Cyclic Hematopoiesis: Effects of Lithium on Colony-Forming Cells and Colony-Stimulating Activity in Grey Collie Dogs

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Cyclic hematopoiesis represents a disorder of hematopoietic stem cell regulation in which periodic bone marrow cell production results in cyclic fluctuations of blood leukocytes, reticulocytes, and platelets. The regularly recurring neutropenia occurs at 11-13-day intervals and results in severe infections and premature death. In the grey collie this disorder is an autosomal recessive disease and can be cured or transferred to a normal littermate by bone marrow transplantation.

Lithium carbonate causes leukocytosis in man. Blood neutrophil turnover and bone marrow neutrophil reserve studies have shown that lithium increases neutrophil production, while studies of granulocytic precursor cells have shown increased growth during lithium therapy in man. The mechanism whereby lithium alters neutrophil production has not been fully elucidated. Some investigators have reported that lithium causes an increase in colony-stimulating factor, a putative granulopoietic substance, while others have suggested a direct effect of lithium on granulocytic precursor cells. The recent report of effects of lithium on mouse CFU-S numbers in long-term liquid cultures raises the possibility that lithium alters neutrophil production by effects on the more primitive pluripotent stem cell.

We recently reported that lithium abrogates the recurrent neutropenia in canine cyclic hematopoiesis and ameliorates the fluctuations in other blood cell numbers. In the studies reported here, we have examined potential mechanisms by which lithium therapy might alter blood neutrophil numbers in this stem cell disease.

MATERIALS AND METHODS

Dogs

Male and female grey collie dogs from 2 to 30 mo of age and weighing from 5 to 20 kg were housed in individual cages or runs for these studies as described previously. Normal mongrel dogs or normal littermates of the grey collies served as controls. Blood specimens were routinely drawn from the cephalic vein of unanesthetized dogs between 7:30 and 9:30 a.m. and intravenous injections were given, when indicated, after specimen withdrawal.

Blood Counts and Bone Marrow Aspirates

Blood counts were performed by standard laboratory methods. For marrow aspirates, dogs were anesthetized with intravenous thiamylal sodium (Surital, Parke Davis & Co., Detroit, Mich.). Bone marrow was aspirated from the long bones or iliac crests into sterile syringes containing 100-200 U of beef lung heparin (Upjohn Co., Kalamazoo, Mich.). Five-hundred cell differential counts of Wright-Giemsa stained coverslip smears were made. For serial bone marrow aspirate studies, the sites were rotated so that no bone was aspirated more often than once per week.

In Vitro Bone Marrow Culture

Growth of granulocyte-macrophage precursor cells (CFU-C) was performed as described before. Briefly, freshly aspirated heparinized marrow was diluted in 5 ml of supplemented tissue culture medium (medium 199 with Hanks, Microbiological Associates, Walkersville, Md.), pelleted into auffy coat by centrifugation at 600 g for 12 min, washed 3 times, and resuspended in fresh media. Alternatively, marrow was subjected to a density gradient cut using Ficoll-Hypaque (density 1.074-1.076, osmolality 295-300 mosmole) and cells from the interface zone collected, washed 3 times, and resuspended. Cells were counted by hemacytometer and cell suspensions diluted to 1-2 x 10^6 nucleated cells/ml.

Final plating suspensions contained 1-2 x 10^5 nucleated cells/ml, 10% fetal calf serum, 5%-10% postendotoxin dog serum (as source of colony-stimulating activity) or test dog serum (for CSA assays), and 0.3% agar (Bacto-Agar, Difco Laboratories, Detroit, Mich.).

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One milliliter of this mixture was placed in each 35-mm plastic dish, allowed to gel at room temperature, and then placed in a 37°C, 5% CO₂, high humidity incubator for 8-10 days. All assays were performed in triplicate. Colonies of 50 cells were counted with an inverted microscope and results expressed as colonies per 10³ nucleated marrow cells or as percent of control colony growth. This number is referred to here as total granulocyte-macrophage colony (CFU-C) growth.

Colony Identification

To identify the particular cell types within each colony grown in the agar, a method was devised to fix and stain the entire contents of the 35-mm petri dish. After counting total numbers of colonies with the inverted microscope, the edges of the agar were scored and the contents of the dish slipped onto a glass slide (Clay Adams, Parsippany, N.J.). The moist agar was oriented on the slide, overlaid with two layers of no. 1 Whatman filter paper (VWR Scientific Inc., Seattle, Wash.), and air dried for 1 hr. The upper filter paper was removed, and the lower filter containing the agar was flooded with 5% glutaraldehyde in PBS for 5 min. The lower filter paper was carefully peeled off, the slide allowed to sit in tap water for 2-3 min, and then air dried with a small fan. The agar plate was stained with plain Giemsa for 10 min, rinsed with tap water, and air dried. The agar was covered with a glass cover slip and Preservaslide (Matheson, Coleman and Bell, Norwood, Ohio) for later reading.

Each colony was subsequently classified as neutrophil, macrophage, mixed neutrophil-macrophage, or unclassified by the morphological appearance of individual cells within it. In a "neutrophil colony," at least 80% of the cells were small with condensed lobulated nucleolated chromatin. In a "macrophage" colony, at least 80% of the cells were large, with round or oval nuclei (often containing nucleoli), a low nuclear-cytoplasmic ratio (< 1:3), and prominent granules in the cytoplasm. Mixed neutrophil-macrophage colonies were those containing less than 80% of a single cell type. Only 0%-3% of all colonies on any single agar plate could not be classified as neutrophil, macrophage, or mixed and were labeled "unclassified." One-hundred consecutive colonies (or all of the colonies present if less than 100) were classified as to type and the percentage of each colony type calculated. The number of each type of colony was determined by multiplying its percentage by the total colony number.

Tritiated Thymidine (³H-TdR) Suicide Studies

An estimate of the cell cycle status of CFU-C was made as previously described. Briefly, marrow cell suspensions were incubated with 100 µCi of high specific activity ³H-TdR for 20 min at 37°C. The reaction was stopped by washing with 12 ml supplemented medium containing unlabeled thymidine (100 µg/ml) three times and plating cells for CFU-C growth as described above. Percent thymidine suicide was calculated by subtracting ³H-TdR-treated CFU-C growth from similarly treated cells not exposed to ³H-TdR ("control") and dividing by "control" colony growth. Control cell suspensions to which both tritiated thymidine and excess unlabeled thymidine were added gave CFU-C suicides of zero, demonstrating that nonspecific toxicity was not involved.

Assay for Colony-Stimulating Activity (CSA)

Serum CSA was determined by comparing the total number of CFU-C stimulated to grow by a test serum to the number of colonies stimulated by the standard postendotoxin dog serum (PEDS) and by a normal dog serum (NDS) pool from a given marrow suspension. The "activity" of serum was normalized to allow comparison of different assay runs by the following formula: CSA = colonies grown with test serum/colonies grown with PEDS in same assay x mean number of total colonies grown with PEDS on the 12 test assays. To relate the CSA of a test serum to that of a normal dog serum, the "percent of normal CSA" was calculated by dividing the test serum CSA "activity" by the CSA "activity" of the normal dog serum (NDS) pool assayed concurrently. Marrow cell CSA production was determined by measuring the number of CFU-C grown when 10% marrow cell conditioned medium (see below) was added to triplicate plates of normal dog marrow cell cultures.

Bone Marrow Conditioned Media

To measure the elaboration of CSA from bone marrow cells with and without lithium treatment, bone marrow cell conditioned media (marrow LCM) were prepared. Bone marrow cell suspensions were obtained from normal dogs as for culture, but instead were placed in supplemented media with 10% fetal calf serum in liquid culture flasks without agar or dog serum. Ten milliliter volumes containing 1-2 x 10⁶ cells/ml with or without LiCl at 2 x 10⁻³M were placed in the tissue culture incubator for 7 days, removed, and centrifuged at 1000 g for 20 min to remove all cellular material. This lithium concentration, equal to 2.0 meq/liter, was chosen on the basis of preliminary data demonstrating an optimal effect of lithium on submaximally stimulated unseparated dog bone marrow CFU-C at 2 x 10⁻³M. The supernatant, referred to as marrow LCM, was stored at -10°C until assayed for CSA.

Lithium Administration

Lithium carbonate tablets were given to 7 grey collies and 5 normals once or twice daily, initially at 150 mg then at 300 mg/dose. Serum lithium levels were determined by flame photometry, and attempts were made to maintain the levels between 0.8 and 1.5 meq/liter by adjustment of the lithium dosage.

Endotoxin Administration

Grey collies and normal dogs were given single injections of S. typhosa endotoxin (lipopolysaccharide W from S. typhosa 0901, lot 3124-25, Difco Laboratories, Detroit, Mich.) at 0.1 µg/kg as previously described. Blood neutrophil counts and serum CSA determinations were made prior to and following these endotoxin injections in dogs on and off lithium treatment.

Statistical Methods

CFU-C and CSA values were expressed as arithmetic means of colony counts for triplicate plates. For comparison of data from cycle to cycle in each collie and for summarizing data for our group of cyclic dogs, the values were averaged by "cycle day," defining "cycle day 1" as the first day of a dog's cycle when the neutrophil counts fell below 1000/cu mm. Each day was then numbered in sequence until the next occurrence of cycle day 1. Individual data points occurring on a given cycle day were averaged and standard errors calculated. During lithium treatment periods, cycle day definition was derived in similar manner by extrapolation of prior and subsequent cycle periodicities. Groups means were compared by the Student's t test.

RESULTS

Blood Counts

The regularly recurring severe neutropenia and associated monocytosis of the grey collies was abrogated by lithium treatment as previously reported.
Data were averaged by cycle day for neutrophils, monocytes, and in vitro colony counts from 2-wk periods on and off lithium treatment during which bone marrow aspirates were carried out (Fig. 1). Some apparent fluctuation in the neutrophil counts remained (Fig. 1, right), although the depth and duration of neutropenia was markedly decreased compared to untreated groups (Fig. 1, left). Of note, the monocytosis seen most strikingly on cycle days 2 through 4 was eliminated by lithium treatment. In contrast, normal dogs' neutrophil and monocyte counts were not altered during lithium therapy. Neutrophil counts prior to lithium were 5338 ± 339/µl (34 determinations in 3 dogs ± SEM), while neutrophil counts during lithium were 5621 ± 168/µl (115 determinations in the same 3 dogs ± SEM, p > 0.10). Monocyte counts prior to lithium were 783 ± 60/µl (34 determinations in 3 dogs ± SEM), during lithium, 917 ± 40/µl (115 determinations in the same 3 dogs ± SEM). During the lithium treatment periods, the measured serum lithium levels varied from 0.5 to 1.9 meq/liter in both normal and grey collie dogs.

**Bone Marrow Colony Counts**

Total granulocyte-macrophage colony numbers (CFU-C/10^5 nucleated marrow cells) in cycling dogs showed high levels on cycle days 2 and 3 compared to lows in cycle days 6 and 7 (Fig. 1, left), as shown before. The CFU-C numbers in lithium-treated dogs showed less apparent cyclic changes (Fig. 1, right). When mean CFU-C numbers per 10^5 nucleated marrow cells were calculated for all cycling grey collies and for all lithium-treated grey collies, no difference was demonstrable (cycling greys, CFU-C = 154 ± 11 (n = 50); lithium greys, CFU-C = 141 ± 11 (n = 33); p > 0.10). Lithium treatment did not increase and in fact slightly decreased total CFU-C numbers in normal dogs (normal CFU-C = 103 ± 11 [21 experiments in 3 dogs]; lithium normals CFU-C = 75 ± 12 [15 experiments in 3 dogs] p < 0.05).

Neutrophil colony numbers, however, demonstrated a remarkable change with lithium therapy in the grey collie. In every cycle the greys had at least one marrow with zero neutrophil colonies. In the lithium-treated grey collies this did not occur. When the data were averaged by cycle day, this difference was even more apparent. The cycling greys had zero neutrophil colonies on cycle days 6 or 7 (Fig. 1, left), while the lithium-treated greys had detectable neutrophil colonies throughout the cycle (Fig. 1, right). In the normal dogs, neutrophil colony numbers were not significantly altered by in vivo lithium treatment (normal, 10 ± 3 col/10^5 versus lithium 6 ± 2 col/10^5 cells, p > 0.10).

**Tritiated Thymidine (¹H-TdR) Suicide Studies**

No effect of lithium treatment on the cell cycle status of CFU-C in the grey collie could be demonstrated. The mean ¹H-TdR suicide rate for untreated grey collies was 46% ± 2%, for lithium-treated grey
collies 42% ± 4%, for normal dogs 40% ± 3%, and for lithium-treated normal dogs, 42% ± 3%.

CSA and Lithium

There was no increase in serum CSA levels when grey collies receiving lithium were compared to those not receiving lithium therapy (Fig. 2). In fact, mean CSA decreased slightly from 107% to 81% of normal dogs serum CSA values. Mean serum CSA of lithium-treated normal dogs was 106% of the normal dog serum pool value assayed concurrently. Normal dog marrow LCMs colony-stimulating activity was 48 ± 9 colonies per plate; when Li at 2 × 10^{-3} M (2 meq/liter) was present, the activity was 48 ± 10 colonies per plate.

Endotoxin Response

Increments in blood neutrophil counts and serum CSA values were determined following injections of S. typhosa endotoxin. Peak neutrophil counts occurred 6–8 hr following the injections in normal dogs and untreated grey collies (Fig. 3, left). Of note, the grey collies’ responses to endotoxin depended on the cycle day: during neutropenia the increment was zero, while during neutrophilia a large, possibly supranormal, response occurred (Fig. 3, lower left), as previously reported.2,19 During lithium treatment the normal dog’s response to endotoxin was unaltered (Fig. 3, upper right). Lithium treatment of the grey collie produced a definite change in endotoxin responsiveness. The increments in neutrophil counts were generally small and very large increments no longer occurred: but at all phases of the cycle some increase in the counts occurred when the grey collies were on lithium (Fig. 3, lower right).

Changes in serum CSA following these small endotoxin injections had been documented previously for untreated grey collies and normals.19 The lithium-
treated dogs, both normal and grey collies, respond to endotoxin challenge with an increase in serum CSA, which peaks between 4 and 6 hr after the injections (Table 1). The mean peak increment in CSA in lithium-treated greys or normals is not different from untreated greys or normals.

DISCUSSION

Cyclic hematopoiesis is an autosomal recessive disease in the grey collie dog in which periodic interruption of marrow cell production causes cycling of the blood cell counts. The recent demonstration that lithium, an agent known to produce neutrophilia in other species, will abrogate the severe recurrent disease in the grey collie dog in which periodic interruption of marrow cell production causes cycling of the blood cell counts permitted us the opportunity to examine some potential mechanisms whereby lithium affects granulopoiesis.

The studies reported here confirm our prior report that lithium therapy prevents severe neutropenia in the grey collie. The periodic monocytosis was clearly eliminated even when some residual periodicity was evidenced in the neutrophil counts (Fig. 1). Our data on normal dogs failed to confirm a preliminary report that lithium increases blood neutrophil numbers in dogs. Blood monocyte counts in the normal dogs also were not significantly altered by lithium therapy.

Our data on marrow colony growth showed that the cycling in total CFU-C numbers demonstrated previously in cycling grey collies was not apparent in the lithium-treated grey collies. These numbers, it must be emphasized, represent the proportions of nucleated marrow cells that form colonies and do not necessarily reflect the total body content of CFU-C. Since the total marrow cellularity of the grey collie in certain parts of the cycle may be significantly different from the cellularity of a normal dog, we have avoided direct comparisons of CFU-C numbers in greys and normals. We do believe that the data from a group of untreated dogs may be appropriately compared to the same group on treatment and indeed have shown that the mean CFU-C numbers and 3H-TdR suicide rates in grey collie marrow specimens were not significantly altered by lithium treatment. There also was no effect of lithium on normal dog CFU-C numbers or suicide rates. These results differ from the report of Rossof et al., in which total CFU-C increased, but may be explained by the fact that our normal dogs were on chronic rather than short-term lithium therapy.

Because increased CSA levels have been thought to mediate lithium’s effects on granulopoiesis, we examined serum CSA levels in dogs treated chronically with lithium and found no increase (Fig. 2). Lithium-treated grey collie dogs as well as normal controls were shown to be normally responsive to small doses of endotoxin in terms of serum CSA elevations. Lithium-treated grey collies also had at least some marrow neutrophil reserve response at all points in a cycle (Fig. 3).

Since local, intramedullary elaboration of CSA could explain lithium’s effects without altering serum CSA, we examined the CSA generation of bone marrow cells. Marrow cell conditioned media with and without lithium added in vitro did not differ in CSA values. We also found no differences in CSA elaboration by grey collie marrow cells obtained prior to or during lithium therapy in vivo (data not shown). These data strongly suggest that if CSA is an important in vivo regulator of neutrophil production, its production and release are not the primary regulatory abnormality in cyclic hematopoiesis.

The principal new findings in these studies were the demonstration that neutrophil colonies (>80% neutrophils) were fluctuating markedly in the cycling grey collie, consistently falling to undetectable levels during each period of neutrophilia, and that these fluctuations were abrogated by lithium treatment (Fig. 1). These observations would be consistent with at least three possible mechanisms of lithium action. One mechanism could be that lithium directly alters the pluripotent hematopoietic stem cell in such a manner that the

| Table 1. Endotoxin Effects on Serum Colony-Stimulating Activity (CSA) in Normal and Grey Collie Dogs During Lithium Therapy |
|-------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| CSA†        | Pre*      | 1 hr      | 2 hr      | 4 hr      | 6 hr      | 8 hr      | 24 hr     | Peak Increment‡ |
| Grey collie | 35 ± 4    | 28 ± 5    | 39 ± 4    | 38 ± 4    | 54 ± 7    | 40 ± 6    | 44 ± 3    | 20 ± 5†      |
| (n = 12)§  |          |           |           |           |           |           |           |             |
| Normal     | 37 ± 6    | 42 ± 7    | 47 ± 4    | 54 ± 6    | 51 ± 7    | 43 ± 8    | 31 ± 4    | 23 ± 7†      |
| (n = 10)§  |          |           |           |           |           |           |           |             |
| *Hours following endotoxin injection. |
| †Colonies per plate (10⁶ marrow cells stimulated by test serum) ± SEM. |
| ‡Average of largest increases in CSA following each injection. |
| §Number of individual injections in 3 lithium-treated dogs. |
| ||Values significantly different from “pre” values, p < 0.05. |
| ††Increments significantly different from zero, p < 0.05, and not significantly different from untreated dogs’ increments (as published in reference 19), p > 0.10. |
flux of committed precursor cells into all cell lines is normalized from a variable to a constant rate. This mechanism could explain the correction of the anemia and thrombocytosis as well as the neutropenia of these dogs. A second mechanism could be that lithium indirectly alters the pluripotent stem cell by altering the production or release of a feedback signal (most likely intramedullary) from differentiated cells to the stem cell, thereby abrogating cycling. Among the candidate feedback regulators that could be altered are granulocyte products, lactoferrin, prostaglandin E2, and granulocyte chalone. Implicit in this mechanism is the assumption that the feedback regulator affects all of hematopoiesis or that the erythrocyte, platelet, and other leukocyte cycles are secondary to the neutrophil disorder. Or third, lithium could act directly on the committed granulocyte-macrophage stem cell to cause differentiation into neutrophils rather than monocytes and macrophages. Since our data did not show a shift from the monocyte-macrophage colonies to neutrophil colonies in normal dogs treated with lithium, we cannot conclude that this represents a general mechanism whereby lithium increases neutrophil production. However, such an abnormal switch mechanism within the granulocyte-macrophage progenitor cell directing excessive differentiation into monocytopoiesis in the grey collie could be normalized by lithium treatment and could produce the findings reported here.

Although these studies do not yet define the precise cellular mechanism for cyclic hematopoiesis, they do point clearly to a specific defect in the early phases of neutrophil proliferation and differentiation. Since both lithium and endotoxin can correct the severe neutropenia in these dogs but appear to have somewhat different effects on other marrow cell populations, it should be possible to determine a more precise mechanism for cycling by studying the biochemical and physiologic properties of the early precursor cells of grey collie dogs treated with endotoxin and lithium.

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