Induction in Rabbits of Activity Against Human Blood Thromboplastin

By Theodore H. Spaet and Jose R. Cintron

The concept that blood cells contain thromboplastic activity similar to that obtained from tissue extracts has given way to the view that blood thromboplastin is formed from the interaction of platelets and plasma clotting factors. The historical development of this transition has been well reviewed by Quick. It is now generally accepted that thromboplastic activity of tissues is preformed; that derived from blood results from a series of complex chain reactions. Whether the end product of blood thromboplastin formation is chemically related to tissue thromboplastin, and what the nature of any such relation may be, is presently a matter of conjecture. Biggs and associates suggested the hypothesis that tissue thromboplastin might be the end product of blood thromboplastin formation, and Nour-Eldin and Wilkinson were able to prepare active antihemophilic factor (AHF) from thromboplastic brain preparations. Gollub et al. showed that bacterial thromboplastinase, which was capable of destroying tissue thromboplastic activity, was also active against certain blood thromboplastin intermediates. An important difference between the two thromboplastins is that tissue thromboplastin requires factor V, factor VII and Stuart factor as co-factors for optimal prothrombin conversion; blood thromboplastin works equally well in the absence of these co-factors. It was the purpose of the present study to explore the relationship between blood and tissue thromboplastin by immunologic methods. The results appear to indicate antigenic dissimilarity.

Methods and Materials

All biologic reagents were of human origin, unless otherwise stated. Brain thromboplastin was prepared and stored as described by Quick, and crude cephalin was extracted from this brain material according to the method of Bell and Alton. Plasma and serum reagents for use in blood thromboplastin generation were made as described by Pool and Robinson and were diluted in isotonic saline brought to pH 7.2 with imidazole buffer.

Blood thromboplastin was sedimented with phosphatide as reported elsewhere. The total thromboplastin prepared was derived from plasma, serum, cephalin, and Ca$^{2+}$ reagents added in 50 ml volumes each, and the resulting sediment was suspended in a final volume of 10 ml of isotonic saline. As was previously shown, blood thromboplastic activity could be sedimented and washed at 20,000 rpm, leaving a supernatant without clotting activity. The thromboplastin generation test was performed according to the method in use by this laboratory.

Experimental animals were New Zealand rabbits of about 3 Kg. in weight. These were given weekly intraperitoneal injections of 2 ml of human blood thromboplastin suspension. Originally 4 animals were so injected, but following the second injection 1 died. Of the

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remaining 3, only 2 developed significant antithromboplastin, and the data to be described are derived from these animals. Definite activity against blood thromboplastin was first noted after four injections, but maximal activity appeared after 5 to 7 injections. In each case sera were obtained for testing 1 week after the last injection. Test sera were obtained from blood collected from the central artery of the ear. The blood was allowed to flow into an uncoated glass tube, clotted at 37 C. and given an additional hour of incubation. Serum was drawn off after appropriate centrifugation and was then adsorbed with aluminum hydroxide to remove the “serum factors.” Serum similarly prepared was also obtained from untreated control rabbits.

“Plasma clotting factors” were concentrated by precipitation from adsorbed plasma, according to the method of Ratnoff and Conley. The precipitate was dissolved in a final volume of buffered saline equal to one-fifth of that of the starting plasma. “Serum clotting factors” were obtained from serum by adsorption with barium sulfate, as described by Ware and Stragnell. The barium sulfate was then washed three times with isotonic saline and reuspended in a volume of 10 per cent sodium citrate equal to one-fifth that of the starting serum. The suspension was incubated for one hour at 37 C., and the barium sulfate removed by centrifugation. The supernatant was dialysed for 24 hours against isotonic saline.

RESULTS

Following repeated intraperitoneal injections of human blood thromboplastin, two rabbits developed activity against this reagent not found in the sera of the untreated controls. Figure 1 shows the effect of the experimental sera when added into the generating mixture of the thromboplastin generation test. In each case, 0.1 ml. of test serum was added in the initial mixture following the plasma, serum and cephalin reagents, but before the calcium. It is evident that the sera from injected rabbits were markedly inhibitory, and their effect was strikingly greater than sera of the controls. When rabbit plasma and serum reagents were used in the generating mixture in place of human reagents, sera from the injected rabbits were not inhibitory: evidently the induced anticoagulant activity was specific against human reagents.

Figure 2 demonstrates the effect of the sera from injected rabbits against sedimented human blood thromboplastin prepared as described above and diluted so that it clotted recalcified substrate plasma in 12 seconds. Equal volumes of test sera were incubated with the thromboplastin reagent, and the clotting activity of the mixture was tested against recalcified substrate plasma at the intervals shown. Again it is evident that the experimental sera were considerably more potent than the control in their antithromboplastic activity. In both the two previous experiments the control sera presented some antithromboplastic activity, which would normally be anticipated; it is not certain how much of a role this naturally occurring thromboplastin inactivator played in the progressive loss of thromboplastic activity seen in figure 2.

Anti-tissue thromboplastin activity was investigated by incubation of sera with brain thromboplastin. Equal volumes of human brain thromboplastin and serum were mixed and tested for clotting activity against recalcified human plasma. As shown in figure 3, the experimental sera had no greater antithromboplastic activity than that of the control, the inhibition present probably representing the naturally occurring inhibitor.

The sera were similarly tested for antithrombin activity against human thrombin (Cutter). Mixtures were evaluated for clotting activity with plasma
Fig. 1 (top, left).—Inhibition of thromboplastin generation by rabbit sera. The shaded area shows the effect of 5 sera from control animals on human reagents. A and B: Sera from experimental rabbits with human TGT reagents; C: Saline control with human TGT reagents; a and b: Sera from experimental rabbits with rabbit plasma and serum, but human cephalin TGT reagents.

Fig. 2 (top, right).—Inactivation of sedimented human blood thromboplastin by rabbit sera. Thromboplastin incubated with: Sera from experimental rabbits (A and B); serum from control rabbit (C); saline control (D).

Fig. 3 (at bottom). Inactivation of human brain thromboplastin by rabbit sera. Thromboplastin incubated with: Sera from experimental rabbits (A and B); serum from control rabbit (C); saline control (D).

in the absence of calcium. Both experimental and control sera had highly potent antithrombin activity, and no differences could be detected.

Sera were tested for activity against human “plasma factors” or “serum factors” as follows: Equal volumes of test sera were mixed with the respective concentrates. The mixture was incubated at 37 C. for 15 minutes, diluted
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with 9 volumes of buffered saline, and then used as appropriate reagent in the thromboplastin generation test. Figure 4 shows that the “serum factors” were unaffected by incubation with any of the sera, indicating lack of activity by the inhibitor against PTC, Stuart factor or factor X. Figure 5 demonstrates the effect of the sera on the “plasma factors.” As compared to the controls, the experimental sera showed a slight, and possibly insignificant inhibitory effect. When experimental sera were added to a thromboplastin generation mixture containing human plasma reagent and rabbit serum reagent, no inhibition was detected. It is therefore likely that the inhibitor was not active against the “plasma factors” AHF or factor V.

Commercial Coombs’ serum (Ortho) had no demonstrable activity against human blood thromboplastin.

Attempts to concentrate inhibitor by ammonium sulfate fractionation were unsuccessful.

DISCUSSION

Rabbits given repeated injections of human blood thromboplastin developed activity against this reagent significantly greater than that found in the sera of untreated control animals. This induced inhibitory activity appeared to be active only against human blood thromboplastin; it had little or no effect on

Fig. 4.—Activity of human “serum factors” in the thromboplastin generation test following incubation with: Sera from experimental rabbits (A and B); serum from control rabbit (C); saline control (D).
human blood thromboplastin precursors, human tissue thromboplastin or rabbit blood thromboplastin. The present data are in agreement with the recent findings of Shirakura and associates, who reported inhibitory activity in sera of rabbits given intramuscular injections of tissue or blood thromboplastin. The sera were active mainly against the reagent injected, and little cross reaction was evident. During inhibition complement fixation was demonstrated, and inhibitory activity could be specifically absorbed by the appropriate reagent. It is of interest that the Japanese investigators have obtained data similar to those reported here with use of a human blood thromboplastin prepared by the ether precipitation method of Nour-Eldin and Wilkinson. The present method of thromboplastin preparation is based on quite different principles and appears to yield a product of different properties.

The findings of the present study and those of Shirakura et al. suggest that the inhibitory activity induced in the injected rabbits is an antibody, as shown by typical development during the course of "immunizing" injections, species and reagent specificity, and demonstration by the latter investigators of complement fixation and specific absorption. The selective activity of the inhibitors against blood and tissue thromboplastin would thus appear to reflect antigenic dissimilarity between these two reagents. Differences between blood and tissue thromboplastin have been previously noted in their clotting activity and their requirement for plasma co-factors. It is accordingly suggested that tissue thromboplastin does not represent a storage or clearance form of blood thromboplastin, but is rather a true tissue product. Although the two forms of thromboplastin are probably lipoproteins with similar phosphatide composition, each appears to have its own unique protein moiety.

**SUMMARY**

Rabbits were injected with sedimented human blood thromboplastin, and two animals developed serum activity against human blood thromboplastin.
in significant excess over naturally occurring inhibitor. This antithromboplastic activity was specific against human reagent, failed to affect thromboplastin precursors and had no activity against tissue thromboplastin.

**Summario in Interlingua**

Conilios esseva tractate con injectiones de sedimentate thromboplastina de sanguine human. Duo del animales disveloppava un activitate seral contra le thromboplastina de sanguine human significativamente in excesso del occurrentia natural de activitate inhibitori. Iste activitate antithromboplastic esseva specific pro le reagente human. Illo non afficeva precursors de thromboplastina. Illo disveloppava nulle activitate contra thromboplastina tissutal.

**REFERENCES**

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