Studies of Neutrophil Production and Turnover in Grey Collie Dogs with Cyclic Neutropenia

D. C. DALE, S. B. WARD, H. R. KIMBALL, and S. M. WOLFF

From the Department of Health, Education and Welfare, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Laboratory of Clinical Investigation, Bethesda, Maryland 20014

ABSTRACT 12 grey collie dogs had cyclic neutropenia with the neutropenia recurring at 11.8±0.1-day intervals. The recovery from neutropenia was accompanied by a single wave of myeloid proliferation, an increase in marrow myeloid-labeling indices, and an increase in serum muramidase levels. After recovery from neutropenia during the period when blood neutrophils (PMN) were normal or increased, marrow myeloid precursors became scarce. The decline in marrow precursors and marrow PMN reserves heralded the recurrence of neutropenia. Neither diisopropyl fluorophosphate ($DF^{32}P$) leukokinetic studies nor the rate of development of neutropenia suggested shortened PMN survival as a mechanism for the neutropenia. These studies indicate that the cyclic neutropenia is due to a regularly recurring failure in PMN production.

INTRODUCTION

In 1967 Lund, Padgett, and Ott reported that cyclic neutropenia occurs as an autosomal recessive disorder of grey collie dogs (1). They observed that grey collies develop severe neutropenia at 12-day intervals and suggested that these dogs might serve as a model for studying cyclic neutropenia in man. Since an understanding of cyclic neutropenia may also provide information concerning the control mechanisms of leukopoiesis, we have initiated detailed studies of the formation and fate of neutrophils (PMN)[1] in grey collies (2). The present studies indicate that the cyclic neutropenia is due to a regularly recurring failure of PMN production.

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[1] *Abbreviations used in this paper:* $DF^{32}P$, diisopropyl fluorophosphate; ^{3}HTdR, tritiated thymidine; PMN, blood neutrophils; WBC, white blood cells.

METHODS

Dogs. 12 grey collie dogs of both sexes were acquired from eight breeders in six states. There were four pairs of littermates. Serious and often fatal pyogenic infections frequently occurred when the dogs were neutropenic. Consequently, only three dogs survived longer than 6 months. Most of the observations reported were made on these longer surviving animals. Normal purebred collies with no history of grey forebears were used as controls except as indicated.

Blood counts. Venous blood samples anticoagulated with EDTA were obtained daily at 1:00 p.m. White blood cell (WBC) counts were made using an electronic particle counter[2] and 100 cell differential counts were done on air-dried, Wright's stained smears.

Bone marrow. Under light sodium thiamylal[3] anesthesia, aspirates of bone marrow were taken from the iliac crest or upper femur on 8 days of the 12-day cycle of one grey collie and at 3-day intervals of a cycle of two other grey collies. Differential counts of 300–500 nucleated cells were made on Wright's stained smears. Bone marrow biopsies were obtained from similar sites at 3-day intervals in one dog. Formalin-fixed, decalcified, Giemsa-stained specimens were examined.

Marrow neutrophil reserves. Hourly blood PMN counts were made for 6 hr following intravenous administration of *Escherichia coli* -127:B8 endotoxin[4] (3). A dose-response curve determined by testing eight mongrel dogs at weekly intervals indicated that 0.1 μg/kg produced an initial granulocytopenia and marked increase in the number of circulating nonsegmented PMN. The maximum increase in the blood PMN over 6 hr following this dose of endotoxin was used as a measure of the marrow PMN reserves.

Tritiated thymidine (^{3}HTdR)-labeling indices. Marrow precursor-labeling indices were determined by the method of Mauer (4) with minor modifications. The nucleated cell concentration of heparinized marrow-aspirate samples was adjusted with Hank's solution to 2.0×10^6/ml. 1 ml of cell suspension was incubated with 2.5 μCi/ml ^{3}HTdR[5] (SA 27.8 μCi/ml) for 1 hr at 37°C in gently rocked

[2] Model Fn, Coulter Electronics, Industrial Div., Hialeah, Fla.

[3] Surital, Parke, Davis & Company, Detroit, Mich.

[4] Difco Labs, Detroit, Mich.

[5] New England Nuclear, Boston, Mass.

Leighton tubes. The cells were washed thrice in Hank's solution and fixed in Carnoy's ethanol-acetic acid fixative. Slides covered with Kodak[6] AR10 stripping film were developed after 7 days. The per cent of 200 myeloid precursors (myeloblasts, promyelocytes, and myelocytes) labeling with more than five grains per cell was determined using the marrow morphologic criteria for dogs of Patt and Maloney (5).

Serum muramidase (Lysozyme). Serum was obtained from blood samples allowed to clot at room temperature for 2 hr and was kept frozen at −20°C before use. Muramidase concentrations were determined by the turbidimetric method of Litwack (6), using a *Micrococcus lysodeikticus* substrate[7] and expressed as micrograms per milliliter of egg white standard.[7]

Circulating leukocyte kinetics. Diisopropyl fluorophosphate ($DF^{32}P$) leukocyte kinetic studies were performed by the methods of Athens, Mauer, Ashenbrucker, Cartwright, and Wintrobe (7). Because PMN are more highly labeled by $DF^{32}P$ than other leukocytes (7), these methods were assumed to measure PMN distribution and survival under the circumstances tested (blood PMN of 65–85%). These methods have also been shown to be applicable to neutropenic patients (8). Under light sodium thiamylal anesthesia, approximately 10% of the animal's blood volume was collected in a plastic bag[8] containing 37 ml of acid citrate dextrose anticoagulant. After the addition of 100 μCi of $DF^{32}P$[9], the blood was gently mixed at room temperature for 1 hr and then reinfused over 15 min. 12-ml heparinized venous blood samples were obtained from the bag before reinfusion and at 1, 3, 6, 12, and 24 hr. Dextran-sedimented leukocytes were separated from platelets by centrifugation at 200 *g* for 3 min and the erythrocytes removed by hypotonic lysis. The number of separated WBC were counted and the cells then digested in 0.2 N NaOH for 2 hr at 60°C. The digested material was acidified with 10% acetic acid and solubilized in a Bio-Solv (BB5-3)[10]-Liquifluor[5]-Toluene cocktail[11] and the sample radioactivities were measured with a liquid scintillation counter[12]. The specific activity for each sample was expressed as counts/minute per cell. The slope and half-life for the leukocyte specific activity decay curves were calculated by the method of least squares (9). The total body PMN pool, marginating and circulating pools, and PMN turnover rates were measured by extrapolating the specific activity decay curves to time zero and applying the formulae of Athens et al. (10).

Combining the data on 18 cycles for four grey collies, a rate of decrease of the blood PMN count was calculated for the 3 days before the most neutropenic day of the cycle using PMN counts made at the same time each day. The line of best fit was determined by the method of least squares (9).

[6] Eastman Kodak Co., Rochester, N. Y.

[7] Worthington Biochemical Corp., Freehold, N. J.

[8] Fenwal Laboratories, division of Travenol Laboratories, Morton Grove, Ill.

[9] Radiochemical Centre, Amersham, England.

[10] Beckman Instruments, Fullerton, Calif.

[11] Bio-Solv 10%, Liquifluor 3.6%, Toluene 86.4% by volume.

[12] Beckman Liquid Scintillation Spectrometer, model LS-250, Beckman Instruments.

RESULTS

Clinical aspects. All grey collies studied had cyclic neutropenia. The collies regularly became ill 1 or 2 days after the onset of severe neutropenia (less than 500 PMN/mm^3). The severity of the illness with each neutropenic episode varied considerably. At times there was almost nothing noticeable but frequently the dogs had fever, lethargy, anorexia, and overt pyogenic infections. The degree of illness with each episode bore no apparent relation to the degree of neutropenia. In four pairs of littermate puppies, the cyclic neutropenia occurred simultaneously and the cycles remained synchronous throughout their lives (to maximum age of 5 months). In another litter with three grey collies, only two puppies had synchronous cycles, although the third also had cyclic neutropenia.

Blood counts. Daily neutrophil counts for one dog over a 75 day period are shown in Fig. 1. Similar studies have been done in two dogs for more than 6 months and in all dogs for periods greater than two cycles. For the dog in Fig. 1, the neutropenic phase occurred at 11.2-day intervals. For all 12 dogs the mean cycle length was 11.8±0.1 days. Neutrophilia followed the neutropenic phase and a single minor dip and peak of blood PMN generally anteceded the recurrence of neutropenia (Fig. 1). On those occasions when the dogs developed overt infections, transient excessive neutrophilia usually occurred without altering the period between the neutropenic episodes.

Daily blood neutrophil counts were also performed in two normal collies (one male, one female) for 120 days from the age of 3 months. The geometric mean PMN counts were 8900 and 6200, respectively. Cyclic variation in PMN counts was not observed.[13]

Bone marrow. In Fig. 2 are shown the differential counts for one series of frequent bone marrow aspirates in one dog and the corresponding blood PMN and reticulocyte counts during an infection-free cycle. Less frequent examinations of two other dogs and the same dog during a different cycle showed similar changes (all three dogs were free of infections during these studies). A wave of myeloid development occurs with each cycle with marked marrow proliferation of PMN precursors before the return of blood PMN. On successive days of the neutropenic period, myeloid elements are progessively more mature. During the period of normal blood PMN counts, the per cent of marrow myeloid precursors declines. Similarly, marrow erythroid precursors, particularly normoblasts, fluctuate with the cycle as do blood reticulocytes. More precise studies of the regulation of erythropoiesis in these animals are currently underway.

[13] Dale, D. C., D. W. Alling, and S. M. Wolff. In preparation.

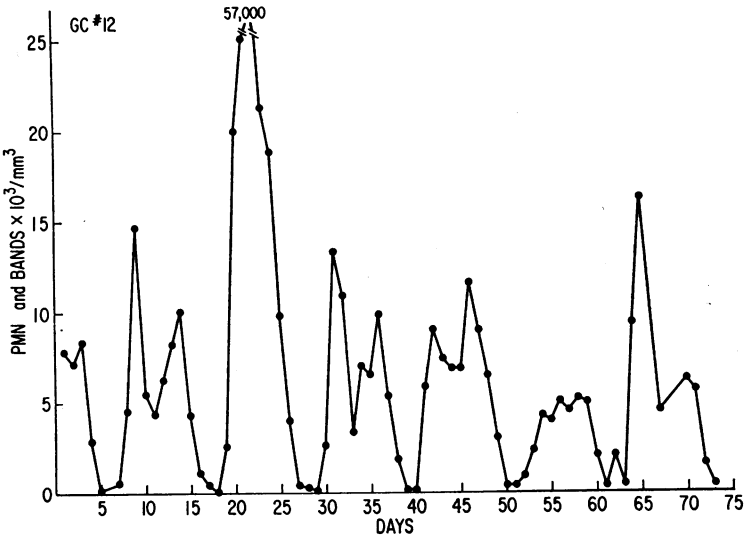

FIGURE 1 Circulating neutrophil counts over a 75 day period in a grey collie dog.

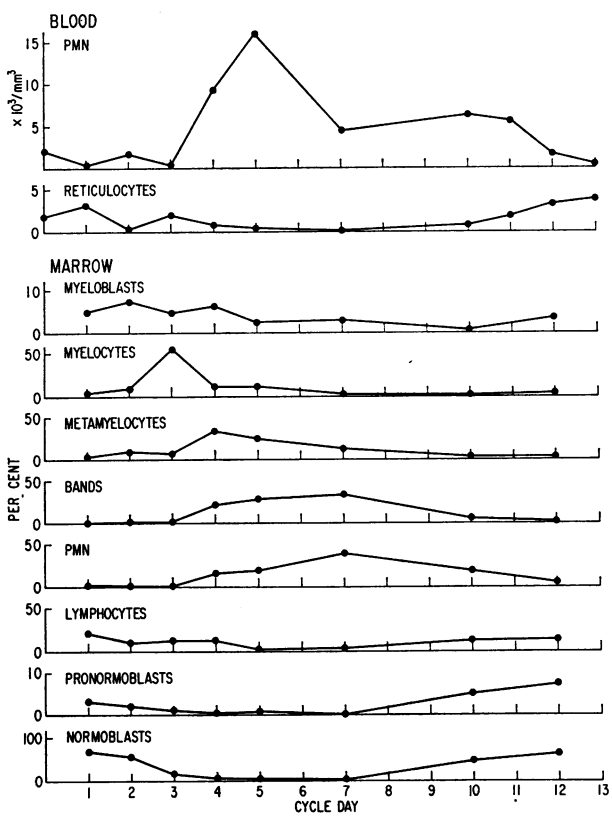

FIGURE 2 Circulating neutrophil and reticulocyte counts during a 12 day cycle in a grey collie dog (upper two graphs). Differential counts of bone marrow nucleated cells during the same period in the same dog (lower eight graphs).

Because no marrow element seems to remain constant, it is not possible to express the absolute quantitative fluctuations in the marrow cell populations. Nevertheless, examination of many aspirates as well as marrow biopsies taken at 3-day intervals strongly suggest that there are fewer myeloid precursors in the period before neutropenia occurs.

Marrow reserves. The neutrophil response to endotoxin on different cycle days for one grey collies is illustrated in Fig. 3. These studies were performed no more than once per week to avoid the development of endotoxin "tolerance" (11). As can be seen, there was no response during the neutropenic phase. The response was maximal just after the return of blood PMN and gradu-

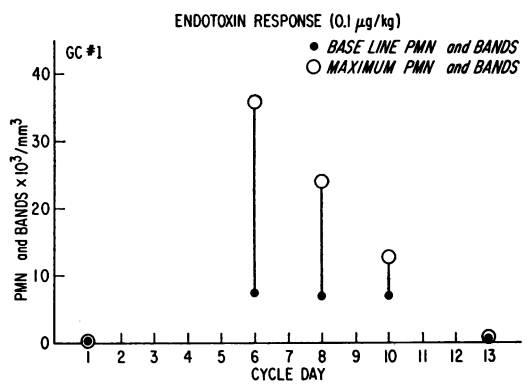

FIGURE 3 Circulating neutrophil responses to the intravenous injection of *E. coli* endotoxin on different cycle days in a grey collie. Closed circles are base line counts of PMN and bands and open circles are maximum responses during a 6 hr period after endotoxin.

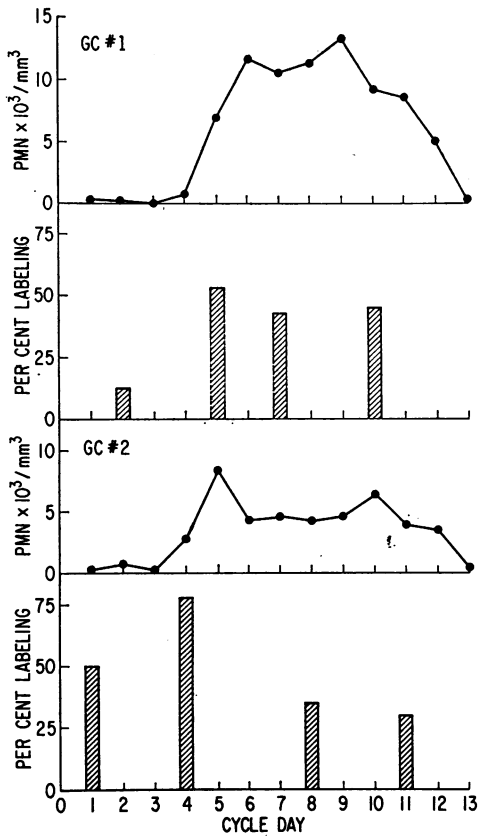

FIGURE 4 In vitro bone marrow myeloid precursor ^{3}HTdR-labeling indices and corresponding blood neutrophil counts during a 13 day cycle in two different grey collies.

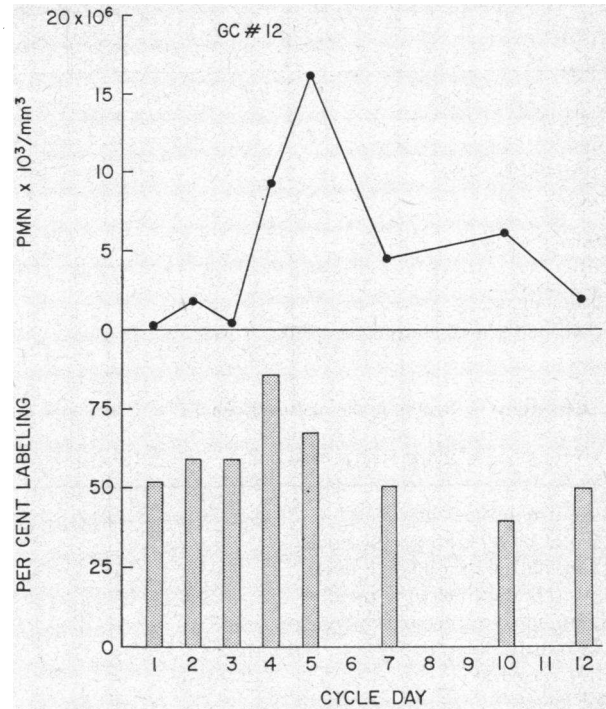

FIGURE 5 In vitro bone marrow myeloid precursor ^{3}HTdR-labeling indices and corresponding blood neutrophil counts during a 12 day cycle in a grey collie.

ally diminished as the dog approached the neutropenic phase. Four dogs have been similarly studied, and the results are the same as shown in Fig. 3.

^{3}HTdR-labeling indices. Marrow myeloid precursor-labeling indices and the corresponding blood PMN counts for three dogs are shown in Figs. 4 and 5. The increase in labeling indices just before or at the time of the rapid rise in blood PMN was uniformly observed. No period of markedly decreased labeling was found.

Serum muramidase. Fig. 6 demonstrates the pattern of the serum muramidase levels and blood PMN in one dog. Regularly, muramidase levels began to rise before the blood PMN counts peaked or before the phase of neutrophilia, and fell to low levels several days before the blood PMN began to fall. Similar studies in five other

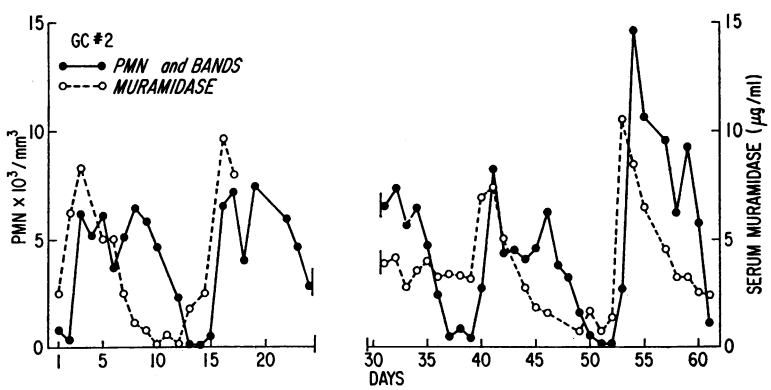

FIGURE 6 Serial circulating PMN and bands (closed circles) and serum muramidase levels (open circles) in a grey collie.

TABLE I

Leukokinetic Studies in Grey Collie Dogs

Dogs	Number	$t_{\frac{1}{2}}$*	TBGP‡	CGP§	MGP‖	GTR¶
		hr	$\times 10^7$/*kg*	$\times 10^7$/*kg*	$\times 10^7$/*kg*	$\times 10^7$/*kg per day*
Normal mongrels**	31	5.6±0.17‡‡ (4–8)§§	102±6.2 (48–168)	54.8±3.7 (27–98)	48.0±4.2 (10–86)	305±20.0 (150–560)
Normal collies	5	6.4±0.29 (5.5–7.2)	132.4±13.9 (93–173)	50.8±5.8 (32–68)	81.7±8.5 (61–105)	346±41.6 (216–459)
Grey collies—						
Normal phase (autologous cells)	2	7.4 8.7	197 173	66.5 28.4	130.5 144.6	436 331
Becoming neutropenic (transfused cells)	1	5.9	51.5	19.7	31.8	145
Neutropenic phase (transfused cells)	1	5.9	9.7	3.6	9.9	36.6

* Neutrophil half-life.
‡ Total blood granulocyte pool.
§ Circulating granulocyte pool.
‖ Marginal granulocyte pool.
¶ Granulocyte turnover rate.
** From Rabb et al. (12)
‡‡ Mean±1 SE.
§§ Range.

dogs gave the same results as the data presented in Fig. 6.

Leukokinetic studies. DF^{32}P leukokinetic data are shown in Table I. Grey collies were studied by autologous labeling when their blood leukocyte differential counts were similar to the normal control dogs (12). At this time the blood PMN half-lives were slightly longer than the normals (Table I). In one grey collie entering the neutropenic phase and in another already neutropenic, the injection of PMN from normal collies had a normal survival. The number of dogs large enough for these investigations has been limited; nevertheless, the studies performed have not suggested a shortened neutrophil survival to be a mechanism for the development of neutropenia. The total blood PMN pool, the circulating pools, and PMN turnover rates were normal for autologous cells in the normal phase, but the marginating pool was increased. In the neutropenic phase, transfused normal collie cells had a normal half-life, but the various pools sizes were decreased (Table I).

The rate of decrease of the blood PMN, expressed as PMN half-life as the dogs enter the neutropenic period, is slower than the normal collie PMN half-life measured by the DF^{32}P technique. This slow rate of decrease of the blood PMN count is further evidence, albeit indirect, that neutropenia does not occur by a mechanism that rapidly decreases the circulating PMN count. In Fig. 1 it can also be seen that the PMN count declines steadily for about 3 days before each neutropenic period.

DISCUSSION

These studies clearly indicate that cyclic neutropenia occurs in grey collie dogs because they fail to maintain a steady rate of PMN production. Myelopoiesis in these animals is characterized by periodic bursts in myelopoietic activity. They regularly develop neutropenia when they have exhausted their marrow neutrophil reserves. Then, as if stimulated by the development of neutropenia, they repeat the burst of myelopoietic activity.

Most of the data of these studies relate to the periodic increases in myelopoiesis. This activity is reflected by several parameters. First, the marow histology shows the orderly appearance of myeloid precursors of increasing maturity during the neutropenic phase. These cells rapidly mature to metamyelocytes and mature PMN. Second, the labeling indices of the myeloid precursors are increased above that expected for normal dog marrow (5) and above that observed at other times for the same dogs. Third, the serum muramidase, which indirectly reflects marrow myelopoietic activity (13), rises just before the return of blood PMN. These dogs also have increased numbers of monocytes in the blood heralding the return of PMN, as seen in a recovering marrow under many circumstances (14). Under these circumstances, the rise in serum muramidase might be due to either the change in PMN or monocyte production or both. Finally, we have recently reported (15) that these dogs have high levels of urinary colony-stimulatory activity for mouse marrow (16) during this phase of active myelopoiesis.

After the recovery from neutropenia, during a second phase of the neutrophil cycle, the dogs have a normal or increased blood PMN count for 6 or 7 days. During this period the PMN make a slight dip and peak before neutropenia recurs (Fig. 1). No explanation for this "biphasic" phenomenon is apparent, but it is conceivable that this is an attempt to regulate the blood PMN count toward the normal level (17). Marrow reserve tests at this second phase indicate a very large marrow neutrophil reserve, consistent with the marrow histology for this phase of the cycle. The half-life and distribution of circulating PMN are near normal, and at this time the dogs are also generally well.

The decline in PMN before neutropenia recurs is gradual. The rate of decrease (half-life = 11.4 hr) is very similar to that observed by Thomas for lethally irradiated dogs (half-life = 13 hr) (18). These data, the normal survival of homologous transfused PMN, and the fact that serum muramidase falls to low levels several days before the PMN count begins to fall, all indicate that the development of neutropenia is not related to accelerated cell removal.

The data presented here and other studies of myelopoiesis in dogs (19, 20) suggest that the failure of proliferation occurs during the phase when blood PMN counts are normal. In these studies, marrow promyelocytes and myelocytes are scarce during the latter part of this phase. This concept is also supported by the detailed studies in dogs of Maloney and Patt (19) and Boggs et al. (20), which indicated that 5–8 days are required for the generation of blood PMN from early precursors. Although a failure of the marrow to maintain PMN production seems to be the primary defect, the mechanism for the marrow failure is presently unknown.

In man, cyclic neutropenia occurs generally with 21-day cycles. Several studies have shown, primarily by serial marrow examinations (21, 22), that a single wave of myeloid proliferation occurs with each blood PMN cycle. Meuret and Fliedner recently reported (22) that ^{3}HTdR administered intravenously during the neutropenic phase appeared more rapidly than normal in the circulating PMN, indicating a more rapid than normal movement of cells from the proliferative through the maturational marrow pools in the recovery phase. The other concomitants of the proliferative phase seen in the dogs have not been reported for human cyclic neutropenia; however, cyclic fluctuations of serum muramidase have been seen in patients with induced periodic marrow failure and recovery due to intermittent cytosine-arabinoside therapy (23).

We,[14] and others, have observed that blood PMN half-life is normal or slightly prolonged in patients with cyclic neutropenia when they have their highest PMN counts (22, 24). Kinetic studies in the neutropenic phase are technically difficult due to the lack of cells to label, but the blood PMN count in man, as in the dogs, falls gradually with the neutropenic phase. Despite minor differences between the cyclic neutropenia of grey collie dogs and man, these peculiar dogs are an excellent model for the study of the human disease.

[14] Dale, D. C., and S. M. Wolff. In preparation.

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