PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 163, 421–424 (1980)

Effect of Neutropenia on Colony Stimulating and Inhibiting Activity of Dog Serum (40790)

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Acute neutropenia produced by endotoxin (1), antineutrophil serum (2), or irradiation (3, 4), is accompanied by increases in serum colony stimulating factor (CSF) (5), but there is little information on inhibitors in serum that might block the formation of mouse bone marrow colonies *in vitro*. A CSF inhibitor decreases in the serum of mice after whole body irradiation (3, 6), and from urine of mice treated with methotrexate (7) but others have not been able to confirm these findings (8).

In the present study, the kinetics of serum CSF and inhibitors after induction of neutropenia by an antineutrophil serum (ANS) were investigated in the dog. Since the kinetics of dog granulocytes are similar to those of humans, it might be expected that the granulopoietic control mechanism would also be similar.

Materials and methods. Five dogs, 1–3 years of age, weighing 11 to 16 kg were used. ANS was prepared by a modification of a method previously described (9). Six to eight hr after injection of 2 USP units/kg body weight of ACTH (Adrenomone, Armour-Baldwin Lab.), blood was taken in 10% EDTA solution (0.1 ml/ml blood). The circulating leukocytes (about 90% neutrophils) were isolated by lysing the erythrocytes in 3 vol of 0.87% ammonium chloride (10). Leukocytes were pelleted by centrifugation (150*g* for 10 min) and washed twice in 0.9% saline. The cells were suspended in 0.5 ml of saline and mixed with an equal amount of "complete Freund's adjuvant" or "incomplete Freund's adjuvant" (Difco Laboratories) for the first and second injections, respectively. A total of $1-3 \times 10^8$ cells were injected subcutaneously at four sites in 3.2 to 3.6 kg New Zealand White rabbits three times a week. Fourteen days after the injections, the rabbits were bled from ear veins and the sera harvested. Each serum was heated at 56°C for 30 min to inactivate complement and then absorbed four times with washed dog red blood cells. The absorbed antineutrophil (ANS) had a leukoagglutination titer of 2 plus at 1:1280 dilution (11).

The experimental dogs were injected ip with ANS, 0.35–0.50 ml/kg body weight. Blood with and without EDTA was obtained by venipuncture before and at different times after ANS administration. On each blood sample, packed cell volume, plasma protein, white blood cell, and differential counts were performed by standard methods. Serum was harvested by centrifugation at 3000*g* for 10 min. One-half of each serum sample was filtered (Millipore, 0.45-μm) and stored at −20°C until assayed for inhibitors (untreated serum). The other half was treated by dialysis, ether plus dialysis, or by heating at 53°C for 30 min to remove or inactivate serum inhibitors. The serum was then filtered as above and stored at −20°C until assayed for CSF activity.

The ability of the sera to stimulate or inhibit colony formation by mouse bone marrow cells was assayed by an adaptation of the soft agar technique (12, 13) using methylcellulose (14) instead of agar. Bone marrow cells from 8- to 16-week-old male mice (C57B6/bJ) were cultured in 1.4% methylcellulose solution containing McCoy's 5a culture medium, 15% fetal calf serum, 1.5% glutamine (29.2 g/liter), and 1% antibiotics (penicillin 10,000 U/ml and streptomycin 10,000 μg/ml) (Pacific Biologicals). The final concentration of nucleated cells was 2×10^5/ml of media. Usually, 5 ml of this mixture plus 0.25 to 0.50 ml of treated dog serum was well mixed and plated in 1-ml aliquots into four

0037-9727/80/030421-04$01.00/0

35 × 10-mm plastic petri dishes (Falcon Plastic) and incubated at 37°C in a humidified atmosphere continually flushed with 5% CO_2 in air. After 7 days, colonies, defined as aggregates of at least 30 cells, were counted using a dissecting microscope (30–40 X). Serum inhibiting capacity was assayed by mixing 0.05 ml of the untreated dog serum and 0.05–0.10 ml of a known active dog serum and cultured under the same conditions as above. Results are given as percentage inhibition of colonies produced by the active serum.

In both assays, control groups with and without standard stimulatory activity were cultured in parallel. A medium "conditioned" by incubation with dog blood leukocytes using the method for human leukocytes (15) was used as the standard control. The optimum number of cells incubated and the dog sera volume used in this investigation were determined. Statistical comparisons were by Student's *t* test.

Results. Serum colony stimulating factor (CSF) and inhibitors in normal dogs. No colonies were observed in the initial experiments using 23 untreated dog sera when they were mixed with known active dog serum and then added to the *in vitro* mouse bone marrow culture. The known active dog serum normally induced the formation of 50–65 colonies per dish. When the same sera were heated, however, a variable degree of colony stimulating activity was observed ranging from 2 to 54 colonies per 0.1 ml of serum. Dialysis or ether–dialysis of the sera had the same effect as heating. At low concentrations the activity of the treated and active dog sera increased almost linearly with increasing volumes of serum as measured by the number of mouse bone marrow colonies formed (Fig. 1).

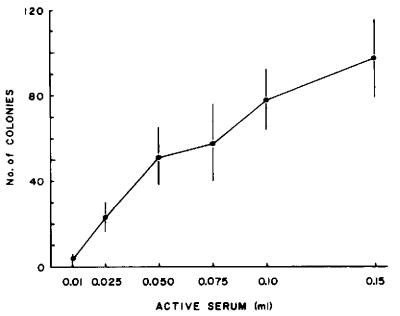

FIG. 1. The dose–response curve of a known active dog serum. Points at 0.05, 0.1 and 0.15 ml of serum represent the mean value for eight replicates (two experiments). The other points (0.01, 0.025, and 0.075) are the mean value of four replicates (one experiment). Vertical bars are the standard deviations.

Effect of ANS on dog serum CSF and inhibitor. After the ip injection of ANS (Fig. 2), neutrophils decreased markedly (approximately sixfold) at 1.5 hr, remaining low for the next 24 hr and then returning to its initial value. Band neutrophils were constantly observed after 6 hr and metamyelocytes were low in number after 24 hr of the ANS injection. Lymphocytes, eosinophils, and monocytes also decreased but proportionately less than neutrophils. The serum CSF activity progressively increased after the ANS and reached a maximum between 6–9 hr (threefold, $P < 0.01$), and then decreased to a nadir at 48 hr. The colonies at the peak appeared to be larger than the colonies at 0 hr, and more clusters were present when the CSF was high. Serum inhibitor activity decreased significantly at 6 hr ($P < 0.01$) and returned to its original value at 12 hr.

Discussion. It has been shown (16) that the mouse bone marrow cell culture system in solid media is a sensitive system to detect colony-stimulating activity in dog urine. Using this system, dog serum was shown in this study to contain substances stimulatory and inhibitory for colony formation. The inhibitor was removed or inactivated from the sera in about the same proportions by dialysis, ether–dialysis, or by heating. A similar phenomenon has been described in human (17) and mouse (18) sera, indicating that the substance in the dog serum is likely to be the same as the inhibitory substance described in humans and mice.

Mature neutrophils are known to contain specific antigens which stimulate production of specific antibodies that cause the disappearance of band and segmented neutrophils from blood and bone marrow shortly after administration (9, 2). In the present study, ip injection of ANS produced a marked decrease of blood neutrophils at 1.5 hr after administration (Fig. 2) followed by a marked decrease in inhibitor

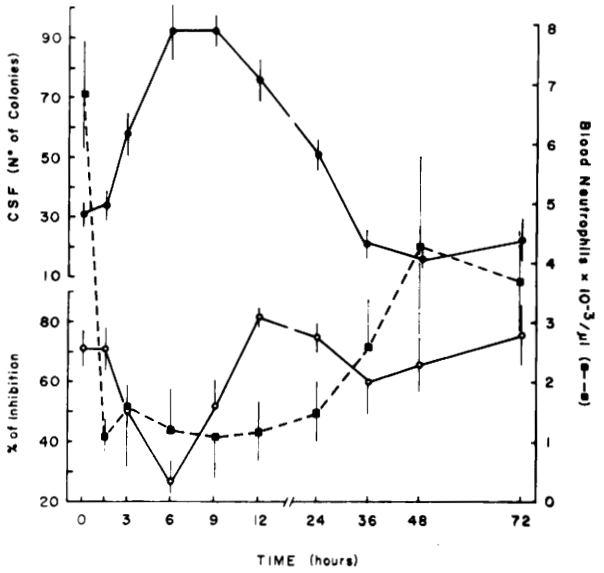

FIG. 2. Blood neutrophils (■), serum CSF (●), and inhibitor (○) levels at various time intervals after ip injection of ANS. The inhibitor values are the percentage of inhibition produced by 0.05 ml of untreated serum on the colony stimulatory activity of a known sera (54–93 colonies/dish). They are the mean ± SE of 12 to 16 replicates (four dogs). CSF values are the number of colonies stimulated by 0.1 ml of treated serum (heating) and are the mean ± SE of 16 to 20 replicates (five dogs).

and a marked increase in CSF, both with their maxima at about 6 hr. Later, during the return of blood neutrophils, serum CSF levels decreased and the inhibitor increased. This is in contrast to a report in neutropenic rats, in which no changes in serum inhibitory levels were observed at the time of maximum CSF response (8). This may be a reflection of differences in the systems used.

The results of the present study using ANS and those which show that irradiation (4, 6) or methotrexate (7) produce an acute fall or disappearance or serum or urine inhibitor, suggest that a direct relationship between levels of the serum inhibitor and blood neutrophil exist. Previous *in vitro* studies have demonstrated that the main source of inhibitors is the mature neutrophil (19–21) which further suggests that a controlling relationship exists between a serum inhibitor and the blood neutrophil.

The reports on the chemical nature of the serum inhibitors are conflicting. In human serum (17), the inhibitors might be present in at least two forms, a high MW lipoprotein and a low MW protein. Others (22) have shown that mouse cell derived inhibitors have a MW of less than 10,000 a.m.u. and do not contain lipids.

The serum CSF levels have also been shown to be inversely related to the number of blood neutrophils in rats injected with ANS (2), in irradiated mice, (3) in cyclic neutropenia of man (23), and dog (24), and dogs in the present study injected with ANS. The temporal relationship between the blood neutrophil count and CSF levels, however, is also at present unclear, since results are conflicting (25). Seventy-two hours after ANS injection, the neutrophils were still below their original values (Fig. 2). This would indicate that the marrow granulocyte reserve (MGR) was almost depleted by the action of the ANS. It is known in dogs that the mean transit time to the post mitotic neutrophils is 82.1 hr (26), which means that total blood recovery would occur after the 72 hr of this study. Therefore, the increased CSF activity observed after the administration of ANS could be a response to (i) a decrease in the total blood granulocyte pool, (ii) a decreased marrow granulocyte reserve

(MGR), (iii) the activation of the monocyte-macrophage system by neutrophil destruction (27), or (iv) still other mechanisms.

Summary. Acute neutropenia was induced in five dogs using an antineutrophil serum prepared in rabbits. Sera obtained at various times after injection were assayed for their capacity to stimulate or inhibit mouse bone marrow cell colony formation *in vitro*. The results indicated that serum colony stimulating factor (CSF) in the dog is inversely related to the number of blood neutrophils and that a serum inhibitor closely paralleled the change of neutrophils in blood. These results suggest that both CSF and a serum inhibitor are involved in the regulation of granulopoiesis.

The authors acknowledge the support of the University of Chile–University of California Cooperative Program.

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Received July 30, 1979. P.S.E.B.M. 1980, Vol. 163.