
Afibrinogenemia and a circulating antibody against fibrinogen in a Bichon Frise dog

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Abstract: A 1.5-year-old female Bichon Frise dog was evaluated for a life-threatening hemorrhagic condition that occurred after ovariohysterectomy, requiring 4 whole-blood transfusions. A hemostatic profile, including activated clotting time (ACT), one-stage prothrombin time (OSPT), activated partial thromboplastin time (APTT), buccal mucosal bleeding time, and specific assays (heat-precipitation microhematocrit method and electroimmunoassay) for fibrinogen, were performed to investigate the coagulopathy. Clotting times for all tests having a fibrin clot endpoint (ACT, OSPT, APTT) and buccal mucosal bleeding time were prolonged. Plasma fibrinogen was not detected by heat-precipitation microhematocrit method or electroimmunoassay. Using the Ellis-Stransky method, a mixture of patient plasma and normal canine plasma with known fibrinogen content yielded substantially less than the calculated fibrinogen concentration, indicating the presence of an interfering substance. The interferent properties of the patient's plasma were retained following heat precipitation at 56°C indicating the absence of a pyroglobulin or an abnormal fibrinogen molecule. Radial immunodiffusion assay using the patient's plasma and activated thrombin confirmed the existence of an inhibitor to the formation of fibrin. Western blot analysis using the patient's plasma identified an IgG antibody that reacted with the β - and γ - but not the α -subunits of canine fibrinogen. Antibody was detected in samples taken 8, 16, and 68 days after the surgery; peak titers were evident at day 16. These results supported a diagnosis of afibrinogenemia with a circulating antibody inhibitor to fibrin clot formation that developed secondary to blood transfusion. (*Vet Clin Pathol.* 2005;34:148-155)

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Key Words: Acquired, afibrinogenemia, blood coagulation tests, congenital, fibrinogen antibody

A female 1.5-year-old Bichon Frise dog was referred to the Veterinary Medical Teaching Hospital at the University of Missouri-Columbia, College of Veterinary Medicine, for uncontrolled hemorrhage following ovariohysterectomy. The dog was referred because the bleeding was not controlled despite 2 whole-blood transfusions (150 mL each) given at 4 and 6 days after surgery. The owners had recently acquired the dog and had little knowledge about its medical history. Approximately 1 month before the ovariohysterectomy, the referring veterinarian noted excessive bleeding at a site of venipuncture and a deep swelling hematoma in the quadriceps 1 week later, which initiated submission of blood to a referral laboratory for coagulation studies. Laboratory findings suggested hypofibrinogenemia (<100 mg/dL, performed by heat-precipitation microhematocrit method) and prolonged values (>2 minutes) for one-stage prothrombin time (OSPT; control value 8 seconds) and activated partial thromboplastin time (APTT; control value 13.8 seconds).

At presentation (on the 7th day after surgery), the dog had pale mucous membranes, erythematous ears, depression, ventral abdominal bruising, and blood oozing from venipuncture sites (right cephalic and jugular veins). At admission, HCT was 44% (reference interval 37-55%) and plasma total protein concentration was 5.8 g/dL (reference

interval 5.7-7.9 g/dL), however, the dog was dehydrated. The following day, the HCT decreased to 18%, and platelet concentration fell from an adequate blood film estimate ($\geq 150,000$ platelets/ μ L) to approximately 25,000 platelets/ μ L (hemocytometer). Biochemical results were within reference intervals except for mild hyponatremia (143 mmol/L, reference interval 144-154 mmol/L) and hypokalemia (3.3 mmol/L, reference interval 4.0-5.5 mmol/L). Abdominal radiographs demonstrated fluid in the abdomen that was considered to be hemoperitoneum. Vitamin K₁ (7.5 mg, SC) was given for 7 days. Nine days after surgery, the dog spiked a temperature of 103°F and had a green discharge from the venipuncture site on the right forelimb. Amoxicillin (25 mg, PO, BID) was prescribed for 14 days. Several tests were performed to determine possible causes for the hemorrhagic episode. A Coombs' test result was negative at 1:2 to 1:32 dilutions of Coombs' reagent. Thin-layer chromatography was performed on a blood sample for warfarin-like anticoagulants, and the results were negative. Because the thrombocytopenia could have been the result of immune-mediated destruction and commonly is seen in patients with systemic lupus erythematosus (SLE), an antinuclear antibody test (ANA) was performed on patient's serum; the ANA test result was negative. Because both the

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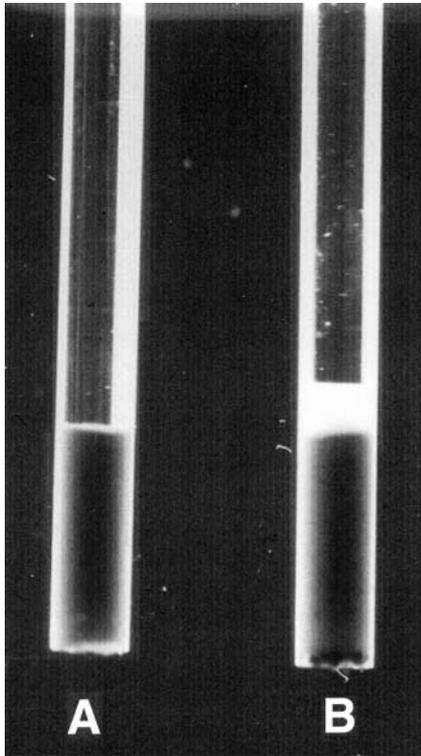


Figure 1. Endpoint of the heat-precipitation procedure to detect fibrinogen in plasma samples from (A) the afibrinogenemic dog and (B) a healthy dog. The patient plasma sample lacks the opaque protein precipitate that forms over the clay plug after heating and centrifugation of a plasma sample from a healthy dog.

Coombs' and ANA test results were negative, an immune-mediated cause for the anemia and thrombocytopenia was ruled out.

The modified heat-precipitation method of Millar was performed to detect fibrinogen.^{1,2} Microhematocrit tubes were filled with plasma, centrifuged in a microhematocrit centrifuge, heated to 56°C for 3 minutes and recentrifuged. The results demonstrated the absence of a fibrinogen precipitate in the sample from the patient when compared with a sample from a healthy dog (Figure 1). Activated clotting time (ACT) was >11 minutes (reference interval 60–90 seconds).³ Buccal mucosal bleeding time was prolonged at 6.3 minutes (reference interval 2.1–3.1 minutes).⁴ The OSPT assay using rabbit brain thromboplastin reagent (Simplastin, Organon Tekninka, Jessup, MD, USA) and APTT assay using Platelin Plus (Organon Tekninka) were performed according to manufacturer's specifications, using a mechanical clot detection device (FibroSystem, BD Diagnostics, Franklin Lakes, NJ, USA). Both clotting times were prolonged (>2 minutes). Because the dog's hematocrit was 18%, 9.5 days after the initial surgery, and the venipuncture site on the right forelimb continued to bleed, 150 mL of whole blood from a donor dog (dog erythrocyte antigen, DEA, 1.1 negative) was given over a period of 3 hours, followed by administration of a second aliquot of 150 mL during the next 3 hours. Component therapy was not available at the time. Von Willebrand factor (vWF) antigen

concentration was 121 U/dL (reference interval 48–159 U/dL, based on 220 healthy dogs).⁴ A fibrinogen defect was suspected based on the history of abnormal bleeding associated with hypofibrinogenemia (referral data), failure to detect heat-precipitable fibrinogen on admission, and prolonged clotting times for tests that used a fibrin clot endpoint (ACT, OSPT, APTT).

During the 10 days following surgery, the dog was given a total of 4 whole-blood transfusions of 150-mL aliquots. By 15 days after surgery, the patient's attitude and appetite improved and the hematocrit reached 43% with reticulocytosis (390,000/ μ L), and a platelet concentration of 176,000 platelets/ μ L. Although all internal bleeding and oozing from venipuncture sites had stopped, buccal mucosal bleeding time was prolonged (9.5 minutes). Vitamin K₁ was discontinued, and even though the patient's clotting times remained abnormal throughout its stay in the hospital, the dog was discharged 18 days after admission.

The dog remained asymptomatic from the time of discharge until 2 months later (68 days after surgery) when she was examined at home. Buccal mucosal bleeding time at that time was prolonged (5.7 minutes, reference interval 2.1–3.1 minutes). Excessive bleeding under the skin was noted at a venipuncture site necessitating a small pressure bandage. Unfortunately, the dog died 2.5 months later from trauma-induced hemorrhage caused by another dog.

Methods for Detection of Fibrinogen and a Circulating Interferent

Eight, 16, and 68 days after ovariohysterectomy, blood samples from the patient (6-mL aliquots) were drawn from the external jugular vein into plastic syringes containing 0.13-M trisodium citrate (1 part to 9 parts of blood). Samples were centrifuged at 2000g for 20 minutes, and harvested plasma was stored in plastic tubes at –70°C until analysis. A normal dog plasma pool (NDPP) was prepared by pooling identically prepared plasma samples from 10 clinically healthy client-owned dogs. The dogs were considered healthy on the basis of physical examination and CBC results and a negative Knott's test.

To further evaluate the bleeding diathesis and apparent lack of fibrinogen, the patient's plasma was analyzed for fibrinogen concentration using a modification of the Ellis and Stransky assay.⁶ The Ellis–Stransky method detects turbidity in a plasma sample when fibrinogen in plasma is polymerized and converted to fibrin by the action of thrombin. Turbidity is measured photoelectrically using a semiautomated instrument (Fibrinogen analyzer, Lancer, St. Louis, MO, USA). The method was adapted for use on canine plasma by adding bovine thrombin (0.25 units, Miles Scientific, Naperville, IL, USA) diluted in 550 μ L of barbital buffer (1 M NaCl, 0.02 M sodium barbiturate, pH 7.2, 50% NaOH, 0.25 M CaCl₂, 3 mM NaN₃) in a cuvette containing 50 μ L of canine plasma. The analyzer was calibrated by previously described methods with NDPP, which contained 220 mg/dL of fibrinogen.⁷ To prepare a standard curve, NDPP containing 220 mg/dL of fibrinogen was analyzed neat (no dilution) and diluted with barbital

buffer by 25%, 50%, and 75%. Barbitol without plasma was tested as a negative control. The final fibrinogen concentrations of the NDPP were equivalent to 53, 100, 163, and 220 mg/dL indicating that recovery was not exactly 100% compared with expected values (55, 110, and 165 mg/dL) for each dilution.

To further determine if the failure of the assay to detect fibrinogen in the patient's plasma was the result of an interferent (ie, a heat-stable or heat-labile protein), 2 mixture studies were performed. In the first mixture study, NDPP was diluted with patient plasma by 25%, 50%, and 75%. This experiment was designed to demonstrate an inhibitor in the patient's plasma that interfered with the formation of fibrin in NDPP. To determine if pyroglobulins in the patient's plasma interfered with the formation of fibrin in the NDPP, the patient's plasma was heated to 56°C for 15 minutes and centrifuged. The resulting supernatant was used to dilute NDPP in similar proportions as described. Fibrinogen concentrations were determined in each of the 2 mixture studies by the modified Ellis-Stransky method and compared with a standard curve created from dilutions of NDPP in barbitol buffer.

To determine if fibrinogen subunits could be detected in the patient plasma, the plasma was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) containing 5% (stacking) and 10% (running) acrylamide, and the migration pattern of the proteins in the plasma were compared with NDPP and purified canine fibrinogen following staining of the gel with 0.2% Coomassie-Blue R-250 (BioRad, Richmond, CA, USA), 50% methanol, and 10% acetic acid.

The absence of fibrinogen in the patient's plasma was confirmed by a very sensitive rocket electroimmunoassay.⁵ Dilutions of patient plasma and NDPP were electrophoresed in 0.8% agarose gel prepared as previously described except calcium lactate was omitted.⁵ The gel also contained 20 μ L of a 1:10 dilution of rabbit antihuman fibrinogen (Accurate Chemicals, Westbury, NY, USA). Visualization of the precipitin rockets composed of antibody-fibrinogen complexes produced by the electroimmunoassay was accomplished by second-antibody autoradiography. Briefly, the agarose slabs were compressed, then submerged in 25 mL of phosphate-buffered saline (PBS) detergent (0.35% Tween 20 in 0.15-mL NaCl buffered at pH 7.4 with 0.02-M sodium phosphate) containing 2 μ Ci of ¹²⁵I-labeled goat antirabbit immunoglobulin G (IgG) F(ab')₂ fragments (New England Nuclear, Boston, MA, USA).⁵ Precipitin rockets were visualized by incubating the dried gels with Kodak XAR-5 x-ray film (Picker International, Cleveland, OH, USA).

Western blot assays were performed to identify the antibody class to fibrinogen protein. Purified canine fibrinogen was used as a positive control and was precipitated from fresh frozen dog plasma with 95% ethanol. The precipitate was formed on cooling to -3°C for 30 minutes and collected by centrifugation.⁸ Selected fractions obtained by chromatography were assayed for fibrinogen by the Ellis-Stransky method. The purified canine fibrinogen (7.8 μ g) was fractionated on SDS-PAGE containing 5% (stacking) and 10% (running) acrylamide by electrophoresis then transferred to nitrocellulose.⁹ The nitrocellulose membrane was incubated with the

patient's plasma diluted 1:100 in blocking buffer. Following washes in buffer, the membrane was probed with a biotinylated affinity-purified goat antidog IgG or immunoglobulin (Ig) M antibody (Vector Labs, Burlingame, CA, USA) diluted (1:500 in PBS, pH 7.4), and bands were visualized using peroxidase conjugated avidin followed by cobalt chloride, diaminobenzidine, and hydrogen peroxide (ABC kit, Vector Labs, Burlingame, CA, USA). As a negative control for nonspecific reactivity, the blots were probed with biotinylated goat antidog IgG or IgM antibody without the primary antibody (patient plasma).

A radial immunodiffusion assay was performed to titer the antibody with reactivity to fibrin formation in initial and convalescent samples using a modification of the method described by Cassidy et al.¹⁰ The agarose gel coagulation plate was prepared in a manner described previously.¹¹ Five milliliters of NDPP diluted 1:12 in 0.05-M imidazole-buffered saline (pH 7.3) was placed into 14 \times 100-mm plastic tubes containing 7 mL of dissolved low electroendosmosis-agarose and incubated at 56°C in a water bath. After 3 minutes, 0.2 mL of thromboplastin (Sigma, St. Louis, MO, USA) was added, immediately followed by addition of 5.0 mL of dissolved 2% agarose maintained at 56°C. The mixture was poured into a mold and allowed to solidify and cool in a humidified chamber for 2 hours. Circular sample wells were cut with a 3.0-mm diameter suction punch (BioRad, Richmond, CA, USA). Rabbit antihuman fibrinogen was used as a positive control (Accurate Chemical, Westbury, NY, USA). Test plasma samples (initial and convalescent) or rabbit antihuman fibrinogen undiluted or serially diluted 1:2 to 1:32 with imidazole buffered saline were applied to wells (8- μ L aliquots). Imidazole buffer was applied to wells as a negative control. The plates were immersed in 0.025-M CaCl₂ prewarmed to 37°C, removed from the CaCl₂ solution, and incubated at room temperature (20°C) for 3 minutes to allow fibrin formation. The plates were then immersed in 100 mL of 4% formalin at 4°C for 5 minutes to inhibit further fibrin formation. The presence of an inhibitor to fibrin formation was noted by a clear zone surrounding the wells, indicating the absence of fibrin formation. The diameter of the clear zones surrounding the inhibitor standard (rabbit antihuman fibrinogen) and patient wells was measured with a Mancini ruler.

Results

Based on the modified Ellis-Stransky method, the patient's plasma fibrinogen concentration applied neat was below detectable limits (<5 mg/dL, reference interval 180-280 mg/dL) established by Hurst et al.¹² The same result was obtained at 16 and 68 days after surgery.

Fractionation of NDPP and patient plasma on SDS-PAGE identified multiple protein bands. The migration pattern of the proteins in the patient plasma by SDS-PAGE was similar to that of NDPP and of purified canine fibrinogen except that a band migrating in the region of the β -subunit was not apparent in the patient plasma. Migration of the purified canine fibrinogen sample defined the migration pattern for fibrinogen subunits (α , β , and γ). Purified canine fibrinogen

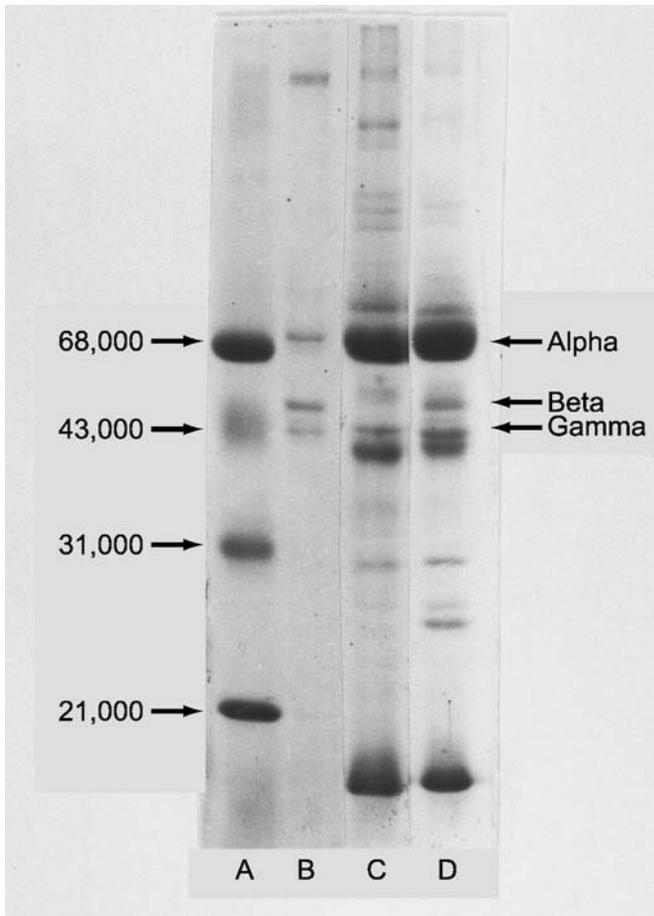


Figure 2. SDS-PAGE stained with Coomassie blue. (A) Molecular weight markers include bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 31,000), and soybean trypsin inhibitor (M_r 21,000). (B) Purified canine fibrinogen (7.8 μ g) fractionated into 3 separate protein bands representing the α -, β -, and γ -subunits. (C) Patient plasma (20 μ L of a 1:10 dilution), and (D) normal dog plasma pool (NDPP) (4.0 μ g) fractionated into multiple protein bands. The patient's plasma and NDPP have a broad band that migrates in the region of the α -chain (presumptive albumin). The patient's plasma lacks the protein band that migrates in the region of the β -subunit of fibrinogen, but has a protein band that comigrates in the region of the γ -subunit of fibrinogen.

had a faint doublet in the γ -chain region (Figure 2b); this heterogeneity of the γ -chains has been observed in human and canine fibrinogen molecules.¹³ Confirmation of the absence of the α - and γ -subunits of fibrinogen in the patient plasma could not be confirmed by this method because of comigration of other proteins in these regions. In particular, a high molecular weight heavy band was apparent in both the patient plasma and control plasma, most likely attributable to albumin, which has a relative molecular mass (M_r) of 68,000, similar to the α -chain of fibrinogen (66,000 d; Figure 2).

The apparent absence of fibrinogen in the patient's plasma sample was confirmed by electroimmunoassay (Figure 3). No precipitin rockets formed in the patient's plasma at any dilution, whereas precipitin rockets were evident in

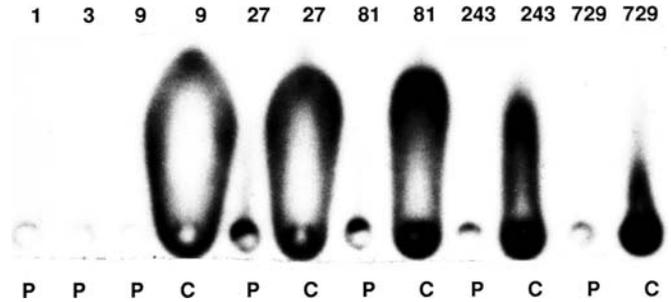


Figure 3. Second-antibody autoradiograph of precipitin rockets. C indicates electroimmunoassay for fibrinogen in normal dog plasma pool (NDPP) diluted 1:9 to 1:729 with imidazole buffer; and P, patient plasma samples undiluted (1) or serially diluted 1:3 to 1:729 with imidazole buffer. Gel contains rabbit anti-human fibrinogen. NDPP and patient plasma is added to the wells at the bottom and electrophoresed. The rockets (C) indicate the production of an immunoprecipitate between the rabbit antihuman fibrinogen antibody and fibrinogen in the plasma. Rocket length is proportional to the concentration of fibrinogen. Serial dilutions are noted at the top of the gel. Rockets are not seen in the patient plasma (P) samples.

normal dog plasma serially diluted to low concentrations of fibrinogen ranging from 24 mg/dL to 0.3 mg/dL.

Mixture studies with the patient's plasma and NDPP indicated that measured fibrinogen concentrations of diluted NDPP determined by the Ellis-Stransky method were lower than expected (Figure 4). For example, a 50% mixture of the 2 plasma samples gave a value of 38 mg/dL compared with the expected concentration of 110 mg/dL when NDPP was diluted 50% with barbital buffer. The fibrinogen concentration remained low at this level after diluting the NDPP with supernatant prepared after heat precipitation of the patient's plasma. This observation suggested that the patient's plasma imparted negative interference to the formation of fibrin in the NDPP. The fact that the interferent remained active after heat treatment of the patient's plasma suggested that the interference was likely not a result of an abnormal fibrinogen or pyroglobulin. Therefore, an antibody inhibitor to fibrinogen or fibrin formation was considered likely.

Western blot analysis of the patient's plasma (obtained on days 8 and 16 after surgery) identified IgG antibodies that reacted with 2 bands that comigrated with the β - and γ -chains of NDPP and the purified canine fibrinogen (Figure 5). The same reactivity, albeit weaker in strength, was present in the convalescent plasma sample obtained 68 days after surgery (data not shown). The test plasma also reacted with a wide band in the NDPP sample in the region of M_r 18,000–19,000, which likely represented fibrinogen breakdown products. The patient's plasma did not react with the secondary antibody to canine IgM (data not shown).

Radial immunodiffusion assay results indicated there was interference in the fibrin formation of the NDPP when patient plasma was in the well. Clear zones surrounded the wells that contained the rabbit antihuman fibrinogen (top row) and the patient wells (rows 2–4). A clearing in the cloudiness of the gel surrounding the patient wells indicated the absence of fibrin formation. Plasma samples obtained from the Bichon Frise

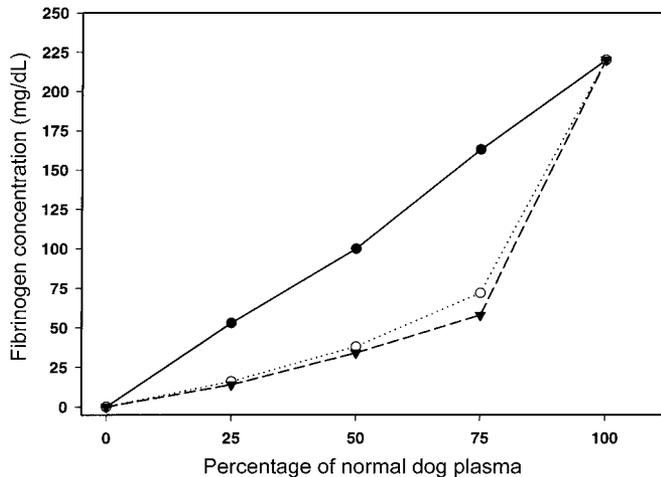


Figure 4. Results of the Ellis–Stransky method to determine fibrinogen concentrations in normal dog plasma pool (NDPP) diluted with either barbital buffer or patient plasma. NDPP containing 220 mg/dL of fibrinogen was analyzed neat or diluted to 25%, 50%, and 75% with barbital buffer solution (closed circles). NDPP was analyzed neat or diluted to 25%, 50%, and 75% with patient plasma (open circles). Normal dog plasma was analyzed neat or diluted 25%, 50%, and 75% with the supernatant of the patient plasma after heat precipitation (closed triangles). The concentration of fibrinogen in the NDPP diluted with either the patient’s plasma or the supernatant of heat–precipitated plasma was lower than the expected concentration.

dog at 8, 16, and 68 days after the ovariohysterectomy had the interferent (Figure 5). The antibody titer peaked at 16 days after surgery and nearly approached the ring diameter of the rabbit antihuman fibrinogen, which was applied neat as the positive control. The convalescent sample (68 days after surgery) contained only about 12% of the peak amount of interferent observed in the second sample.

Discussion

The Bichon Frise dog in this study had a mild clinical condition that was not exacerbated until after a relatively minor, invasive surgery. The ecchymosis observed on several occasions and the massive hemorrhage into the abdominal cavity suggested either an anticoagulant affecting the common pathway or a multiple clotting factor anomaly. Thin-layer chromatography revealed no evidence of warfarin-like anticoagulants, favoring the latter hypothesis. Because the ACT was prolonged, a defect in the intrinsic pathway was possible. Although the patient had marked thrombocytopenia at the time of testing (25,000 platelets/ μ L), prolongation of ACT by reduced availability of phospholipids is reported not to occur until platelet concentrations fall below 10,000/ μ L.¹⁴ Prolongation of ACT, OSPT, and APTT, which have fibrin clot endpoints, indicated a common pathway defect, multiple factor defects, or the presence of inhibitors. Although there was insufficient plasma to perform individual factor assays (other than fibrinogen) or fibrinolysis studies, a multiple factor deficiency, such as disseminated intravascular coagulation,

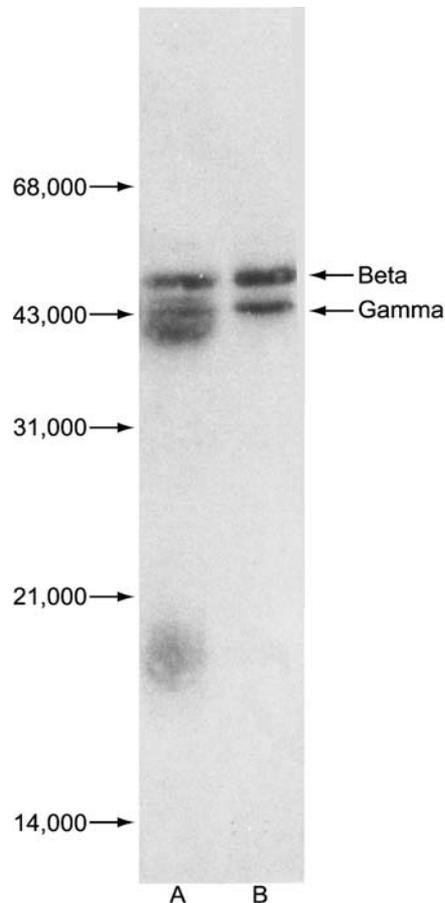


Figure 5. Western blot of fibrinogen subunits using patient plasma diluted 1:500 in PBS (16 days after surgery) as the primary antibody. Normal dog plasma pool (NDPP) (2 μ g) was fractionated by SDS-PAGE and immunoblotted with patient plasma. Purified canine fibrinogen (7.8 μ g) was fractionated by SDS-PAGE, then immunoblotted with patient plasma. The patient’s plasma reacted with proteins in (A) NDPP and (B) purified canine fibrinogen subunits migrating the same distance as the β - and γ -subunits, but not the α -subunit of canine fibrinogen. Biotinylated relative molecular mass markers included bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (not shown).

was eliminated because the patient continued to have abnormal coagulation times and lacked detectable fibrinogen in the convalescent sample obtained when the patient was clinically healthy. The inability to detect fibrinogen in the plasma of this dog by the heat–precipitation method or electroimmunoassay indicated a severe deficiency of clottable fibrinogen, either afibrinogenemia or dysfibrinogenemia. Although not performed in this study, other assays that are available as screening tests to detect fibrinogen deficiency and dysfunction include the thrombin clotting time and clottable (Clauss) fibrinogen test.^{15,16} In people, the Clauss method is considered the method of choice by the National Committee on Clinical Laboratory Standards (NCCLS).¹⁷

The possibility that the patient had dysfibrinogenemia cannot be completely excluded; however, several points make

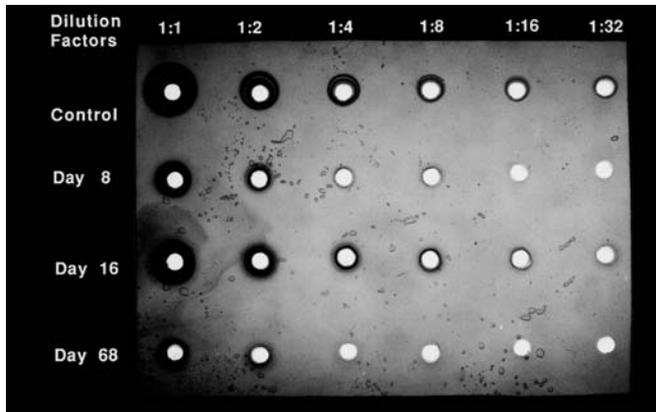


Figure 6. Results of the radial immunodiffusion assay demonstrate inhibition to fibrin formation of normal dog plasma pool (NDPP). Agarose plate contains a 1:12 dilution of NDPP. Positive control wells (top row) contain serial dilutions of rabbit antihuman fibrinogen (control). Initial and convalescent patient plasma samples obtained at 8, 16, and 68 days after surgery were serially diluted (shown in the next 3 rows). Dilutions were neat (1:1), 1:2, 1:4, 1:8, 1:16, and 1:32. The clearing in the gel surrounding the sample well indicates the presence of an interferent that prevented fibrin formation, whereas cloudiness is the result of fibrin formation within the gel. The diameter of the zone decreases with dilution of the plasma sample and titer of the antibody. The patient's antifibrinogen antibody titer is greatest at day 16 and approaches the diameter of the neat positive control sample.

this conclusion less attractive. If dysfibrinogenemia was present, then: 1) the abnormal fibrinogen in this dog did not have the thermal properties of fibrinogen (ie, heat precipitation), 2) the abnormal fibrinogen was so bizarre that the patient's immune system recognized transfused canine fibrinogen as foreign, and 3) thrombin was unable to initiate fibrin clot formation. Therefore, we concluded the dog had afibrinogenemia because fibrinogen was not detectable by heat-precipitation, the Ellis-Stransky method, or the most sensitive method, electroimmunoassay. The best evidence for afibrinogenemia was that the patient's immune system recognized fibrinogen as foreign following exposure to blood transfusions.

Because of the patient's age, it was thought that the dog had afibrinogenemia since birth. Congenital afibrinogenemia is a rare, autosomal, recessive disorder characterized by the complete absence of fibrinogen. The disease originally was described in 1920 by Rabe and Solomon¹⁸ with an estimated prevalence of approximately 1 in 1,000,000 people.¹⁹ Afibrinogenemia usually is detected at birth with umbilical cord hemorrhage, the first clinical sign. Bleeding of the gums, epistaxis, menorrhagia, gastrointestinal bleeding, and hemarthrosis occur with varying severity. As in our report, overt clinical signs may not be apparent in adolescents or young adults until surgery or traumatic events.²⁰ Although functional assays of clot formation are markedly prolonged, the coagulation defect is no more severe than in the hemophilias A and B, varying from severe to mild. This is partially explained by the presence of functional vWF, which allows platelet aggregation and

adhesion even in the absence of fibrin.²¹ The buccal mucosa bleeding times in this Bichon Frise dog were slightly prolonged, even in the face of normal vWF antigen concentration, indicating that a lack of fibrinogen influences the efficiency of primary hemostasis. Mild deficiencies in primary hemostasis have been documented in human patients and goats with afibrinogenemia.^{20,22} Canine hypofibrinogenemia has been reported in Saint Bernard dogs; canine dysfibrinogenemia has been reported in a Borzoi.²³

Formation of antifibrinogen antibodies after transfusion has been reported in several cases of human afibrinogenemia.²⁴⁻²⁶ We concluded that the Bichon Frise dog developed a circulating antibody to fibrinogen based on antibody-mediated interference in the thrombin-induced activation of fibrin formation of normal dog plasma in the Ellis-Stransky assay and in the radial immunodiffusion assay. The interferent was determined to be an antibody rather than a pyroglobulin or abnormal fibrinogen molecule because of its heat stability and reactivity to purified canine fibrinogen.

Although we concluded that afibrinogenemia in this dog was the result of a congenital defect, without genetic studies to identify the inheritance or gene defect, we can not definitively rule out an acquired condition that occurred secondary to exposure to a toxin or drug or to development of a circulating inhibitor. Acquired afibrinogenemia without circulating inhibitors has been reported in cases of induction of the fibrinolytic system by rattlesnake venom or drugs.^{27,28} The Bichon Frise dog in this report had no history of rattlesnake venom or drug exposure nor any history of blood transfusion before the ovariohysterectomy.

Circulating anticoagulants have been classified as 1) specific to clotting factors, 2) nonspecific, but able to interfere with certain steps in the coagulation process, and 3) global inhibitors that inhibit multiple steps (ie, heparin or heparin-like substances). Specific inhibitors typically are IgG antibodies that arise in multitransfused individuals with genetic coagulation factor defects or in normal individuals secondary to transfusions.²⁹ Inhibitors to Factor VIII are by far the most commonly observed circulating anticoagulant observed in canine and human hemophilia A patients following exposure to plasma, cryoprecipitate, or Factor VIII concentrates.³⁰ SLE anticoagulants have been shown to be IgG antibodies that interfere with fibrinogen-fibrin polymerization,³¹ however, the Bichon Frise dog in this report did not have clinical signs consistent with SLE nor in vivo thrombosis, and was negative for erythrocyte and nuclear antibodies by Coombs' and ANA tests. Antifibrinogen antibodies are not classified as lupus anticoagulants. Furthermore, lupus anticoagulants are associated with prolongation of in vitro clotting times in phospholipid-dependent assays.^{32,33}

Western blot analysis demonstrated an IgG antibody to purified canine fibrinogen in the dog's plasma. Curiously, the antibody did not react with the fibrinogen α -chain, even though the polypeptide band was clearly visible in SDS-PAGE of purified canine fibrinogen. In the convalescent plasma sample obtained 68 days after surgery, the antibody concentration was less than 12% of that found in the initial plasma sample suggesting a reduction in the antibody concentration after 2 transfusion-free months.

We cannot explain why the antibodies bound to the reduced β - and γ -polypeptides of fibrinogen exclusive of the α -polypeptide. Perhaps the patient's genetic defect prevented expression of the β - and γ -chains but not the α -chains. The α -chains, alone, would be too unstable to accumulate in the blood. Traces of α -chain antigens, however, might be sufficient to desensitize the immune system to this protein. Another possibility is that the immunoreactive epitopes of the α -chain dissociated upon linearization and denaturation by fractionation in SDS-PAGE. There are other methods, such as preparative isofocusing, that may be useful in confirming or ruling out the presence of anti- α -chain antibodies in this patient.^{34,35}

Investigations in people, rats, and dogs reveal that the 3 polypeptide chains of fibrinogen have separate mRNA.³⁶⁻³⁸ Although the loci of the α -, β -, and γ -genes are adjacent to one another in mammalian genomes, restriction endonuclease analysis of human genomic DNA demonstrates that the α -gene is between the β - and γ -genes.³⁹ The majority of human patients with afibrinogenemia have mutations in the fibrinogen α -gene that involve partial gene deletions and splice site mutations.^{40,41} It would be difficult to explain how a deletion encompasses both β - and γ -genes without affecting the α -gene in this model.

Acknowledgments

The authors thank Dr. Don Schmidt for translating the German articles and Dr. Marilyn Mikiciuk for bringing the case to our attention.

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