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Nucleotide and Prostaglandin Cycling in Canine Cyclic Hematopoiesis (41962)

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Abstract. Bone marrow cells were collected from normal dogs, normal dogs made neutropenic with cyclophosphamide, and 11 dogs affected with cyclic hematopoiesis (CH) on 3 consecutive days of separate 12- to 14-day cycles. The mononuclear marrow cells from both groups of control dogs and from the CH dogs on each of 12-cycle days were cultured for 2.5 hr in serum-free media. The amounts of prostaglandins ($PGF_{2\alpha}$ and PGE) and cyclic GMP (cGMP) measured in the media were found to vary with the cycle in the CH dog. $PGF_{2\alpha}$ was highest as the dogs recovered from the neutropenia and lowest 4 days before the onset of the next neutropenic episode. Cyclic GMP was lowest 4–5 days before the onset of neutropenia, then dramatically increased as the neutropenic period approached. Cyclic GMP was highest when $PGF_{2\alpha}$ was lowest. Normal dogs, made neutropenic with a single dose of cyclophosphamide, had elevations of $PGF_{2\alpha}$ but not PGE or cGMP during the recovery period of active granulopoiesis.

Steady-state hematopoiesis appears to be intricately controlled by an array of stimulators and inhibitors that originate within the marrow compartment and in distant organs. Canine cyclic hematopoiesis is a genetic disease that can be either induced or abrogated with appropriate bone marrow transplantation (1–3). In dogs affected with cyclic hematopoiesis (CH), periods of neutropenia occur at 12- to 14-day intervals as do intercurrent, but not simultaneous, cycles of reticulocytosis, thrombocytosis, and monocytosis. Extensive *in vitro* studies of marrow collected on each of the cycle days have revealed a wave of *in vitro* proliferation beginning 3–4 days before the onset of neutropenia and continuing for 6–7 days (4–6). What causes the marrow to alternately stop and start cellular proliferation is an unanswered question that relates to many hematologic diseases. Recently, we have shown that the adherent portion of marrows from CH dogs alternately stimulates and inhibits the formation of granulocyte–macrophage colony formation of normal dog marrow (7). The importance of the adherent layer in long-term cultures (8) and of the monocyte in prostaglandin colony-stimulating factor production (9) led us to study prostaglandin production in short-term cultures of marrow collected on each cycle day. We also included studies of cyclic nucleotides since some investigators have shown both cyclic nucleotides and prostaglandins affect the proliferation of myeloid (10) and erythroid (11, 12) progenitor cells. Reported herein are changes in levels of cyclic nucleotides and prostaglandins in either plasma or medium collected from CH marrow cultures initiated on each day of the cycle.

Materials and Methods. *Bone marrow collection.* Bone marrow was aspirated from the femurs and iliac crests of 11 different CH dogs on 3 consecutive days of numerous cycles. The dogs were of both sexes and varied in age from 3 months to 2 years. Hematologically normal littermates and kennelmates were used as controls. Four control dogs were given a single dose of cyclophosphamide (200 gm/m^2) and their marrows were aspirated for study on post-treatment Days 1, 5, and 8. Institutional guides for the care and use of animals were followed and the animals were housed in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care. All bone marrow cell collections were carried out under very heavy sedation with oxymorphone and xylazine. The marrow was collected in α minimal essential medium (αMEM) containing heparin and separated on Ficoll–Paque. Bone marrow cells used for cAMP determinations were collected in calcium-free phosphate buffered saline contain-

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ing 1.0% of 0.5 *M* ethylenediaminetetraacetate (EDTA). The resulting mononuclear cells were washed twice in αMEM.

Preparation of conditioned media. Test portions of 1×10^7 mononuclear cells were incubated in 1 ml of serum-free αMEM in 35-mm plastic Petri dishes for 2.5 hr at 37°C and 7.5% CO_2. Quadruplicate plates were prepared for each cycle day. Each cell suspension was centrifuged at 1500 rpm for 10 min and the conditioned medium was divided into two lots. One lot was frozen for cyclic nucleotide assays and the second lot was extracted for prostaglandin assays.

For studies of plasma cyclic nucleotides, 10 ml of whole blood was added to 1.0 ml of 0.5 *M* EDTA, centrifuged immediately, and the plasma was separated and stored at −20°C.

For both the PGE and $PGF_{2\alpha}$ assay, each 1 ml sample of conditioned medium was extracted with 3 ml of a solution containing ethyl acetate:isopropanol:0.2 *N* HCl (3:3:1) and mixed; the materials were mixed again with 2 ml of ethyl acetate and 3 ml of distilled water and then centrifuged. Three milliliters of the organic phase was removed and dried at 55°C in an airstream. Each sample was resuspended in 0.6 ml of gelatin Tris buffer and frozen.

Cyclic nucleotide and prostaglandin assays. Replicates of two samples were used for each prostaglandin or nucleotide determination. PGE and $PGF_{2\alpha}$ were quantitated by use of radioimmunoassay kits based on a competitive binding principle, and purchased from Clinical Assays.[1] The procedure allows for the composite measurement of prostaglandins E_1 and E_2. The antibody to $PGF_{2\alpha}$ used in the measurement process limits cross-reactivity with PGE. Cyclic AMP and cyclic GMP were also quantitated by radioimmunoassay based on the same principle using a kit produced by Amersham.[2]

Results. In Fig. 1A, the levels of PGE and $PGF_{2\alpha}$ found in the media of CH marrow cultures are graphed relative (cycle day) to the peripheral neutrophil and monocyte cycles (Fig. 1B). The neutropenic cycles occurred at 12- to 13-day intervals relative to a Day 1 which was arbitrarily designated as the day the peripheral neutrophil count fell below 1600 mm^3. The PGE and $PGF_{2\alpha}$ values fluctuated from levels comparable to those of normal dogs to levels well above those of unaffected dogs. Three days before the beginning of neutropenia (Day 9 of the 12-day cycle), the levels of both prostaglandins were quite low compared to those of other cycle days. The $PGF_{2\alpha}$ values cycled distinctly from the low values found on Days 9–11 to values much larger than those found in normal dogs.

Levels of cGMP found in the conditioned media are graphed in Fig. 2. The cGMP fluctuated in a cyclic pattern with the lowest values occurring during neutrophil proliferation in the marrow (Days 4–8 (13)). The nadir was followed by a dramatic increase in cGMP which persisted 4–5 days. During the period when cGMP was highest, wide differences occurred among the numerous samples evaluated. As the dogs began to exhibit more normal peripheral neutrophil counts (Cycle Days 4–7), cGMP values became less variable and more comparable to those of normal control dogs' marrows.

Repeated evaluations of cAMP in marrow conditioned media revealed wide fluctuations among CH dogs studied at comparable cycle days but no clear cyclic fluctuation was apparent. Values for normal dogs ranged from 0.05 to 0.7 pmole/100 μl while values for CH dogs fluctuated from 0.05 to 1.25 pmole/100 μl.

In the plasma of CH dogs, cGMP fell to its lowest value (0.8 ± 0.18 pmole/100 μl) 4–7 days after the onset of neutropenia. The cGMP levels of CH dogs were always higher than those of normal dogs (0.48 ± 0.14 pmole/100 μl) and, during neutropenic episodes, reached mean values of 1.58 pmole/100 μl. The plasma cAMP values for each day of the cycle, as determined by studying six CH dogs, were not significantly different from those of seven normal dogs.

The four additional normal dogs given a single dose of cyclophosphamide developed neutropenia 10–12 days following treatment (Fig. 3). The $PGF_{2\alpha}$ values in media conditioned by their marrows were significantly elevated on post-treatment Day 5 while PGE

[1] Clinical Assays, Cambridge, Mass. 02193.

[2] Amersham Corp., Arlington Heights, Ill. 60065.

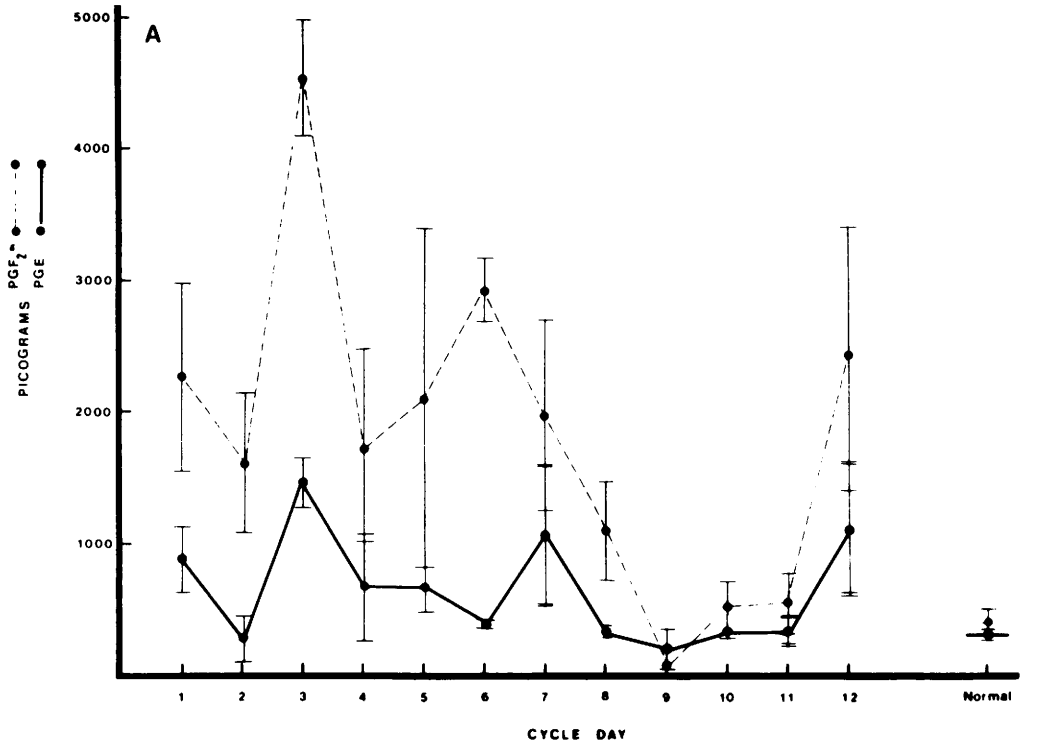

FIG. 1A. Picograms of $PGF_{2\alpha}$ and PGE in 100 μl of medium collected from 2.5-hr duplicate cultures of 1×10^7 mononuclear bone marrow cells collected on each day of the cycle and from normal dogs. Each cycle day represents a mean of three separate experiments for PGE values and a mean of four separate experiments for the $PGF_{2\alpha}$ values. Six normal dogs were used to establish the normal dog's value. The bars represent the standard error of the mean.

values were not significantly different from those of untreated control dogs. The cGMP values in media conditioned by the marrows of cyclophosphamide treated dogs were consistently below 0.1 pg (not graphed) and are comparable to normal dog values but quite different from those of CH dogs (Fig. 2). The plasma cGMP values (Fig. 3) were higher than those found in untreated controls.

Discussion. The data presented (Fig. 1) showed that the CH bone marrow conditioned media contained $PGF_{2\alpha}$ that fluctuated in a cyclic pattern. The lowest values occurred some 4–5 days (Cycle Days 9–11) before the onset of peripheral neutropenia and, during these cycle days, cGMP in the conditioned media was increased but somewhat more variable than during cycle Days 4–8. These data, pointing to Cycle Days 9–10 as critically important to the cycle, are in agreement with two other recent reports (7, 14). In the first of these reports (14) unknown agents in media, collected from 5-hr cultures of CH dog marrows, cyclically caused the mouse multipotential stem cell to divide. The change in the magnitude of [^{3}H]thymidine killing of the mouse stem cell was greatest at CH Cycle Days 9–10. In the second study (7), the CH marrow adherent cell was shown to cyclically influence *in vitro* granulopoiesis of normal dog marrow. The CH adherent cells stimulated normal marrow granulopoiesis of all cycle days except Days 9–10 and possibly 3. On the latter days, inhibition occurred. The cyclical prostaglandin pattern shown in Fig. 1A is most striking when considered together with the data from these two reports in that on Days 9–10, prostaglandin levels are quite different from those noted for the remainder of the cycle.

Hammond *et al.* (15) have recently suggested that PGE production and inhibition of hematopoiesis in CH dogs are normal. Their data showed that CH dog granulocytic

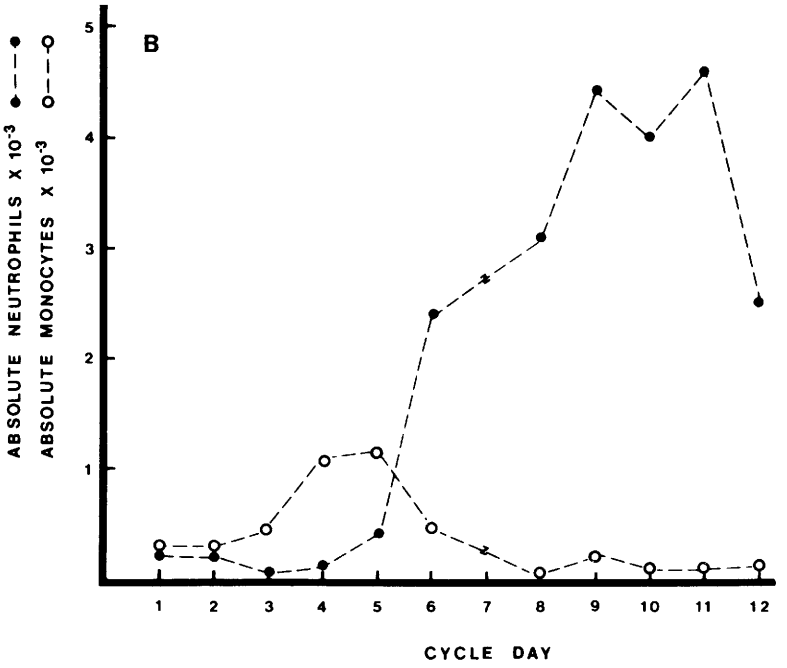

FIG. 1B. Daily absolute peripheral neutrophil and monocyte counts of a typical CH dog illustrating cycle Days 1 through 12.

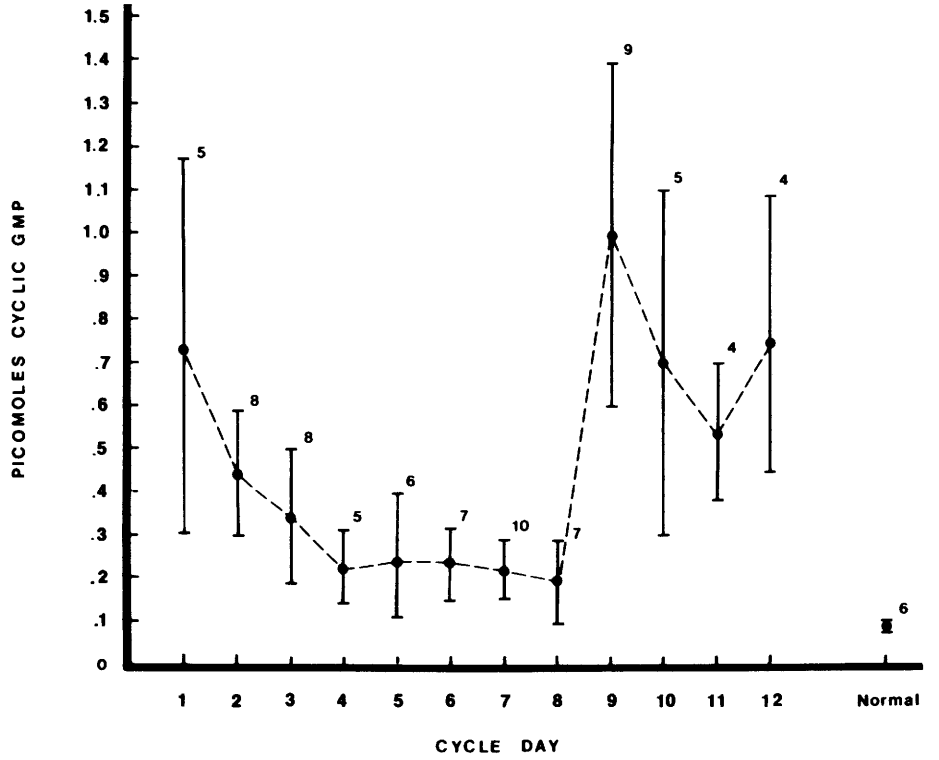

FIG. 2. Picomoles (±SEM) of cGMP in 100 μl of medium collected from 2.5-hr cultures of 1×10^7 mononuclear bone marrow cells collected on each day of the cycle and from six normal dogs. The arabic numeral represents the number of separate experiments with each experiment run with quadruplicate cultures. The bars represent the standard error of the mean of all experiments conducted for each cycle day.

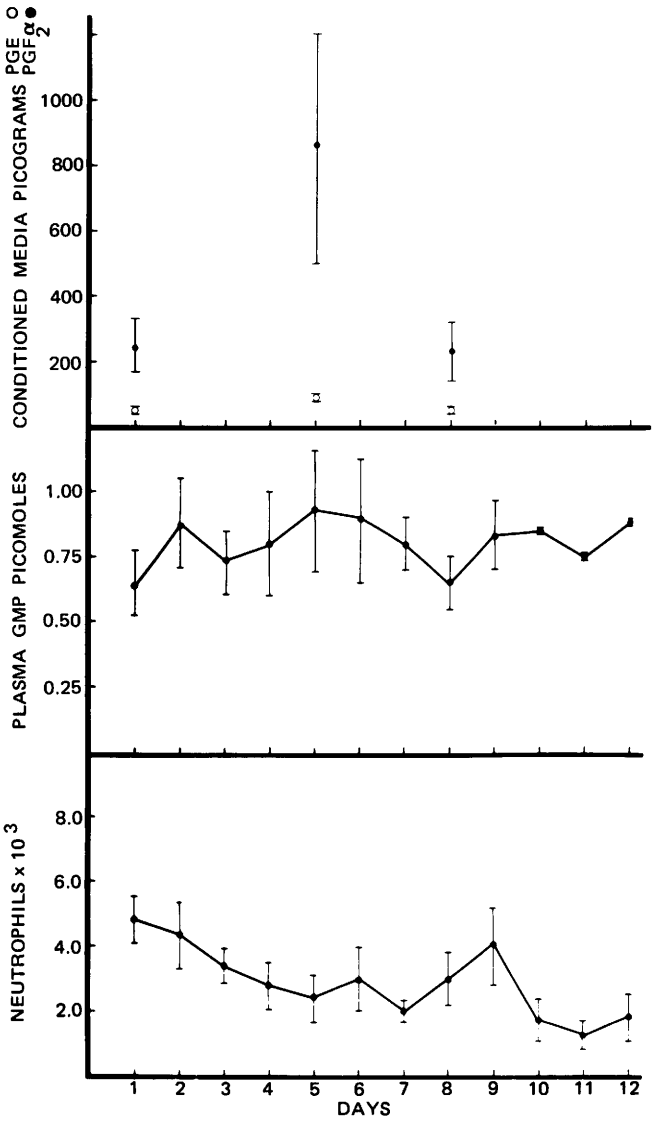

FIG. 3. Top panel: picograms of PGE and $PGF_{2\alpha}$ in 100 μl of medium collected from 2.5-hr cultures of 1×10^7 mononuclear bone marrow cells collected from four normal dogs 1, 5, and 8 days after a single dose of cyclophosphamide. The cGMP in the media (not shown) was not different from untreated controls. Middle panel: picomoles of cGMP in 100 μl of plasma collected from four dogs 1 through 12 days after a single dose of cyclophosphamide. The plasma cGMP values for untreated controls were 0.48 $\pm$ 0.14. The bars in the top and middle panels represent the standard error of the mean for four experiments. Lower panel: the mean absolute daily neutrophil count of the four dogs following a single dose of cyclophosphamide.

colony forming units reacted to PGE and prostaglandin inhibitors in the same way as did those of normal dogs. However, they measured PGE production in peripheral blood leukocyte conditioned media, not bone marrow conditioned media. Local marrow production and local influence of prostaglandin on marrow granulopoiesis was, therefore, not studied.

The changes in levels of $PGF_{2\alpha}$ and cGMP found in Cycle Days 9–10 culture medias could reflect the effects of changes in cellular

proliferation rather than the cause for the upcoming wave of proliferation. *In vitro* studies in this laboratory (4–6) and elsewhere have shown that proliferation begins to increase around Cycle Day 10 and is well above that of normal controls on Cycle Days 12–13. The question of increased proliferation was addressed in the studies of normal dogs made neutropenic with single dose cyclophosphamide treatment whose marrow subsequently became quite active. Medias from their marrow cultures contained elevated amounts of $PGF_{2\alpha}$ but not of PGE or cGMP. The rising $PGF_{2\alpha}$ values may be characteristic of more active cellular proliferation since it occurred in both the CH dogs and the dogs with drug induced neutropenia. However, the striking changes in cGMP levels in the culture medias of CH marrows were not found in culture medias of cyclophosphamide treated dogs and indicate cGMP changes are not simply a result of more active cellular proliferation.

The fluctuations of $PGF_{2\alpha}$ and cGMP graphed in Fig. 1 are of interest because previously these chemicals had been shown to influence *in vitro* hematopoiesis (10, 11). The cyclic changes of $PGF_{2\alpha}$ and cGMP in an animal undergoing rigid cycles of hematopoiesis are of unknown significance but may relate to certain *in vitro* studies. For example, Miller *et al.* (16) showed that direct addition of $PGF_{2\alpha}$ to granulocyte cultures led to increased colony formation. In studies in which endogenous CSF producing cells were removed (10), cGMP and compounds known to elevate intracellular cGMP (carbamylcholine, $PGF_{2\alpha}$) increased granulocyte formation. The data in Figs. 1, 2 show that *in vivo* events in the CH dog are accompanied by measurable changes of $PGF_{2\alpha}$ and cGMP in conditioned media, while in dogs with cyclophosphamide induced neutropenia, only the $PGF_{2\alpha}$ was found to be elevated. The elevated cGMP found in the CH dog but in neither the normal nor the cyclophosphamide treated dog indicates that cyclic fluctuations of this compound are important in the cycle of hematopoiesis. The cause of the cGMP cyclic elevation remains speculative.

The culture media concentrations of cyclic nucleotides graphed in Fig. 1 may be due to cyclic nucleotides leaking from the cells (17), and although media levels may indirectly reflect what is happening in the cell, this possibility is not at all certain. The media levels of prostaglandins, on the other hand, may be quite important since prostaglandins appear to activate cell membrane adenylate cyclase (18, 19). Adenylate cyclase activation by prostaglandins, hormones, or neurotransmitters provides for a theoretical chain of events leading to a physiologic response such as cell division (18). The wave of cell division previously documented in dogs with cyclic hematopoiesis (4–6) is accompanied by marked changes in $PGF_{2\alpha}$ and cGMP and even though a cause and effect relationship cannot be stated, the data are provocative.

Recently, Osborne *et al.* reported cyclic fluctuations of purine and pyrymidine nucleotides in erythrocytes of CH dogs (20). The highest values were found during periods of neutropenia. The data in Fig. 2 show that the elevated cGMP in bone marrow culture media first occurred 3–4 days before the onset of peripheral neutropenia, although it persisted into the neutropenic period. Since Osborne *et al.* were reporting fluctuations in nucleotides found in peripheral erythrocytes, their data should be considered in a somewhat different context from our data collected using bone marrow conditioned media. It is not surprising that changes in bone marrow culture fluids can be detected some 3–4 days earlier than changes in peripherally circulating cells.

The CH dog's unique cycle of hematopoiesis, with its accompanying cycles of prostaglandins and cGMP, provides an avenue to approach the intriguing problem of exactly how peripheral blood cell numbers are either maintained or become aberrant.

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