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Research Article

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Hematopoiesis in the Grey Collie Dog

STUDIES OF THE REGULATION OF ERYTHROPOIESIS

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ABSTRACT Hematopoiesis in the grey collie dog undergoes periodic fluctuations which involve reticulocytes, granulocytes, platelets, lymphocytes, and monocytes. This syndrome is inherited in an autosomal recessive manner and can be transmitted or abolished by appropriate bone marrow transplantation experiments, thus demonstrating this to be a primary marrow defect. Investigation of humoral regulation in this setting indicates that serum erythropoietin (ESF) also undergoes cyclic fluctuation and that shortly after the increase and peak in serum ESF levels recognizable red cell precursors appear in the marrow. Erythropoiesis in the grey collie is reciprocally related to the blood O₂ carrying capacity. With phlebotomy, ESF activity and reticulocytes increase but continue to cycle, while hypertransfusion eliminates reticulocyte production completely. Neither phlebotomy nor hypertransfusion alter the underlying cycle time (11–12 days) nor influence the peaks of peripheral blood granulocytes. Thus, in these experiments, no direct evidence of competition between reticulocyte and granulocyte production is observed. In vitro studies of canine hemoglobin synthesis fail to demonstrate evidence of an inhibitor to ESF. These results indicate that periodic fluctuation of serum ESF is an integral part of the grey collie syndrome and are most consistent with some form of feedback regulation of ESF production.

INTRODUCTION

The grey collie dog is characterized by a genetically determined cyclic variation in the level of blood neutro-

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phils, reticulocytes, platelets, monocytes, lymphocytes, and eosinophils with a cycle time of 11–12 days (1, 2). The patterns of fluctuation of the blood elements suggest that this disorder is due to periodic marrow proliferation or cyclic hematopoiesis. Morphologic studies of the bone marrow indicate that recovery from neutropenia results from a wave of myelopoiesis beginning with the earliest recognizable forms. The proliferative recovery of the bone marrow is accompanied by increased tritiated thymidine labeling of myeloid precursors and an increase in serum muramidase levels (3). Since blood granulocyte survival is normal, the data indicate a failure of granulocyte production occurring at regular intervals (3). Examination of the bone marrow reveals that changes in erythropoiesis parallel those in granulopoiesis; that is, there is a cyclic variation in the percentage of recognizable erythroid precursors with a cycle length identical to that of the myeloid precursors (3).

Although the basic defect responsible for the periodic marrow proliferation in this disorder is not known, recent studies have demonstrated that the urinary excretion of a stimulator of in vitro granulocyte colony formation (colony-stimulating activity; CSA)¹ also cycles, with peak activity coinciding with the nadir and rising phase of the peripheral blood neutrophil count (4). Since controversy exists as to whether CSA represents a primary humoral regulator (5), it becomes important to examine the behavior of other humoral regulators in this setting.

The studies reported here were designed to determine the role of erythropoietin (ESF) in the regulation of erythropoiesis in the grey collie and to examine the relationship of the blood O₂ carrying capacity to ESF and red cell production. In addition, in vitro assays for pos-

¹Abbreviations used in this paper: CSA, colony-stimulating activity; ESF, erythropoietin; IRP, International Reference Preparation.

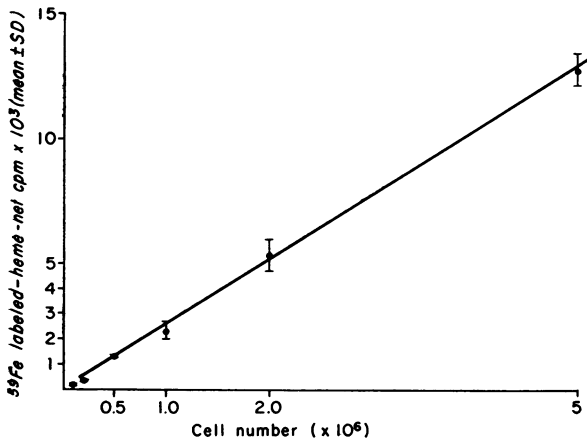


FIGURE 1 Heme synthesis in vitro. The effect of varying numbers of dog bone marrow cells on the recovery of ^{59}Fe -labeled heme from normal marrow cultures. A constant dose of ESF was employed and the results expressed as net heme labeled (ESF-stimulated minus controls).

sible inhibitors of ESF or hemoglobin synthesis were performed.

METHODS

Dogs. Four grey (two males, two females) and two normal collies (one male, one female) were studied. Their handling and care have been described previously (2).

Hematology. Blood samples (2–8 ml) were obtained from the cephalic vein at the same time each day for serum and cell counts. The hematocrit and white blood counts were done by standard techniques, and the reticulocyte percentage was determined from air-dried smears by counting the number of reticulocytes in 1,000 red cells, as previously described (2). Iron determinations were performed on each serum specimen by the method of Schade, Oyama, Reinhart, and Miller (6).

ESF bioassay. The *in vivo* quantitation of ESF was performed by using ex-hypoxic polycythemic mice (7). Grey collie or normal dog serum was given as a single intraperitoneal injection in a volume of 0.4 ml to groups of five mice on the 4th day after the removal of the assay animals from the hypobaric chamber. 48 h later, ^{59}Fe as ferrous citrate in saline was injected intraperitoneally. The red cells were harvested by retro-orbital bleeding 48 h after isotopic administration, washed, and analyzed for radioactivity in a well-type NaI crystal scintillation counter (Nuclear-Chicago Corp., Des Plaines, Ill.). Quantitation of serum ESF was achieved by comparing mean values of ^{59}Fe incorporation in groups of mice receiving test material with a simultaneously determined ESF International Reference Preparation (IRP) dose/response curve.

Erythropoietic response to phlebotomy and hypertransfusion. During the course of study, two normal and two grey collies were phlebotomized approximately 10 and 20% of their blood volume; two additional grey collies were hypertransfused with washed homologous red cells. Daily blood samples were obtained to determine the effects of the phlebotomy and hypertransfusion on reticulocyte and granulocyte cycling and on ESF production.

In vitro studies of hemoglobin synthesis. The bone marrow cell culture technique utilized was modified from that of Krantz, Gallien-Lartigue, and Goldwasser (8) and employed the incorporation of ^{59}Fe into heme as marker. Marrow cells were obtained by sterile needle aspiration from the iliac crest of randomly bred dogs anesthetized with sodium Pentothal. The aspirated cells were placed immediately into 5 ml of cold balanced salt solution (Hanks' BSS; Grand Island Biological Co., Grand Island, N. Y.) containing 20 U/ml of sodium heparin. The cells were washed twice in BSS and then resuspended in a concentration of 0.5 or 1.0×10^6 trypan blue dye-excluding cells/ml in NCTC-109 (Microbiological Associates, Inc., Rockville, Md.). 1 ml of cell suspension and 0.1 ml of dog serum were added to 10×35 -mm tissue culture dishes (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.). No fetal calf serum or antibiotics were employed in any of these experiments. A minimum of three plates was set up for each test serum. Where indicated, human urinary ESF³ in microliter quantities was added to identical cultures established in parallel.

The plates were incubated for 36 h in a 100% humidity, 95% air-5% CO_2 tissue culture incubator (National Appliance Co., Portland, Ore.). $0.5 \mu\text{Ci}$ of ^{59}Fe tagged to autologous serum in a volume of 0.1 ml (40% serum/60% NCTC-109) was added to each plate, and the incubation was continued for 12 h.

At the end of the 48-h culture period the cells were harvested with a Pasteur pipette, suspended in phosphate buffered saline (pH 7.4), and centrifuged at 200 *g* for 10 min at 4°C. The lysate was then acidified with 0.1 ml 2 N HCl and vortex mixed, and cyclohexanone was added. The aqueous and organic layers were thoroughly vortex mixed and centrifuged at 2,000 *g* for 30 min at 4°C, and then the organic layer was removed for determination of radioactivity.

Basic aspects of the dog marrow culture system are shown in Figs. 1 and 2. The relationship between labeled heme recovered and cell number plated was linear over a range of 10^5 – 5×10^9 cells/dish, and the regression of this relationship passed through the origin (Fig. 1). Appropriately, increased heme synthesis was observed in direct relationship to the dose of ESF added (Fig. 2). The dog marrow culture system is sensitive to added ESF at levels of 0.001–0.002 IRP U/dish, and the response is generally linear above 0.003 U. In all of the experiments employing ESF, doses were chosen to fall well within the linear portion of the dose/response curve. Reproducibility of the assay gives estimates of activity of serum specimens which vary by no more than 25% on repeated measurement.

Endogenous levels of ESF in grey collie sera were assayed *in vitro* by this method. A direct comparison of multiple serum samples was possible after equalizing the cold iron pool (serum iron concentration) in each sample. This was necessary since previous studies in this laboratory demonstrated the profound influence of the serum iron concentration on ^{59}Fe uptake. Since this is a complex relationship and not strictly dilutional,³ it was necessary to add elemental iron in appropriate amounts to the various dog

² The ESF employed was prepared in this laboratory from the lyophilized urine of a patient with aplastic anemia. Biological activity of the material was approximately 50–100 IRP U/mg protein. Doses used *in vitro* ranged from 0.1–0.25 U/plate.

³ J. W. Adamson. Unpublished observations.

sera. To accomplish this, ferric chloride in normal saline was prepared in a concentration of 1 mg of elemental iron/ml and citrated. Microliter quantities of the iron salt solution were then added to the sera. The iron concentration chosen was the highest concentration observed among the specimens to be compared. The cultures were then processed as described above.

To confirm that any fluctuations found in endogenous hemoglobin-stimulating activity were, in fact, likely due to ESF, antiserum capable of neutralizing the biological activity of ESF was added to selected sera in a separate experiment. The antiserum had been prepared in rabbits against human urinary ESF after the method of Schooley and Garcia (9). A neutralizing capacity of 5 IRP U/ml of antiserum had been established by bioassay techniques. Normal rabbit serum was added in equal volume (25 μ g) to control cultures.

To examine the possible role of inhibitors of ESF or hemoglobin synthesis, serum obtained at various times in the 11–12-day cycle was added to normal dog marrow cells in vitro up to a final concentration of 83% by volume, and the effect on basal and ESF-stimulated heme synthesis was measured. Simultaneously established cultures with normal collie serum of equal iron content and at equal serum concentrations served as controls.

The results determined from the in vitro experiments were expressed as basal and net labeled heme recovered (ESF-stimulated minus basal activity).

RESULTS

Hematology. The hematocrits and reticulocyte counts of the normal control collies averaged 42.7 and 1.2%, (SEM 0.3 and 0.1, respectively) (Fig. 3). Granulocyte counts ranged from $4\text{--}9 \times 10^8/\text{mm}^3$, and there was no evidence of cyclic fluctuation in any of these parameters. In contrast, as shown in Fig. 4, the expected fluctuation of circulating granulocytes and reticulocytes was observed in the grey collies. Hematocrits in these animals were consistently lower than control values, averaging 31.5%. Reticulocyte counts ranged through the cycles from 0.0–2.7% and granulocyte counts ranged from $0.3\text{--}16 \times 10^9/$

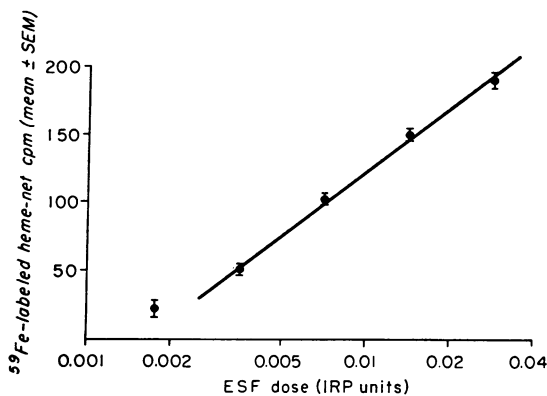


FIGURE 2 Heme synthesis in vitro. An ESF dose/response curve obtained by using 2.5×10^6 nucleated dog marrow cells/plate; the ESF dose range is approximately 0.002–0.04 IRP U/plate.

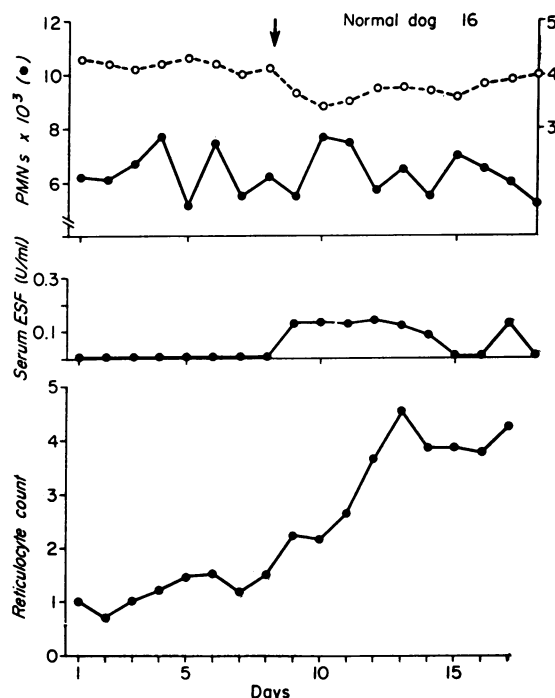


FIGURE 3 Response to phlebotomy in the normal collie. Daily serum ESF levels, hematocrit, and granulocyte and reticulocyte counts in a normal dog are shown pre- and post-phlebotomy. No cycling of any of these parameters was observed. In this and Fig. 4, the arrowhead indicates the time of phlebotomy.

mm^3 with cycle times of about 12 days. As previously described (2), a secondary peak in the granulocyte count was observed, and the reticulocyte and granulocyte cycles were out of phase with one another.

ESF bioassay. Basal levels of ESF in 13 of 14 normal dog serum specimens were undetectable (Fig. 3). Saline and normal dog serum rarely stimulated ^{59}Fe incorporation values above 1.5% in the polycythemic mice. In contrast, levels of ESF in the grey collies were frequently measurable and tended to cycle as well. This was particularly evident for grey collie 22 (Fig. 4a) which was the more anemic. ESF activity appeared to cycle out of phase with the reticulocyte count, tending to peak when the reticulocyte count was lowest.

Erythropoietic response to phlebotomy and hypertransfusion. After phlebotomy of the control dogs (Fig. 3), the reticulocyte count (corrected for hematocrit) rose to a peak of about 4% and serum ESF levels, although not exceeding 0.2 IRP U/ml, became consistently measurable. Similar results were seen in the grey collies (Fig. 4). There was a rise in the reticulocyte count in response to phlebotomy, particularly in dog 28 which was bled the more vigorously, and serum ESF levels rose. Significantly, there appeared to be continued cycling of

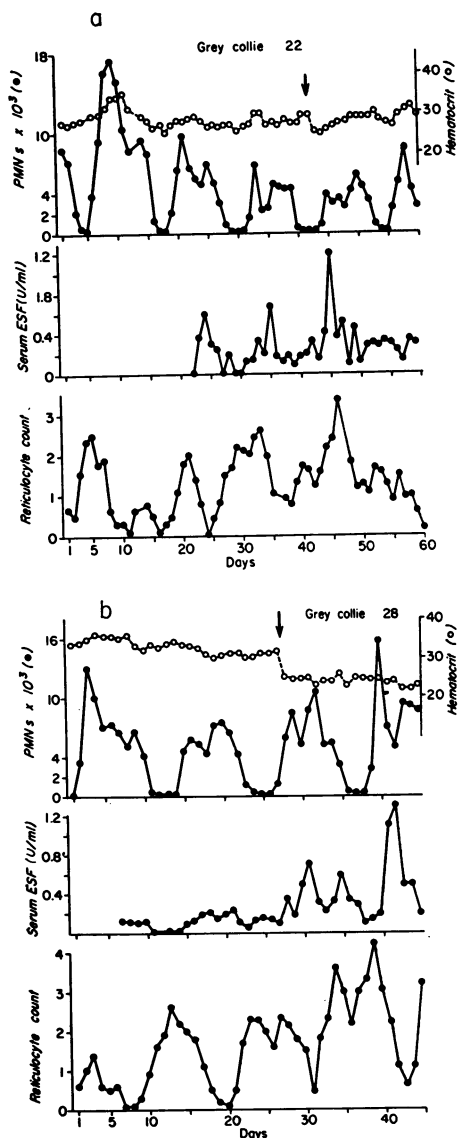


FIGURE 4 Response to phlebotomy in the grey collie. Daily ESF levels, hematocrit, and granulocyte and reticulocyte counts in two grey collies are shown pre- and post-phlebotomy. Cycling of several of these parameters is seen and there is an increase in ESF and reticulocytes in response to phlebotomy, particularly in dog 28.

ESF activity in the grey collies, and neither the reticulocyte count nor ESF levels became unmeasurable during the nadir of their cycles. No consistent alteration in the height of the granulocyte peaks or the underlying periodicity was seen.

In the hypertransfused grey collies, the reticulocyte count fell to unmeasurable levels (Fig. 5). As long as the hematocrit was substantially elevated no reticulocytes were observed in circulation. As the hematocrit returned

toward pretransfusion levels, reticulocytes reappeared and cycling activity commenced again. Through the period of hypertransfusion and recovery there was no detectable alteration in the height of the peaks of granulocyte counts or in the granulocyte cycle time, and once the reticulocyte cycles were reestablished, they retained their previous temporal relationship to the granulocyte cycles.

In vitro studies of hemoglobin synthesis. To demonstrate the responsiveness of the *in vitro* system to physiologic changes in ESF, sera obtained from the normal dog pre- and post-phlebotomy were adjusted for iron content and added to cells in culture. As shown in Fig. 6 there was a prompt rise in ^{59}Fe -labeled heme stimulated by post-phlebotomy samples, indicating a rise in endogenous ESF. The addition of rabbit anti-ESF serum to post-phlebotomy serum samples completely inhibited the increase in heme labeling; normal rabbit serum had no effect.

Similar studies with grey collie serum confirmed the cycling nature of ESP much more precisely, as shown in Fig. 7. The cycle activity, as predicted by bioassay, fell out of phase with the reticulocyte count and had the characteristic interval of about 12 days. As estimated by simultaneously determined ESF dose/response curves *in vitro*, serum ESF activity varied from 0.02 to 0.4 U/ml. When normal dog serum was assayed in an analogous manner, no consistent or periodic change in assayable ESF levels was observed (Fig. 8).

The addition of anti-ESF serum to separately established cultures completely blocked the variation in basal heme synthesis (Fig. 9), implying that, in fact, the stimulating activity being measured was ESF. Normal rabbit serum had no effect on heme synthesis.

As the concentration of normal or grey collie serum was increased in culture there was first a rise, followed by a decline in basal (no exogenous ESF) heme synthesis. At no concentration did grey collie serum inhibit hemoglobin synthesis to levels below those seen with normal dog serum. When exogenous ESF was added to the cultures, no apparent inhibition of hormone effect was observed.

DISCUSSION

Hematopoiesis in the grey collie dog undergoes predictable and uniform cycling that involves multiple hemato-poietic cells including granulocytes, lymphocytes, and reticulocytes. The common denominator appear to be periodic marrow hypoplasia during which myeloblasts, pronormoblasts, and lymphocytes are markedly reduced in number.

Recently, experiments have shown the primacy of the marrow nature of this disorder, first by demonstrating that cyclic neutropenia in grey collies can be abolished by appropriate marrow allografting (10), and second,

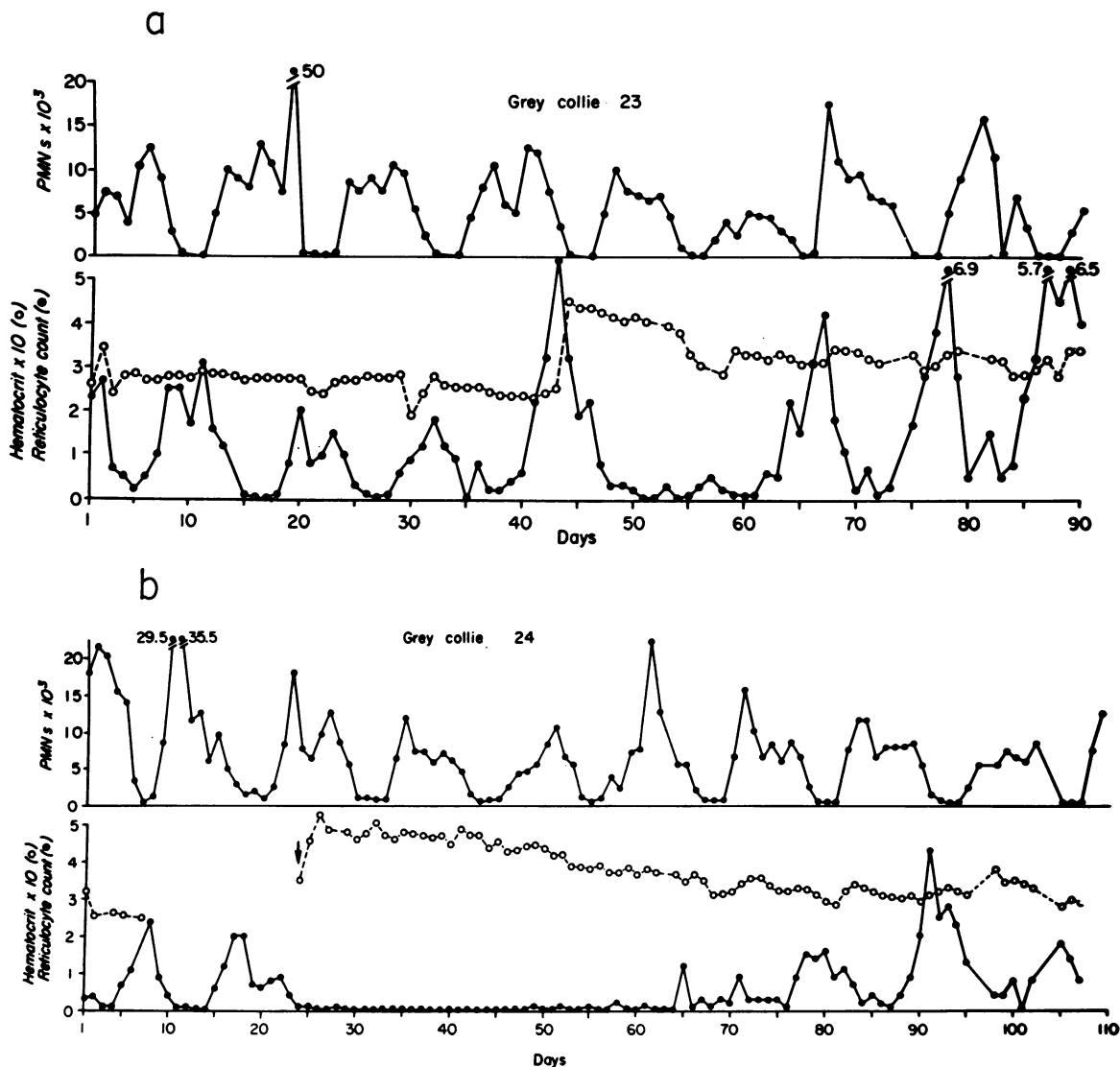


FIGURE 5 Effect of hypertransfusion on the reticulocyte count in two grey collies. Persistent suppression of reticulocyte production for a period of up to 40 days was observed after hypertransfusion with washed homologous red cells.

by the demonstration that cyclic hematopoiesis can be established in normal dogs by transplantation of grey collie marrow (11). Although these findings appear to remove host factors as possible determinants of the syndrome, the exact nature of the regulatory defect remains unknown.

While it has been shown previously that urinary CSA cycles in these animals, the state of CSA as a primary regulator of granulopoiesis, analogous to ESF, remains unresolved. Consequently, the studies reported here have concentrated on the question of humoral regulation in the observed cyclic erythropoiesis by both direct mea-

surements of ESF levels as well as the effect of phlebotomy and hypertransfusion on ESF and reticulocyte production. Before phlebotomy, ESF appears to cycle in grey collies in a fashion not observed in similarly studied normal dogs. Serum ESF levels in the grey collies were generally higher than levels in normal dogs, however, clear-cut cycling of ESF was not always discernible in the steady state because of the relative insensitivity of the polycythemic mouse assay. A more precise definition of ESF cycling was achieved with an *in vitro* assay employing normal dog marrow cells and grey collie serum. This technique, suggested by others

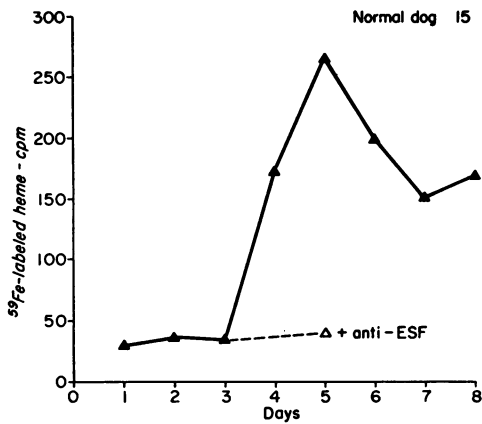


FIGURE 6 The effect of phlebotomy on endogenous ESF levels in normal collie serum as assayed in vitro. The sera employed had identical (adjusted) unlabeled iron levels and were cultured with 1.0×10^6 normal dog bone marrow cells in the absence of exogenous ESF. In the neutralization experiment, 25 μg of rabbit anti-ESF serum were added to the serum specimen showing the greatest in vitro activity.

(8, 12, 13), is sensitive, reproducible, and able to recognize concentrations of as little as 0.001–0.002 IRP U/culture dish, a degree of sensitivity similar to that reported by Wardle, Baker, Malpas, and Wrigley, (13) using mouse fetal liver cells. The application of the assay requires equalizing the pool of unlabeled iron in the test samples, and it promises to be most useful in providing information about serial changes in ESF in the same animal studied over a period of time.

Confirmation of ESF cycling was also obtained, however, by bleeding the grey collies. With a hematocrit reduction of 4–8 points, both trough and peak levels of serum ESF rose, with as much as 1.3 IRP U/ml seen post-phlebotomy; however, the cycling pattern of serum ESF levels was maintained. Such patterns were not observed in the normal dogs similarly stressed. Of significance, after the immediate response to phlebotomy, both ESF and the reticulocyte cycling fell back into phase

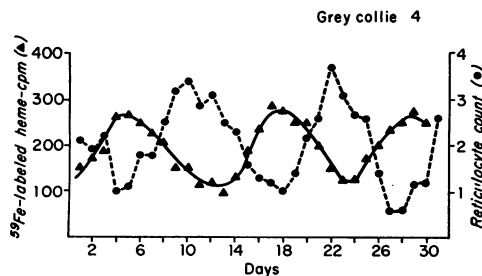


FIGURE 7 The relationship of daily endogenous serum ESF levels as assayed in vitro to the reticulocyte count in the grey collie.

with the previously determined periods. Further evidence for the contribution of ESF to erythropoiesis in these animals was shown by the hypertransfusion experiments which completely eliminated reticulocyte production.

Studies of ESF cycling both pre- and post-phlebotomy indicate that ESF and the reticulocyte count are out of phase. By replotting marrow and reticulocyte count data from the previously published studies (2, 3) ESF levels appear to be highest at a time when few pronormoblasts are evident in the marrow. Thus, serum ESF levels rise and approach maximum values 1–2 days before the appearance of recognizable red cell precursors in the bone marrow (Fig. 10). It is possible to speculate that the in vivo bone marrow response to ESF is intact and that the reticulocyte cycles are a direct consequence of erythropoiesis arising in response to varying levels of ESF stimulation. That ESF is, indeed, necessary for reticulocyte production in this animal is clearly demonstrated by the effects of hypertransfusion. In concert with this interpretation is the fact that after bleeding, both ESF and reticulocyte levels, while continuing to cycle, usually do not return to the low values observed in the basal state. This is particularly apparent in dog 28 (Fig. 4). Thus, with anemia, ESF production may remain elevated and the reticulocyte cycle reset at a higher level. Consequently, in the post-phlebotomy animal, the absence of reticulocytes and, presumably, marrow erythroid precursors is not seen.

The mechanism underlying ESF cycling in the grey collie is unknown. One possibility is that inhibitors of ESF or hemoglobin synthesis fluctuate in this setting. In order to assess this, the effect of increasing concentrations of serum on basal and ESF-dependent hemoglobin synthesis was measured. The grey collie serum utilized was from the high and low points of already established ESF activity. Comparison of the control and grey collie sera revealed no significant differences except those expected from differences in endogenous levels of ESF. Grey collie sera supported basal and ESF-dependent hemoglobin synthesis as well as normal sera. If inhibitors to ESF or hemoglobin synthesis are operative in this setting, then they must exist in delicate

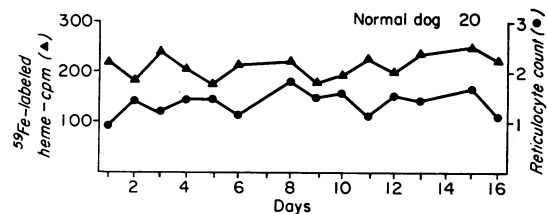


FIGURE 8 The relationship of daily endogenous serum ESF levels as assayed in vitro to the reticulocyte count in a normal dog. No evidence of cycling of ESF is observed.

balance with existing hormone, and their excess cannot be detected by these means.

Because of the nature of the periodicity, peaks of granulocytes and reticulocytes appear nearly out of phase, and thus ESF cycles in phase with the neutrophils. In adult dogs, the time of severe granulocytopenia is occasionally accompanied by fever and infection and this might account for the decline in ESF. However, this does not appear to be the case for two reasons. First, reticulocyte cycling is seen in young animals at a time when infection is rare, and cycles were chosen for analysis in our study when animals appeared clinically well. Secondly, similar changes in ESF and CSA have been described recently in a human with cyclic neutropenia (14). Here, the cycle times (21 days) are such that ESF and neutrophil peaks are not coincident and the nadir of neutrophils is not associated with infection or fever. Thus, we feel that the cycling of ESF is a property of the regulatory system and not an artifact of superimposed infection.

Marrow utilization of ESF is another possible explanation for the cycling of the hormone in this setting (15). It has long been recognized that the initial ESF response to anemia or hypoxia is muted within a period of hours or days (16-18). It has been postulated that as erythroid proliferation progresses, ESF is utilized by the expanding marrow and serum levels fall. This possibility, although attractive in many ways, again is made unlikely by several observations. First, since this thesis was advanced, a number of direct attempts to document marrow utilization of ESF have failed, including studies in dogs (19, 20). Secondly, the pattern of the ESF response to hypoxia has recently been shown to be due to the gradual enhancement of tissue oxygen delivery achieved by a decreased hemoglobin affinity for oxygen—a rightward shift in the oxygen-hemoglobin dissociation curve (21). Consistent with this observation are studies over a wide hematocrit range in humans which have failed to show differences in ESF excretion on the basis of the degree of marrow erythroid proliferation (22). Finally, studies in human cyclic neutropenia clearly demonstrate that ESF rises and remains elevated for a period of time seemingly inconsistent with marrow utilization as the cause for eventual hormone decline (14). Nonetheless, marrow utilization of ESF remains a possibility.

Another entirely speculative but possible explanation for ESF cycling is that there is regulatory feedback from proliferating marrow cells on the synthesis of humoral regulators. Thus, as the bone marrow achieves a certain state of proliferation or a particular cell compartment attains critical size, there is suppression of the biogenesis of ESF. This interpretation would be in concert with several observations relating to serum or urine ESF levels in patients around the time of

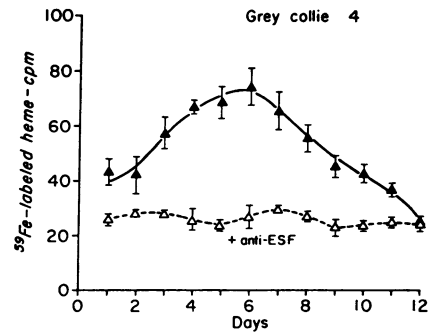


FIGURE 9 The effect of anti-ESF serum on fluctuations in endogenous serum ESF levels in the grey collie as assayed *in vitro*. Neutralization of the erythropoietic stimulating activity of grey collie serum was achieved in each case. Normal rabbit serum had no inhibitory effect on heme synthesis.

recovery from aplastic anemia (23) or during the conversion to normoblastic erythroid hyperplasia after correction of folate or vitamin B₁₂ deficiency (24, 25). In these instances, ESF levels fall before any increase in hemoglobin or hematocrit. In the cases of marrow aplasia, it seemed reasonable to hypothesize increased utilization of ESF with the onset of erythroid proliferation. This explanation seems less tenable, however, in those patients whose marrow already shows marked erythroid hyperplasia at the outset. Feedback regulation of ESF biogenesis could account for both of these observations.

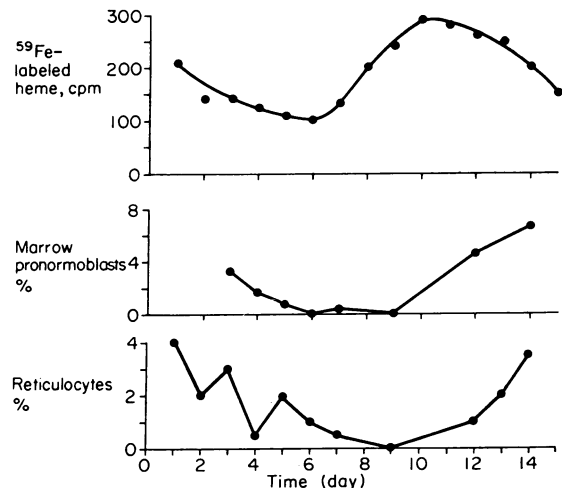


FIGURE 10 The temporal relationship between endogenous ESF, marrow pronormoblasts, and the reticulocyte count in the grey collie dog. The relationship between ESF and reticulocyte peaks was derived from the study shown in Fig. 7 and then extrapolated to the relationship between marrow pronormoblasts and reticulocytes for other grey collies as previously published by Dale, Ward, Kimball, and Wolff (3).

Cycling of reticulocytes in normal dogs (26) and of granulocytes in cyclophosphamide-treated dogs (27) has been reported by Morley and Stohlman. They were able to shift the phase of the underlying cycles of reticulocytes and granulocytes in their animals by phlebotomy/retransfusion and the administration of antineutrophilic serum, respectively. The maneuvers described here failed to alter the basic periodicity or the temporal relationship between reticulocytes and neutrophils. In addition, the administration of antineutrophilic serum to grey collies left unaltered the underlying neutrophil cycles.⁴ Consequently, the periodic nature of this syndrome appears much more deeply entrained than that reported in the normal or marrow-suppressed animal.

A final point derived from these studies relates to the question of stem cell competition or demand for granulocytic or erythrocytic production (28, 29). In this study, none of the procedures employed to manipulate peripheral counts affected the levels or periodicity of other blood elements. This included a period of 40 days of virtually complete erythroid suppression achieved by hypertransfusion (Fig. 5). While such competition for expression may be the nature of the defect, as suggested by Patt, Lund, and Maloney (30), these studies would not support that hypothesis.

While the mechanism of ESF cycling in this syndrome and in human cyclic hematopoiesis is not known, the data clearly show that periodic fluctuation of long-range humoral regulators is an integral part of the syndrome.

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