

## Lack of Antibodies to Clotting Factors in the Blood of Hemophiliacs Refractory to Transfusions\*)

Charlotte Drake Cardeza Foundation Jefferson Medical College Philadelphia, Pennsylvania

Jorge A. Penalver, R. R. Holburn, R. T. Carroll  
and L. M. Tocantins

The refractory state to blood transfusions observed in some Hemophilia "A" patients has been attributed to the presence of antibodies against certain clot-accelerating fractions of normal plasma (Fraction I of Cohn, particularly) (1—8). These antibodies are said to develop as the result of an immunization process following repeated transfusions of blood, plasma or plasma derivatives. Most workers, while tending to regard this explanation as plausible, have nevertheless been unable to demonstrate, conclusively, the existence of such antibodies (9—10). The present paper is a report of the results of experiments designed to test the validity of this hypothesis.

### Material and Methods

Precipitation tests were used to demonstrate the presence or absence of immune antibodies in the serum of the patients. Artificial antisera, produced by immunization of rabbits with human Fraction I, were utilized as positive controls. All antigens were employed at full strength and, in most cases, diluted to various concentrations.

The *human sera* were obtained from clotted blood, collected under sterile conditions from subjects fasting for at least 6 hours. The blood samples after clotting were allowed to stand at 37° C for 4 to 6 hours; they were then centrifuged, the sera were separated and stored at 5° C from 2 to 4 days until used.

The *antigens* were: 1) Fraction I of Cohn, 2) Defibrinated Fraction I of Cohn and 3) Normal human plasma. *Fraction I of Cohn*\*\*) was used as a 4% solution in distilled water buffered to pH 7.2—7.4. This solution was designated full strength. Several dilutions

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\*\*) Supplied by Cutter Laboratories as "Purified Fraction I", a dried material prepared from pooled normal human plasmas.

in buffered physiological saline solution were prepared, usually as follows: 1/5, 1/50, 1/500 and 1/5000. *Defibrinated Fraction I of Cohn* was prepared from the 4% solution by heating at 56° C, in some tests for 3 minutes and in other tests for 15 minutes. After centrifugation at 3000 rpm for 10 minutes, the clear supernatant obtained constituted the antigen, which was used for the tests either undiluted or diluted as described for unheated Fraction I. *Normal human plasma* was prepared as follows: from a careful venipuncture 9 parts of blood were rapidly drawn into a syringe containing 1 part of 0.1 M sodium citrate; the citrated blood was then centrifuged at 2000 rpm for 10 minutes, the plasma separated and used in the tests. All antigens were utilized immediately after preparation.

*Precipitin tests.* a) Ring test: In a small culture tube (75 mm × 10 mm) 0.2 ml of serum was placed and then carefully overlaid with 0.2 ml of antigen.

b) *Simple mixture* of serum and antigen: 0.2 ml of both serum and antigen were placed in test tubes (75 × 12 mm) and mixed immediately, by gentle shaking. Control tests using the serum against plain buffered saline were carried out simultaneously. The tubes stood undisturbed at room temperature and readings were made at 30 minutes, 60 minutes and 24 hours. Only sera and antigens perfectly clear and free of particulate matter proved suitable for the tests. Scrupulously clean, unscratched glassware was employed for accuracy in reading the results.

*Artificial antisera.* The immunizing antigen consisted of 1% solution of Fraction I in distilled water (1 ml equals 10 mg of Fraction I). It was prepared in the same manner as the unheated Fraction I for the precipitin tests and administered to the animals shortly after preparation. Each of 2 rabbits (wt. 2 kg) received a total of five injections of 4 ml each, given intramuscularly on alternate days and occasionally every third day. Twenty days after the final injection, blood samples were drawn from the animals by heart puncture, the sera were separated, allowed to stand at 5° C overnight, and stored at -20° C until used. All procedures were carried out under sterile conditions. Three months later, both rabbits were re-immunized, one of them receiving 2 and the other 4 additional doses of the same antigen, identically administered. Five days after the last injection, the animals were exsanguinated and the sera stored at -20° C. Precipitin tests similar to those used for human sera were employed in testing the rabbit antisera.

*Other determinations.* The fibrinogen content of the antigens was determined as follows: The fibrinogen in a 0.1 ml aliquot of the antigen was clotted by excess thrombin in a diluted buffered system. The clot was washed thoroughly with 0.85% NaCl and digested in 10% NaOH. The tyrosine content of the digested clot was measured according to the method of Folin-Ciocalteu (11).

The clot-accelerating activity of these antigens on Hemophilia "A" plasma was measured in the following system: 0.1 ml Hemophilia "A" plasma, 0.1 ml antigen (or control), 0.1 ml 1% purified cephalin, 0.1 ml CaCl<sub>2</sub> (molarity dependent on citrate content of plasma and antigen), in silicone tubes at 37° C. All fractions and particularly the unheated, were active in shortening the clotting time of hemophilic plasma.

The precipitin tests were done with sera of patients with Hemophilia "A", Hemophilia "B" and miscellaneous conditions without hemorrhagic manifestations; a group of normal individuals was included. Thirty nine human sera were studied (Table). Hemophilia "A" patients were classified into Grades I, II, III and IV indicating the severity of the coagulation defect (12). The most severe cases, those of Grades III and IV, are comparable to the so-called "refractory hemophiliacs" (4), that is, those who derive little or no beneficial effect from transfusions of blood or blood derivatives.

## Results

Negative precipitin tests were obtained in all cases using human sera (Table). No difference was observed between the refractory or non-refractory Hemophilia "A" patients, and the other groups.

Heated Fraction I is the only antigen that was used in all cases. When unheated Fraction I (or normal human plasma) was used as antigen, fine clots appeared constantly. This phenomenon markedly interfered with reading of the tests, and may be a cause for error in interpreting results. The possibility of error is greatest with the Ring Test. The fibrin ring formed at the interface simulates closely a true precipitation ring. Gentle agitation of the tubes, however, clearly shows the presence of a thin, tenuous clot.

*Testing of the Various Sera Against Heated and Unheated Fraction I and Normal Human Plasma*

Source of sera tested	No. of cases	Unheated Fraction I		Heated Fraction I		Normal Human Plasma	
		No. of tests	Result	No. of tests	Result	No. of tests	Result
Hemophilia "A"	21	13		21		1	
Grade I	1	1	Negative	1	Negative		
Grade II	9	5	Negative	9	Negative		
Grade III*)	8	5	Negative	8	Negative		
Grade IV*)	3	2	Negative	3	Negative	1	Negative
Hemophilia "B"	2			2	Negative		
Miscellaneous conditions	5	5	Negative	5	Negative		
Normal Persons	11	9	Negative	11	Negative	1	Negative
Total	39	27		39		2	
Anti-Fraction I Rabbit serum	2	6	Positive	22	Positive	2	Positive
Normal rabbit serum	2	3	Negative	3	Negative	1	Negative

This phenomenon usually appears 5 to 10 minutes after the mixture is made, is most evident at the end of 30 to 60 minutes and was observed in all sera studied, including those from normal subjects. The phenomenon seems to originate from interaction of the fibrinogen present in the unheated Fraction I with some of the residual thrombin in the serum. In support of this interpretation are the following facts: 1) it takes place only when a fibrinogen-rich antigen is used (i. e. unheated Fraction I), whereas it is not observed with fibrinogen-poor antigens (heated Fraction I); 2) it is not detected when the serum utilized for the tests is thrombin-free, as by the addition of heparin to either normal or

\*) Refractory to transfusions

hemophilic serum (1.1 unit of heparin per ml) which results in complete inhibition of formation of fibrin rings; 3) there is apparently a close relationship between the amount of residual thrombin and the intensity of the clot-formation. The slow evolution of thrombin in Hemophilia "A" serum may account for the fact that the phenomenon is more marked in hemophilic than in normal serum. Even after standing for 6 hours, the sera from hemophilic clots will often contain unconverted prothrombin which slowly converts to thrombin on more prolonged standing. When this difficulty was, however, avoided by the use of heated Fraction I as antigen, there was no clotting of the mixture and the tests were clearly negative.

*Rabbit antisera* — Control precipitin tests performed with the rabbit anti-Fraction I sera were invariably positive. True precipitation appeared in a matter of seconds in both the ring test and the simple mixture of serum and antigen, regardless of the type of antigen used. The two antisera from the first immunization of the rabbits exhibited a similar potency (positive tests with antigens diluted to 1/500). Heating of the antisera at 65° C for one hour or storing them at — 20° C for more than 3 months did not destroy their activity. When unheated Fraction I (or normal human plasma) was used as antigen, the development of tenuous clots similar to those observed with human sera, interfered with the reading of tests. However, in the case of the artificial antisera, a true precipitation could be clearly detected in addition to the fibrin clots. Simultaneously performed control tests utilizing normal rabbit serum were invariably negative, giving identical reactions to those of the normal and pathologic human sera previously mentioned. However, when normal rabbit serum was mixed with unheated antigens, the phenomenon of clot-formation was observed to be somewhat stronger than when human sera were used.

### Comment

All precipitation tests performed with the sera of Hemophilia "A" patients gave negative results and no difference was observed between the sera of "refractory" and "non-refractory" hemophiliacs or between these sera and any of the other human sera studied. It should be noted that all Hemophilia "A" patients studied, with one exception, had in the past received multiple transfusions of blood or plasma.

Interference by clot-formation in reading the precipitin tests using unheated antigens (unheated Fraction I, normal human plasma) is noteworthy. This phenomenon closely simulates true precipitation and may be a source of error in interpretation of the results. In contrast, precipitin tests using heated Fraction I which (though having lost its fibrinogen) still maintained its clot-accelerating activity, gave clear cut results and no interference from clot-formation.

These and perhaps other factors explain the difference between our results and those of other authors (1—8) using similar precipitin tests. No antibodies against clot-accelerating factors contained in Fraction I of Cohn could be demonstrated in any of the hemophilic patients studied by our methods.

### Summary

1. The blood of 11 Hemophilia "A" patients refractory to transfusions has been studied for the presence of precipitins against certain clot-accelerating fractions. The tests were invariably negative. Identical results were observed in 10 Hemophilia "A" patients non-refractory to transfusions, in 2 cases of Hemophilia B, in 5 patients with miscellaneous conditions and in 11 normal individuals.

2. When unheated Fraction I of Cohn is used as antigen, false positive results due to formation of fibrin clots were observed with all human sera tested. This may be a source of error in the interpretation of the tests, especially in the blood of refractory Hemophilia "A" patients with an unusually long clotting time and slow prothrombin conversion.

3. These observations do not support the hypothesis that the refractory state to blood transfusions observed in some Hemophilia "A" patients is due to the presence of antibodies against certain clot-accelerating fractions of normal plasma.

### Résumé

1) Des essais de mise en évidence de précipitines contre certaines fractions favorisant la formation du caillot ont été effectuées sur le sang d'hémophiles A réfractaires aux transfusions. Les tests sont invariablement négatifs. Des résultats identiques ont été obtenus dans dix cas d'hémophilie A non réfractaires aux transfusions, dans deux cas d'hémophilie B, chez cinq patients souffrant d'affections diverses et chez onze individus normaux.

2) Quand la fraction I de Cohn, non chauffée, est utilisée comme antigène on obtient des résultats faussement positifs dus à la formation de caillots de fibrine avec tous les sérums humains testés. Cela peut être une source d'erreur dans l'interprétation des tests, spécialement avec le sang d'hémophiles A réfractaires aux transfusions qui ont un temps de coagulation très prolongé et une conversion de la prothrombine très lente.

3) Ces observations ne supportent pas l'hypothèse qui veut que l'incompatibilité aux transfusions observées chez certains hémophiles A soit due à la présence d'anticorps contre certaines fractions du plasma normal, accélératrices de la formation du caillot.

### Zusammenfassung

1. Die Autoren untersuchten 11 gegenüber Transfusionen refraktäre Hämophilie-A-Patienten auf das Vorhandensein von Präcipitinen gegen Fraktionen, die die Bildung des Fibringerinnsels beschleunigen. Die Teste fielen immer negativ aus. Gleiche Resultate wurden bei 10 gegenüber Transfusionen nicht refraktären Hämophilie-A-Patienten, bei 2 Hämophilie-B-Patienten, bei 5 Patienten mit verschiedenen Affektionen und bei 11 Normalen beobachtet.

2. Braucht man nicht erhitzte Fraktion I nach Cohn als Antigen, so werden bei allen geprüften menschlichen Seren falsche positive Resultate beobachtet, verursacht durch Bildung von Fibringerinnsel. Dies kann eine Fehlerquelle in der Interpretation der Versuche sein, vor allem mit Blut von refraktären Hämophilie-A-Patienten mit langer Gerinnungszeit und langsamem Prothrombinverbrauch.

3. Diese Beobachtungen sprechen gegen die Hypothese, daß Antikörper gegen gewisse im normalen Plasma vorhandene gerinnungsbeschleunigende Faktoren für das bei einigen Hämophilie-A-Patienten beobachtete refraktäre Verhalten gegenüber Bluttransfusionen verantwortlich sind.

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