Canine Cyclic Neutropenia: Erythropoietin and Platelet Cycles After Bone Marrow Transplantation

By J. B. Jones, R. D. Lange, T. J. Yang, H. Vodopick, and E. S. Jones

The marrow of a dog affected with cyclic neutropenia (CN) was transplanted into an unaffected supralethally irradiated littermate. Prompt engraftment occurred, and cyclic rises and falls in numbers of platelets, reticulocytes, and granulocytes were noted in the recipient soon after engraftment. Prior to transplantation and under hypoxic conditions, the donor had serum erythropoietin (ESF) peak levels at 11–12-day intervals. Following transplantation and under hypoxic conditions, cyclic peaks of ESF occurred in the transplanted dog.

Canine cyclic neutropenia (CN) is characterized by profound fluctuations in the neutrophil count, and affected dogs have an unusual grey coat color.1,2 The disease is inherited in an autosomal recessive pattern.1 Cyclic changes of all cellular hematologic elements occur in CN dogs, and it is likely these animals experience cyclic hematopoiesis.4 Successful bone marrow transplants of CN dog marrow into a normal dog5 and normal marrow into a CN dog6 have shown that the disease may be established or abrogated by appropriate allografting. The grafting studies5,6 suggest that the disease is caused by a primary marrow disorder. Marrow responses to hematopoietic stimuli have not been studied in the animals bearing marrow grafts. In CN dogs, serum7 and urinary8 colony-stimulating factor (CSF) levels have been shown to vary cyclically. In this report, data are presented to demonstrate cyclic variations of granulocytes, reticulocytes, and platelets, as well as to show cycles of erythropoietin (ESF) production in a CN dog and in the normal littermate following the transplantation of marrow from the CN dog.

MATERIALS AND METHODS

The three dogs (CN No. 217, normal No. 219, and normal No. 220) used in these studies were female littermates of the collie-beagle genotype9 born in this laboratory. The dog identified as CN No. 217 was used as the marrow donor and normal No. 220 as the recipient. This pair of dogs, typed at the Mary Imogene Bassett Hospital, Cooperstown, N.Y., was found to be DL-A matched.10 One-way mixed leukocyte culture studies11 using irradiated cells as stimulators showed very low stimulatory activity; a stimulation index (S.I.) of only 1.08 was found when normal No. 220 irradiated cells were used to stimulate CN No. 217 cells and of only 1.30 when CN No. 217 cells were used to stimulate normal No. 220. (A S.I. of greater than 2 is considered significant).

At the time of transplantation, the dogs were 8 mo of age; No. 220, the normal recipient of CN marrow, weighed 10 kg, and the CN marrow donor weighed 7 kg. The recipient was irradiated with 1250 R total body irradiation at 9 R/min using 60Co sources.12 Four hours later,
3.24 x 10^9 viable marrow cells from the CN dog No. 217 were infused intravenously into No. 220. The donor cells were collected from the four long bones of the donor following exsanguination under pentobarbital anesthesia. Cell collection was made in serum-free Medium 199 (Grand Island Biological Co., Grand Island, N.Y.), and marrow cells were defined as those approximately 9 μ or larger. Viability was determined with trypan blue exclusion test.

Beginning the day before transplantation and continuing for 5 days following the transplantation, the recipient dog was given no food and was sustained by subcutaneously administered Ringer’s solution. Principen/N (E. R. Squibb and Sons, New York, N.Y.), 250 mg twice daily for 12 days, Polymagma (Wyeth Laboratories, Philadelphia, Pa.), twice daily for 6 days, and methotrexate (Eli Lilly Co., Indianapolis, Ind.), 5 mg, given only on day 4 post-transplant, were the only drugs administered to the recipient during the post-transplantation period. Each morning, 1-2-cc samples of blood were collected in EDTA for total leukocyte counts, determinations of platelets, and hematocrits as well as differential leukocyte counts. Erythrocyte counts were not performed. Leukocyte counts were made using an Autocytometer II (Fisher Scientific Co.), and differential counts were made on 100 leukocytes from smears stained with Wright’s stain. Platelets and reticulocytes were enumerated according to standard techniques,9 and hematocrits were determined by the microhematocrit technique. Total granulocyte counts are the product of the leukocyte count and the sum of the per cent polymorphonuclear leukocytes and immature forms.

Sixty to ninety days before the transplantation experiment, CN dog No. 217 (donor) and normal No. 219 were subjected to hypoxia (in a partial vacuum, 0.5-0.54 atmosphere) for 24 days, 23 hr each day. During the daily 1-hr period outside the hypoxic chamber, 22-25 ml of blood were collected for cell counts and serum ESF studies. The serum was separated from cells by centrifugation and stored at -20°C until assayed for ESF. These sera were used for pre-transplantation ESF studies of the CN dog and the normal littermate.

On post-transplant day 45, dog No. 220 showed no evidence of graft-versus-host disease and was placed in hypoxia for 24 days as described for the pretransplant marrow donor. Daily blood and serum samples were collected for cell counts and serum ESF determinations.

Serum ESF studies were carried out in the previously described bioassay system. Exhypoxic, polycythemic mice were test animals in the ESF bioassay system, and each mouse was injected subcutaneously with 1.0 ml of the test serum. Appropriate standard ESF preparations were used in each assay. The 59Fe RBC iron incorporation values were converted to units of ESF by a computer program.

RESULTS

The recipient dog’s total leukocyte count dropped to 15% of the pretreatment count by the second postirradiation day and began to recover by day 13. However, the platelet count did not drop until day 6 but similarly started to recover

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Fig. 1. Daily granulocyte, platelet, and reticulocyte values for normal dog No. 220 bearing a marrow graft from CN No. 217.
Fig. 2. Daily hematocrit, reticulocyte, and ESF values during 24 days of hypoxia for (A) CN No. 217, (B) dog No. 220 following a marrow graft from CN No. 217, and (C) normal dog No. 219. The arrows in (A) and (B) indicate neutropenic episodes.

After day 13. In Fig. 1 are illustrated two neutropenic episodes in the normal recipient during the 27th to the 46th day following transplantation of the CN marrow. Also shown in this figure are the cyclic rises and falls of platelets and reticulocytes.

Pretransplantation serum ESF levels for serum samples collected from the donor CN dog on 24 consecutive days of hypoxic stimulation are graphed in Fig. 2A together with the daily reticulocyte counts and hematocrit values. Erythropoietin peaks occurred at 11–12-day intervals and were followed by peak reticulocyte values 1–3 days later. Hematocrit values fluctuated irregularly and decreased slightly towards the end of the study. Figure 2B depicts the 24 daily ESF values found in the sera of the transplanted dog that was placed in hypoxia on post-transplant day 45. Also shown are daily reticulocyte counts and hematocrit values during that time. Peaks of ESF activity were found on days 2, 10, 15, and 19, but levels were not as high as in the intact CN dog (Fig. 2A). Hematocrit values fluctuated irregularly between 32% and 42% but remained essentially at the prehypoxic level. In Fig. 2C are shown the serum
ESF values and daily reticulocyte counts for normal dog No. 219 exposed to hypoxia within the same chamber but 30 days prior to hypoxic exposure of CN No. 217. In the normal dog, only the initial rise in ESF level was noted, and there were no secondary peaks as shown in Figs. 2A and 2B. The normal dog's reticulocyte counts fluctuated irregularly but remained elevated throughout the hypoxic period. Hematocrit values of the normal dog fluctuated irregularly between 38% and 48% but were probably essentially unchanged.

During hypoxia the neutrophil counts of CN No. 217 and the transplanted dog No. 220 continued to show regular cyclic fluctuations typical of CN dogs in room air environments. The normal dog (No. 219) had irregular neutrophil counts during hypoxia, but the values were within the normal range.

**DISCUSSION**

In extensive transplantation studies in dogs, Storb et al. have shown that circulating leukocytes and platelets begin to return to normal levels soon after the 12th post-transplantation day. This was shown to occur in the present study of the transplanted dog. The CN donor's cycles of neutropenia were quickly established in the normal recipient dog and became evident as soon as the CN donor cell engraftment was capable of producing significant quantities of leukocytes (Fig. 1). Earlier studies of CN marrow transplantation have not included platelet counts, but Fig. 1 clearly shows these elements also cycle when CN marrow is transplanted into a normal dog. The pre- (CN-dog) and post-transplantation (CN-normal chimera) dogs' cycles of granulocytes, platelets, and reticulocytes closely resembled cycles of those elements in nongrafted dogs. Reports from three laboratories show that CN dogs have cycles of neutropenia accompanied by platelet and reticulocyte cycles that reach a peak near the onset of severe neutropenia (Fig. 1). Granulocyte and reticulocyte values continued to cycle in the hypoxic CN dog. The normal dog transplanted with CN marrow (Fig. 2B) had regular cycles of neutropenia during the period of hypoxia, but cyclic reticulocyte counts were not as well defined as in CN No. 217. The stress of hypoxia experiments may have affected reticulocyte cycling but had minimal effect on the cycles of neutropenia.

Hematocrit values did not increase in any of the three dogs during hypoxia and in fact declined slightly in the CN dog (Fig. 2). Dogs with CN are known to have a microcytic anemia. Significant quantities of blood were withdrawn daily, and it is likely that daily bleeding offset the effects of hypoxia on red cell mass. Hematocrits of all three dogs fluctuated within a ten-point range, and changes in reticulocyte counts could not be related to changes in the hematocrit. All of the dogs had the expected initial ESF response to hypoxia.

Cyclic fluctuation of CSF found both in the urine and in the serum of CN dogs has been demonstrated. Previously, Adamson et al., in an abstract, have reported cyclic variations in serum erythropoietin levels in CN dogs. Our report confirms these observations of erythropoietin fluctuations in CN dogs. Serum ESF levels in CN dog No. 217 and in the post-transplantation chimera No. 220 followed cyclic patterns when the dogs were exposed to hypoxia. The ESF peaks in these two dogs were not completely synchronous. In fact, the peaks of ESF in the transplanted dog seemed to occur at 4-5-day intervals and
are difficult to explain in relationship to cycles of granulocytes and reticulocytes. The shortened cycles could reflect a change in a delicate feedback control system. Dale and Graw have suggested that the cyclic variations of erythropoietin and colony-stimulating factor are normal physiologic responses in an animal with defective marrow cells. The cyclic fluctuations of ESF in CN No. 217 and its chimera No. 220 lend credence to this suggestion. However, somewhat similar cyclic variations have been found in mice exposed to irradiation and hypoxia. The relationship between the stimulating factors and the response in the bone marrow and peripheral blood are still unresolved. Indeed, the cyclic variations in the ESF levels seen under hypoxic conditions in the CN dog and the CN transplant chimera but not in the normal dog would suggest that some still unknown factors influence the regulation of erythropoietin in these animals.

In general, two defects known to occur in mice could explain the occurrence of cyclic hematopoiesis in the CN dogs. One defect is in the stem cell and the other in the environment. Two previous papers describing engraftment studies involving CN collies inferred that the defect was in the marrow pluripotent cells. With the additional evidence for cyclic behavior of the blood platelets presented in our report, the evidence for a defect in the pluripotent stem cell is strengthened. Either periodic failure of marrow cell production or competition for limited numbers of stem cells seem to be likely causes of CN disease. Although available data point to a stem cell defect, internal environmental influences have not been excluded as a contributory factor. Studies reported herein and now in progress in this laboratory indicate that CN disease is a complex phenomenon that will require careful studies before conclusions can be drawn as to the exact nature of the defect.

One current model of hematopoietic cell kinetics envisions a committed stem cell compartment, individual elements of which can be stimulated to form differentiated cells. For instance, the erythroid-committed cell under the influence of erythropoietin differentiates into mature red blood cells. The committed stem cell compartment is thought to be largely self-sustaining but, if needed, can be repopulated by pluripotential cells which are capable of differentiating into either myeloid, megakaryocytic, or erythroid elements. There is evidence to support such a model, and the evidence may be augmented with the use of CN dogs.

In the present experiments, the littermate receiving the CN dog's marrow may have been heterozygous for the CN defect. Presently, CN heterozygosity is detectable only by test mating, and the shortened life span of CN dogs precludes a delay in transplantation until the heterozygosity of potential donors can be determined by this means. That heterozygosity may influence the behavior of granulocyte cycles cannot, as yet, be eliminated, since slight fluctuation of granulocytes occurred even in the normal dog No. 219. Dale and Graw's experiments dealing with the transplantation of marrow from a normal unrelated dog into a CN dog, whose granulocyte cycles then ceased, suggest that homozygosity is a prerequisite for the cyclic neutropenia of dogs with the CN phenomenon.

Several authors have pointed out similarities between human and canine
cyclic neutropenia. As marrow transplantation in human subjects begins to be conducted extensively, the dog transplantation experiments may point the way to the possible amelioration of this disease afflicting dog and man.

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