Thrombocytopenia in a Mouse Model of Human Granulocytic Ehrlichiosis

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Despite its apparently strict granulocytotropism, thrombocytopenia is a consistent hallmark of infection with the agent of human granulocytic ehrlichiosis (HGE), regardless of host species. Laboratory mice are valuable models of HGE agent infection kinetics and immunity, but initial studies of HGE infection in mouse models have failed to demonstrate thrombocytopenia. More thorough analysis of platelet kinetics, however, reveals a consistent and rapid, marked decrease (50% decline by day 2–4 after infection) in circulating platelet number in both C3H/HeN and C57BL/6J mice during infection with the HGE agent. The roles of splenic consumption and immune-mediated destruction were evaluated as potential mechanisms of the thrombocytopenia. Both splenectomized mice and mice with severe combined immunodeficiency (lacking B and T lymphocytes) became similarly thrombocytopenic in response to infection with the HGE agent. This study validates the appropriateness of the mouse as a model of HGE, including its usefulness for the investigation of thrombocytopenia.

Human granulocytic ehrlichiosis (HGE) is an emerging, potentially fatal tickborne zoonotic disease caused by an obligate intracellular bacterium. *Ehrlichia* species infect a wide array of mammalian hosts and long have been recognized to be significant veterinary pathogens [1]. Most *Ehrlichia* species have an exclusive intracellular tropism for a single, specific hematopoietic cell lineage. In spite of this, thrombocytopenia is a consistent hallmark of ehrlichial infection, regardless of the pathogen’s intracellular venue, in human beings and a variety of animals [2–4]. We hypothesize that the mechanisms underlying this thrombocytopenia are a central feature of disease pathogenesis.

Mechanisms underlying the pathophysiology of ehrlichial thrombocytopenia are not well understood. Decreased hematopoietic production of platelets certainly contributes to the marked pancytopenia noted in association with chronic canine monocytic ehrlichiosis. However, in human monocytic ehrlichiosis, cytopenias occur in conjunction with normal or hypercellular marrow [5], with only scattered case reports of bone marrow hypoplasia [6]. Discrepancies may be due in large part to the timing of bone marrow evaluation in relation to disease progression and severity. Increased peripheral destruction of platelets [7] and decreased platelet life span [8] with splenic consumption [9] have been reported in association with acute canine monocytic ehrlichiosis, and the presence of antiplatelet antibodies has been described as early as 7 days after infection [10, 11].

Laboratory mice are useful for investigation of HGE agent pathogenesis and infection kinetics that are not accessible with in vitro or cell culture systems. It has been demonstrated that both C3H/HeN (C3H) and C57BL/6J (B6) mice are susceptible to infection, as measured by blood smear examination, polymerase chain reaction, and xenodiagnosis [12]. In addition, these mice show hematologic, immunologic, and pathologic responses to infection that mimic infection in humans and other models of disease. However, the kinetics of infection with the HGE agent, including infection burden and rate of bacterial clearance, differ in these 2 mouse strains. Concurrent evaluation of infection with the HGE agent in these strains permits a more complete and rigorous analysis of murine host responses that may be dependent on or independent of infection burden. One study confirmed that C3H mice show typical leukopenia (both neutropenia and lymphopenia) and mild anemia in response to infection. However, thrombocytopenia was not documented by blood smear examination [12]. This deviation from the classic hematologic alterations associated with HGE has been seen as a potential weakness of the mouse model.

In the present study, we speculated that, because of high circulating platelet numbers in mice, blood smear evaluation may not be sufficiently sensitive to detect thrombocytopenia. Therefore, we investigated platelet kinetics in the murine model of HGE.
infection more critically, including the roles that the spleen and T and B lymphocytes may play in altering platelet number during acute infection with the HGE agent.

Materials and Methods

Mice. Female, 5–8-week-old, specific pathogen–free C3H, C3H/Scid, and B6 mice were purchased from the National Cancer Institute Animal Production Program (Frederick Cancer Research Center, Frederick, MD), Harlan Sprague-Dawley (Indianapolis, IN), and Jackson Laboratory (Bar Harbor, ME), respectively. Mice were maintained according to approved institutional animal use and care protocols and euthanized with CO2.

HGE agent and in vivo experimental infection. The HGE agent was isolated from peripheral blood samples from a patient with HGE from Nantucket, Massachusetts (NCH-1 isolate) [13], and maintained through serial intraperitoneal passage of infected blood in SCID mice, as described elsewhere [12]. Pooled blood from these mice served as the source of infectious material in all experiments. All experimental mice were inoculated intraperitoneally with 100 μL of infected blood. Control mice were inoculated intraperitoneally with 100 μL of uninfected blood. Because all experimental mice were young, were euthanized within 6 days of inoculation with the HGE agent, and had no previous exposure to blood transfusion, no effort was made to passage the HGE agent in congeneric SCID mice for experiments in which B6 mice were used.

Hematology. Blood was collected by cardiocentesis and placed into tubes containing EDTA (Becton Dickinson), except for samples obtained from the retro-orbital sinus (eyeblood) in experiments with SCID mice. Platelet counts were made within 6 h of blood collection on a Serano Baker 9000 automated cell-counting instrument (Biochemical Immunosystems).

Splenectomy. Twenty-four C3H mice were splenectomized and 24 C3H mice were sham-splenectomized under aseptic surgical conditions. Mice were anesthetized with 0.15–0.20 mL of ketamine/xylazine (10 mg/mL ketamine [Vetamine; Schering-Plough Animal Health] and 1 mg/mL xylazine [TranQuilVed; Vedco]), administered intraperitoneally. In sham-splenectomized mice, a single ligation was made with 3-0 silk around splenic vasculature, and spleens were excised. In sham-splenectomized mice, spleens were simply exposed and then replaced into the peritoneal cavity. A single 3-0 silk suture was placed to close the peritoneum, and a skin staple was used to close the skin incision. Because splenectomy results in alterations in peripheral blood cell counts (primarily reactive thrombocytosis and postsurgical neutrophilia), mice were allowed to recover for 12 days before experimental infection was carried out. Pilot experiments showed that peripheral blood counts normalized in mice by 10 days after splenectomy (data not shown).

Experiment with SCID mice. Twelve SCID mice were infected with the HGE agent, as described in the section “HGE agent and in vivo experimental infection,” and 12 SCID mice were injected with uninfected whole blood to serve as negative controls. Both infected and uninfected mice were separated into 3 groups of 4 mice. Blood samples were obtained from the retro-orbital sinuses of mice in each group 2 times during infection on a rotating basis. Mice were lightly anesthetized with isoflurane (Halocarbon Laboratories), and ~50 μL of blood was collected from each mouse by means of an EDTA-coated 100-μL capillary tube. Samples were obtained on postinfection days 0 and 6 for group 1, days 2 and 8 for group 2, and days 4 and 10 for group 3. Platelet counts were made as described in the section “Hematology.”

Statistical analysis. Statistical comparisons between infected and uninfected mice were made by Student’s t test. Multiple-comparison analysis was done with 1-way analysis of variance followed by a least-squares difference post hoc test (SPSS, version 6.1 for Windows). P < .05 was considered to be significant.

Results

Four infected and 2 uninfected C3H mice were evaluated on days 0, 1, 2, 3, 4, and 6 after infection with the HGE agent. Platelet counts were made as described in the section “Hematology” in Materials and Methods. C3H mice had a significant decrease in platelet number on days 3, 4, and 6 after infection, compared with the number in uninfected control mice (P = .017, P = .005, and P = .011, respectively; figure 1A). Four infected and 2 uninfected B6 mice were evaluated on days 1–4 after infection with the HGE agent. B6 mice had a significant decrease in platelet counts on days 2–4 after infection, compared with counts in uninfected control mice (P = .02, P = .006, and P < .0001, respectively; figure 1B). No significant difference in platelet number was seen between infected B6 mice and infected C3H mice on day 2 after infection.

Twelve splenectomized C3H mice and 12 sham-splenectomized C3H mice were infected with the HGE agent, and the remaining 12 mice in each group were injected with uninfected blood to serve as negative controls. On days 2, 4, and 6 after infection, blood samples were obtained from 4 mice in each group (infected, splenectomized; infected, sham-splenectomized; uninfected, splenectomized; uninfected, sham-splenectomized), and platelets were counted (figure 2A). A tendency was seen for uninfected, splenectomized mice to have higher platelet counts than did their sham-splenectomized counterparts. However, the difference in platelet counts between uninfected animals was significantly different (P = .004) only on day 4 after infection (day 16 after splenectomy). Results of statistical analyses were the same whether data for uninfected animals that were splenectomized or sham-splenectomized were combined or kept separate (the numbers reported reflect analysis for separate comparisons). In both groups of infected mice, platelet numbers were significantly lower than those in uninfected control mice on days 2, 4, and 6 after infection (sham, P = .0003, P = .0009, and P = .0001, respectively; splenectomized, P = .014, P = .00001, and P = .0009, respectively). However, there was no difference in platelet numbers for infected, splenectomized mice and numbers for infected, sham-splenectomized mice on any day of infection.

Platelet counts over the course of the first 10 days of HGE-agent infection in SCID mice are shown in figure 2B. Platelet
numbers were significantly lower in infected SCID mice on days 4, 6, 8, and 10 after infection than in uninfected control mice ($P = .002, P = .008, P = .0007$, and $P = 0.028$, respectively). A trend toward higher platelet numbers was seen on the second eyebleed of uninfected mice, likely because of reactive thrombocytosis. Because infected mice were similarly eyebleed, the relative increase in platelet number in uninfected mice does not confound the data.

Discussion

We have shown that mild thrombocytopenia, as demonstrated by a 50% decrease in circulating platelet numbers 2–4 days after infection, developed in 2 strains of mice infected with the HGE agent. This degree of platelet loss parallels that noted in naturally occurring acute disease and in other animal models of ehrlichial disease. Further study of HGE agent–infected splenectomized mice and SCID mice showed that thrombocytopenia occurred independent of the presence of the spleen and functional B and T lymphocytes.

Other work with C3H mice documented hematologic alterations over the course of the first 25 days of infection with the HGE agent; however, samples were not evaluated during the first 5 days of infection, when bacterial numbers in the blood increase exponentially [12]. In addition, platelet numbers were estimated only qualitatively, from examination of blood smears, and were deemed “adequate.” Because of the normally high platelet counts that are found in mice, a 50% decrease in platelet number still results in levels of 300,000–500,000 platelets/µL. In our study, platelet counts were made on an automated instrument that confirmed an acute reduction in platelet numbers in infected animals.

In this study, platelet kinetics differed between mouse strains; B6 mice showed a significant decrease on day 2 after infection, and C3H mice did not show a significantly lower platelet count until day 3 after infection. This difference may not be biologically significant but, rather, may reflect individual variation in response to infection. Although important differences have been documented between C3H and B6 mice with regard to platelet count, megakaryocyte ploidy, platelet life span, and spleen weight [14, 15], further work is needed to confirm the potential strain differences in the kinetics of HGE agent–induced thrombocytopenia. Ongoing work in our laboratory suggests that both strains of mice become consistently thrombocytopenic by day 2–3 after infection and that no strong, reproducible trend toward an earlier decrease in platelet count exists in B6 mice. Regardless, the fact that mice of both strains became comparably thrombocytopenic in light of known differences in platelet counts and infection kinetics adds further credibility to the rigor of the mouse model of infection with the HGE agent and its associated hematologic abnormalities.

Mechanisms underlying thrombocytopenia in ehrlichiosis are largely speculative. In general, a decrease in circulating platelet number may be caused by decreased or ineffective bone marrow production, increased intramedullary destruction (hemophagocytic syndrome), increased peripheral destruction (immune-mediated or non–immune-mediated mechanisms), altered distribution of circulating cells (splenic consumption or endothelial sequestration), or decreased cellular life span. Each of these mechanisms has received some research attention.

Given the acute nature of the thrombocytopenia associated with HGE-agent infection, it is unlikely that decreased hemopoietic production or immune-mediated destruction plays a significant role. Indeed, bone marrow evaluation in mice (data not shown), as well as in humans with acute monocytic ehrlichiosis, generally shows normal or hypercellular marrow [5]. Quantitative assessment of hemopoiesis has not been done
and would be needed to fully rule out the possibility that alterations in cell production significantly contribute to these cytopenias. In addition, although antiplatelet antibody levels were not directly measured, thrombocytopenia occurs by day 2–4 after experimental infection, before an effective acquired immune response can be mounted. In support of this, we have shown that SCID mice, which lack functional B and T lymphocytes, become thrombocytopenic in the same time frame and to the same extent as do infected immunocompetent mice.

Although it has been suggested that immune-mediated destruction of platelets after the first 7 days of infection is a primary mechanism of thrombocytopenia in chronic or severe ehrlichial thrombocytopenia caused by *Ehrlichia canis* [10, 11], data from this study suggest that alternative mechanisms are at work during early granulocytic ehrlichiosis in mice.

The role of the spleen in thrombocytopenia has been investigated in experimental canine monocytic ehrlichiosis caused by *E. canis*. In the dog model, splenectomized animals developed transient and less severe clinical and hematologic abnormalities. In those studies, splenectomized animals showed a decrease of as much as 50% in circulating platelets by day 20 after infection. This decrease was transient, and, by day 35 after infection, platelet counts had recovered in splenectomized, infected animals but not in nonsplenectomized dogs [9]. These findings suggest that a biphasic decrease in platelet number occurs, likely as a result of 2 entirely separate mechanisms. The spleen clearly played a role in the thrombocytopenia associated with subacute and chronic canine ehrlichiosis. However, as in our findings, a rapid decrease in platelets during acute disease was also seen that was independent of splenic consumption. Because *E. canis* is monocytotropic and frequently associated with chronic disease characterized by marked pancytopenia and, ultimately, bone marrow aplasia, only cautious comparison between the mouse and dog models is warranted. Furthermore, although we have not evaluated a mouse model for chronic ehrlichial disease, platelet numbers in nonsplenectomized, immunocompetent mice infected with the HGE agent eventually return to normal. Nonetheless, the similar dynamics of the early thrombocytopenia in both canine monocytic and murine granulocytic ehrlichiosis suggest a possible common pathogenesis that does not involve splenic consumption.

Thrombocytopenia was found to be a consistent hallmark of murine infection with the HGE agent in both C3H and B6 mice. In addition, thrombocytopenia was not secondary to either splenic consumption or immune-mediated destruction. Thus, the mouse is a valid model for hematologic alterations, including thrombocytopenia, associated with infection with the HGE agent.

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**References**