17. A male 200-g rat normally consumes around 20 g of laboratory chow each day. Since 0.3 percent by weight of this feed is tryptophan (Big Red Animal Food, Country Best, Agway, Inc., Syracuse, N.Y.), the animal ingests 60 mg of tryptophan daily. The injection of 2.5 mg of tryptophan (12.5 mg/kg) thus represents less than one-twentieth of the normal daily intake of the amino acid.
18. V. R. Young, M. A. Hussein, E. Murray, N. S. Scimshaw, *Am. J. Clin. Nutr.* **22**, 1563 (1969).
19. J. L. Kostyo and S. E. Schmidt, *Am. J. Physiol.* **204**, 1031 (1963); T. Pozefsky, P. Felig, J. D. Tobin, J. S. Soeldner, G. F. Cahill, Jr., *J. Clin. Invest.* **48**, 2273 (1969); R. L. Landau and K. Lugibihl, *Metabolism* **18**, 265 (1969); W. W. Bromer and R. E. Chance, *Diabetes* **18**, 748 (1969).
20. J. D. Fernstrom, F. Larin, G. Schonfeld, R. J. Wurtman, *Fed. Proc.* **3**, 250 (1971).
21. A. E. Gordon and B. S. Meldrum, *Biochem. Pharmacol.* **19**, 3042 (1970).
22. P. Albrecht, M. B. Visscher, J. J. Bittner, F. Halberg, *Proc. Soc. Exp. Biol. Med.* **92**, 703 (1956); B. N. Dixit and J. P. Buckley, *Life Sci.* **6**, 755 (1967); A. H. Friedman and C. A. Walker, *J. Physiol. (London)* **197**, 77 (1968); D. J. Reis, A. Corvelli, J. Conners, *J. Pharmacol. Exp. Ther.* **167**, 328 (1969); W. B. Quay, *Am. J. Physiol.* **215**, 1448 (1968).
23. A. L. Beckman and J. S. Eisenman, *Science* **170**, 334 (1970); F. Hery, J.-F. Pujol, M. Lopez, J. Macon, J. Glowinski, *Brain Res.* **21**, 391 (1970).
24. G. K. Aghajanian, A. W. Graham, M. H. Sheard, *Science* **169**, 1100 (1970).
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Cyclic Urinary Leukopoietic Activity in Gray Collie Dogs

Abstract. *The urinary activities for bone marrow colony formation were measured on consecutive 24-hour urine samples from two gray collie dogs with cyclic neutropenia and from two normal collies. The activity varied cyclically in the gray collies with a peak activity developing during the neutropenic phase, which antecedes the return of blood neutrophils. The activity fell to undetectable levels after the blood neutrophil counts returned to the normal range. The urine of normal dogs showed no activity. Since the dogs with cyclic neutropenia have been shown to have periodic hematopoiesis, these data suggest a regulatory hormonal role for the substance measured by this assay.*

The recently developed methods of short-term marrow culture in soft agar (*1*) or methylcellulose (*2*) have permitted the identification and partial characterization of a factor present in animal and human serum and urine which stimulates single bone marrow cells to form granulocytic and mononuclear colonies (*3–5*). This factor [referred to as colony-stimulating activity (CSA)] has been characterized in human urine to be a heat-stable glycoprotein having a molecular weight of about 190,000 (*6*). Its physicochemical properties closely resemble erythropoietin but its biological properties are distinctive (*7*). The role of this factor in the control of leukopoiesis is still unclear.

An animal model of naturally occurring periodic myelopoiesis occurs in gray collie dogs (*8*). In these dogs, neutrophils are virtually absent from the peripheral blood for 2 or 3 days at 12-day intervals. This defect is related primarily to intermittent neutrophil production. The cyclic neutropenia is a component of a more general marrow defect involving cyclic production of erythrocytes, monocytes, and platelets (*9*).

A central question in evaluating the physiologic role of the colony-stimulating substance measured in the in vitro assay of colony formation has been whether the serum or urine levels vary with the peripheral blood neutrophil count or with marrow myelopoietic activity. Since gray collie dogs have regularly recurring cycles of myelopoiesis without the complicating influences of cytotoxic drugs or malignancy, it was thought that their pattern of urinary CSA, if found, might be of general physiologic import.

All urine samples were obtained from two unrelated gray collies (one male, one female) and two normal collies. Consecutive 24-hour urine samples were collected while the dogs were housed in stainless steel metabolic cages. Urine was collected and frozen as it drained from the bottom of the cages into polyethylene plastic bottles supported beneath the cages in Styrofoam boxes filled with Dry Ice. A modest contamination of the urine with feces was unavoidable.

The urines were kept at −10°C for 1 to 2 weeks, thawed at room temperature, and prepared for dialysis by centrifuging for 20 minutes at 3500 rev/min at 4°C. Urine (25 ml) was dialyzed in Visking tape at 4°C against 1 liter of distilled water for 72 hours, with three changes of water. The volume of urine present in each dialysis bag at the completion of the dialysis was measured. The specimens were then centrifuged at 3500 rev/min at 4°C for 20 minutes and the sediment was discarded. The supernatant was filtered through Millipore filters (pore size, 0.45 nm) and stored at −10°C until CSA was measured.

Colony-stimulating activity in each urine specimen was assayed with C57B1/6N mouse bone marrow as target cells (*5*). A modification of the methylcellulose technique for bone marrow culture, reported by Worton *et al.* (*2*), was employed. Femoral bone marrow plugs from three mice were obtained and pooled in 3 ml of McCoy 5A (modified) medium. A nucleated cell count was performed on the pooled specimens and a sample was diluted to a concentration of 750,000 nucleated cells per milliliter. Three-fourths milliliter of each dialyzed urine sample was added to separate plastic test tubes (17 by 100 mm; Falcon Plastics, Los Angeles) containing 0.5 ml of the diluted mouse marrow, 2.5 ml of 1.6 percent methylcellulose, 0.5 ml of fetal calf serum, and 1.25 ml McCoy medium supplemented with additional sodium bicarbonate, amino acids, vitamins, calf serum, and 6 percent (weight/volume) bovine serum albumin (*10*). For purposes of assuring that proper conditions for mouse marrow colonization existed, appropriate controls with a supernatant from an L cell culture of known stimulatory activity were run in parallel with the experimental assays. After the addition of all ingredients, the test tubes were vigorously shaken to produce a homogeneous single-cell suspension. From each test tube, four 1.1-ml samples were removed and plated in separate plastic petri dishes (35 by 10 mm). Thus, 75,000 nucleated cells and 0.15 ml of dialyzed urine were contained in each dish. The dishes were placed in a humidified incubator containing 10 percent CO_2 in air at 37°C. Seven days later, each dish was examined with an inverted microscope at a magnification of × 50, and all colonies with 20 or more cells were counted. The colony-stimulating activity was expressed as the mean number of colonies from four replicate dishes per 0.15 ml of a standardized daily urine output of 300 ml, with adjustment of the CSA being made for dilution or concentration of the urine sample during dialysis. White blood cell and differential counts were performed by

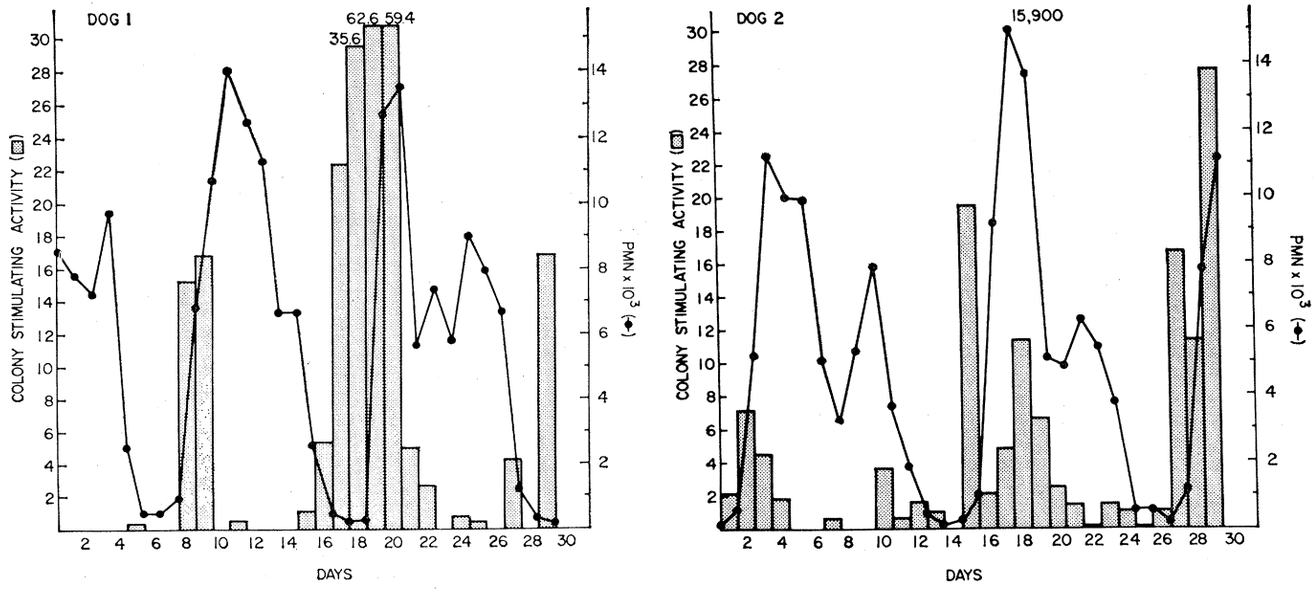

Fig. 1 (left) and Fig. 2 (right). Cyclical pattern of circulating neutrophils (solid line) and urinary colony-stimulating activity (bars) of gray collie dogs for 30-day periods. *PMN*, polymorphonuclear neutrophil.

standard techniques on venous blood samples collected at 1 p.m. daily.

Figures 1 and 2 show the urinary colony-stimulatory activities and corresponding absolute neutrophil counts for 30 consecutive days in two gray collies. On six of six occasions when the dogs became neutropenic the CSA began to rise with the increase in blood neutrophils or before the rise in neutrophil count occurred. At four of these times, CSA rose on the day that blood neutrophils began to appear. On the other two occasions, CSA was measurable during the nadir of neutrophil counts, but rose to even higher levels with the return of blood neutrophils. Further, the CSA fell before the peak neutrophil count was reached and remained absent or at the level of unstimulated marrow until the dog again entered or began to recover from its neutropenic phase.

Urine from normal collie dogs prepared identically and assayed simultaneously, did not stimulate colony formation.

Considerable evidence is available to support the occurrence of periodic leukopoiesis in gray collie dogs (*8*). Bone marrow aspirates show a single wave of developing myeloid cells with each cycle. Serum muramidase cycles strikingly with peak levels anteceding the return of blood neutrophils. Labeling indices of myeloid precursors varies cyclically and bone marrow reserves of neutrophils rise and fall with each cycle. The nature of the underlying marrow defect and consequent failure to maintain a pool of immature dividing precursors is not known. The dramatic cyclic nature of the hemapoietic process in these dogs affords an opportunity to examine the possible homonal role of the substance measured by this assay. In this series of observations it would appear that the timing of the appearance, peak, and disappearance of CSA is in keeping with a hormonal regulatory function in neutrophil production for the substance measured.

This bioassay system does not afford, however, an absolute measure of the biological activity of this leukopoietic substance. Hence, it is not possible to determine at exactly what time in the dog's cycle the CSA first begins to rise. Concentration of CSA by chemical means might increase the sensitivity of the assay (*6*). The possibility that the periods of lower activity are due to the presence of an inhibitor have not yet been examined. The present data suggest that the levels of CSA fell to normal when the neutrophil counts became normal. Similarly, the present data do not demonstrate whether the fluctuations in CSA are a causative or secondary feature of the cyclic myelopoiesis, but the implication for a regulatory role for CSA would nevertheless be the same.

Since these dogs have cyclic erythropoiesis as well as cyclic myelopoiesis, it may be possible to measure fluctuations in their erythropoietin levels which could be correlated with these observations on CSA. The physiological significance of CSA may be further elucidated by such parallel studies of the analogous hormone whose influence on erythropoiesis has been so firmly established.

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References and Notes

1. T. R. Bradley and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.* **44**, 287 (1966).
2. R. G. Worton, E. A. McCulloch, J. E. Till, *J. Cell Physiol.* **74**, 171 (1969).
3. W. A. Robinson, D. Metcalf, T. R. Bradley, *ibid.* **69**, 83 (1967).
4. W. A. Robinson, T. R. Bradley, R. Foster, D. Metcalf, *Brit. J. Haematol.* **15**, 147 (1968).
5. W. A. Robinson, E. R. Stanley, D. Metcalf, *Blood* **33**, 396 (1969).
6. E. R. Stanley and D. Metcalf, *Aust. J. Exp. Biol. Med. Sci.* **47**, 467 (1969).
7. W. A. Robinson and B. L. Pike, *N. Engl. J. Med.* **282**, 1291 (1970).
8. D. C. Dale, H. R. Kimball, S. M. Wolff, *Clin. Res.* **18**, 402 (1970).
9. D. C. Dale, S. B. Ward, S. M. Wolff, in preparation.
10. W. A. Robinson and B. L. Pike, in *Hematopoietic Cellular Proliferation*, E. Stohlman, Ed. (Grune and Stratton, New York, 1969), p. 249.
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