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Cell Proliferation of Canine Cyclic Hematopoietic Marrow in Diffusion Chambers (40137)

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Canine cyclic hematopoiesis (CH) is an inherited disorder characterized by a grey coat color and recurrent fluctuations of peripheral blood neutrophils, monocytes, reticulocytes and platelets (1–3). Recent studies (4, 5) in dogs not subjected to any known external stimuli have shown a cyclic variability in serum Colony Stimulating Activity (CSA) and Erythroid Stimulating Activity (ESA). Committed granulocytic progenitor cells, (Colony Forming Units-Culture or CFU-C) have also been shown to cycle in the marrow of dogs afflicted with CH as have Erythropoietin Responsive Cells (ERC) studied in suspension cultures (5). Furthermore the two progenitor cell populations cycled in the same phase as one another. These observations together with those showing that CH can be abrogated (6, 7) or produced (6, 8) by appropriate bone marrow transplantation, suggest that CH is a disorder of the multipotential hematopoietic stem cells. There is some evidence, at least within a murine system, that cell proliferation in diffusion chambers can be attributed to multipotential hematopoietic stem cells (9, 10) or to a combination of these cells and the CFU-C (11). We therefore initiated a study of canine CH in which the diffusion chamber technique was employed expecting results which would add further to our knowledge on the nature of the defect in this disorder.

Materials and methods. Dogs used in these studies were born in the colony maintained at this institution (12). Marrow for the control studies was obtained from four hematologically normal dogs. Two dogs afflicted with typical CH were studied on representative days of the erythroid and myeloid phases of the cycle during five hematopoietic cycles. Cycle day 1 was designated as the day that peripheral blood neutrophil counts fell below $1600/mm^3$ (5). Intervals between recurrent days 1 varied from 11 to 13 days.

Analgesic drugs were used in each marrow collection procedure. Marrow was aspirated from the head of the humerus into heparinized Medium 199 supplemented with 10% heat inactivated fetal calf serum. The buffy coat cells were separated by centrifugation and nucleated cell numbers determined with a hemocytometer.

The diffusion chamber technique has been described in detail previously (13). The 0.22 μMillipore filters were heat-sealed to a plastic ring and autoclaved for 15 min at 15 psi, of steam pressure. The chambers were loaded with nucleated cells, sealed with paraffin wax and surgically implanted, two per mouse, into the peritoneal cavity of male C3H mice irradiated with 800R (250kV, 85–90 R/min, HVL 2.7 mm Cu, added filtration 1mm Al) 1–4 hr prior to surgery. Seven days after implantation the chambers were removed from the mice and agitated in 0.5% pronase and 5% Ficoll solution for 1–2 hr. The contents of the chambers were aspirated and cell counts made with a hemocytometer. Cytocentrifuge preparations were prepared and stained with Wright's Stain for morphological analysis.

At each study day, groups of not less than six chambers were loaded with nucleated bone marrow cells at a minimum of four different cell numbers between $0.1–10 \times 10^5$ cells/chamber. Over this range of cell inocula the number of cells harvested from the chambers was linearly related to cell input with highly significant correlation coefficients (see later). As described by Breivik and Benestad (14), the slope (and its standard error) of the regression line for each marrow specimen was calculated (15, 16); and we have termed this the Cytopoietic Potential of that marrow. Student's t test was used to determine the degree of significance of differences in the Cytopoietic Potential of the marrows.

Results. A typical cycle of peripheral blood

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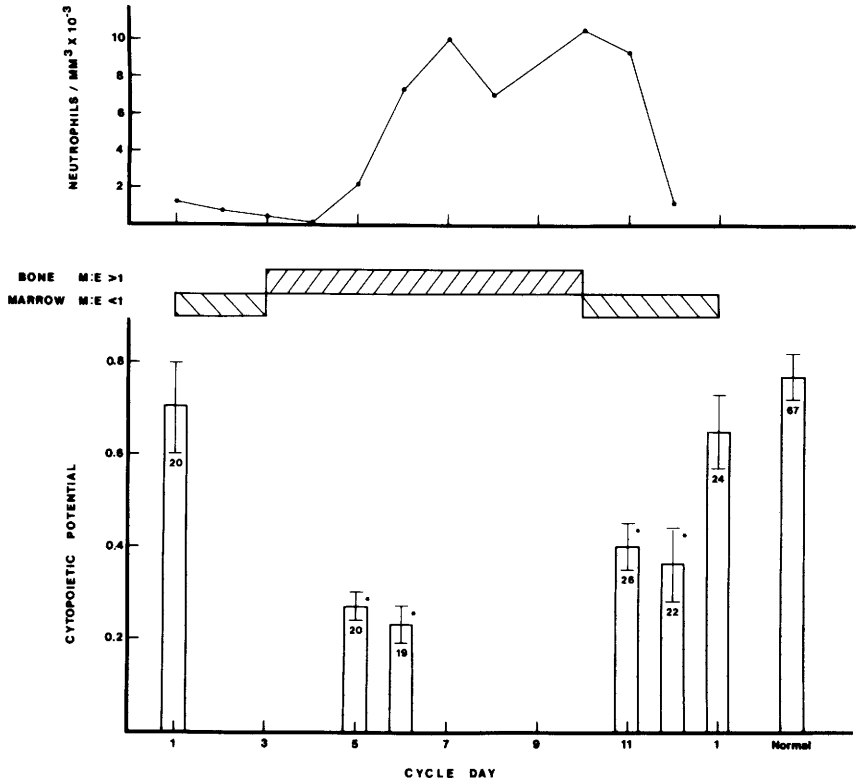

FIG. 1. The Cytopoietic Potentials of dog marrow in diffusion chambers implanted into 800R whole-body irradiated mice. The Cytopoietic Potential is defined as the slope (± SE) of the calculated regression line relating number of cells harvested from the chambers to the number of cells initially inoculated. Numbers on bars indicate number of diffusion chambers. Vertical bars indicate ± SE. * = $P < 0.05$ from the normal marrow. For comparative purposes typical changes in marrow morphology and peripheral blood neutrophil counts are shown in the upper two panels. M:E = Ratio of myeloid:erythroid cells in the marrow.

neutrophils in a dog afflicted with CH is shown in Fig. 1. As documented previously (5), changes in the peripheral blood reflect events in the bone marrow which was predominantly myeloid on cycle days 3–10 and showed a definite erythroid phase during cycle days 10–1 (Fig. 1).

The Cytopoietic Potentials of marrow from four normal dogs studied on four separate occasions did not differ significantly. Therefore, the results from a total of 67 diffusion chambers were combined to give a mean Cytopoietic Potential of normal dog marrow of 0.77 ± 0.05 (Fig. 1) and a correlation coefficient of 0.90 ($P < 0.001$).

The Cytopoietic Potential of CH marrow on day 1 of the cycle, i.e., at the beginning of the neutropenic episode was comparable to that of normal dog marrow. However, the Cytopoietic Potentials at all other time points studied were significantly below normal (Fig. 1). Correlation coefficients of CH marrow varied between 0.74 (day 5) and 0.94 (day 12) and were all highly significant ($P < 0.001$).

Although the composition of the cell inoculum varied markedly from overtly erythroid (days 1, 11 and 12) to definitely myeloid (days 5 and 6) during the cycle (Fig. 1), the morphology of the cells harvested from the chambers was similar in all instances. Mature granulocytes, megakaryocytes and monocytes were occasionally seen. However, large cells, with a dark-staining nucleus and homogenous "foamy" cytoplasm, which were not morphologically recognizable as belonging to any one particular hematopoietic cell line constituted the vast majority of cells harvested.

Discussion. The proliferation of normal dog marrow in diffusion chambers implanted into irradiated mice was comparable to that seen when human marrow was cultured under similar conditions (17, 18).

The Cytopoietic Potential of the marrow of dogs afflicted with CH showed a clearly defined periodicity over the hematopoietic cycle. Normal proliferative potentials were observed at the beginning of each cycle when peripheral blood neutrophil counts were low and the marrow was in a distinctly erythroid phase (Fig. 1). When the marrow was predominantly myeloid (days 5 and 6) and peripheral blood neutrophil counts approximated those seen in normal dogs, reduced Cytopoietic Potentials of the marrow were observed (Fig. 1). Lower than normal Cytopoietic Potentials of CH marrow were also observed on cycle days 11 and 12 when peripheral blood neutrophils were decreasing in number. This periodicity of Cytopoietic Potential was in a similar phase to both CFU-C and ERC (5). Unlike CFU-C, however, which fluctuated from well below to well above levels found in normal dog marrow, the Cytopoietic Potentials fluctuated only downwards from the levels found in normal dog marrow. The reasons for this difference are not readily apparent. It is possible that the diffusion chamber environment sets a limit in the amount of cell proliferation that can be supported and thus a supranormal growth potential might not be detected. Highly significant correlation coefficients, however, argue against this possibility. Rather, the results suggest that the low numbers of cells harvested from chambers containing CH marrow from days 5, 6, 11 and 12 of the cycle may reflect a real reduction of the Cytopoietic Potential of the marrow at these times. The reduced Cytopoietic Potential of days 5, 6, 11 and 12 marrow could be related either to a reduced number of progenitor cells or to increased inhibition of cell growth in this cell population.

Recent evidence is confirming earlier studies (9) that the cells principally involved in the population of diffusion chambers are the multipotential hematopoietic stem cells. Thus diffusion chamber progenitor cells have a similar proliferation rate to hematopoietic stem cells as assessed by the spleen colony technique (10). Furthermore, production of CFU-C and ensuing granulocytopoiesis can occur in diffusion chambers initially inoculated with marrow suspensions containing low numbers of CFU-C as a result of cytotoxic agent therapy (19). If cell proliferation in diffusion chambers does indeed detect multipotential hematopoietic stem cells, the results now reported suggest that changes in the Cytopoietic Potential of CH marrow result from inhibition and/or reduction of this cell population. Periodic proliferation of the multipotential hematopoietic stem cells might then account for the observation (5) that granulocytic and erythrocytic precursor cells cycle in the same phase as one another, although the mature peripheral blood elements cycle 180° out of phase.

The investigations reported herein confirm data obtained from bone marrow transplantation studies (6–8) that the basic fault in canine CH probably resides within the multipotential hematopoietic stem cell compartment. However, the bone marrow transplantation studies have not distinguished between: (i) a decrease in the number of stem cells, (ii) a decrease in their proliferative capacity or (iii) a decrease in the sensitivity of the stem cells to humoral and/or cellular stimuli. The studies now described suggest that stem cells may be reduced in number in canine CH. The other possibilities can be more readily studied using the diffusion chamber technique than by bone marrow transplantation between littermates.

Summary. The ability (Cytopoietic Potential) of marrow from dogs with cyclic hematopoiesis to proliferate within diffusion chambers that had been implanted into irradiated mice has been studied. Cytopoietic Potentials fluctuated over the hematopoietic cycle from near normal levels during the period of peripheral neutropenia to significantly below normal when peripheral blood neutrophils were elevated. Although interpretation of the results has to be tempered by the lack of knowledge concerning the nature of the cell responsible for cell generation in diffusion chambers, the data appear to suggest a compromised multipotential stem cell compartment in cyclic hematopoiesis. Periodic proliferation of these stem cells may account for the previously recognized in phase cycling of

committed granulocytic and erythrocytic progenitor cells.

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