells in a concentration of $3 \cdot 10^5$/ml or higher. With the minimal cell concentration ($3 \cdot 10^5$/ml) about 2.4 spontaneous CFUc to 10^5 cells were found.

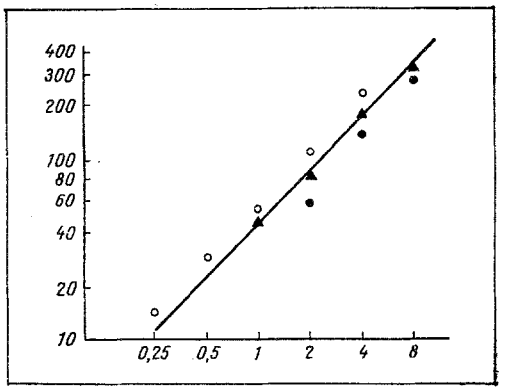

Fig. 2. Effect of concentration of explanted cells on number of colonies formed. Empty circle) experiment 1, triangle) experiment 2, filled circle) experiment 3. Abscissa, number of explanted cells ($\cdot 10^5$); ordinate, number of colonies developing.

When all the sources of CSA studied were used, proliferation of hematopoietic cells began during the first days of cultivation; by the third to fourth day developing colonies consisting of 3 to 20 cells could be seen. In the course of cultivation the colonies increased in size and they became diffuse in character. During further cultivation degeneration of the cells in the colonies, followed by death, was observed. When canine serum was used as the source of CSA, the maximal number of colonies was observed on the 9th day, whereas with human serum it was on the 13th day.

Examination of the cultures on the 6th-12th day revealed three types of colonies (Fig. 1). The number of committed precursor cells of the granulocytic series could be determined only after establishment of the cell concentrations within the zone of which the number of explanted cells was a linear function of the number of clonal colonies formed. The results (Fig. 2) indicate that the zone of this linear function in the system used was wide – from $0.25 \cdot 10^5$ to $8 \cdot 10^5$ cells/ml. On average 1 CFUc was contained in 1800-2100 bone marrow cells.

On the whole, the cultivation system described above is suitable for determining the number of CFUc in canine hematopoietic tissues and for investigating the possible humoral regulators of granulocytopoiesis in dogs.

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