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Platelet Kinetics in Canine Ehrlichiosis: Evidence for Increased Platelet Destruction as the Cause of Thrombocytopenia

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A significant ($P < 0.025$) increase in the mean platelet diameter occurred in five *Ehrlichia canis*-infected dogs when platelet numbers decreased to 100,000/μl or less. Maximal incorporation of [^{75}Se]selenomethionine into platelets of six uninfected dogs was 0.080 ± 0.019% (mean ± standard error) and occurred 5 to 6 days after dosage, whereas maximal incorporation was 0.036 ± 0.004% within 2 to 3 days after dosage in seven chronically infected dogs that had thrombocytopenia. Analysis of the [^{75}Se]selenomethionine curves yielded a platelet lifespan of 9 days in uninfected dogs versus 4 days in chronically infected dogs. Thus, megakaryocyte maturation and/or platelet release occurred at an accelerated rate in infected dogs, whereas increased destruction of newly produced labeled platelets diminished their number of peripheral blood. [^{51}Cr]sodium chromate-labeled platelet survival was exponential, with a half-life of approximately 1 day in two dogs at 2 to 4 days postinfection and three chronically infected dogs. Platelet survival time was 8 days and rectilinear in four uninfected dogs. Platelet recovery was 39.43 ± 2.86% in infected dogs as compared with 68.2 ± 10.72% in uninfected dogs. Whole-body scans of one dog prior to and 7 days after infection showed that labeled platelets were destroyed primarily in the spleen. It is concluded that the thrombocytopenia in *E. canis*-infected dogs is the result of increased platelet destruction which begins within a few days after infection.

Canine ehrlichiosis (tropical canine pancytopenia), caused by the rickettsial agent *Ehrlichia canis*, is a febrile, tick-borne disease manifested by pancytopenia, particularly thrombocytopenia (17, 18). Dogs frequently undergo a clinical recovery from the acute illness, but hematologic abnormalities persist and infective organisms continue to circulate in the blood (17, 35). The persistent thrombocytopenia of chronic canine ehrlichiosis often precedes a hemorrhagic crisis and death, especially in German shepherd dogs.

Histopathologic studies of laboratory and field infections have shown decreased marrow cellularity (P. K. Hildebrandt, D. L. Huxsoll, and R. M. Nims, Fed. Proc. **29**:754, 1970; 15, 22). Survival of ^{32}P-labeled platelets in normal and infected dogs indicated that decreased platelet production may account for the thrombocytopenia of canine ehrlichiosis (36). The occurrence of many megathrombocytes in the peripheral blood, however, suggested that thrombopoiesis had increased in thrombocytopenic dogs.

In the present study platelet production and destruction were compared in normal dogs and in thrombocytopenic dogs infected with *E. canis* by measurement of megathrombocyte production, rate, and percentage of incorporation of [^{75}Se]selenomethionine into newly formed platelets and rate and site of ^{51}Cr-labeled platelet destruction.

MATERIALS AND METHODS

Animals and inoculation procedure. A total of 21 mixed-breed dogs 1 year old or older, weighing 9.1 to 21.3 kg, were used in the study. The animals were infected with *E. canis* by intravenous inoculation of 5 ml of blood from a carrier dog.

Megathrombocyte production. Peripheral blood was collected in syringes containing disodium ethelenediaminetetraacetic acid. Wright-stained blood smears were prepared, and the mean platelet diameter was obtained with a calibrated ocular micrometer. Serial pre- and postinfection smears were examined from five dogs, and 50 platelets were

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randomly selected and measured on each blood smear.

Platelet measurements were also made on smears from a dog treated with rabbit anti-dog platelet antiserum to induce thrombocytopenia. One dog was injected intravenously with 1.0 ml of undiluted rabbit antiserum. This antiserum was absorbed five times with washed erythrocytes from the recipient prior to use.

Rate and percentage of incorporation of [^{75}Se]-selenomethionine into circulating platelets. Approximately 50 μCi (0.12 to 0.3 μg) of [^{75}Se]selenomethionine (Sethotope, E. R. Squibb and Sons, New Brunswick, N.J.) was injected intravenously into normal and thrombocytopenic *E. canis*-infected dogs.

Platelets were periodically isolated and quantitated by procedures previously described (35), with the exception that platelet radioactivity was measured with a gamma well scintillation spectrometer. Canine blood volume was assumed to be 74.8 ml per kg of body weight (23).

Rate and site of ^{51}Cr-labeled platelet destruction. ^{51}Cr in the form of sodium chromate (sterile solution in isotonic saline; 50 to 400 mCi/mg of Cr; Amersham/Searle, Arlington Heights, Ill.) was used for in vitro labeling of platelets from uninfected and *E. canis*-infected dogs. Two of the dogs were studied 2 to 4 days after infection when thrombocytopenia was commencing. The remaining infected dogs were given labeled platelets 2 to 5 months postinfection.

The in vitro labeling procedure was that of Abrahamsen (1) with minor modifications. Approximately 170 ml of whole blood was collected aseptically for platelet separation. Siliconized glass bottles and polyethylene centrifuge tubes were used in place of plastic bags, and erythrocytes and leukocytes obtained by centrifugation procedures were transfused into the donor dog. Labeling was achieved with 100 to 200 μCi of [^{51}Cr]sodium chromate, and ascorbic acid was eliminated from the procedure.

One milliter of the platelet suspension was removed for preparation of standards, and 19 ml was injected into the dog via the cephalic vein. Blood samples (2 ml) were collected from the jugular vein at 15 min, 1 h, 2 h, and 24 h after administration and daily thereafter for determination of radioactivity using a well-type gamma scintillation spectrometer. Approximately 10% of the label was taken up by dog platelets.

The location of the spleen and liver of one dog prior to infection was ascertained by injecting 500 μCi of technetium 99m sulfur colloid (Tesuloid; E. R. Squibb and Sons, Princeton, N.J.) into the cephalic vein and visualization with a gamma camera (Nuclear Chicago, Des Plaines, Ill.). Two weeks before inoculation with *E. canis*, the dog was injected with autologous platelets labeled with 400 to 600 μCi of [^{51}Cr]sodium chromate. When 90% of the ^{51}Cr-labeled platelets had been removed from the circulatory system, the dog was examined with the gamma camera. Four days postinoculation, when the thrombocyte count began to decrease, the procedure was repeated. Pre- and postinoculation images obtained were compared with the images obtained by injection of technetium 99m sulfur colloid to locate the site of platelet destruction.

Statistical analysis of data followed standard recommended procedures (16).

RESULTS

Before measuring changes in platelet diameter during infection, thrombocytopenia was induced in a normal dog by injection of undiluted rabbit anti-dog platelet antiserum. The platelet count dropped precipitously from 251,000/μl to 42,000/μl within 1 h. A simultaneous increase in mean platelet diameter occurred. Platelet numbers subsequently increased and platelet size decreased as thrombopoiesis normalized.

A megathrombocyte response was elicited by all five *E. canis*-infected dogs after platelet numbers decreased below 100,000 per μl of blood. Megathrombocyte release was sufficient to significantly increase ($P < 0.025$) the mean platelet diameters of all dogs. The preinfection platelet diameter (mean $\pm$ standard error) was 3.224 $\pm$ 0.119 μm compared with 3.844 $\pm$ 0.119 μm in thrombocytopenic dogs.

Thrombopoiesis, as measured by percentage of incorporation of [^{75}Se]selenomethionine into platelet proteins, was reduced in thrombocytopenic *E. canis*-infected dogs (Fig. 1). Maximal incorporation of label in uninfected dogs was 0.080 $\pm$ 0.019% (mean $\pm$ standard error) as compared with 0.036 $\pm$ 0.004% in infected dogs. Maximal uptake of label occurred between 5 and 6 days after label administration in uninfected dogs and 2 to 3 days in *E. canis*-infected dogs.

Platelet survival in uninfected and infected dogs was extrapolated from the ^{75}Se labeling curve by measuring the time interval between the 50% labeling index on the ascending and descending slopes (7, 27). By this method, platelet survival was approximately 4 days in infected dogs versus 9 days in uninfected dogs.

^{51}Cr-labeled platelet survival in chronically infected thrombocytopenic dogs differed from that of uninfected dogs (Fig. 2). The uninfected dog platelet survival curve was slightly curvilinear and did not conform to a linear or exponential equation. Mean platelet survival was 8 days in all uninfected dogs.

The platelet survival curve for chronically infected dogs was exponential, with a half-life of approximately 1 day. The maximum percentage of recovery of labeled platelets in the peripheral blood of infected dogs was only 39.43 $\pm$ 2.86% as compared with 68.2 $\pm$ 10.72% in infected dogs.

^{51}Cr-labeled platelet survival in two dogs 2 to 4 days after infection was similarly reduced (Fig. 3). Platelet survival was considerably

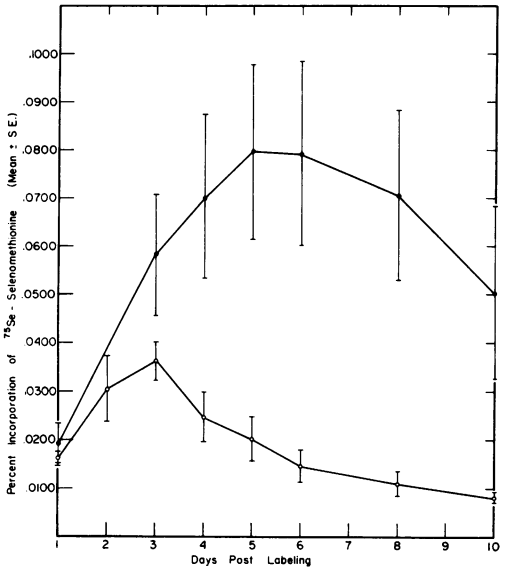

FIG. 1. *Percentage of incorporation of [^{75}Se]-selenomethionine into newly formed platelets of six uninfected (●) and seven thrombocytopenic E. canis-infected (○) dogs during the chronic phase of infection. (Bars represent ± 1 standard error.) Platelet count (mean ± standard error) of uninfected dogs was 292,500 ± 35,000 per μl versus 41,570 ± 7,640 per μl in infected dogs.*

shortened and exponential shortly after infection when the platelet count was declining, but prior to the onset of clinical signs. Whole-body scanning of one of these dogs prior to and 7 days after infection (3 days postlabeling) showed that the labeled platelets were destroyed principally in the spleen at both times (Fig. 4).

DISCUSSION

Studies in dogs (21, 24) and man (2, 37) have shown that thrombocytopenia stimulates thrombopoiesis. Presumably thrombopoiesis is stimulated by thrombopoietin, which results in the appearance of an increased proportion of large young platelets in the peripheral circulation (19, 20, 33). Quantitation of the response has been measured and expressed as the percentage of megathrombocytes (13), platelet volume and density (24, 37), diameter (31), and surface area (21). Blood smears, electronic particle counters, and density gradients have been used.

Mean platelet diameter was found to be an easy and accurate method for detecting changes in platelet size in response to both antibody- and *E. canis*-induced thrombocytopenia. The finding that canine platelet size increased after experimental thrombocytopenia agrees with the findings of others (21, 24). The fact that a similar response occurred in thrombocytopenic dogs infected with *E. canis* indicated that a feedback mechanism was sensitive to thrombocytopenia and that the megakaryocytes were capable of responding to stimulation. The presence of megathrombocytes during canine ehrlichiosis was reported previously (32).

Although thrombopoiesis is stimulated by the thrombocytopenia in canine ehrlichiosis, the bone marrow response appeared to be inadequate, as determined by incorporation of [^{75}Se]-selenomethionine into platelets by uninfected and infected dogs. However, the decrease in platelet survival time, observed with ^{51}Cr, may have prevented the selenomethionine from reaching its peak activity due to early destruction of newly produced, labeled platelets. Such an occurrence was also suggested when platelet survival was extrapolated from the 50% labeling index on the upward and downward slopes of the [^{75}Se]selenomethionine curve. ^{75}Se labeling in *E. canis*-infected dogs was initiated after they had recovered from the initial infection. Aside from hypergamma-globulinemia, blood chemistry was normal during this phase of the disease (17) and it is unlikely that alterations in

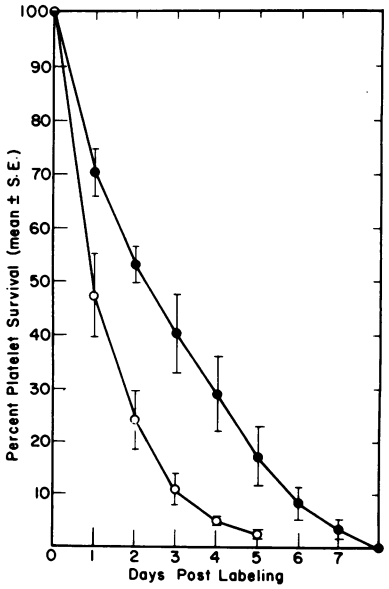

FIG. 2. *^{51}Cr-labeled platelet survival in four uninfected (●) and three E. canis-infected (○) dogs during the chronic phase of infection. (Bars represent ± 1 standard error.) Platelet count (mean ± standard error) of uninfected dogs was 273,000 ± 2,900 per μl versus 49,000 ± 8,000 per μl in infected dogs.*

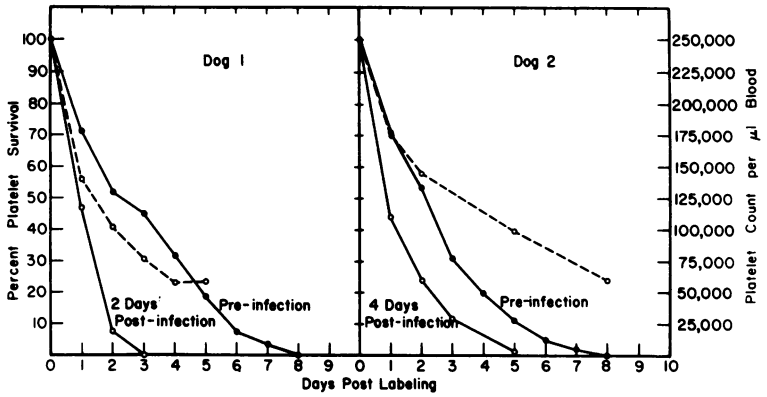

FIG. 3. *^{51}Cr-labeled platelet survival in dogs prior to (●——●) and 2 to 4 days after (O——O) intravenous inoculation of 5 ml of E. canis carrier blood. Peripheral platelet count (O- -O) is depicted from the day platelet labeling occurred.*

splenic or hepatic function or blood volume occurred which might have affected the calculation of [^{75}Se]selenomethionine uptake.

The rate of maturation of platelet precursors was in agreement with the findings of others (10). Accelerated maturation of platelet precursors during canine ehrlichiosis was comparable to findings in experimentally induced immune thrombocytopenia (30) and thrombocytopenia due to exchange transfusions (14). In these cases, however, incorporation of radioactivity was much greater than normal. The labeling pattern with [^{75}Se]selenomethionine in chronically infected dogs is consistent with a model of decreased numbers of physiologically active platelet-producing cells in the marrow.

The results of platelet survival studies with ^{51}Cr were not in complete agreement with earlier findings utilizing ^{32}P-labeled diisopropyl fluorophosphate (35). Both ^{32}P- and ^{51}Cr-labeled platelets were rapidly removed from the circulation early in the disease when platelet numbers were decreasing. Platelet survival in chronically infected dogs was only moderately reduced and linear with ^{32}P, whereas it was short and exponential with ^{51}C. [^{75}Se]selenomethionine data also indicated that platelet survival was considerably decreased.

One possible explanation for the observed discrepancy between the ^{32}P-labeled diisopropyl fluorophosphate and ^{51}Cr labeling data is the reported effect of high doses of ^{32}P-labeled diisopropyl fluorophosphate upon platelet behavior in vitro and in vivo (9, 26). An alternate explanation may be reutilization of ^{32}P-labeled blood components for the production of new platelets (11, 25). This phenomenon accounts for the "tailing" of ^{32}P-labeled platelet survival

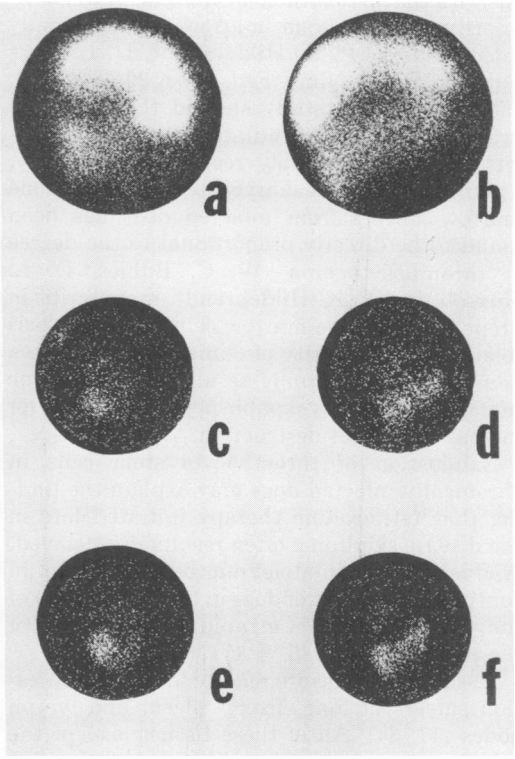

FIG. 4. *Appearance of the liver and spleen on dorso-ventral (a) and left lateral (b) views with a gamma camera after intravenous injection of 500 μCi of technetium 99m sulfur colloid into an uninfected dog. The liver appears as a dense image on the upper portion of each scan, whereas the spleen is a ball in the lower left region of (a) and a band across the lower portion of (b). ^{51}Cr-labeled platelet destruction occurred principally in the spleen prior to (c, d) and 7 days after intravenous injection of 5 ml of E. canis-infective blood (e, f).*

and differences between lifespan estimates when comparing ^{32}P and ^{75}Se with ^{51}Cr, which is not reutilized (4, 27).

An accurate assessment of platelet production from ^{51}Cr-labeled platelet survival requires that the effect of platelet sequestration or pooling upon platelet mass and survival be approximated. In this study it was not clear whether initial platelet loss was due to sequestration and pooling or should be considered part of platelet survival. These considerations have hampered assessment of thrombokinetics in platelet destructive syndromes in man (5, 6). Platelet destruction did occur at an accelerated rate during *E. canis* infections and was the primary cause of thrombocytopenia in affected dogs.

The radioisotopic evidence for decreased numbers of platelet-producing cells was consistent with the histopathologic findings of hypocellularity in the bone marrow of chronically infected dogs (P. K. Hildebrandt, D. L. Huxsoll, and R. M. Nims, Fed. Proc. **29**:754, 1970; 15). The present study showed that the cells present were being stimulated to produce platelets and were probably responding normally. The number of megakaryocyte present in bone marrow smears from infected dogs has been found to be directly proportional to the degree of thrombocytopenia (W. C. Buhles, D. L. Huxsoll, and P. K. Hildebrandt, manuscript in preparation). Bone marrow cellularity also was related to the severity of clinical signs. Thus, a hemorrhagic crisis may result when the bone marrow no longer is capable of compensating for increased platelet destruction.

Exhaustion of thrombocyte stem cells in chronically infected dogs may explain the finding that tetracycline therapy initiated late in the disease syndrome often results in a delayed, gradual return of platelet numbers to normal. In contrast, treatment of dogs in the acute phase of the disease results is a rapid normalization of the platelet counts (3, 8, 17).

E. canis morulae are readily found in impression smears of lung, liver, spleen, and lymph nodes (17, 34). All of these tissues, except the spleen, were negative for ^{51}Cr-labeled platelets during the period of increased platelet destruction. It appeared that direct involvement of platelets in the inflammatory processes in these organs did not occur. Destruction of platelets principally in the spleen was similar to that which occurred in immunologically mediated idiopathic thrombocytopenic purpura of man (29). The decrease in platelet survival time 2 to 4 days after inoculation with *E. canis*-infective blood, however, occurred too rapidly for an antibody-mediated response. The possible role of other factors (12, 28), i.e., circulating immune complexes, endotoxin, or vascular endothelial injury, as causes of thrombocytopenia in canine ehrlichiosis should be investigated.

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