Reticuloendothelial Clearance of Blood Thromboplastin by Rats

By Theodore H. Spaet, Herbert I. Horowitz, Dorothea Zucker-Franklin, Jose Cintron, and Jerzy J. Biezinski

It is traditionally considered that in vivo blood fluidity is normally preserved by the action of circulating anticoagulants combined with minimal activation of blood coagulants. However, preliminary evidence has been presented to support the hypothesis that these mechanisms are inadequate to account for the degree of protection afforded under diverse circumstances and that a cellular mechanism functions to remove clotting intermediates as they form. It has been demonstrated that experimental animals tolerate large amounts of intravenous blood thromboplastin precursors without generalized intravascular clotting; highly active coagulants failed to elicit generalized intravascular clotting provided circulatory flow was maintained. The present study is concerned with the clearance of blood "thromboplastin." The data indicate that this coagulant is selectively and rapidly removed by the reticuloendothelial system (RES).

Materials and Methods

Experimental animals were Sprague-Dawley male rats weighing about 250–300 Gm. All procedures were undertaken with ether anesthesia.

Biological reagents were of rat origin except that in some experiments, as indicated below, crude "cephalin" was obtained from human brain. Necessary dilutions were made with isotonic saline brought to pH 7.2 with imidazol buffer. Thromboplastin generation reagents were prepared as previously described from blood collected by cardiac puncture. Platelets were isolated by differential centrifugation, and were suspended in a volume of buffered saline equal to one tenth that of the original blood specimen. Platelet lysate was obtained from such a platelet suspension which was frozen by immersing its container in acetone held at a temperature of -20 C. in a deep freezer. After thawing, sedimentable material was removed by centrifugation at 20,000 rpm for 30 minutes in a PR-2 International centrifuge supplied with a multi-speed attachment.

Blood thromboplastin was washed and sedimented according to our published method. Rat phosphatides were labeled with P as follows: Radioactive sodium orthophosphate (Abbott) was given intraperitoneally to rats in a dose of 5–15 mc per animal. Five days to one week after injection the animals were sacrificed and brain phosphatides were extracted as described by Bell and Alton. Each batch of radioactive phosphatides represented the pooled extracts of 3 to 5 brains. In some experiments the crude phosphatides were used directly; in others a partially purified mixture of phosphatidyl serine and ethanolamine was substituted.

Purification was achieved by placing a chloroform solution of crude cephalin on a silicic acid column prepared by the method of Hirsch and Ahrens. All nonphosphatide-

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Lipids were removed by consecutive elutions with 40 cc. of petroleum ether, 40 cc. of 1 per cent diethyl ether in petroleum ether, 40 cc. of 25 per cent diethyl ether in petroleum ether, and 40 cc. of pure diethyl ether. The phospholipids were eluted with methanol-chloroform 1:3 (v/v) and collected in 3 cc. aliquots by means of a Technicon automatic fraction collector. The fractions were evaporated to dryness under vacuum at about 40 C. and each was dissolved in a small volume of chloroform. The composition of each fraction was determined by chromatography on silicic acid-impregnated paper and staining with Rhodamine 6 G, ninhydrin, and phosphomolybdic acid. In a number of fractions in the early tubes all the spots detected with Rhodamine 6 G were also ninhydrin positive: choline and inositol phosphatides were not detected. These were pooled and mixed with buffered saline. Prepurified nitrogen was then bubbled through the mixture until all the chloroform was evaporated. The resulting suspension was then used as platelet reagent in the thromboplastin generation test, at various dilutions, to determine its optimal activity. When used for the preparation of sedimented blood thromboplastin each dose of this reagent contained 10.5 μM of phosphatide, determined as phosphorus, in which 58 per cent was phosphatidyl ethanolamine, 29 per cent phosphatidyl serine, and 10 per cent phosphatidyl choline. No attempt was made to evaluate plasmalogens.

Prothrombin times and fibrinogen levels were estimated by published technics.

**Experiments and Results**

*Effect of Injection Route on Defibrination Induced by Blood Thromboplastin*

The basic protocol of these experiments was similar to that of earlier studies. Each rat was given 4 cc. of thromboplastin generation mixture which had reached maximal activity and after clotting had occurred. Blood specimens were collected prior to and 10 minutes after injection. Injections were given into vessels dissected free, and the material was injected in a period of about 20 seconds. Routes of injection were the jugular vein, a branch of the mesenteric vein, or the abdominal aorta below the origin of the renal arteries. A fresh thromboplastin generation mixture was prepared for each injection, and the routes being compared were tested on alternate animals. The various generation mixtures achieved thromboplastic activity such that they clotted rat substrate plasma in 10–13 seconds, and there was little variation from day to day.

The combined results are presented in figure 1. The data are in agreement with those previously published where it was shown that intravenous blood thromboplastin produced a profound fall in prothrombin and fibrinogen. Defibrination was also obtained when injections were given into the aorta, demonstrating that there is no difference whether the pulmonary or the peripheral capillary bed is the first microcirculation encountered by the coagulant. When the thromboplastin was given into a mesenteric vein so that it first encountered the portal circulation, considerably less defibrination resulted. This is evident from the diminished reduction in prothrombin and fibrinogen depicted in figure 1. The differences in fibrinogen reduction as compared to the intrajugular data are highly significant, giving a p value of <0.0001. In addition, the animals given thromboplastin by the portal route showed none of the symptoms of defibrination characteristically presented by the intra-jugular and intra-aortic injected rats. Evidently, when thromboplastin encountered the hepatic capillary bed prior to reaching the general circulation, protection against defibrination was afforded. This protection was not the result of the coagulant
being grossly trapped in the liver because of local clotting, as illustrated by the following experiment: To the usual dose of blood thromboplastin was added 0.1 cc. of human albumin labeled with $^{131}$I (Abbott), representing 0.2 μG of isotope. The addition of the tagged albumin did not affect thromboplastic activity. Injections of the mixture were given either into the jugular or mesenteric veins; after five minutes blood was collected by cardiac puncture and its radioactivity was determined. Were the portally injected material trapped with clots in the liver, the portally injected animals should have had less blood radioactivity than those injected by the jugular route. Since no differences were obtained, it may be assumed that this type of trapping did not occur.

The source of phosphatide used in the thromboplastin generation mixture did not materially affect the differences obtained between jugular and portal thromboplastin injection. As seen in figure 1, similar results were obtained with whole platelets, and with platelet lysate which presumably provided the phosphatide as lipoprotein. With platelet lysate, the difference between jugular and portal injection was greater than with whole platelets; but the difference in each instance was statistically significant (platelet lysate $p = 0.004$; whole platelets $p = 0.01$). Possibly whole platelets are removed with greater difficulty because per unit of clotting activity a greater mass of material is presented.

The protective effect of the liver against blood thromboplastin-induced
defibrination could result from the activity either of parenchymal or RE cells. The latter system was studied by application of RE "blockade." The blockading agent used was a suspension of carbon particles at a concentration of about 10 per cent in Gunther-Wagner Pelikan Ink. This preparation is free of noxious agents present in India Ink, and it has been found to be suitable for use in studies of the RES. Animals subjected to blockade were given the ink into the dorsal vein of the penis at a dose of 1.5 cc. per animal. It was found that larger doses of ink alone produced defibrination. Thromboplastin was injected into the mesenteric vein about six hours after ink administration, at which time the carbon was seen to be completely cleared from the plasma. As compared to non-injected control animals, those with RE blockade suffered significantly greater defibrination ($p < 0.001$) following intraportal blood thromboplastin, as shown in figure 1. This reduction in protection suggests that the RE cells are the components of the liver responsible for the protection demonstrated.

**Fate of Injected Blood Thromboplastin**

Blood thromboplastin was sedimented on crude or purified cephalin labeled with $^{32}$P, as noted above. The original starting amount was 50 cc. of each thromboplastin generation reagent, and the final product was suspended in 10 cc. of buffered saline. This preparation clotted recalcified substrate plasma in about 12 seconds, indicating that some loss of activity had occurred during the procedure. Additional aliquots of crude cephalin were similarly processed except that calcium was omitted throughout. Such cephalin developed no thromboplastic activity, and served as a control for exposure to the blood proteins. Four cc. of blood thromboplastin or non-thromboplastic cephalin were injected into the dorsal vein of the penis, and after 20–40 minutes the animals were sacrificed in ether. Blood samples were drawn into heparin by cardiac puncture and visceral organs were promptly removed for determination of radioactivity. Blood radioactivity was evaluated on 1 cc. aliquots, and total blood radioactivity was calculated based on an estimated blood volume of 20 cc. In the case of lungs, kidney and spleen, counts were obtained from the total organs. Small sample sections of liver and gut were counted, and the total organ radioactivity was calculated based on adjustment to organ total weight. In some experiments organs were liquified by digestion to 1 cc. of 10 per cent NaOH. All counts were performed by means of an end-window geiger tube attached to a decade scaler.

The results expressed as per cent of radioactivity in total organs are presented in figure 2. Most of the radioactivity was found in the liver in all animals, with no other organ showing any appreciable accumulation. Although the liver is, of course, the largest organ studied, its specific activity was correspondingly increased over other viscera except the spleen. The spleen, because of its small size had relatively little total activity, although its specific activity was high. Similar results were obtained both with crude cephalin and the mixture of partially purified serine and ethanolamine phosphatide. The data also indicate that non-thromboplastic cephalin, although similarly dis-
tributed among the organs, was somewhat less effectively cleared from the blood than was the thromboplastic reagent.

It is to be noted that the distribution of radioactivity obtained was quite similar to that of carbon suspension given intravenously, and is again consistent with the hypothesis that the blood thromboplastin reagent is cleared by the RES.

**Effect of Intravenous Blood Thromboplastin on Carbon Clearance**

Carbon clearances were studied as described by Biozzi and associates, and all injections were given into the dorsal vein of the penis. Animals were given 5 mg. of heparin just prior to each experiment. Pelikan Ink was injected in a dose representing 10–12 mg. of carbon/100 Gm. of animal. Blood samples were obtained at about one minute intervals from the retro-orbital plexus by puncture at the medial angle of the eye with heparinized capillary tubes standardized so that 0.05 cc. of blood was used for each sample. Each blood sample was diluted with 2 cc. of 0.1 per cent NaHCO₃, and the optical density was read in a Coleman Junior spectrophotometer at 675 Mₐ. A blood specimen taken prior to carbon injection served as the blank. Test reagents were injected 10 minutes after injection of the carbon; the effect on carbon clearance was followed for an additional 15 minutes. Test reagents were: (1) Sedimented

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blood thromboplastin prepared from 50 cc. of each ingredient. (2) An equivalent amount of crude cephalin. (3) An equivalent amount of crude cephalin exposed to plasma and serum reagents, but with 0.038 per cent sodium citrate substituted for calcium reagent. All test reagents were given in 2 cc. volumes and contained 8 mg. of phospholipid per dose.

The results of these experiments are presented in figure 3. In animals given carbon alone, a $t_{1/2}$ of about 7 minutes was obtained in each run. Subsequent injection of blood thromboplastin caused marked inhibition of carbon clearance, as demonstrated by the flattened slopes thereafter. Quantitation of these differences is given in figure 4. When the original slope of carbon clearance was termed $K_1$ and the slope following injection of test material $K_2$, the $K_1/K_2$ ratio represented an index of inhibition. The mean inhibition index following blood thromboplastin injection was 2.82 and was significant with a p value of <0.01 as compared to control injections. Non-thromboplastic cephalin injection also depressed carbon clearance, but to a lesser degree; and both preparations gave similar results. A mean $K_1/K_2$ ratio of 1.63 was obtained, and the values obtained gave no overlapping either with the blood thromboplastin injections or with the saline controls.

These findings further support the hypothesis that the thromboplastic reagent was cleared by the RES, since inhibition of carbon clearance indicates the presence of a particle competing with carbon for phagocytosis.17

**Discussion**

Four distinct lines of evidence have been presented which favor the view that the blood thromboplastin reagent under study is cleared in vivo by the reticuloendothelial system (RES).

1. Blood thromboplastin given into the portal circulation so that it is first presented to the major RE organ caused less defibrination than the same reagent given into the general circulation.

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**Fig. 3.—Alteration of carbon clearance following intravenous injection of blood thromboplastin.**
2. The protective effect of portal injection was partially reversed by RE blockade.

3. Radioactive blood thromboplastin injected into the general circulation rapidly accumulated in the liver, and generally was distributed as agents known to be phagocytosed by the RES.

4. Injection of blood thromboplastin into the general circulation caused inhibition of carbon clearance, a reaction associated with agents susceptible to RE uptake.

Granted these data and conclusions, the question remains as to their physiological significance. The primary problem concerns the relationship between the reagent here under study and the coagulant activity which develops in the living organism not subjected to artificial laboratory conditions. Although no answer can be provided by the present data, certain findings suggest that physiological generalization may be warranted. Of particular interest was the observation that injection of blood thromboplastin into the portal circulation was protective as compared to injection into the general circulation, irrespective of the source of phosphatide. The protective effect of the portal circulation has also been reported in two other experimental studies: It has been demonstrated that defibrination from incompatible blood transfusion is greatly reduced when the incompatible cells are given via the mesenteric route; and the thrombogenic effect of serum is less pronounced on administration through the portal circulation.

As discussed in previous publications, clearance of clotting intermediates

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**Fig. 4.** Quantitation of carbon clearance alteration following intravenous injection of blood thromboplastin.

- The protective effect of portal injection was partially reversed by RE blockade.
- Radioactive blood thromboplastin injected into the general circulation rapidly accumulated in the liver, and generally was distributed as agents known to be phagocytosed by the RES.
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As discussed in previous publications, clearance of clotting intermediates
is probably an important factor in the preservation of in vivo blood fluidity. As regards physiological blood thromboplastin, the RES is well adapted for such clearance because of its wide distribution throughout the organism, in structures with abundant blood flow. Moreover, the RE cells have a high affinity for particulate material, and there is a suggestion that blood thromboplastin is itself particulate, or is carried on appropriate particles. In support of the concept that blood thromboplastin is particulate are several suggestive lines of evidence: (1) Blood thromboplastin is sedimentable at relatively slow centrifugal speeds both on platelet material and on phosphatide preparations. (2) Phosphatides appear to participate as active reagents insofar as they can form micelles of appropriate size and shape; indeed latex particles of the proper diameter may form effective platelet substitutes. (3) Clotting activity of the platelets has been localized to the granulomere, which is extruded during the clotting process.

It is evident that hemostatic homeostasis cannot allow highly potent coagulants to remain long in the circulation. Evidence has been provided that blood clotting intermediate product 1 is rapidly cleared from the circulation in a manner far more effective than can be accomplished in vitro. The present data suggest that RE cells are responsible for removal of blood thromboplastin.

**Summary**

Data have been presented suggesting that blood thromboplastin given intravenously to rats is cleared by the reticuloendothelial system. (1) The reagent given into the jugular vein or aorta caused profound defibrination, whereas injection into the portal circulation caused a milder reaction. These differences were obtained irrespective of the source of phosphatide in the thromboplastin generation mixture. (2) Reticuloendothelial blockade reduced the protective effect of portal administration. (3) Radioactive blood thromboplastin given intravenously was rapidly cleared from the blood, and showed an organ distribution resembling that of agents known to be removed by reticuloendothelial cells. (4) Injection of the blood thromboplastin during a carbon clearance caused depression of the carbon disappearance slope.

**Summario in Interlingua**

Es presentate datos que pare indicar que thromboplastina de sanguine, administrate a rattos per via intravenose, es eliminate per le sistema reticuloendothelial.

1. Le reagent administrate in le vena jugular o in le aorta causava un forte disfibrination, durante que su injection in le circulation portal resultava in un plus leve reaction. Iste differentia esseva evocate sin reguardo a qual esseva le origine del phosphatido in le mixtura de generation de thromboplastina.

2. Blocage reticuloendothelial reduceva le effecto protectori del administration portal.

3. Radioactive thromboplastina de sanguine per via intravenose esseva eliminate rapidemente ab le sanguine e revelava un distribution in le organos
simile a illo de agentes del quales on sape que illos es eliminate per le cellulas reticuloendothelial.

4. Le injection de thromboplastina de sanguine durante un clearance de carbon causava un depression del coefficiente de direction in le curva de disparition de carbon.

REFERENCES


Four cases of obstetric defibrination are described, and the laboratory tests used for their investigation are critically evaluated. The estimation of fibrinogen by ammonium sulphate precipitation and by the micro-Kjeldahl method gave normal or near-normal results in three of the four patients, but methods based on the reactivity of plasma to purified thrombin (thrombin titer, thrombin time) gave abnormal results in each case. In no case could any active fibrinolysin be demonstrated. Antihemophilic globulin depletion was demonstrated in three, and factor V deficiency in one case. All four cases had thrombocytopenia. The reactivity of the plasma of three of the cases to thrombin was restored to normal by the addition of a small proportion of normal plasma. The 25 per cent ammonium sulphate fractions of the plasma of three of the cases clotted abnormally slowly on the addition of thrombin, but the appearance of the clots suggested that this was not due to simple dilution. It is concluded that the defect in the early cases of this syndrome may consist in a qualitative alteration in the reactivity of fibrinogen to thrombin, rather than a simple quantitative depletion of fibrinogen.—R. M. H.

RELATION OF ATHEROSCLEROSIS TO HEMOCOAGULATION AND SERUM LIPIHS. J. Polčák, B. Sel and M. Skálović. From the Second Medical Clinic, University Brno, Czechoslovakia. Vnit.lék. 4:388-393, 1958.

A group of arteriosclerotics and group of young healthy individuals were studied following administration of 13 Gm. of cream. In all subjects an acceleration of blood coagulation occurred, more pronounced in the young individuals. The serum cholesterol level did not change. Serum lipid levels in arteriosclerotics reached the peak 3 hours after the ingestion of fat and in the majority of cases was significantly increased after 5 hours. Further it was found that there is no direct relation between the blood coagulability and serum lipid level.—L. D.
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