Development of Factor VIII:C Antibodies in Dogs With Hemophilia A (Factor VIII:C Deficiency)

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Classic hemophilia A (factor VIII:C deficiency) was diagnosed in a miniature Schnauzer dog and a breeding program established. Inbreeding and crossbreeding produced 16 hemophilic animals. All were initially treated with canine cryoprecipitate, as required, for sporadic hemorrhagic events. Five animals developed potent antibodies to canine factor VIII:C. All were the offspring of obligate carriers, resulting from the mating of a hemophilic purebred miniature Schnauzer male to a normal female Brittany spaniel. The mean age at first treatment and factor VIII exposure at the time of inhibitor development was 10.3 wk and 286.3 U, respectively. The remaining hemophilic animals have not developed antibodies, despite receiving a mean factor VIII dosage of $1.5 \times 10^7$ U. This group includes animals derived from a mating between the same purebred miniature Schnauzer hemophilic male and a purebred miniature Schnauzer carrier female. In each case, the antibodies recognize both canine and human but not porcine VIII:C. They are nonprecipitating IgG immunoglobulins. Following inhibitor development, infusion of canine cryoprecipitate was hemostatically ineffective and factor VIII:C recovery at 30 min was negligible. Infusion of a concentrate of porcine factor VIII resulted in a correction of the hemostatic defect and optimal factor VIII:C recovery. All animals receiving porcine factor VIII:C subsequently developed antibodies to this protein. The chance occurrence of this complication should facilitate further studies directed at elucidating the pathogenesis and management of hemophilia complicated by the development of antibodies to factor VIII:C.

ANTIBODIES TO FACTOR VIII:C develop in approximately 10% of all patients with classical hemophilia A (factor VIII:C deficiency) who receive factor VIII replacement therapy. Together with the risk of transmission of infective agents, such as hepatitis B, by multiple transfusion, it represents one of the most serious problems in the current management of hemophilia. Consequently, both the pathogenetic mechanisms involved in their development and alternative modes of management to conventional factor VIII replacement have received considerable attention. The incidence of inhibitors is highest in hemophiliacs with severe disease, but this may relate only to reduced exposure to factor VIII therapy in patients with mild disease. Familial studies have demonstrated an apparent inherited propensity for inhibitor development, but a suggested association with major histocompatibility loci was not confirmed subsequently by more intensive study.

In recent years, we have developed a breeding program of hemophilic dogs in order to facilitate the development of factor VIII:C bypassing therapy. In this article, we present information relating to the development of antibodies to factor VIII:C in a defined arm of our breeding program where transfusion exposure of all the animals concerned has been confined to homologous proteins.

MATERIALS AND METHODS

Animals

The family tree of the animals currently available for study is shown in Fig. 1. The breeding program was initiated in a specially constructed facility at Queen’s University, using breeding stock kindly provided by Drs. W. Bowie and D. Fass of the Department of Hematology Research at the Mayo Clinic, Rochester, MN. These included 4 obligate carrier bitches (III.2, III.3, III.4, III.5), 1 hemophilic male (II.4), and 1 hemophilic female (III.8). The hemophilic animals were purebred miniature Schnauzers from the original stock donated to the Mayo Clinic by Dr. Richard Davis of Omaha, NE. The obligate carriers were crossbreds obtained by mating a purebred miniature Schnauzer with a purebred Brittany spaniel (II.1). The obligate carrier bitches were mated with a single purebred beagle sire (III.1). The same purebred beagle was mated with the purebred hemophilic female miniature Schnauzer (III.8). This animal was also mated back to her hemophilic uncle (II.4). All animals were maintained on water ad libitum and regular dry puppy/dog chow (Ralston Purina, St. Louis, MO).

Cuticle Bleeding Time (CBT)

The cuticle bleeding time was performed as previously described, but the period of observation was reduced to 12 min. Cuticles still bleeding at that time were cauterized with silver nitrate, and the cuticle protected by the application of collodion. In animals treated with factor VIII replacement (see below), a pretreatment CBT was performed and then repeated 30 min after the administration of the factor VIII preparation used. The cuticle bleeding time should not be confused with the skin bleeding time, as used in clinical practice. Whereas the latter specifically tests for the integrity of primary hemostasis, the CBT is sensitive to discrete coagulation factor deficiencies. Its role in evaluating defects in primary hemostasis has not been assessed.

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Factor VIII:C Replacement Therapy

All hemophilic animals were treated with canine cryoprecipitate for hemorrhagic events as necessary. In addition, this was given as part of an experimental protocol (see Results). Some animals were treated with porcine factor VIII concentrate (Hyate, Speywood Labs., Nottingham, U.K.). This was provided as a lyophilized powder, which was reconstituted with water for injection immediately prior to use. Cryoprecipitate was prepared from canine whole blood as previously described. In each case, the prescribed dose was determined using a standard formula. Random quality control of the canine cryoprecipitate produced suggested a factor VIII:C content of 5 U/mL. Assay of the reconstituted porcine material against pooled normal canine plasma (see below) suggested that 1 U of Hyate (manufacturer’s specification) was equivalent to 0.125 U of canine factor VIII:C. In the studies described, the dose of porcine factor VIII was adjusted accordingly.

Coagulation Assays

Blood for coagulation assays was anticoagulated with sodium citrate (3.8% w/v), 9 vol of blood to 1 vol anticoagulant. All blood samples were taken by a two-syringe technique. The prothrombin time, partial thromboplastin time, thrombin clotting time, and factor VIII:C assays were performed as previously described. Normal canine pool plasma (collected from 25 normal animals) was used as a reference standard throughout and considered to contain 1 U of factor VIII:C activity/mL plasma. The presence of inhibitors to canine factor VIII:C was identified by incubating equal volumes of pooled normal canine plasma with varying dilutions of the test plasma for 2 hr at 37°C. When identified, an inhibitor was expressed in Bethesda units (BU) by determining the residual factor VIII:C level following the incubation described. When a particular animal was found to have an inhibitor to canine factor VIII:C, the assays were repeated, substituting both human or porcine plasma for canine plasma in the initial incubation. In each case, the standard curve used in assaying residual factor VIII:C activity was derived using a source of factor VIII:C of the same species as that used in the initial incubation. The antibodies were classified according to criteria established by Biggs and coworkers. They were defined as precipitating or nonprecipitating by the gel diffusion technique of Ouchterlony, using canine, human, and porcine factor VIII:C. Both plasma and concentrates were used in each case.

Immunoglobulin Characterization of Factor VIII:C Inhibitor

Immunoglobulin fractions were prepared from serum collected from animals with factor VIII:C inhibitors by ammonium sulfate precipitation and anionic exchange chromatography. Individual fractions were checked for immunoglobulin class homogeneity by immunoelectrophoresis against rabbit anti-canine class-specific immunoglobulin antisera (Miles-Yeda Ltd., Israel). Each was then reassayed for factor VIII:C inhibitory activity in the Bethesda assay, as previously described.

RESULTS

All the hemophilic animals, both male and female, are severely affected. Clinically, they suffer from spontaneous hemorrhagic events similar to those seen in human hemophilia A. Scalp hematomas and hemorrhage in the hind legs are the most frequent, but two animals have had major intraabdominal bleeds (see below). The factor VIII:C levels are <1% in all but two animals, who have levels of <4%. Initially, we elected to treat all hemorrhagic events aggressively with canine cryoprecipitate. The dose administered was adjusted according to the assessment of clinical risk following the general principles established in clinical practice. The therapeutic protocol used included the collection of postinfusion plasma specimens for factor VIII assays. Although not always processed immediately, they were stored at −70°C and were available for retrospective evaluation.

Development of Inhibitor to Canine Factor VIII:C

As can be seen from Fig. 1, 5 of 15 available affected animals have developed inhibitors to canine factor VIII:C. This was first recognized in one animal who failed to show an adequate therapeutic response to canine cryoprecipitate given for a hemorrhage in a
hind leg. Subsequent assay of postinfusion specimens revealed negligible factor VIII:C recovery 30 min postinfusion, and inhibitor assays demonstrated the presence of a potent anti-canine factor VIII:C antibody. Subsequently, inhibitor assays were performed routinely on all animals at 3-mo intervals or sooner if response to therapy appeared to be inadequate. The mean age and factor VIII exposure at the time of first demonstration of a factor VIII:C inhibitor was 10.3 ± 5.6 (SD) wk and 286.3 ± 1.56.1 U, respectively. All the animals with inhibitors are the offspring derived from the initial mating of the normal Brittany spaniel (II.1) and the hemophilic purebred miniature Schnauzer (II.2). In contrast, none of the other hemophilic animals have demonstrated this propensity, despite having received a mean total factor VIII dosage of 1.5 x 10^6 U.

Characterization of Factor VIII Inhibitor

Each inhibitor was assayed for its potency and species specificity using the Bethesda assay as described under Materials and Methods. The results are shown in Table 1. In each case, initially, the inhibitor recognized both canine and human but not porcine factor VIII:C. The apparent paradoxically higher activity against human factor VIII:C is explained by the increased level of this clotting factor per unit volume of canine plasma in comparison to human. In each case, the antibodies appear to be type II, according to the Biggs classification, i.e., residual factor VIII:C activity can always be measured even following incubation with undiluted inhibitor plasma. Similarly, all animals developing inhibitors had detectable levels of circulating factor VIII:C (range 1%-4%) after transfusion of factor VIII. A typical example of the inactivation of factor VIII:C by varying dilutions of the antibody is shown in Fig. 2.

Preparation of immunoglobulin fractions demonstrated that the inhibitory capacity resided in the IgG fractions. Subclass identification could not be determined due to the unavailability of subclass-specific anti-canine antibodies. The antibodies developing after exposure to only canine cryoprecipitate were nonprecipitating against both canine and human factor VIII:C. Similarly, the antibodies recognizing porcine VIII:C, following porcine factor VIII concentrate infusion, were also nonprecipitating against all three species of factor VIII:C.

In Vivo Response to Factor VIII:C Infusion

First demonstration of antibody development occurred following the apparent suboptimal response to canine cryoprecipitate. The same animal subsequently developed a catastrophic intraabdominal hemorrhage, with the hemoglobin level falling from 12 to 5 g/dl overnight. In view of the lack of specificity of the antibody for porcine factor VIII:C, the animal was

![Fig. 2. Dilutions of the inhibitor serum were made in saline and incubated with an equal volume of pooled canine plasma for 2 hr at 37°C. The volume of inhibitor plasma is plotted against the factor VIII:C remaining after 2 hr. Assayed as described under Materials and Methods.](image-url)
treated with porcine factor VIII concentrate. This resulted in a dramatic improvement in his clinical condition. Postinfusion studies demonstrated optimal factor VIII recovery. He subsequently had a recurrence 2 wk later, but an identical protocol, using porcine factor VIII, resulted in an initial suboptimal clinical response and insignificant factor VIII:C recovery at 30 min. Further transfusion of porcine factor VIII resulted in an initial suboptimal recovery. He subsequently had a recurrence 2 wk later, but an identical protocol, using porcine factor VIII, resulted in an initial suboptimal recovery. Figure 3 shows these events related to factor VIII:C antibody activity. It can be seen that, at the time of recurrence, a low titer antibody recognizing porcine factor VIII:C was now detectable. This became indetectable for 2 days following porcine factor VIII infusion, but subsequently rose to significant levels. We assume that the initial lack of response was associated with the presence of this newly developed antibody and that the subsequent response, following further infusion, followed its saturation.

The above experience prompted us to perform controlled studies using the CBT in order to obtain more definitive data as to the in vivo activity of different species of factor VIII:C in the presence of the antibody to canine factor VIII:C. The results of a typical experiment are shown in Fig. 4. The effects of infusing canine cryoprecipitate into a hemophilic animal, uncomplicated by inhibitor development, was compared with an animal with a demonstrable inhibitor. In each case, an equivalent dose of factor VIII:C, determined on the basis of body weight, was given. Canine cryoprecipitate produced prompt correction of the CBT in the noninhibitor animal, whereas no correction occurred in the animal with the inhibitor. Similarly, factor VIII:C recovery was optimal in the noninhibitor animal, whereas no factor VIII:C was recovered 30 min postinfusion in the animal with an inhibitor. In contrast, infusion of porcine factor VIII concentrate into this animal resulted in an optimal response in the CBT, together with an equivalent recovery of factor VIII:C. Similar results were obtained following the infusion of canine cryoprecipitate into the animal whose clinical course, following porcine factor VIII concentrate, is illustrated in Fig. 3. Despite the inhibitor level having fallen to a low of 1.75 BU from a high of 27 BU over a period of 42 wk, negligible factor VIII:C recovery was recorded 30 min after a dose calculated to give a 50% increase. The prolonged CBT (>12 min) was not corrected at 15 or 30 min. Despite this observation, no anamnestic response in the anticanine factor VIII:C antibody has occurred 13 wk later.

**DISCUSSION**

The development of antibodies to factor VIII:C in hemophiliacs treated with factor VIII replacement represents one of the major problems in the care of hemophiliacs today. This relates not only to the management of individuals with the complication, but also to the counseling of their relatives with regard to the potential for other affected family members to acquire this complication. Although epidemiologic data suggest a familial tendency, we remain completely ignorant as to the precise pathogenetic mechanisms involved. Consequently, the occurrence of this complication in an animal model of the human disease promotes the opportunity to study the possible mechanisms involved that goes beyond the data currently presented.

Study of the inheritance pattern suggests that the propensity for inhibitor development may not be inherited in direct association with the primary genetic abnormality, but rather as a result of its chance...
association with a genetically expressed function acquired from an unaffected individual, in this case, the Brittany spaniel. We cannot exclude the possibility that the propensity is associated with the miniature Schnauzer (II.2) and that segregation has resulted in its limited expression. This mating will be repeated to reexamine that possibility.

The species specificity of the antibody is of both practical and basic interest. The lack of specificity for porcine factor VIII:C allowed us to treat what would have otherwise been a fatal intraabdominal hemorrhage in one animal. This is in line with clinical experience using this preparation. 

We were subsequently able to confirm the efficacy of porcine factor VIII concentrate in more adequately controlled studies using the CBT model previously described.

These data suggest that the epitope recognized by the canine/anti-canine factor VIII:C antibody is either not present or is protected in the porcine factor VIII:C molecule. Our present data do not allow us to come to any firm conclusions with regard to the canine anti-porcine factor VIII:C antibody resulting from the infusion of the porcine factor VIII concentrate. Our present data do not allow us to come to any firm conclusions with regard to the canine anti-porcine factor VIII:C antibody resulting from the infusion of the porcine factor VIII concentrate. Our present data do not allow us to come to any firm conclusions with regard to the canine anti-porcine factor VIII:C antibody resulting from the infusion of the porcine factor VIII concentrate.

Our impression is that it is recognizing a different epitope with different functional properties, but further studies will be required to confirm this. Gawryl and coworkers have recently demonstrated that type II (Biggs classification) anti-factor VIII:C antibodies appear to recognize an epitope on factor VIII:C at, or close to, its binding site to factor VIII:Ag/von Willebrand factor. We have not yet performed similar characterization studies of the canine/anti-canine factor VIII:C antibodies, due to the requirement for relatively pure preparations of canine factor VIII:C. Such studies are in progress. Factor VIII:Ag/von Willebrand factor appears to promote or stabilize the normal function of the factor VIII complex. Confirmation of Gawryl and coworkers' findings in the canine/anti-canine system could explain the association of detectable factor VIII levels with negligible recovery following cryoprecipitate transfusion. It may be expected that an antibody with this epitopic specificity may exert its predominant effect on in vivo survival rather than factor VIII:C coagulant function.

Of additional interest was the absence of an anamnestic antibody response in the face of negligible factor VIII:C recovery following the infusion of canine cryoprecipitate in one animal with a low titer antibody. In the clinical management of hemophiliacs with inhibitors, it has been suggested that failure to mount an anamnestic response is an indicator of a good prognosis for future management. Our results lend support to anecdotal clinical reports that this is not necessarily the case. Results from both this and previous studies employing the CBT support the view that clinical response is closely related to postinfusion recovery of factor VIII:C.

We conclude that the development of canine/anti-canine antibody in animals with classical factor VIII:C deficiency closely mimics many features of the human disease with this complication. Consequently, further studies should allow us to both investigate the pathogenetic mechanisms involved and to derive information that may be used to more effectively manage hemophiliacs with this difficult complication.

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