THE RELATION OF CERTAIN FRACTIONS OF THE PLASMA GLOBULINS TO THE COAGULATION DEFECT IN HEMOPHILIA

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A SERIES of reports from the Thorndike Memorial Laboratory have indicated that normal cell-free human plasma contains some factors which are deficient in hemophilia. There is evidence that by the parenteral administration of derivatives of normal cell-free plasma in hemophilia, the coagulation defect can be modified toward normal. The present communication reviews some of the older work on this subject and presents new evidence accumulated during the past three years.

One of the earliest studies on the use of fresh whole blood in the treatment of hemophilia was made by Minot and Lee in 1916. They ascribed the beneficial effects of transfusion of whole blood to the platelets thus added to the patient's circulating blood. In the same year, Addis showed that the administration of whole blood or of serum reduced the coagulation time of hemophilic blood.

Patek and Stetson reinvestigated this problem in 1935 and found that small transfusions, as little as 30 cc., of fresh whole blood were as effective as large amounts in the initial reduction of the coagulation time in hemophilia, but that the reduction of the coagulation time was much more transient when these small amounts were used. These authors found that the beneficial effects of fresh whole blood could be duplicated by the use of fresh normal human plasma. They then deprived the plasma of its platelet content and found that the filtrates so obtained were equally effective in reducing the coagulation time of hemophilic blood in vivo. From the foregoing evidence it seemed reasonable to conclude that fresh normal whole blood or platelet-free plasma contained a material which was effective in shortening the coagulation time of hemophilic blood. Transfusions of hemophilic blood or of hemophilic plasma failed to have such a beneficial effect. In other words, there seemed to be evidence that hemophilic blood was deficient in some factor or factors present in normal blood.

Competent investigators have shown that the coagulation defect in hemophilic blood could not be related to deficiency in calcium ion, prothrombin.
bin\textsuperscript{14, 15, 16, 17} or fibrinogen\textsuperscript{18, 19, 8, 11, 12} of the plasma. Consequently, our investigations were directed toward an attempt to determine where the deficiency, if any, resided. It was determined that certain globulin fractions prepared by acid precipitates of diluted cell-free plasma at pH 5.5 and also similar globulin preparations obtained by simple dialysis against distilled or tap water contained all the antihemophilic properties of the parent plasma.\textsuperscript{2} Similar preparations from hemophilic plasma had little or no coagulation properties.\textsuperscript{3} The acid-precipitable globulins, unfortunately, produced a refractory phase during which second, third and subsequent injections given within six hours were inactive. This method of preparation was therefore discarded.\textsuperscript{4} Euglobin fractions obtained by dialysis could be repeatedly administered without the development of the refractory period.\textsuperscript{3} Similar preparations of antihemophilic globulins were prepared independently by Bendien and Van Creveld.\textsuperscript{176} Later these observations were confirmed by Howell.\textsuperscript{17}

It would appear that the antihemophilic properties of these globulin preparations were not due to the removal of inhibitory materials, since the parent plasmas were active and the supernatant fluids from the globulin precipitants had no inhibitory activity and only minimal coagulation activity. Furthermore, hemophilic plasma treated by the same method did not acquire any increase in antihemophilic properties.

Protein fractions prepared as we have just indicated contain both prothrombin and fibrinogen. Since the administration of excessive amounts of these materials might possibly have an effect in reducing the coagulation time, plasmas relatively free of prothrombin and of fibrinogen were prepared by heating normal cell-free plasma to 56 C. for two minutes and subsequently passing the filtrate five times through a Seitz filter. Such plasma preparations retain their antihemophilic properties unimpaired.\textsuperscript{6} Euglobin preparations of such plasmas, while not as potent as the parent plasmas, retain a considerable amount of their power to reduce the clotting time of hemophiliic blood in vitro and in vivo.\textsuperscript{22}

The exact nature of the antihemophilic material is not known. It is definitely associated with the plasma globulins, but whether or not it is a lipoprotein has yet to be determined. Howell\textsuperscript{2} termed the material "plasma thromboplastin," but evidence is lacking as to whether or not this nomenclature is acceptable. Studies concerning this matter are at present under way.

As part of the blood substitutes program conducted during the war, large quantities of human plasma have been subjected to multiple fractionation by Dr. E. J. Cohn and the workers in the Department of Physical Chemistry at the Harvard Medical School. Various subfractions of the globulin group have been extensively tested for antihemophilic activity in the Thorndike Memorial Laboratory.\textsuperscript{23} The antihemophilic activity was found to be largely concentrated in fraction I and subfraction II of fraction III of the plasma proteins.\textsuperscript{*} A small amount was found in fraction IV. Fraction I contains above 60 per cent of fibrinogen, with small amounts of the other globulins. Subfraction II of fraction III contains 75 per cent

\* Nomenclature of Cohn and associates.\textsuperscript{23, 24}
beta globulin and most of the prothrombin. Fraction IV contains 55 per cent alpha globulin and 28 per cent beta globulin.24

Extensive studies on the antihemophilic properties of fraction I are being carried out on adult patients in the Boston City Hospital. So far it has been found that single intravenous doses of 200 to 600 mg. of fraction I in volumes of 5 to 10 cc. of isotonic solution of sodium chloride shorten the coagulation time of hemophilic blood as much as do therapeutic doses of whole blood or plasma,25 which reduce the clotting time of such blood to normal. Intramuscular administration of the fraction has been found to give inconstant results and frequently causes painful hematomas.25 Repeated intravenous doses of fraction I are not followed by a refractory period.26 As has been stated, fraction I contains at least 60 per cent of fibrinogen. If it is possible to subfractionate it further in such a manner as to retain its antihemophilic properties while removing the fibrinogen, perhaps even more potent antihemophilic material can be made. It has been found26 that the removal of fibrinogen from fraction I by simple solution and heating to 56 C. for two minutes does not destroy the antihemophilic properties of the fraction, although subsequent tests with thrombin show that no fibrinogen remains in the preparation. Thus, it may be possible to obtain preparations sufficiently potent to make small daily injections feasible, and in this way to maintain the clotting time of hemophilic patients within normal limits.

A pool of hemophilic plasma is now being fractionated by the Harvard Pilot Plant.* So far only fraction I of this plasma has been tested. Although the total nitrogen and fibrinogen content of the fraction are within normal limits, no antihemophilic activity has been found in vitro. Sufficient hemophilic fraction I was obtained to make one test in vivo, and in the subject used little or no clot-promoting ability was detected. Details of these observations will be published elsewhere. In the evidence at hand at present there seems to be considerable confirmation of the theory that hemophilic blood is deficient in a factor or factors which are closely associated in chemical fractionation with prothrombin and fibrinogen and which may be separated with some losses from both of these proteins.

Normal human cell-free plasmas when treated with chloroform develop strong proteolytic activity.27–31 It has been shown that a true protease is obtained with an optimum pH between 7 and 8 and that the enzyme is capable of digesting fibrinogen, fibrin, gelatin and casein. The enzyme can also apparently produce thrombin from prothrombin.32,33 In relation to blood coagulation, small amounts of the enzyme produce coagulation; larger amounts produce coagulation with subsequent fibrinolysis and, in still higher concentrations, fibrinogenolysis.33,34

On the other hand, hemophilic plasma as compared with normal plasma develops little proteolytic activity following treatment with chloroform.35

Globulin preparations possessing antihemophilic activity likewise can act as progenitors of such an enzyme system as has been described. Globulin preparations made by simple dialysis do not as a rule have proteolytic activity prior to treat-

* Courtesy of Drs. J. T. Edsall, L. E. Strong and S. M. Armstrong, with whom the complete observations will be published elsewhere.
ment with chloroform. However, fraction I and subfraction II of fraction III of Cohn have on occasion shown preformed lytic activity.

Upon treatment with chloroform fraction I, subfraction II of fraction III and fraction IV all develop proteolytic activity capable of digesting both fibrin and fibrinogen as well as gelatin and casein. The distribution of the activity between the fractions varies, but it is markedly reduced in subfraction IV of fraction IV and is greatest in subfraction II of fraction III, which likewise contains free enzyme. The parent plasmas have been better sources of enzymes after chloroform treatment than have any of the individual fractions of the globulins. However, when the yield of enzyme of all the fractions is considered collectively, as measured by production of nonprotein nitrogen from casein, there is a definite increase in the nitrogen produced per milligram of original protein. This may be due to the removal of inhibitor substances or of competitive substrates by the fractionation process. It has already been determined in a few experiments that plasma albumin,* which is not itself digested by the protease, markedly lowers the nonprotein nitrogen produced from gelatin by the plasma protease. Fraction IV of Cohn has been found to contain both enzyme progenitors and also inhibitor materials.

The foregoing facts suggest that normal human cell-free plasma contains the progenitor of an enzyme system, which may be elaborated by treatment with chloroform. In hemophilic plasma this enzyme system may be present in reduced amounts. The facts also suggest that the enzyme system plays an as yet unknown role in the process of the coagulation of blood. Further fractionation of the plasma globulins and further studies on the characteristics of the system are necessary to determine the true physiologic action of the enzyme.

Of equal importance with the shortening of the coagulation time of the circulating blood in hemophilia is the local control of hemorrhage when this occurs. The antihemophilic globulin substance discussed earlier in this paper acts as a local hemostatic when applied in dry form with adequate dressings to the bleeding point. For some time preparations of this globulin substance from bovine plasma were used in this clinic. Local hemorrhages may be arrested by such preparations in a few minutes. However, antihemophilic globulin preparations act apparently through the normal coagulation reaction and depend for their activity on the release of thrombin from prothrombin; hence instantaneous arrest of hemorrhage does not occur.

Another globulin preparation may be obtained from human, bovine, rabbit or swine plasma by a 'salting-out' procedure. Parfentjev first described such a preparation from rabbit plasma. Subsequent investigators found that these 'pseudoglobulin' preparations were 'thrombic' in nature; that is, they acted directly on fibrinogen without the intervention of calcium ion or prothrombin. Since no thromboplastin was added in the preparation, it must be concluded that the 'salting-out' process caused the spontaneous conversion of prothrombin to thrombin. Such spontaneous conversions have been described by Milstone. *

* Provided by Dr. J. T. Edsall.
From the practical point of view this hemostatic globulin is definitely superior to the antihemophilic globulin in the control of hemorrhage, since its action is immediate. In persons with hemophilia it has been used to control bleeding following amputations, debridements and dental extractions. In normal persons also it has been widely used, a notable example being its employment as a "physiologic glue" in skin grafting.\(^4\)\(^3\)\(^4\)

At the present time much fractionation of human plasma proteins is being carried on and thrombin of human origin is available. However, the fact that hemostatic globulin can be obtained not only from human but also from bovine, rabbit and swine plasma may be of great economic importance in the postwar period, when human plasma products will probably not be as available as they are at present.

The question of possible toxic reactions to animal globulin preparations applied locally immediately arises. The last word on this has not yet been written. It may be reported that using a rabbit hemostatic globulin* we have noted no untoward local effects or systemic reactions in many multiple local applications of the substance in the control of hemorrhage in hemophilic and normal subjects.

From the academic point of view, it is of interest to note that while active antihemophilic globulin preparations give rise to a proteolytic enzyme system, either spontaneously or upon treatment with chloroform, hemostatic globulin preparations are devoid of this property. This has been found true not only of a thrombic preparation made by Parfentjev's method but also of thrombin prepared by conversion of prothrombin by the addition of thromboplastin as described by Astrup and Darling\(^4\)\(^6\) and Seegers.\(^4\)\(^6\) Thrombin preparations from human plasma prepared by the technic used in the plasma fractionation plant at the Harvard Medical School are likewise devoid of this property; moreover, they do not spontaneously lyse fibrin clots.

**CONCLUSION**

Hemophilic blood appears to be deficient in some activity associated with the globulin fraction of the plasma protein. The chemical identity of the missing factor or factors is not at present known. For want of a precise name for this factor, we have called it "globulin substance," and in this report it has been termed "antihemophilic globulin," which term will probably be used in studies now being undertaken by other investigators. However, it should be remembered that until final proof is obtained both of these terms imply an association rather than an identification of the antihemophilic activity with the plasma globulins.

Normal cell-free plasma and the antihemophilic globulin preparations derived therefrom will on treatment with chloroform give rise to a proteolytic enzyme system having some as yet undefined role in blood coagulation. The globulin preparation, but not the original plasma, except under conditions described elsewhere,\(^1\)\(^7\) will spontaneously produce such an enzyme system. In fractionations of human globulins such spontaneous production of lytic agents has been found in subfraction II of fraction III, and occasionally in fraction I.

* Hemostatic globulin donated by the Lederle Laboratories.
Hemophilic plasma does not produce such proteolytic enzyme systems in optimal amounts. Whether this is due to the lowered amount of the precursor (possibly antihemophilic globulin) or to the presence of inhibitor materials is at present unknown.

The intravenous injection of antihemophilic globulin results in a marked acceleration of the clotting time of hemophilic blood. So far only fraction I of the plasma globulin has been used clinically. The dose of the material has not yet been determined, but single injections of 200 to 400 mg. of the material will keep the blood of a hemophilic patient at low coagulation levels for from eight to twelve hours. Repeated injections may be used without the development of a refractory phase.

For local hemostasis a pseudoglobulin may be prepared from human, bovine, swine or rabbit plasma by a ‘‘salting-out’’ procedure. In this clinic thrombin from rabbit plasma has been used without untoward local or systemic reactions in amputations, debridements and dental extractions in hemophilic patients.

BIBLIOGRAPHY

9 Unpublished observations.
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22. Unpublished observations.
36. Unpublished observations.
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