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A Canine Model of Hemophilic (Factor VIII:C Deficiency) Bleeding

By Alan R. Giles, Shawn Tinlin, and Ronald Greenwood

A model of bleeding due to clotting factor deficiency has been developed in dogs. Normal and hemophilic (factor VIII:C deficient) animals were used. Bleeding was induced in lightly anesthetized animals by severing the apex of the nail cuticle using a guillotine device. In normal animals, bleeding usually ceased spontaneously after 2–8 min. In contrast, in hemophilic animals, bleeding continued for up to 20 min and necessitated either cauterization or the application of topical thrombin to achieve hemostasis. Pretreatment of the hemophilic animals with canine cryoprecipitate corrected the cuticle bleeding time to within

the range noted for normal animals. The method is simple and reproducible and has the advantage that a number of observations can be made sequentially on the same animal. Rebleeding of the cauterized cuticle of the hemophilic animals did not usually occur. This model has considerable potential for the preclinical testing of products considered to bypass or replace factor VIII:C in patients with acquired inhibitors of factor VIII:C and may be adapted to the study of other mechanisms involved in normal and abnormal hemostasis.

THE DISCOVERY and subsequent characterization of various congenital deficiencies of blood coagulation factors has led to many of the major advances in the understanding of the mechanisms involved in normal and abnormal hemostasis. Although many examples of similar discrete clotting factor deficiencies have been described in animals,¹ the development of models of bleeding relating to these disorders has received relatively little attention. Nonetheless, such models would have considerable potential in the development of more innovative approaches to clotting factor replacement. For example, prothrombin complex concentrates, both “activated” and “nonactivated,” appear to bypass the inhibition of factor VIII:C in hemophiliacs who have acquired antibodies to factor VIII:C.^{2,3} The clinical benefit does not appear to be optimal, however, and is hampered by uncertainty with regard to the putative factor(s) responsible.⁴ In vivo studies in a suitable animal model could well assist in delineating this and result in the development of more effective therapy.

In order to achieve these goals, an animal model must closely mimic the human disease to be studied. There must also be a reproducible means of identifying both the consequences of the deficiency state, i.e., bleeding, and its response to replacement therapy. The following report describes the development of such a model in dogs.

MATERIALS AND METHODS

Animals

Six normal male mongrel dogs weighing between 10 and 12 kg were obtained from the Queen's University Animal Facility. Each animal was screened for coagulation abnormalities (see below) prior to being used in the studies described. The two hemophilic (factor VIII:C deficient) animals used were pure bred miniature Schnauzers, weighing approximately 6 kg, and were the generous gift from Drs. Bowie and Fass of the Mayo Clinic, Rochester, Minn. The four female obligate hemophilic carrier bitches were crossbred miniature Schnauzer-Brittany spaniels, weighing between 10 and 12 kg, and were also supplied by Drs. Bowie and Fass. All animals were

maintained on water ad libitum and regular dry dog chow (Ralston-Purina, St. Louis, Mo.).

Cuticle Bleeding Time (CBT) Determination

All animals were lightly anesthetized with a rapid-acting intravenous barbiturate [Bio-Tal (MTC Pharmaceutical, Mississauga, Canada) 5%–18 mg/kg body weight]. A continuous infusion was established via a 21-gauge butterfly needle (Abbot-Ireland Ltd., Sligo, Ireland) in the cephalic vein using isotonic saline for injection to keep the vein open. All medications were given via this route.

Prior to injuring the cuticle, all hair was carefully removed by clipping the vicinity of the claw. Silicone grease (Dow Corning, Midland, Mich.) was applied to the claw in order to prevent blood from tracking back beneath the nail. Where the apex of the cuticle could be visualized, it was severed as shown in Fig. 1 and blood allowed to fall freely by positioning the paw over the edge of the operating table. A spring-loaded sliding blade guillotine nail clipper (Resco, Detroit, Mich.) was used. In some animals, with dark colored nails, the cuticle could not be accurately visualized. It was found by trial and error that the dorsal nail groove generally terminated at the apex of the cuticle, and this was used as the anatomical marker in such cases. Where necessary, bleeding was arrested either with silver nitrate applicators (75%, Ingram and Bell, Ltd., Don Mills, Ontario) or the application of topical thrombin (Parke Davis, Detroit, Mich.).

In hemophilic animals treated with cryoprecipitate, a pretreatment CBT was performed and bleeding stopped as described above. The prescribed dose of cryoprecipitate (see below) was then given and the test repeated 30 min after the administration of cryoprecipitate. Factor VIII:C determinations were performed pre- and post-testing and, in the case of the hemophilic animals, pre- and post-administration of cryoprecipitate.

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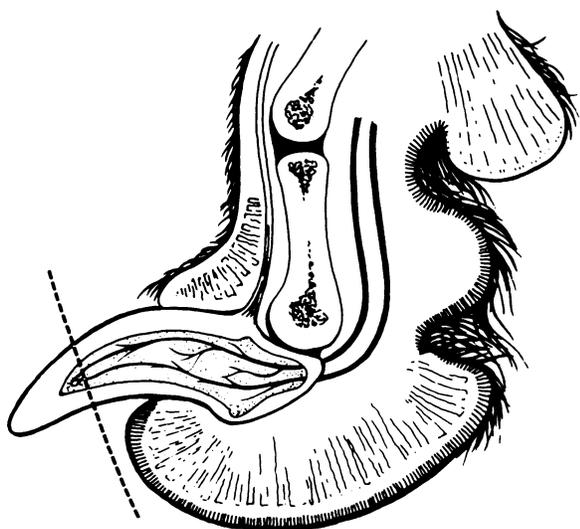


Fig. 1. Sagittal section of paw through the foot pad and nail. The dotted line shows the plane of incision in the nail cuticle.

Canine Cryoprecipitate

Cryoprecipitate was prepared from canine whole blood taken from normal dogs into plastic double bags (Fenwall Laboratories, Division Baxter Travenol, Malton, Ontario) and anticoagulated with CPD (USP), 387 ml blood to 63 ml anticoagulant. The cryoprecipitate fraction was separated by conventional blood banking techniques and frozen and stored at -70°C until used. Random quality control of the cryoprecipitate prepared suggested an average factor VIII:C content of 5 U/ml. To obtain a prescribed percent increment (I) of factor VIII:C, the dose in units to be administered was calculated according to body weight in kilos $\times 0.4 \times I$.

Coagulation Assays

Blood for coagulation assays was anticoagulated with sodium citrate (3.8% w/v), 9 volumes of blood to 1 volume anticoagulant. The prothrombin time (PT) was measured on a Coag-A-Mate 2001 using General Diagnostic reagents (General Diagnostics, Division Warner Lambert Co., Morris Plains, N.J.). The partial thromboplastin time (APTT) was measured manually using cephalin (rabbit brain, Sigma, St. Louis, Mo.) and kaolin (acid washed, Fisher Scientific Co., Fair Lawn, N.J.) as an activator. The thrombin clotting time (TCT) was measured by a modification of the method of Fletcher and coworkers⁵ on a fibrometer (B.B.L., Division Becton Dickinson Co., Cockeysville, Md.) following the addition of 0.5 NIH U thrombin (Parke Davis, Detroit, Mich.).

Factors XII, XI, IX, and VIII:C were all assayed using a one-stage activated partial thromboplastin time technique using congenitally deficient human plasmas and a normal canine pool plasma (obtained from 10 healthy dogs) as a reference standard. The pool plasma was defined as having 1 U/ml (100%) of each clotting factor. Factors VII, X, V, and II were determined using standard assays using congenitally deficient human plasmas and normal pool dog plasmas as a reference standard. Factor VIII:Ag was measured as described by Zimmerman and coworkers⁶ using a rabbit anti-human factor VIII:Ag.

All animals were initially screened by a combination of PT, APTT, and TCT tests. The normal range for each test (mean \pm 2 SD) was established for a separate group of normal healthy animals ($n = 13$). They were: PT 10.4 ± 1.4 sec; APTT 19.9 ± 4.8 sec; TCT

23.2 ± 1.4 sec. With the exception of VIII:C, which was performed on all animals, factor assays were only performed on animals showing an abnormality in the screening tests, i.e., the hemophilic animals.

RESULTS

Development of Method

The cuticle was injured as described under Materials and Methods. Initial pilot studies were performed in both normal and hemophilic animals.

In the normal dogs, bleeding invariably stopped within 8 min of injury induction, although the occasional animal bled for as long as 12 min. It was then unusual for bleeding to recommence unless the clot was disturbed by the operator or the animal during recovery from anesthesia. In contrast, the hemophilic animals showed a quite different pattern. Although bleeding occasionally stopped spontaneously, within the same time interval noted for normal animals, it invariably restarted and continued unabated until measures were taken by the operator to arrest bleeding. As the hemophilic animals in the pilot study bled for at least 20 min, whereas it was rare for a normal animal to bleed longer than 12 min, initially the protocol involved a 20-min observation period. Occasionally, a drop of blood formed at the site of injury but did not fall. Failure to do so within 1 min of forming invariably signalled the conclusion of bleeding. Consequently, to facilitate comparisons, the cuticle bleeding time was "rounded up" from the time of incision to the minute following cessation of bleeding. When rebleeding occurred, a second stopwatch was started at the time bleeding began again and the time of bleeding "rounded up" as above. Cautery was performed at 20 min if required. This was usually only necessary in the hemophilic animals.

Determination of Cuticle Bleeding Time (CBT)

The mean CBT of the normal dogs (mean factor VIII:C 117.3 ± 30.8) was 6.0 ± 3.7 min (SD). In obligate hemophilic carrier females (mean factor VIII:C $54.5\% \pm 5.5$) it was 4.47 ± 5.9 min (not significant, $n = 15$, unpaired t test). In the hemophilic animals (factor VIII:C $< 4\%$), bleeding generally continued uninterrupted for 20 min in all cases and had to be arrested with either the application of silver nitrate or topical thrombin. In some cases, bleeding resulted in a substantial reduction in hematocrit, suggesting that it would be preferable to shorten the period of observation. Figure 2 shows the differences observed between normal and hemophilic animals as a percentage of cuticles bleeding after successive periods of time following injury induction. It can be seen that the

ANALYSIS of CUTICLE BLEEDING TIME DATA

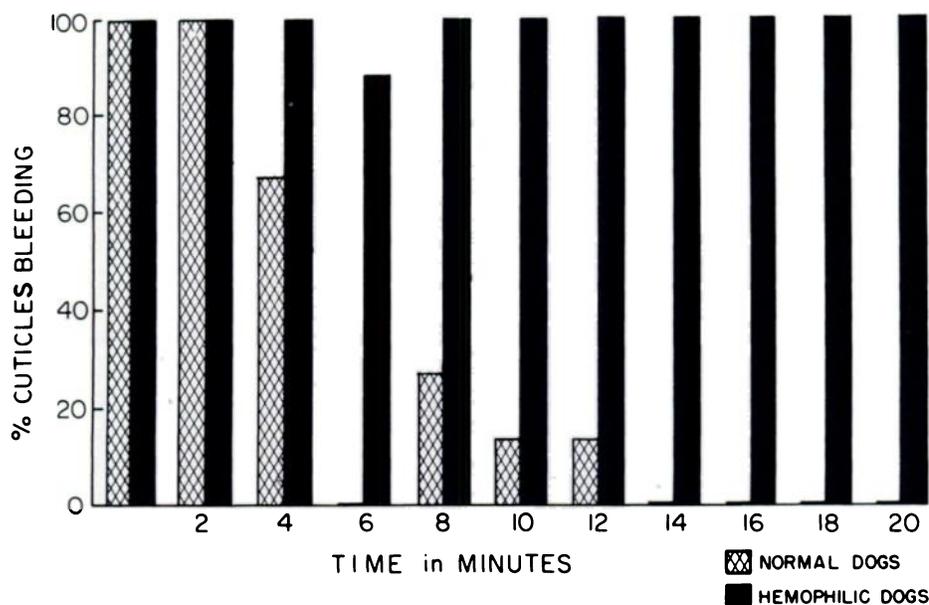


Fig. 2. Comparison of CBT determined in normal and hemophilic animals. Each vertical bar shows the percentage of nails bleeding at sequential 2-min intervals following the injury to the cuticle at zero time ($n = 15$).

percentage differences between the 2 groups is significant at 12 min. The occasional rebleed noted in the normal animals invariably followed disturbance of the clot due to involuntary movement by the animal. This occurred as the level of anesthesia lightened, usually when 3 or more observations were made in the same period. Thus, shortening of the observation period from 20 to 12 min resolved this problem. Four observations could easily be made during the deepest plane of anesthesia, and the power of discrimination was increased as rebleeding did not occur in the normal animals.

Response to Cryoprecipitate

The CBT was determined in 2 nails prior to the administration of cryoprecipitate to one of the hemophilic animals, and typical results were obtained, i.e., CBT > 20 mins. Thirty minutes following cryoprecipitate administration, the CBT had corrected to within the normal range. The factor VIII:C levels pre- and postcryoprecipitate were <4% and 60%, respectively. This response was highly reproducible on restudy in both animals tested.

Further studies were performed where the effect of various levels of factor VIII:C on the cuticle bleeding time was determined. These were all performed on one animal. The cuticle bleeding time was normal at factor VIII:C levels greater than 25%. At levels below 20%, the cuticle bled throughout the whole observation period, i.e., 20 min.

Coagulation Studies

The hemophilic animals had a discrete deficiency of factor VIII:C with normal levels of factor VIII:R:Ag, and all other clotting factors assayed, i.e., within 2 standard deviations of the mean level for each determined in a group of 6 normal animals. The obligate hemophilic carrier bitches had normal levels of factor VIII:R:Ag, but a mean factor VIII:C coagulant to factor VIII:R:Ag ratio of 0.67 ± 0.05 (SD).

It should be emphasized that, due to the unavailability of canine-deficient substrate plasmas, human congenitally deficient plasmas were used. Canine plasma, in contrast to human, contains substantially higher levels of most coagulation factors, approximately eightfold in the case of factor VIII:C. Consequently, the clotting times of the lower dilutions of the test plasma in the factor assays are influenced by the concentration of clotting factors other than the one specifically being assayed. This lack of specificity results in an overestimate of the level of any given clotting factor. The magnitude of this discrepancy is inversely related to the actual level of the clotting factor being assayed. Nonetheless, the differences noted were marginal. For example, the 1:10 dilution of plasma obtained from one of the hemophilic animals assayed at 4% factor VIII:C against pooled canine plasma whereas the 1:20 assayed at 1.5%. Following infusion of cryoprecipitate into this animal, the same dilutions assayed at 60% and 58%, respectively. It was considered that this degree of nonspecificity was

acceptable in terms of achieving the objectives of the studies described.

DISCUSSION

The hemophilic dogs used appeared to have an identical factor deficiency state to that described in humans and therefore are suitable for the preclinical study of therapy directed at ameliorating this condition. However, the precise quantification of the severity of sporadic clinical hemostatic events, such as muscle hematomas and hemarthrosis, and their response to therapy is difficult. The model of bleeding described circumvents this problem by allowing quantification of the time of bleeding following deliberate injury of the nail cuticle. Using this parameter, clear differences could be determined between both hemophilic and normal dogs and hemophilic dogs and dogs with clinically insignificant reduction in factor VIII:C levels, i.e., obligate carriers. Moreover, restoration of the factor VIII:C level to normal by replacement with normal canine cryoprecipitate resulted in a correction of the cuticle bleeding time defect. Consequently, this model would appear to be suitable both for testing the consequences of modifying factor VIII:C activity, e.g., by infusing factor VIII:C antibodies into normal dogs, and the detection of factor VIII:C bypassing or replacing activity in hemophilic animals.

The model described requires light anesthesia. We are aware of a report describing qualitative platelet defects following the use of barbiturate anesthetics.⁷ Consequently, it is essential that all studies performed should include appropriate controls to exclude this as a contributing factor to the results observed. However, a particular advantage of this model is that more than one observation may be made in any one animal during

any one period of study. Consequently, the animal may be used as its own control by performing tests before and after therapeutic intervention. Each dog has 16 usable claws (the dew claws were not used) and the injury rapidly heals making it feasible to use the same nail 3–4 wk later. This did not significantly modify the results previously obtained for any one nail (results not shown).

Rebleeding from the cauterized cuticle in the hemophilic animals did not usually occur, and no form of surgical dressing was required. This, and the facility for performing multiple tests in one animal, results in advantages for this model over that recently described by Kingdon and coworkers. Block resection of the gum in hemophilic dogs resulted in bleeding that was responsive to factor VIII:C replacement therapy.⁸ However, rebleeding due to salivation and disturbance of the clot by the animal's tongue was apparently a problem in some tests.

In conclusion, we believe that this model closely simulates the human disease of classical hemophilia A (factor VIII:C deficiency). The model described is simple and reproducible and clearly discriminates between normal and abnormal animals. It has obvious applications to the study of other hemostatic disorders and as such may be used both for furthering the understanding of the mechanism of action of specific coagulation factors and the research and development of new therapies for disorders of hemostasis.

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