Hemophilic Dog Model for Evaluating Therapeutic Effectiveness of Plasma Protein Fractions

By Henry S. Kingdon and Thomas M. Hassell

Therapeutic effectiveness of factor VIII inhibitor bypassing materials has been evaluated in dogs with hemophilia A. A standardized template gingival biopsy was performed using local anesthesia. Hemophilic dogs bled extensively from the biopsy site, whereas in normal dogs the wound was sealed within 5 ± 2 min. If untreated, the hemophilic dogs frequently bled for several days. Factor VIII infusion stopped the bleeding promptly. Some experimental preparations of factor VIII inhibitor bypassing materials were shown to be therapeutically effective, whereas others were not. Intravascular thrombi could not be demonstrated histologically. The model should prove useful for evaluating factor VIII inhibitor bypassing materials and also for evaluating their mechanism of action.

A BOUT 15% of patients with hemophilia A develop inhibitors to factor VIII. While these inhibitors do not appear to influence the frequency of bleeding episodes or their severity, they render such patients very difficult to treat, particularly if the inhibitors are of high titer or if there is a strong anamnestic response of inhibitor following treatment with factor VIII.

A decade ago, it was observed that the factor IX clinical concentrates then available appeared useful for treating bleeding episodes in patients with factor VIII inhibitors. Concern was developing at that time regarding the possible thrombogenicity of factor IX concentrates when used as replacement therapy for patients with factor IX or factor X deficiency, especially those with liver dysfunction. The nonactivated partial thromboplastin time was adopted in our laboratory to test for potentially thrombogenic materials. Concomitant with this effort, the nonactivated partial thromboplastin times produced by the final product. Concomitant with this effort, the clinical impression has been that factor IX concentrates have become less and less effective in treating patients with factor VIII inhibitors, although a recent controlled double-blind study indicates that they still are effective in about 50% of cases.

Specifically, in this study the factor IX concentrates were used at a dose of 75 factor IX units per kilogram for early treatment of hemarthroses. Treatment with factor IX was judged to be successful 50% of the time, compared to a 25% success rate with albumin placebo.

Because treatment for factor VIII inhibitor patients is not ideal, materials have been developed specifically to bypass factor VIII inhibitors. Several of these materials are currently available for therapeutic use, though one report has questioned therapeutic efficacy of one of the products. Unfortunately, there is no clear correlation between any standard laboratory coagulation tests and the therapeutic effectiveness of such materials. Whereas one can monitor factor VIII levels in patients without inhibitors undergoing factor VIII replacement therapy and follow reproducible guidelines for the effective therapeutic blood level of factor VIII, no such measurement is available using factor VIII inhibitor bypassing materials. Therefore, we developed an animal model for evaluating such materials with the hope that the model would predict which of the materials would be effective in controlling hemorrhage in humans with hemophilia.

We chose to evaluate the materials in the hemophilic dog, reasoning that any material capable of bypassing a factor VIII inhibitor would also be therapeutically effective in an individual without any inhibitor. We selected gingival biopsy as the standardized technique to the hemostatic system because this is a convenient technique that can be performed under local anesthesia. It also circumvents the problem of lack of reproducibility introduced by hair on the skin of the dog. In this article, we describe the model in detail and report some preliminary information regarding its usefulness in testing factor VIII inhibitor bypassing materials.

MATERIALS AND METHODS

Rabbit brain thromboplastin was obtained from Ortho-Diagnostics, Raritan, N.J. Platelet Plus Activator was obtained from General Diagnostics Corporation, Morris Plains, N.J. Autoplex was...
HEMOPHILIC DOG MODEL

a gift from Hyland Laboratories, Glendale, Calif. Koate was a gift from Cutter Laboratories, Berkeley, Calif.

Procedure

Dog plasma. Citrated plasma was collected from the jugular vein by drawing 4.5 ml of whole blood into a plastic syringe containing 0.5 ml of 0.13 M trisodium citrate. The plasma was separated from cells by centrifugation in the cold and used for the assays described.

Prothrombin time. The standard prothrombin time was modified, because if undiluted brain thromboplastin is used the prothrombin time of the normal or hemophilic dog is 5–6 sec and a shortening of the prothrombin time could not be evaluated. The reagent used was one part rabbit brain thromboplastin diluted with 9 parts of 0.025 M calcium chloride. The prothrombin time was performed with 0.1 ml of the dog plasma, plus 0.2 ml of the diluted thromboplastin reagent.

Partial thromboplastin time. The test was performed using 0.1 ml of citrated dog plasma, 0.1 ml of Platelin Plus Activator (PPA), and 0.1 ml of 0.025 M calcium chloride. The plasma and the PPA were incubated at 37°C for 5 min before the addition of CaCl₂.

Template gingival biopsy. The experimental animals were inbred dogs with hemophilia A; normal dogs without hemophilia served as controls. After infiltration of vasoconstrictor-free Mepivacaine Hydrochloride into the mucobuccal fold region, a standardized 5 x 2 x 1.5 mm section of attached gingiva was delineated superior to the maxillary cuspid using a flexible plastic template and a modified scalpel handle housing two parallel no. 11 Bard-Parker blades (Fig. 1). This circumscribed rectangle of tissue was then removed by sharp dissection and placed in 10% neutral buffered formalin for later histologic evaluation. In normal dogs, bleeding from the wound ceased in 5 ± 2 min, the wound sealed tightly with a concave contour and rebleeding never occurred (4 normal dogs tested). In contrast, hemophilic dogs formed an abnormal convex clot over the wound, and rebled for several days if untreated. The hematocrit routinely dropped by 2–10 percentage points in association with uncontrolled bleeding (12 hemophilic dogs tested). When bleeding was evident in the 10–30-min postoperative period, either factor VIII or an experimental factor VIII inhibitor bypassing material was infused via a foreleg vein over a 10–15-min period. Serial samples for blood coagulation factor assays were obtained by venepuncture before infusion and at the indicated time intervals after infusion. Bleeding status was assessed by direct observation.

Fig. 1. Scalpel and template for standardized gingival biopsy. The main figure shows the twin-bladed scalpel and the template separately. The insert shows the scalpel blades in place in the template, as if initiating the biopsy. The slots in the template were 5 mm long and 2 mm apart, and the scalpel blades were set so that they protruded 1.5 mm beyond the lower surface of the template.
HEMOPHILIC DOG MODEL

Table 1: Coagulation Testing After Infusion With Autoplex*

<table>
<thead>
<tr>
<th>Time After Infusion</th>
<th>APTT</th>
<th>PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>37.4</td>
<td>13.5</td>
</tr>
<tr>
<td>15 min</td>
<td>35.6</td>
<td>9.1</td>
</tr>
<tr>
<td>30 min</td>
<td>36.1</td>
<td>9.3</td>
</tr>
<tr>
<td>1 hr</td>
<td>38.1</td>
<td>10.2</td>
</tr>
<tr>
<td>2 hr</td>
<td>35.0</td>
<td>11.3</td>
</tr>
<tr>
<td>3 hr</td>
<td>35.0</td>
<td>11.1</td>
</tr>
<tr>
<td>4 hr</td>
<td>35.8</td>
<td>10.6</td>
</tr>
<tr>
<td>5 hr</td>
<td>36.0</td>
<td>11.6</td>
</tr>
<tr>
<td>6 hr</td>
<td>35.5</td>
<td>11.2</td>
</tr>
<tr>
<td>24 hr</td>
<td>33.0</td>
<td>11.8</td>
</tr>
</tbody>
</table>

*A dog with hemophilia A infused with 55 U/kg Autoplex after baseline blood sample was drawn. Apparent FVIII level did not change using either human or canine VIII-deficient substrate (data not shown).

and intraoral photographs, and was also assessed by serial determinations of hematocrit. Ten to 15 min after infusion, a second, smaller wedge biopsy was performed in the posterior portion of the mouth; this tissue was also fixed in formalin.

The tissues obtained at biopsy were each divided in half and imbedded in either paraffin or glycomethyl methacrylate (JB4, Polysciences, Warrington, Pa.). The paraffin sections were stained with hematoxylin-eosin (H&E) periodic acid-Schiff (PAS), and phosphotungstic acid-hematoxylin (PTAH). The glycomethacrylate sections were stained only with H&E and PAS.

RESULTS

In Fig. 2 are shown some representative examples of the photographic records obtained after gingival biopsy. The top line demonstrates the response of a normal dog to biopsy. It shows the typical concave permanent seal of the wound formed in a normal dog or in a dog with hemophilia A treated with a single infusion of factor VIII concentrate. The middle line demonstrates the response of an untreated hemophilic dog to biopsy. The bottom line shows the response of a hemophilic dog to infusion with a factor VIII inhibitor bypassing material. Note (frame L) that even in a wound that sealed after infusion with the factor VIII inhibitor bypassing materials, the clot tends to have a convex contour and thus is more susceptible to dislodgement than the clot depicted in frame D. This was a consistent finding.

In Table 1 are given the values for serial studies on the peripheral blood of an animal following infusion of Autoplex. The only consistent change is a transient shortening of the prothrombin time. The activated partial thromboplastin time and the apparent factor VIII level as tested with either human or canine factor VIII deficient substrate did not change. In studies with other materials, a transient drop in platelet count also occurred, but not to a level that would compromise hemostasis (data not shown).

In Table 2 are shown the clinical results obtained from infusion of two different doses of Autoplex. The lower dose gave partial hemostatic effect, but rebleeding occurred. At the higher dose, hemostasis was achieved promptly and was maintained.

Table 3 depicts the results of an experimental factor VIII inhibitor bypassing material ("factor VIII correctional material") generously provided by Dr. David Aronson of the Bureau of Biologics, Food and Drug Administration, Bethesda, Md. The preparation of this material and its in vitro properties are described elsewhere. It was prepared from human serum, was a highly purified lyophilized powder, and when reconstituted contained 400 factor VIII inhibitor correctional units/ml as determined by the ability to correct human factor VIII inhibitor plasma in vitro. Hemostasis in this dog was achieved by subsequent infusion of factor VIII concentrate.

Multiple histologic sections of each preinfusion and postinfusion biopsy were examined for the presence of intravascular thrombi. None were found. Thus, hemostasis was achieved with Autoplex without histologic evidence for intravascular thrombosis.

DISCUSSION

We have developed an animal model for evaluating hemostatic effectiveness of materials designed to bypass factor VIII inhibitors. We have used a standardized challenge to hemostasis by performing a template-controlled gingival biopsy in dogs with hemophilia A. Serial studies are possible in a single dog during a 5-day work week, because immunologic response to the injection of human protein does not
Table 3. Infusion of Factor VIII Correctional Material*

<table>
<thead>
<tr>
<th>Time Infusion</th>
<th>APTT</th>
<th>PT</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>35.6</td>
<td>14.7</td>
<td>Bleeding</td>
</tr>
<tr>
<td>0 min 300 U/kg</td>
<td>—</td>
<td>—</td>
<td>Continued bleeding</td>
</tr>
<tr>
<td>15 min</td>
<td>38.8</td>
<td>14.8</td>
<td>Continued bleeding</td>
</tr>
<tr>
<td>30 min</td>
<td>40.7</td>
<td>14.9</td>
<td>Continued bleeding</td>
</tr>
<tr>
<td>1 hr</td>
<td>36.3</td>
<td>15.1</td>
<td>Continued bleeding</td>
</tr>
<tr>
<td>2 hr</td>
<td>39.2</td>
<td>14.6</td>
<td>Continued bleeding</td>
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<tr>
<td>3 hr</td>
<td>36.9</td>
<td>14.7</td>
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<tr>
<td>5 hr</td>
<td>36.9</td>
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<td>Continued bleeding</td>
</tr>
<tr>
<td>6 hr</td>
<td>40.2</td>
<td>12.9</td>
<td>Continued bleeding</td>
</tr>
</tbody>
</table>

*Supplied by Dr. David Aronson, Bureau of Biologics, FDA; † 40 U/kg and 100 U/kg were also ineffective.

...other materials that appear to be effective in this... (H.S. Kingdon, unpublished observations).

Our studies with the experimental inhibitor bypassing material provided by Dr. Aronson indicate that some materials that are capable of bypassing the factor VIII inhibitor of a human factor VIII deficient patient in vitro are ineffective in vivo, at least in the hemophilic dog. From the studies with Autoplex and other materials that appear to be effective in this model, we would have expected a response to this material at a dose between 25 and 75 U/kg. Instead, no response was seen at 40, 100, or 300 U/kg (Table 3). In contrast, Autoplex was effective in our model, and the effective dose was comparable to doses recommended for use in humans (Table 2). No intravascular thrombi were observed. Factor VIII infusion (25 U/kg) always brought about prompt cessation of bleeding and provided a valuable positive control indicating that the animal under study was capable of responding to appropriate therapy.

This model is being used further to screen materials under development for their hemostatic effectiveness and also to test subfractions of factor VIII inhibitor bypassing materials in order to determine which component or components are responsible for the hemostatic effect.

ACKNOWLEDGMENT

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REFERENCES

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HS Kingdon and TM Hassell