Production of Anti-Human PTC and Anti-Human Proconvertin in Rabbits.* (22778)

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The appearance of circulating anticoagulants has been described in both hemophilia A(1-12) and hemophilia B (PTC deficiency, Christmas Disease)(13,14). Presumably, in both diseases, the inhibitors have arisen as antibodies against "foreign" proteins, either the AHF or the PTC of therapeutically infused normal plasma. In each disease it has been possible to demonstrate that the inhibitor is specific to the factor missing in the particular patient(15). The preparation, in animals, of similar specific antibodies to the various human clotting factors could prove of great value in the study of blood coagulation and, if precipitin reactions occur, in assays of the various factors. Pinniger and Prunty (16) and Hardisty and Pinniger(17) describe the preparation, in rabbits, of an anti-human fibrinogen giving positive precipitin reactions to human plasma and fibrinogen but negative to serum and the plasma of an afibrinogenemic patient. Recently Richards and Spaet (18) have, by immunization of rabbits with human AHF, prepared a rabbit serum highly inhibitory to human AHF, and giving positive precipitin tests to the antigen. However, they do not describe precipitin tests with normal plasma, serum, or hemophilic plasma.

These studies were undertaken in an attempt to prepare rabbit antisera with specific anti-PTC activity. Rabbits injected with our "PTC" preparations formed inhibitors to both PTC and proconvertin. Attempts were made to separate and further purify the antiserum by adsorption with human plasmas from patients congenitally deficient in PTC or proconvertin.

Methods. Preparation of serum PTC. A number of different PTC preparations were made, often with slight modifications of the following: Outdated citrated blood bank plasma was dialysed 48 to 72 hours against 0.01 M sodium oxalate in 0.85% NaCl, recalcified with 1/50 volume 1 M $CaCl_2$, allowed to stand overnight (4°C) and the clot removed. Barium sulfate, 10 mg/ml, was stirred for 10 min. with the serum, centrifuged, washed twice with 0.85% NaCl, twice with distilled water and eluted twice with 1/10 the plasma volume of 0.2 M sodium citrate. The eluates were combined, dialysed 24 hours against water and lyophilized to 1/20 the original plasma volume. The specific resistance was adjusted to that of 0.85% NaCl and the pH to 7.1. Tests on these "PTC" fractions revealed that they contained 5 to 10 times the plasma concentration of PTC, some proconvertin, little prothrombin and no detectable thrombin, AHF, proaccelerin, Hageman factor or fibrinogen. Initial attempts at immunization involved intravenous injections in 3 rabbits, 2 of which convulsed and died within seconds after the fifth injection and showed adherent clots in the ventricles. The remaining 8 rabbits were immunized with intramuscular injections: 1 ml 3 times a week for the first week, 2 ml 3 times a week for the second week, a 10 day rest, followed by a 5 ml injection. Blood was obtained by cardiac puncture 12 and 24 days later. Initial tests, employing rabbit serum, frequently caused clotting when plasma was present in the system. Therefore, in most instances, whole or $BaSO_4$ absorbed oxalated rabbit plasma was used. *Plasma Fractions.* AHF: 0.5 ml rabbit plasma was mixed with 0.1 ml 95% alcohol, chilled 1 hour, the precipitate collected and dissolved in 0.5 ml 0.85% NaCl. *PTC*: 1.5 ml rabbit plasma was mixed with 150 mg $BaSO_4$, centrifuged, the $BaSO_4$ washed once with distilled water,

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drained and eluted with 0.5 ml 0.2 M sodium citrate. This eluate was diluted 1-10 before testing, and contained prothrombin and proconvertin as well as PTC. *Clotting tests.* Most of the methods have been described(19, 20). Hageman factor was measured in a manner similar to AHF, but employing as a substrate plasma from a patient with Hageman factor deficiency(21). This patient was seen through the courtesy of Dr. Oscar Ratnoff. Titers for AHF, PTC, Hageman factor, prothrombin, proconvertin and proaccelerin were carried out on 1-10 dilutions of plasma. Tests for inhibitors to these factors were carried out by incubating 1-5 diluted normal human plasma with equal volumes of normal or injected $BaSO_4$ adsorbed rabbit plasma or saline for 30 minutes and then measuring the residual activity in each test system. When this activity was less than that of the saline-incubated human plasma, inhibition had occurred. Complete inhibition was recorded as 4+. Antifibrinogen was measured by incubating equal volumes of whole human plasma and whole rabbit plasma and, after 30 minutes, measuring fibrinogen concentration of the mixtures turbidometrically(2). This concentration could then be compared to that calculated for the mixtures, the concentration of each component having been measured separately. Anti-thrombin activity was estimated by comparing the clotting times of normal and injected rabbit plasma after addition of human thrombin. Anti-thromboplastin was measured by comparing clotting times of normal and injected rabbit plasmas, mixed with human brain suspension for 30 minutes and recalcified. Anti-human plasma thromboplastin was measured by allowing a normal human thromboplastin generation (modified from(22)) mixture (0.2 ml $BaSO_4$ plasma (1-5) + 0.2 ml serum (1-10) + 0.2 ml platelet suspension + 0.2 ml 0.025 M $CaCl_2$) to incubate 6 minutes, removing 0.1 ml samples, incubating these with 0.1 ml of control or injected rabbit $BaSO_4$ adsorbed plasma or with 0.85% NaCl for 5 minutes and recording the clotting times on addition of 0.1 ml human citrated plasma and 0.1 ml 0.025 M $CaCl_2$.

Results. Blood coagulation studies in the rabbits. (Table I, Fig. 1). Clotting times, prothrombin consumptions and platelet counts carried out on one rabbit from the injected group and one from the control group did not show any significant differences. The 8 injected rabbit plasmas all showed anti-human PTC, so were pooled and compared to pooled control rabbit plasma in the remaining tests. Hageman factor appeared to be normal and proaccelerin and proconvertin far higher in both normal and injected rabbit plasma than in human plasma. Only assays for AHF and PTC were significantly low in the injected group. As these assays are carried out on human substrates, *i.e.* plasmas from patients with hemophilia A or B, it is

TABLE I. Blood Clotting Factors in the Rabbits.

	Normal	Injected
Clotting time (min.)	5	6
Prothrombin consumption, %	95	95
Platelet count (per mm^3)	566,000	662,000
Recalcification time (sec.)	120	120
Prothrombin time (sec.)	7.6	7.3
Prothrombin*	85	105
Proconvertin*	190	225
Proaccelerin*	200	300
AHF*		
on plasma	100	20
on fraction	100	120
PTC*		
on plasma	150	20
on fraction	100	100
Hageman Factor*	100	<100
Fibrinogen*	125	117

* As % of normal human level.

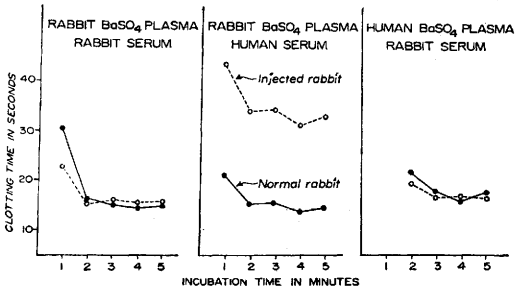

FIG. 1. Thromboplastin generation tests. All tests contained 0.2 ml rabbit platelets plus 0.2 ml 0.025 M $CaCl_2$. Substrate in all was normal human citrated plasma. Part I. 0.2 ml rabbit $BaSO_4$ adsorbed plasma (1-5) plus 0.2 ml rabbit serum (1-10) from normal and inj. rabbits. Part II. 0.2 ml normal or inj. rabbit $BaSO_4$ plasma (1-5) plus 0.2 ml human serum (1-10). Part III. 0.2 ml human $BaSO_4$ plasma (1-5) plus 0.2 ml normal or inj. rabbit serum.

TABLE II. Inhibition of Human Clotting Factors by Rabbit Plasma.

Test	Normal rabbit $BaSO_4$ plasma	Inj. rabbit plasma			
		$BaSO_4$	N*	PTC*	ProC*
Plasma Anticoagulant	0	2+	—	—	—
Anti-Prothrombin	0	tr	0	0	0
" Proconvertin	0	3+	0	0	3+
" Proaccelerin	0	0	0	0	0
" AHF	0	tr	0	0	tr
" PTC	0	4+	0	4+	tr
" Hageman Factor	0	0	0	0	0
" Fibrinogen	0	0	—		
" Thromboplastin	0	0	—		
Brain†	0	0	—		
Plasma	slight	slight	—		

* Samples of $BaSO_4$ treated rabbit plasma incubated 18 hr with equal volumes of normal human plasma (N), PTC deficient plasma (PTC) or proconvertin deficient plasma (ProC) and centrifuged at 20,000 rpm before testing.

† Tested on whole plasma.

possible that the assays are falsely low due to inhibition of PTC and proconvertin in the human substrates. That AHF and PTC in injected rabbits are actually normal is shown by the normal thromboplastin generation (Fig. 1, part 1) and by the assays on rabbit plasma fractions, prepared to contain the factor in question and to be free of inhibitors.

Inhibitory activity of rabbit plasmas to human clotting factors. Table II shows that the normal rabbit $BaSO_4$ plasma had no inhibitory effects other than a slight inhibition on formed plasma thromboplastin. $BaSO_4$ treated plasma from the injected rabbit prolonged normal human plasma recalcification time (plasma anticoagulant) and markedly inhibited human proconvertin and PTC. It also produced a slight diminution of prothrombin and AHF activity, but had no effect on proaccelerin, Hageman factor, fibrinogen or brain thromboplastin. In an attempt to separate the anti-PTC and anti-proconvertin activities of the injected rabbit plasma, equal volumes were incubated 18 hrs. (4°C) with normal human, PTC deficient, and hypoproconvertinemic plasmas. Tests on these mixtures showed that incubation with normal plasma removed all inhibitory effects, incubation with PTC-deficient plasma removed anti-proconvertin and incubation with proconvertin deficient plasma removed most of the anti-PTC.

Fig. 1 also demonstrates the inhibitory effects of the injected rabbit plasma. The first part of the figure shows that $BaSO_4$ plasma and serum from the injected rabbit generate plasma thromboplastin as effectively as those from the normal rabbit. In the second part the normal $BaSO_4$ rabbit plasma is effective when mixed with human serum, showing that rabbit AHF (and proaccelerin?) react normally with human PTC (and proconvertin?). On the other hand, the injected rabbit $BaSO_4$ plasma-human serum mixture does *not* generate thromboplastin. This can be interpreted either as lack of AHF in the injected rabbit or as inhibition of PTC (+ proconvertin) in the human serum. The third part of Fig. 1 shows that the activity of human $BaSO_4$ plasma (AHF + proaccelerin) is not inhibited by the injected rabbit serum.

Precipitin reactions. Normal rabbit plasma did not give precipitin reactions with human plasma. Plasma and serum obtained from the rabbits 12 days after the last injection of "PTC," either whole or $BaSO_4$ treated, gave markedly positive precipitin reactions with the "PTC" fraction, normal plasma or serum and PTC deficient plasma. Plasma obtained 12 days later still had marked anti-PTC and anti-proconvertin activity, but the precipitin reaction was far weaker and only a marked cloudiness without flocculation was observed. Table III shows that when this rabbit plasma was treated with $BaSO_4$ and then adsorbed with human plasmas of various types, the pre-

TABLE III. Precipitin Reactions.

Human material	Inj. rabbit plasma				
	7-11-56	7-23-56			
	$BaSO_4$	$BaSO_4$	N*	PTC*	ProC*
Normal plasma	4+	2+	0	+	+
" serum	4+	+	—	—	—
"PTC" fraction	4+	2+	—	—	—
PTC deficient plasma	4+	2+	0	0	+
Proconvertin deficient plasma	—	+	0	+	0

* See footnote Table II.

cipitin reactions were affected in the same way as the inhibitor reactions.

Discussion. The experiments illustrate clearly that the rabbit and human clotting factors studied have similar and interchangeable biological activities, but that the PTC and proconvertin of man and of rabbits are immunologically dissimilar. Following injections of a human serum fraction containing PTC and small amounts of proconvertin, rabbits developed inhibitors to these human factors but not to their own. Equally heavy precipitin reactions were observed when this rabbit antiserum was incubated with normal or with PTC deficient plasma, indicating the presence in the rabbit antiserum of antibodies other than anti-human PTC. Antiproconvertin was also identified. Separation of the antiproconvertin and anti-PTC activities was achieved by differential adsorption.

These experiments suggest that it may be possible to prepare antisera specific to the various human clotting factors. With sufficient purification of the antigens, antibodies of high specificity and in high titer may result which could be employed in assay methods independent of the formation and titration of thrombin.

Summary. Rabbits injected with human serum "PTC" developed inhibitors to human PTC and also to human proconvertin. These two activities could be separated by differential adsorption.

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