Plasma factor VIII synthesis and control as revealed by canine organ transplantation

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OVER THE PAST 20 YEARS much effort has been invested into discovering the tissues that participate in the synthesis and regulation of plasma factor VIII (antihemophilic factor, AHF). The relatively recent application of organ perfusion and transplantation techniques to this problem has contributed many interesting observations by several research teams. Much of the evidence is conflicting and contradictory; Norman et al. (24) and Dodds (6, 7) place great emphasis on a synthetic role of the spleen, a view not held by Webster (37-39), Peacock (25), Marchioro (18), McKee (21), and their respective co-workers. Marchioro et al. (18, 19) suggest that the liver is the major site of synthesis of factor VIII in mild hemophilia. Tissue culture studies have implicated leukocytes as possible cells of synthesis (42). Dodds (7), on the basis of renal perfusion, and Barrow and Graham (unpublished data), on the basis of chemical extraction of renal tissue, provide evidence that antihemophilic activity may be recovered from kidneys under certain circumstances.

The studies described in this report amplify our knowledge about the relative roles and possible interactions of the liver, spleen, kidneys, and other tissues in the control of circulating factor VIII. They indicate that: 1) under ordinary circumstances, the liver is the principal site of synthesis; 2) there are potentially multiple sites of synthesis; and 3) the spleen apparently serves as a site of storage and selective release of factor VIII upon physiologic demand.

MATERIALS AND METHODS

Normal and hemophilic dogs, selected without regard to sex, were used in these studies. The hemophilic dogs came from a highly inbred strain of animals with severe classic hemophilia (<3% factor VIII) that has been maintained at this institution for more than 20 years (10). Each hemophilic animal had a history of repeated hemorrhages into joints and soft tissues requiring transfusion of factor VIII since birth. Normal dogs were either from the colony of inbred animals or mongrel dogs selected according to body weight (15-20 kg) and erythrocyte compatibility (35) but without regard to age.

Clotting studies included prothrombin time (PT) (32), partial thromboplastin time (PTT) (17), and specific assays for factors I (fibrinogen) (13), VIII (17), and IX (Christmas factor) (1). In the last two procedures, substrate plasma was obtained from dogs specifically deficient in factors VIII and IX, respectively, and results were expressed in terms of percent activity with respect to plasma pooled from five normal dogs. Fibrinolytic activity was measured by the euglobulin lysis time test (16). Bilirubin was assayed using the method of Jendrassik and Grof (8). Transaminase determinations were done by the methods of Karmen (14) and Wroblewski and LaDue (41). Platelets were counted by the method of Brecher and Cronkite (3) and leukocytes were counted by the conventional microscopic method (4).

Transplantation of livers, spleens, and kidneys was performed by previously described techniques (5, 20, 34). Sodium pentobarbital was employed as a general anesthetic agent. Before liver transplantation, hypothermia (32 C) was induced in donor animals. A portocaval shunt was created and the inferior vena cava was decompressed by an exterior bypass tube to an external jugular vein. Donor livers were perfused with cold Ringer lactate solution (4 C). Duration of liver graft ischemia varied between 60 and 90 min. The liver of the recipient was removed and replaced orthotopically by the liver from the donor animal. The spleen of the recipient was left intact. Blood pH, PaO₂, and PaCO₂ were maintained at normal levels during the anhepatic phase by administering NaCHO₃ and mechanically assisted respira-
tion. Spleens were transplanted heterotopically into the pelvis by anastomosing the splenic vessels to the common iliac vessels. Kidneys were transplanted either into the pelvis or groin skin pouches. Spleens and kidneys were not perfused and were ischemic for no longer than 20 min. During surgical procedures and the immediate postoperative periods, blood and fluid volumes were maintained by infusing normal whole blood and Ringer lactate solution. Anticoagulant drugs were not employed. Hemophilic recipients were cross-circulated with normal dogs (36) or transfused with factor VIII concentrates preoperatively to increase circulating plasma levels of factor VIII.

Immunosuppressive drugs employed were azathioprine (Burroughs, Wellcome and Co.) and prednisolone (The Upjohn Co.). Azathioprine, 5 mg/kg body wt, was usually administered preoperatively and the dose was then decreased 0.5 mg/kg per day until a dose of 1 mg/kg was reached. The preoperative dose of prednisolone was 2.5 mg/kg; it was also decreased 0.5 mg/kg per day to a dose of 1 mg/kg per day. The doses of azathioprine and prednisolone were increased whenever the temperature became elevated, the transplanted viscera became palpably enlarged, or specific organ excretory functions became impaired (elevated serum bilirubin or urca). Splenic function was evaluated by observing the response of levels of circulating factor VIII to the administration of 1.0 mg Adrenalin chloride as measured by inserting cannulas into the splenic arteries and veins and assaying factor VIII activity in blood samples withdrawn from these vessels (25) (Fig. 1).

RESULTS

Liver Allo Transplantation

Normal to normal. Considerable technical difficulties were encountered in transplanting the livers of normal dogs to normal canine recipients. Eleven such experiments were performed. Six fatalities were the result of hepatic outflow block as established by observing swollen, congested livers on postmortem examination; four animals died from other technical and metabolic complications. Coagulation and blood chemical determinations performed on the one dog that survived longer than 24 hr are presented in Fig. 2. All values remained within normal ranges except forFactor VIII

FIG. 1. Normal dog with indwelling cannulas in portal vein and aorta. Prior to adrenalin injection difference between aortic and venous samples was small (12%). Immediately following infusion of adrenalin, factor VIII activity increased to 170% in portal blood; a lower level was measured in aortic blood. Difference between values in portal and systemic vessels persisted throughout course of experiment.

normal canine recipients. Four normal livers were transplanted into hemophilic recipients. One dog died during the surgical procedure; two died from hemorrhage postoperatively; the remaining animal survived for 113 days, eventually dying.
TABLE 1. Response of hemophilic spleen to Adrenalin injection

<table>
<thead>
<tr>
<th>Time After Injection, min</th>
<th>Factor VIII, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>15</td>
<td>104</td>
</tr>
<tr>
<td>30</td>
<td>63</td>
</tr>
</tbody>
</table>

Subject was a hemophilic recipient of a normal liver transplant 30 days previously (Fig. 3).

TABLE 2. Splenic allotransplantation: normal to normal

<table>
<thead>
<tr>
<th>Exp No.</th>
<th>Factor VIII, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Posttransplantation time, days</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>83</td>
</tr>
<tr>
<td>2</td>
<td>105</td>
</tr>
</tbody>
</table>

* Experiment terminated at 2 days.

TABLE 3. Splenic allotransplantation: normal to normal

<table>
<thead>
<tr>
<th>Organ</th>
<th>Factor VIII, %, in Splenic Venous Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host spleen</td>
<td>130</td>
</tr>
<tr>
<td>Transplanted spleen</td>
<td>116</td>
</tr>
</tbody>
</table>

* At 80 min Adrenalin chloride was injected into the aorta, and both spleens were observed to contract.

FIG. 5. Normal to hemophilic spleen allotransplantation. Experimental (recipients) and control (nontransplanted) animals were treated with one cryoprecipitate infusion to increase factor VIII levels. Significant circulating levels persisted after transplantation for 96 hr and then fell to near-zero values at 120 hr. Half-life of factor VIII in control experiment is about 15 hr.

FIG. 4. A: hemophilic to normal liver allotransplantation. Animal survived for 13 days. Prothrombin time and PTT remained within normal limits. Note slow decrease in factor VIII to a low of 14% on 10th day and terminal rise to 44%. On 8th day evidence of rejection was observed. Immunosuppressive drugs were increased with temporary clinical improvement, but death eventually resulted from hepatic failure. B: hemophilic to normal liver allotransplantation. Animal survived for 22 days. Prothrombin time and PTT were within normal range during observation period. Factor VIII slowly decreased to a low of 34% with a terminal rise to 86%. Death was attributed to gastrointestinal hemorrhage.

FIG. 3. Spleen Allotransplantation

Normal to normal. Three experiments were performed (Tables 2 and 3). In the resting recipient animal, factor VIII levels in the peripheral circulation were not appreciably altered postoperatively (Table 2). However, measurements of factor VIII efflux from both a recipient's spleen and a transplanted spleen showed similar responses to adrenalin (Table 3).

Normal to hemophilic. Seven normal canine spleens were transplanted heterotopically into hemophilic canine recipients, and the postoperative levels of factor VIII were determined. Three of these survived more than 48 hr (10 and 18 days and 2 years, respectively). The means and ranges of the factor VIII levels in these three animals over the first 120
postoperative hours are plotted in Fig. 5, together with a control curve representing the fall-off rate of factor VIII in a nontransplanted hemophilic control dog receiving cryoprecipitate. In an additional similar recipient, the response of the transplanted normal spleen to Adrenalin infusion was negligible (Fig. 6) in comparison to the response of a normal spleen in a normal animal (Fig. 1 and Table 3).

**FIG. 6.** Normal to hemophilic spleen transplantation. Cannulas were inserted into transplanted spleen vein and a systemic (femoral) vein 45 hr after transplantation. Blood samples were withdrawn before and after adrenalin administration and plasma factor VIII was measured. Although allograft contracted in response to adrenalin, factor VIII rose only 10% in efflux from transplanted spleen.

**DISCUSSION**

The liver is considered to be the site of synthesis of many of the plasma proteins. Most of the coagulant proteins—fibrinogen, prothrombin, factors V, VII, IX, and X—are of hepatic origin (33). Current evidence suggests that factor VIII may be a glycoprotein (15) or a lipoprotein (12). One might anticipate that the liver is also the site of synthesis of factor VIII, and such would appear to be the case from the observations of Marchioro et al. (18).

The site of synthesis of factor VIII and its regulatory mechanisms have been studied by many direct and indirect means, including chemical injury (9, 11, 28), total body irradiation (27), organ ablation (31), hormone administration (26), cross-circulation and splenectomy (36), organ perfusion (7, 39), inhibition of protein synthesis (6, 7), and tissue culture (42). Transplantation of normal and hemophilic organs into hemophilic and normal dogs provides a mechanism for making direct observations that give new
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and interesting insights into the synthesis and regulation of plasma factor VIII.

It has long been felt that inherent in transplantation experiments are potential artifacts that might result from activation of the fibrinolytic system or from diffuse intravascular coagulation, either of which might distort values of circulating clotting factors (28). This is particularly true with respect to liver transplants. In our experiments, simultaneous assays of fibrinolytic activity and consumable clotting factors have been conducted; in none of the experiments was there any evidence that either of these reactions produced significant artifacts. For example, normal-to-normal liver transplants cause no appreciable coagulation imbalance (Fig. 2), although the difficulties encountered in this series of transplants precluded obtaining more than a 48-hr control of these hypothetical reactions. It is conceivable that either immunosuppression or rejection could alter the clotting mechanism. However, the experience of McKee et al. (21) would indicate that the immunosuppressive regimen employed would not have affected the factor VIII levels and, except for the terminal course of the experiment depicted in Fig. 4A, rejection was not a prominent feature in our recipients.

Transplantation of a normal liver into a hemophilic dog completely corrected the inborn metabolic error in the recipient. Clinically, hemostasis after extensive surgery was essentially normal; no evidence of hemorrhage was evident, even though no exogenous factor VIII was administered postoperatively. The in vitro assays of factor VIII demonstrated high levels. Inherent in the design of the experiment is the fulfillment of the ultimate criterion of true antihemophilic activity, namely the effect in a hemophilic recipient of a transfused test material (40). The transplant recipient represented the test subject. The validity of this interpretation has been verified by Marchioro et al. by further transfusing the recipient’s plasma into a second hemophilic subject and observing a proportionate level of factor VIII activity. In similar unreported experiments, the identity of factor VIII obtained from a hemophilic subject and observing a proportionate level of factor VIII activity, namely the effect in a hemophilic recipient of a normal heterotopic liver transplant was further established utilizing a human antifactor VIII. The decrease in factor VIII activity was proportional to the amount of antifactor VIII added to the test material. Our results with severe hemophilia thus agree with those obtained by Marchioro et al. (18, 19) with mild hemophilia. In the mild form of the disease, Marchioro et al. described normal factor VIII levels after transplantation. One of their animals survived 154 days postoperatively. The data presented in Fig. 3 demonstrate that it is possible to obtain protracted correction of the hemophilic defect by replacement of the hemophilic liver with a normal liver, thus establishing unequivocably that hepatic synthesis of factor VIII does indeed occur in the liver. However, the transfused liver was found in 240 and 384 hr, respectively. This observation suggests that the absence of factor VIII is being secreted into the bloodstream from some source faster than it is being catabolized. It is theoretically conceivable that the liver may have been some release of factor VIII that was stored in extravascular spaces. However, it is of extreme interest that terminally, 13 and 21 days after operation, the factor VIII levels were more gradual than one would expect from the factor’s biologic half-life (in dogs, 12-16 hr). On the basis of fall of transfused factor VIII, the curve should have reached values of <5% in about 60 hr. Instead, in these two experiments the low points of 14 and 34% were reached in 240 and 384 hr, respectively. This observation suggests that factor VIII is being secreted into the bloodstream from some source faster than it is being catabolized. It is theoretically conceivable that the liver has some release of factor VIII that was stored in extravascular spaces. However, it is of extreme interest that terminally, 13 and 21 days after operation, the factor VIII levels in these two recipients rose to 44 and 86%, respectively. This observation suggests extrahepatic synthesis in cells that are undergoing adaptations of their enzyme synthesis mechanisms to compensate for the lowered plasma levels that would result from the lack of hepatic synthesis of factor VIII.

That plasma proteins can be synthesized in sites other than the liver is not a novel concept. Miller et al. (23) demonstrated by perfusing eviscerated rat carcasses that extrahepatic synthesis of plasma proteins could occur. One of the most familiar examples of protein synthesis by nonhepatic cells is the formation of α-globulin in plasma cells. It is also claimed that the β₉-globulin component of complement (C₉₅), certain α- and β-globulins, and some lipoproteins may be of extrahepatic origin (33).

In these studies, the spleen was evaluated as a possible extrahepatic site of factor VIII synthesis. Earlier perfusion (7, 39) and transplantation studies (24, 38) had suggested that the spleen might contribute significant amounts of factor VIII to the bloodstream. In the current series of splenic transplants, the response of circulating factor VIII levels was variable in hemophilic recipients of normal splenic allografts; the trend of response is represented by the mean data plotted in Fig. 5. Factor VIII levels of approximately 10% were maintained for as long as 96 hr, but no longer. In contrast, the disappearance rate of factor VIII was faster after administering cryoprecipitate into control animals. This would suggest that the transplanted normal spleen, under these circumstances, was providing a source of some additional factor VIII, but not a continuing source.
The spleen’s failure to release factor VIII over long periods may be related to the difficulty of obtaining long-term parenchymal viability in splenic allografts, although McKee et al. (21) and Marchioro et al. (18) have demonstrated long-term persistence of vascular integrity and radioisotopic scanning patterns. A competitive and lowering effect on levels of circulating factor VIII by the hemophilic recipient’s spleen would appear to have been eliminated because their experimental animals were asplenic at the time of transplantation. The short-term elevations may indicate release of stored factor VIII from spleens rather than synthesis.

Further evidence in support of this thesis is gleaned from the experiments in which the normal spleen is shown to be capable of releasing factor VIII when adrenalin chloride is injected intravenously. These experiments were designed to measure the factor VIII in the effluents of spleens before and after adrenalin administration. The results indicate a responsive rise in factor VIII in the outflow blood of the spleen of an intact normal dog (Fig. 1) and of a normal spleen transplanted into a normal dog (Table 3). In the latter experiment similar values were obtained in efferent samples from both the transplanted and host spleens before and after injection of adrenalin. Both of these spleens presumably were provided with a continuing supply of factor VIII from an extrasplenic source. Inasmuch as all nervous supply to the allografts had been severed during transplantation, stimulation must have been humoral.

That the hemophilic spleen fails to respond by releasing factor VIII is evidenced by the data on a hemophilic dog with a normal liver transplant (Table 1). A significant rise in factor VIII failed to occur after adrenalin injection into a hemophilic recipient of a normal liver, even though one would assume that such a spleen would have had adequate time and opportunity to have become saturated with factor VIII. One can only speculate whether this defect in splenic function is a failure to store or to release factor VIII in addition to being unable to synthesize large quantities of this factor.

In further search for extrahepatic sites of factor VIII synthesis, a normal kidney was transplanted into a hemophilic dog. Figure 8 demonstrates that this graft had essentially no effect on circulating levels of factor VIII. The fall-off rate of preoperatively administered factor VIII (cross-circulation) was the same in the transplanted and control (nontransplanted) animals. On the other hand, it is of interest that autotransplantation of the kidney does produce some response in an animal with an intact mechanism for synthesizing, storing, and releasing factor VIII. Autotransplantation of a kidney (Fig. 7) produced a wide range of response, representing in general an initial postoperative rise followed by a slow fall similar to effects following partial intestinal resection in the experiments of McKee et al. (21). Results such as these raise interesting questions about a possible role of kidneys in the regulation of factor VIII, especially inasmuch as Barrow and Graham (unpublished data) have been able to extract a small molecule that has antihemophilic activity from kidney tissue of both normal and hemophilic dogs.

From the above-described observations and conclusions, it is possible to construct a coherent, working hypothesis about the mechanisms regulating the production and distribution of factor VIII in the body. Probably the synthesis of the fully functioning factor VIII molecule takes place as a series of successive enzymatic linkage steps, and a lack of one of these essential enzymes, most likely operating late in the sequence, represents the basic hemophilic defect. Although synthesis occurs in both hepatic and extrahepatic sites, the liver appears to be the primary site of the final linkage reaction under ordinary circumstances. However, under such abnormal conditions as those created by the replacement of a normal dog’s liver by one from a hemophilic dog, extraneptic tissues appear to be able to adapt their enzyme systems and assume the ability to synthesize a fully functional factor VIII molecule. This response suggests that synthesis is regulated by a feedback mechanism so that low levels of circulating factor VIII are a stimulus for its production. A converse relationship to high levels is implicit in the observation of Pool (30) that accelerated catabolism of factor VIII occurs in normal individuals when exogenous factor VIII is infused at a rate that should elevate circulating factor VIII levels above normalcy.

Several observations support the concept that synthesis may occur in extraneptic cells at a low level of activity at all times: 1) the levels of circulating factor VIII in normal recipients of hemophilic livers did not fall lower than 14% in our experiments or in those of Marchioro et al. (19); 2) hemophilic recipients of normal livers (Fig. 9) (18) usually do not acquire normal levels until enzymatic adaptation takes place in the transplant to permit it to compensate for the lack of extraneptic synthesis; 3) extracorporeal perfusion of spleens and hindlimb tissues—in addition to livers—yields appreciable quantities of factor VIII (39); 4) Barrow and Graham (2) have been able to extract from renal tissues a small molecule capable of exhibiting antihemophilic activity.

Synthesis and enzyme adaptation are time-consuming responses. Hemorrhage, on the other hand, frequently requires rapid physiologic response to provide life-saving hemostasis. To meet such crises, the body is also supplied with sites of storage capable of rapidly releasing factor VIII into the bloodstream under emergency circumstances. The spleen appears to be such an organ. It is interesting that its release of factor VIII may be mediated through adrenalin, a hormone circulating in increased quantities under conditions of stress. Seemingly inconsistent with this concept is the observation that the hemostatic mechanism is not impaired in asplenic normal individuals. However, the normal body is supplied with superfluous quantities of factor VIII capable of coping with minor injuries, and it has been shown (29) that a depressing effect on factor VIII levels is created by splenectomy in hemophilic heterozygotes, even though clinically abnormal hemostasis does not develop.

It is probable that such a working hypothesis represents oversimplification. Conceivably, subunits might originate in many tissues, multiple sites of storage may exist, extraneptic sites might synthesize factor VIII under less drastic conditions than those we have studied, and other types of stimuli for synthesis and release probably operate. These variations require continued study before the complex regulatory mechanisms will be fully understood. The
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regulatory mechanism is thus a complicated but well coordinated one, including increased synthesis, decreased synthesis, release from storage sites, and increased catabolism—all interacting to maintain normal levels within the circulating blood, the "steady state."

The authors gratefully acknowledge the technical assistance of Mr. William Foster, Mr. John V. Alcott III, Mrs. Laura Grimes, and Mrs. Janine Wilson. Dr. J. L. Wagner provided veterinary consultation and unavailing care of experimental animals. The authors are grateful to Dr. E. F. Peacock and Dr. J. W. Machen for their help in the development of some of the transplantation techniques. Dr. K. M. Brinkhous and Dr. R. D. Langdell kindly assisted in preparation of the manuscript.

This investigation was supported, in part, by grants from the John A. Hartford Foundation and National Institutes of Health Grants HF-6350 and HF-5652.

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Received for publication 13 July 1970.

REFERENCES


