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Spontaneous Coagulopathy in Inbred WAG/RijYcb Rats

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Here we describe a series of cases of spontaneous coagulopathy in a colony of inbred WAG/RijYcb (WAG/RijY) rats. This strain previously had been bred at our institution without symptomatology for several decades. The index case was a 10-wk-old male rat that developed a large hematoma at a subcutaneous injection site. Clinicopathologic findings included a decreased RBC count, decreased hematocrit, decreased hemoglobin concentration, normal PT, and prolonged (50% to 70%) aPTT (52 s; reference, 15 to 33 s). Examination of additional WAG/RijY rats that died unexpectedly or had clinical signs of bleeding in the absence of experimental manipulation also revealed normal PT and prolonged aPTT. Histologic examinations of tissues from all rats were unremarkable except for severe acute focally extensive hemorrhage corresponding to the macroscopic findings of acute hemorrhage. Furthermore the aPTT in 8 clinically normal adult rats and 8 clinically normal 4-wk-old WAG/RijY littermates of both sexes was prolonged. We conclude that these WAG/RijY rats have an inherited defect in the intrinsic coagulation pathway.

A breeding colony of WAG/RijY rats was established at Yale University with breeding stock brought from the Netherlands in 1970.[4,5] Over the past 4 decades, these rats have been used extensively in cancer research testing the responses of transplanted tumors and normal tissues to potential new treatment agents, novel combined-modality regimens, and physiologic modulations that might alter the response of tumors and tissues to irradiation.[4,9,11,16] In addition, WAG/RijY rats have been used as a model for absence epilepsy[2,12] and in the development of physiologic imaging techniques.[16] Review of the colony records indicated sporadic cases of unexplained postpartum maternal deaths in this breeding colony as early as June 2004. However, clinical signs of bleeding problems were not noted in these rats until the index case in late July 2007. This index case was followed by investigators' reports of a series of unexpected injuries. Here we describe the results of clinicopathologic studies indicating that the bleeding diathesis in the WAG/RijY rats is caused by a defect in the intrinsic coagulation pathway. The noted in vivo and in vitro signs are unique to WAG/RijY rats and compatible with a de novo mutation that has become fixed in this colony.

Case Report

The initial case sent for evaluation was a 10-wk-old male rat that had been inoculated with 0.2 mL of a sterile suspension of tumor cells (from the BA1112 rhabdomyosarcoma line) by subcutaneous injection with a 25-gauge needle in the dorsal neck; this rat presented with a $4 \times 4 \times 2$ cm hematoma subjacent to the injection site (Figure 1, A and B). This inoculation procedure is not normally associated with any clinical signs other than the development of a palpable tumor 2 to 3 wk later. Since recognition of coagulopathy in the index case, an entire litter (Figure 2, litter A2-5 [designated by #]) of 11 WAG/RijY pups was found dead (8 pups postnatal day 4) or missing and presumed dead and cannibalized (3 pups postnatal day 12) and another 7 WAG/RijY rats (3 male, 4 female; age, 3 to 26 wk) have been found dead or moribund (1 of the rats was pregnant and died en route to necropsy). In addition, a total of 24 rats (7 female and 17 male) between 3 and 46 wk of age have been euthanized for humane reasons because of problems secondary to bleeding (Table 1, Figure 1). The index case and one other rat with a postinjection hematoma were the only affected rats that had undergone any experimental manipulation, including exposure to an experimental or therapeutic drug. Rats with unexplained bleeding or bruising have been found in litters from all of the 4 generations of rats since the index case (Table 1, Figure 2). Further, the dam of the index case died while pregnant with her third litter before submission of the index case (Figure 2, designated by *). Evaluation of feeding, cleaning, and other husbandry practices for maintaining the WAG/RijY colony rats revealed no differences from those used to maintain more than 2000 other rats in the facility. Moreover, none of the WAG/RijMCW rats housed in the same room as the affected WAG/RijY animals have shown any signs of abnormal bleeding. Thus, toxins in the food or water, vitamin K deficiency in the chow, or an environmental cause of an acquired coagulopathy were considered unlikely. Rather, we suspected a hereditary defect unique to the WAG/RijY strain.

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Materials and Methods

Animals. A breeding colony of WAG/Rij rats (*Rattus norvegicus*) was established at Yale University (New Haven, CT) with breeding stock brought from the laboratory of H S Reinhold (Radiobiological Institute TNO, Rijswijk, Netherlands) in 1970 and has been

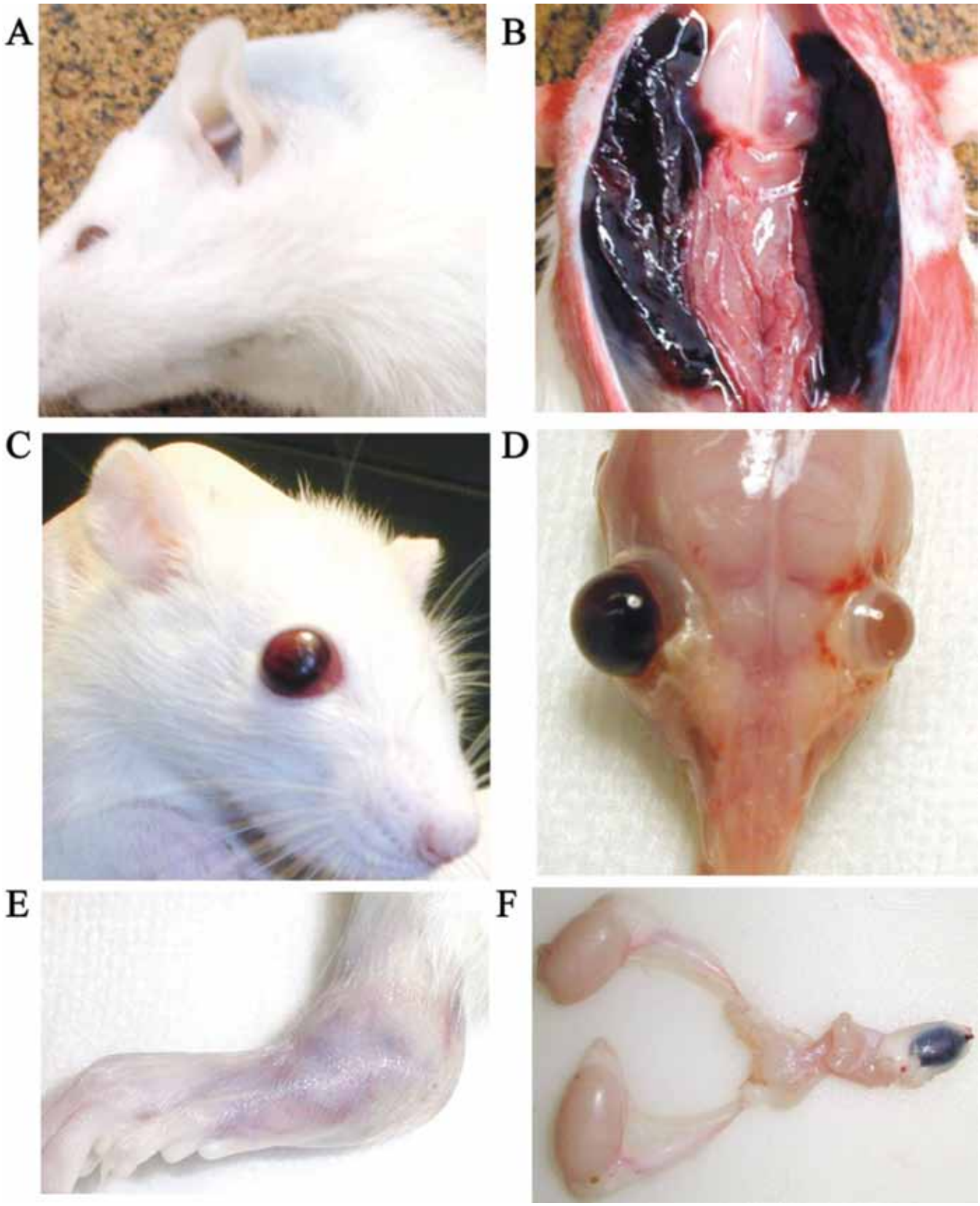

Figure 1. WAG/RijY rats with clinical signs of bleeding. (A) Head. Dorsal surface shaved, with underlying hematoma. (B) Rat in panel A, with skin excised to visualize hematoma. (C) Right eye. Periocular contusion and hyphema. (D) Rat in panel C, with skin removed to visualize normal left eye and hyphemic right eye. (E) Contusion and hemorrhage of right medial tarsal joint. (F) Testes, urinary bladder, and penis with peripenile hematoma.

Table 1. WAG/RijY Rats with clinical signs of bleeding diathesis

Age (wk)	Sex	Generation	Necropsy findings
10	M	B2-1	Postinjection subcutaneous hematoma[a]
7	M	B2-2	Peripenile hematoma
12	F	B2-2	Gastric hemorrhage
3	M	A2	Tarsal joint: contusion and hemorrhage
4	M	A2	Periocular contusion, hyphema
4	M	A2	Periocular contusion, hyphema
4	M	A2	Large intestine: melena
5	M	A2	Postinjection subcutaneous hematoma
5	M	A2	Tarsal joint: contusion and hemorrhage
9	M	A2	Tarsal joint: contusion and hemorrhage
3	F	A2	Periocular contusion, hyphema
7	F	A2	Tarsal joint: contusion and hemorrhage
19	F	A2	Ear injury: excessive hemorrhage
5	M	A3	Thoracic vertebra: contusion and hemorrhage
6	M	A3	Vertebra: contusion and hemorrhage
7	M	A3	Tarsal joint: contusion and hemorrhage
11	M	A3	Postvenipuncture excessive hemorrhage
16	M	A3	Tarsal joint: contusion and hemorrhage
21	M	A3	Periocular contusion, hyphema
46	M	A3	Toenail injury: excessive hemorrhage
46	M	A3	Tarsal joint: contusion and hemorrhage
3	F	A3	Periocular contusion, hyphema
4	F	A3	Periocular contusion, hyphema
19	F	A3	Tail tip injury: excessive hemorrhage
19	M	A4	Periocular contusion, hyphema

[a]Index case.

maintained by Yale Animal Resources Center since that time.[4,5] The Yale WAG/Rij strain recently was registered with the Rat Genome Database as the WAG/RijYcb strain and assigned the identification number 2314861. Because of known sex-associated and interspecies differences in the clinical hematologic parameters and pathology of rats,[8,10] we acquired breeding pairs of WAG/Rij rats from the colony of John E Moulder (Medical College of Wisconsin, Milwaukee, WI); these rats have been referred to in previous publications from that institution as WAG/RijMCW rats but recently were registered with ILAR as substrain WAG/RijCmcr. These additional breeding rats from the Medical College of Wisconsin colony were obtained to provide genetically matched rats to serve as controls in the studies described here and for use in other research. The WAG/RijMCW colony was established with breeders from the Yale WAG/RijY colony in 1978, approximately 35 generations before the identification of the coagulopathy in the WAG/RijY rats. The WAG/RijY and WAG/RijMCW colonies now at Yale are maintained by monogamous breeding pairs selected from siblings from the same litter. A minimum of 2 new breeding pairs is selected from each generation. Once fertility is proven, only 1 pair is chosen to become part of the parental lineage.

At Yale, both colonies are housed in the same room in full-service microisolation (static filter-top) or individually ventilated caging (that, is autoclaved cage, food, and bedding; serviced in a clean-air hood or change station) under barrier conditions. These specific pathogen-free rats are screened by serology for coronavirus, Sendai virus, pneumonia virus of mice, rat parvovirus, and *Mycoplasma pulmonis*; by examination of the pelage for ectoparasites and the cecum and proximal colon for endoparasites; and by microbiologic culture of the nasopharynx and cecum for pathogenic bacteria. Rats are housed on a 12:12-h light:dark cycle and have access to water and a standard autoclaved rodent chow ad libitum. Because of the coagulopathy, WAG/RijY affected rats are housed with double the standard bedding to minimize bruising. Animals are monitored daily by Animal Resources Center or Rodent Services personnel for signs of bruising or injury; any adverse events are reported to the principal investigator and to Veterinary Clinical Service. Animals with minor bruising are monitored and treated as appropriate, and rats are euthanized for humane reasons when clinically indicated. Animals are used in accordance with protocols and policies approved by the Yale Institutional Animal Care and Use Committee.

Necropsy and histopathology. Blood was collected as a terminal procedure from rats rendered unconscious by CO_2 narcosis. For clinically abnormal rats with signs of bruising or bleeding, blood was collected by cardiac puncture and placed into the appropriate tubes for whole blood, plasma, and serum. For coagulation assays on healthy rats, asymptomatic for bleeding or bruising, the abdominal cavity was opened for venipuncture of the caudal vena cava with a syringe preloaded with 3.8% sodium citrate to provide a ratio of 1 part sodium citrate to 9 parts whole blood. After blood collection, rats were euthanized while still anesthetized. Plasma was removed after centrifugation and frozen at –70 °C until assayed. All tissues were fixed in 10% neutral-buffered formalin or Bouin fixative, processed, embedded in paraffin blocks, sectioned at 3 to 5 µm, transferred to glass slides, stained with hematoxylin and eosin, and mounted with glass coverslips by routine methods.

Clinical pathology. Hematology assays (Advia 120 Hematology Analyzer, Bayer Diagnostic Systems, Berlin, Germany) and clinical chemistry assays were performed according to standard methods by Antech Diagnostics (Irvine, CA), except that coagulation assays were performed by the Animal Health Diagnostic Laboratory (Comparative Coagulation Section, Cornell University, Ithaca, NY) under the direction of M B Brooks. Coagulation assays were performed by using an automated instrument with a mechanical endpoint detection method (STA Compact, Diagnostica Stago, Parsippany, NJ), an aPTT reagent with an ellagic acid activator (Actin FS, Dade-Behring, Marburg, Germany), and a rabbit brain thromboplastin PT reagent (Thromboplastin LI, Helena Diagnostics, Beaumont, TX). Clottable fibrinogen (Clauss method) was measured by using a human calcium thrombin reagent (80 NIH units/mL) and human fibrinogen calibrator (STA-Fibrinogen, Diagnostica Stago). WAG/RijY rats with clinical symptoms of bleeding were used for different tests depending on the blood volume that could be collected at necropsy. The tests performed on these rats included CBC, serum chemistry analyses, and a coagulation panel (aPTT, PT, D-dimer, and fibrinogen). Because of the sample volume required, not all tests could be performed on each rat. Blood and plasma samples from adult female Sprague–Dawley or Lewis rats were used as controls before the arrival of the WAG/RijMCW rats.

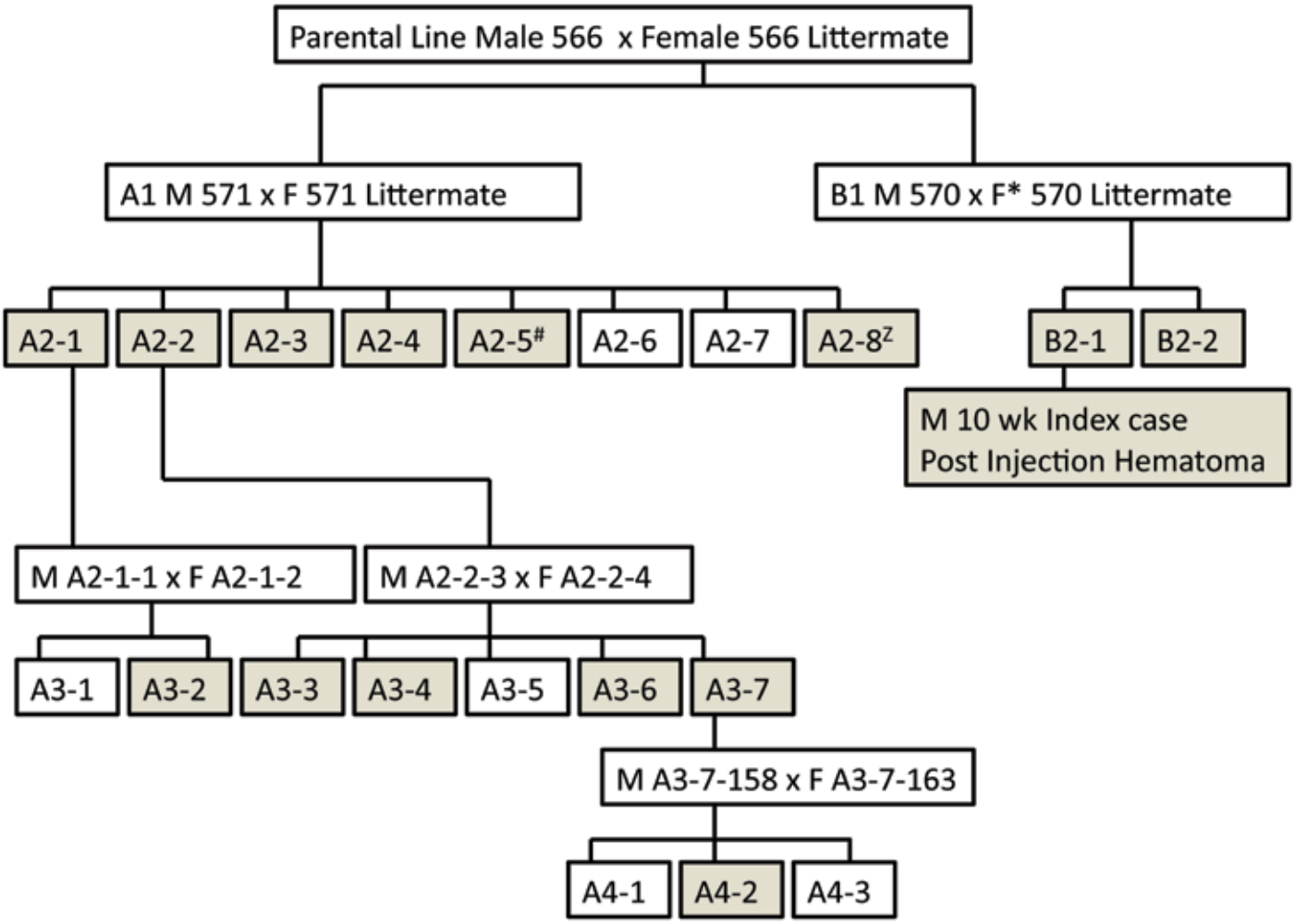

Figure 2. Pedigree of WAG/RijY strain. The colony is maintained by pair breeding of monogamous littermates. All of the affected rats descended from the same parental breeding pair (566). The index case came from the second generation of (B1, 570); this line was discontinued after the dam in the first B generation breeding pair (Female [F] 570, noted by *) died while pregnant with her third litter. All rats now in the colony are derived from the first A generation breeding pair (A1, 571). There were 8 litters in the second A generation (A2), but none of the pups in litter A2-5 survived (#). Two breeding pairs were used to generate generation A3, one of which has been used to generate generation A4. Litters with individual rats manifesting clinical signs of bleeding, death, or prolonged aPTT are shaded. The rats from litters in unshaded boxes were not tested or were euthanized prior to developing clinical signs and are therefore uninformative. Litter A2-8 (Z) was used for aPTT evaluation in Figure 4.

Results

Examination of blood smears from WAG/RijY rats affected with bleeding or bruising revealed no remarkable abnormalities in erythrocytes, leukocytes, or platelets. Histologic examination of tissues from necropsied clinically affected rats were unremarkable except for the severe, acute, focally extensive hemorrhage corresponding to the macroscopic findings (Figure 1), thus eliminating hereditary telangiectasis and cavernous hemangiomas as etiologies.[1] Noteworthy findings in the initial 7 WAG/RijY rats that presented with clinical symptoms of bleeding diathesis included prolonged (by 50% to 70%) aPTT (56 to 70 s; reference, 15 to 33 s),[8] normal or decreased hematocrit (15% to 38%; laboratory reference, 33% to 50%), normal PT (14.4 to 22.8 s; reference, 13.9 to 21.1 s; Figure 3),[8] normal or decreased WBC count (3.4 to 8.8 $\times$ 10^3 cells/μL; laboratory reference, 5.5 to 11 $\times$ 10^3 cells/μL), normal or decreased RBC count (2.45 to 7.9 10^6 cells/μL; laboratory reference, 5.5 to 10.5 $\times$ 10^6 cells/μL), normal or decreased hemoglobin (5.8 to 13.2 g/dL; laboratory reference, 11.4 to 19.2 g/dL), variable platelet count (300 to 1400 $\times$ 10^3 cells/μL), and normal fibrinogen and D-dimer concentrations (data not shown). In addition, MCV, MCH, and MCHC did not differ significantly between affected WAG/RijY rats and adult female Sprague–Dawley rats (data not shown). Reticulocyte counts were 3.5% and 20% for 2 WAG/RijY rats with bleeding and anemia. These findings were compatible with a bleeding diathesis that caused a normocytic, normochromic regenerative anemia secondary to blood loss.

Additional aPTT determinations from an entire litter of 8 apparently healthy 4-wk-old WAG/RijY rats (Figure 2, litter A2-8, designated by Z) revealed marked prolongation (greater than twice the control) for all individual rats (Figure 4). Plasma from 2 of the 4-wk-old WAG/RijY rats was mixed with an equal volume of plasma from healthy Lewis rats to screen for the presence of a coagulation inhibitor (Figure 4). The decreased aPTT of the mixture made the presence of a coagulation inhibitor unlikely. Instead, correction of the clotting time of the mixture was com-

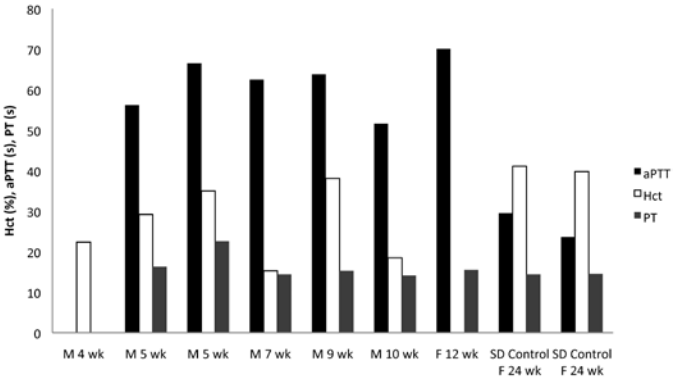

Figure 3. Individual WAG/RijY rats with clinical signs of bleeding have prolonged aPTT (reference, 15 to 33 s), normal or decreased hematocrit (Hct; reference, 33% to 50%), and normal PT (reference, 13.9 to 21.1 s) when compared with control Sprague–Dawley (SD) rats. F, female; M, male.

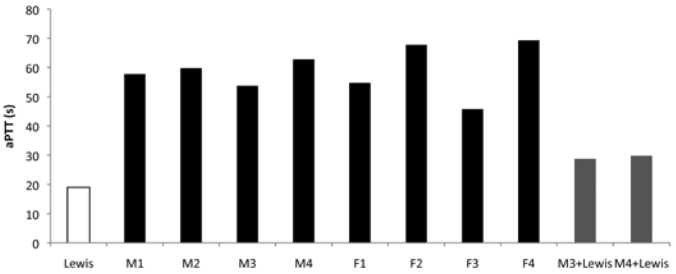

Figure 4. In this litter of clinically normal WAG/RijY rats (F, female; M, male) at 4 wk of age, PT was normal and aPTT was prolonged (46 to 70 s). The aPTT of WAG/RijY rats M3 and M4 were corrected by the addition of normal plasma from a Lewis rat (M3 + Lewis, 29 s; M4 + Lewis, 30 s) to a value closer to that of the Lewis rat (19 s). This result indicates that plasma inhibitors are not responsible for the prolonged aPTT and suggests the absence of a critical clotting factor in the blood of the affected rats.

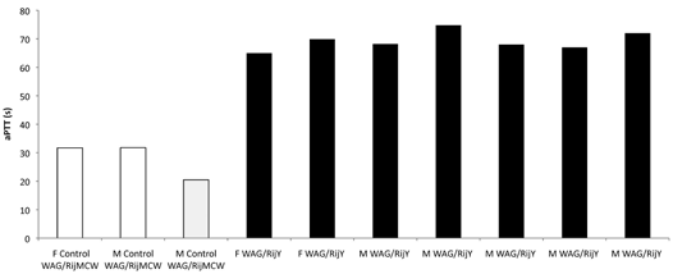

Figure 5. Adult clinically normal WAG/RijY rats have prolonged aPTT (65.0 to 74.5 s) when compared with that of the closely related WAG/RijMCW control strain (20.5 to 31.8 s).

patible with the lack of a critical clotting factor in the WAG/RijY plasma.

We then compared the aPTT of adult WAG/RijY rats with no overt hemorrhagic signs and WAG/RijMCW rats (Figure 5). WAG/RijY rats of both sexes had relatively prolonged aPTT, with values ranging from 2 to 3 times that of the strain-specific WAG/RijMCW controls. The hemostatic defect and associated laboratory finding of prolonged aPTT appear to be unique to the WAG/RijY colony rather than a characteristic common to all WAG/Rij rats.

Acute	Chronic
Gastrointestinal ulcers	Gastrointestinal ulcers
Hemostatic defects:	Hematuria
Toxins	Neoplasia:
Hemophilia A and B	Vascular
Disseminated intravascular coagulation	Gastrointestinal
Neoplasia:	Parasites:
Splenic hemangioma	Ectoparasites
Splenic hemangiosarcoma	Endoparasites
Surgery	Vitamin K deficiency
Thrombocytopenia	
Trauma	

Figure 6. Causes of hemorrhage. Modified from reference 6.

Discussion

We have identified a hemorrhagic diathesis that apparently developed de novo in the WAG/RijY rat colony. This trait is characterized by specific prolongation of aPTT in rats with and without clinical signs of bleeding. In addition, WAG/RijY rats with clinical signs had an associated normocytic normochromic regenerative anemia with decreased hemoglobin attributed to blood loss. The possible causes of acute and chronic blood loss are many (Figure 6);[6] however, most potential acquired coagulopathies have been excluded due to the colony's controlled husbandry and environmental conditions, through histopathologic examination, and through clinicopathologic assays. The limitation of clinical signs of acute hemorrhage to the WAG/RijY colony, as opposed to affecting any of the many other rat colonies at Yale University, rules out toxins and vitamin K deficiency as likely etiologies. The findings of normal PT, normal fibrinogen concentration, and prolonged aPTT are compatible with deficiency of an intrinsic or contact pathway factor. Bleeding diatheses due to hereditary deficiencies of factors VIII, IX, or XI in humans and other species have been described.[7] In contrast, deficiency of contact pathway factors (including, prekallikrein and high-molecular–weight kininogen) typically cause in vitro prolongation of clotting but are not associated with an overt bleeding tendency.

We have eliminated all causes for hemorrhage in the WAG/RijY rats except for a primary hemostatic defect. Hemophilia is among the most prevalent bleeding disorders in humans[13] and with von Willebrand disease, these disorders comprise more than 95% of all known inherited deficiencies of coagulation factors.[14] Excluding von Willebrand disease, rare clotting factor deficiencies of fibrinogen, prothrombin, or factors V, VII, X, XI, and XIII, or combined V+VIII[3] comprise approximately 15% of the reported clotting factor deficiencies. Spontaneous hemophilia has been reported to occur in many species of domestic animals, including cattle, sheep, cats, horses, and various breeds of dogs.[13] However, well-characterized spontaneous hemophilia or von Willebrand disease in rats has not been reported.[13] Animal models are essential for developing new and improved therapeutic options

for treating bleeding disorders, and rats are commonly used for pharmacokinetic absorption, distribution, metabolism, and elimination studies needed to develop new therapies. A rat model would provide a novel and valuable tool for the study of new therapeutics.[15] The ultimate goal of our ongoing studies is to develop the inbred WAG/RijY rat substrain carrying this inherited coagulopathy into a model for use in improving the understanding, management, and treatment of the corresponding human disease. Further work is ongoing to determine which component of the coagulation pathway is defective and responsible for the spontaneous coagulopathy in WAG/RijY rats.

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