Platelet and Fibrinogen Sequestration

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When dogs are exposed to hypothermia their platelet counts drop to approximately 15 per cent of normal as the body temperature approaches 20°C. When the body temperature returns to normal the platelet levels also return to normal. With isotope technics it has been shown that this thrombocytopenia of hypothermia is due to sequestration rather than destruction. By arterial and venous cathetherizations and by removal of organs, the major portion of this sequestration has been shown to take place in the portal circulation and to involve both the liver and spleen.

When isotope technic of platelet tagging were combined with the technics of platelet transfusions, a second example of platelet sequestration was found to occur during the first few post-transfusion hours. The duration of this sequestration varies from 3 to 24 hours, and the proportion of platelets involved varied from 20 to 75 per cent. We have observed that the amount of platelet sequestration following platelet transfusion is related to the amount of difficulty encountered in the phlebotomy. This suggests that platelet sequestration may be due to thromboplastin activity—whether derived directly from tissue juice or indirectly by incipient coagulation occurring in the phlebotomy equipment.

In 1951, Conley and his associates noted thrombocytopenia and fibrinogenopenia following the infusion of thromboplastin intravenously into dogs. Our purpose in this paper is to study this phenomenon with isotope technics to learn what role, if any, platelet and fibrinogen sequestration plays in this phenomenon.

METHODS

All experiments were carried out on healthy mongrel dogs weighing approximately 12 Kg. Thromboplastin was prepared by acetone extraction of human brain as described by Quick. Five-hundred mg. of the dried powder were suspended in 5 ml. of isotonic saline. The suspension was heated at 56°C for 10 minutes, then centrifuged at 2500 rpm for 20 minutes. The clear supernatant is referred to as "stock thromboplastin solution." It was diluted to various concentrations as described under each experiment.

Throughout each experiment the animals were kept under barbiturate anesthesia. Sodium pentothal was used to initiate the anesthesia, and sodium nembutol was used to maintain it.

All platelet counts were carried out by phase microscopy, and all fibrinogen measurements were done by the method of Quick. In brief, 1 mc. of #32p was injected into a normal dog. One week elapsed during which a population of radioactive platelets was produced. With the use of plastic bags with sequestrone-sodium anticoagulant, blood was drawn from the donor animal and injected into the recipient, which had been prepared by prior phlebotomy of an equivalent amount of its own blood. At frequent intervals after the transfusion, samples of platelets were isolated from the recipient's blood, washed and the radioactivity per platelet measured by means of a thin window Geiger tube. The platelet samples contained less than 1 red or
PLATELET AND FIBRINOGEN SEQUESTRATION

white cell per 5000 platelets. The radioactive counts per platelet were multiplied by the platelet count of the peripheral blood, so that total radioactivity of platelets could be expressed per milliliter of whole blood.

$^{131}I$ tagging of fibrinogen was carried out by the method of Bournell et al. by which one or two atoms of $^{131}I$ were attached to each protein molecule. The plasma proteins were all tagged, and the nonprotein bound $^{131}I$ was removed by dialysis. The tagged proteins were Seitz filtered and then injected into the animal from whom the protein was originally obtained. At intervals, samples of blood were drawn, and fibrinogen separated by the addition of thrombin to exactly 1 ml. of plasma diluted 25 times. The resultant clot was removed by filtration through glass wool; the glass wool-fibrin mixture was washed with large amounts of saline and then placed in a counting tube. Two ml. of 10 per cent sodium hydroxide were added, and the tube placed in a water bath at 56 C. for 10 minutes. The $^{131}I$ activity was then counted in a well scintillation counter. Prior experiments had shown no advantage in using purified fibrinogen rather than plasma as a starting point in the $^{131}I$ tagging procedure.

EXPERIMENTS AND RESULTS

Experiment 1. The Effect on Platelet Survival of an Infusion of a Small Amount of Thromboplastin

The thromboplastin solution for this experiment was prepared by diluting the stock thromboplastin solution 1:16 with isotonic saline. This preparation gave a prothrombin time of 12.5 seconds with normal dog plasma. One day prior to the thromboplastin injection the dog received an infusion of $^{51}Cr$-tagged platelets. The next day, the thromboplastin infusion was begun. The solution was given at a rate of 1 ml. per minute for a total of 2½ hours.

Throughout the experiment, fibrinogen levels, platelet counts and platelet radioactivity were followed. Figure 1 summarizes these results. The platelet count dropped to 45 per cent of the original count. The drop in radioactive platelets occurred somewhat more quickly and was more severe (to 35 per cent) than the drop in nonradioactive platelets. Fibrinogen levels at first rose slightly, then fell to 42 per cent of the original. The drop in fibrinogen occurred after the drop in platelets. In all these infusion experiments the platelets in the counting chamber showed a marked tendency to clump 15 minutes after the start of the thromboplastin infusion. In this experiment, the clumping tendency persisted from the 15 minute sample through the 70 minute sample, then subsided. It was also noted in this and the next experiments that while the fibrinogen levels were dropping and rising, the blood in the syringe had a marked tendency to clot, and it was necessary to keep sodium oxalate in the syringe itself in order to avoid this clotting.

When the thromboplastin infusion ended, the platelet and fibrinogen levels quickly returned to normal. The platelet radioactivity also returned, thus proving that the returning platelets were the same ones that had been present prior to the thromboplastin infusion. They had undergone sequestration and not destruction during the infusion.

Experiment 2. The Effect on Fibrinogen Survival of an Infusion of a Small Amount of Thromboplastin

Again a 1:16 dilution of the stock thromboplastin solution was used. One day prior to the thromboplastin injection, the dog received an infusion of $^{131}I$-tagged
fibrinogen. The next day the thromboplastin infusion was begun at a rate of 1½ ml. per minute for a total of 3½ hours. Throughout the experiment, platelet counts, fibrinogen levels and fibrinogen survivals by radioactivity were followed. Figure 2 summarizes these results. As in the first experiment, the platelets dropped to about 45 per cent; the fibrinogen levels first rose, then fell; the fall in fibrinogen occurred after the drop in platelets; and the levels of platelets and fibrinogen quickly returned toward normal after the thromboplastin infusion was stopped. The drop in fibrinogen levels in experiment 2 was greater than in experiment 1—probably because of the larger total amount of thromboplastin injected. The rise in fibrinogen levels after the thromboplastin infusion was stopped was accompanied by a return of radioactive fibrinogen. This means that the fibrinogen which reappeared is the same fibrinogen that was present prior to the thromboplastin infusion. The fibrinogen, like the platelets, had not been destroyed but merely sequestered.

Experiment 3. The Effect on Platelet Survival of an Infusion of a Large Amount of Thromboplastin

As in experiment 1, the thromboplastin infusion was preceded by a transfusion of P³²-tagged platelets. For the first 6 hours of the experiment, a 1:16 dilution of stock thromboplastin was given at a rate of 2 ml. per minute. From 6 hours to 7½ hours, the concentration of thromboplastin was increased to a 1:4 dilution at a rate of 1 ml. per minute. Figure 3 summarizes the re-
Fig. 2.—Effect of small amount of thromboplastin infusion on fibrinogen survival.

Results of the experiment. As in experiment 1, the platelet drop preceded the fibrinogen drop; and the fibrinogen levels rose before they decreased. Unlike experiment 1, the return of platelet levels required 4 days—from day 2 to day 5. Furthermore, the platelet radioactivity did not return at all. This indicates that there had been actual platelet destruction and that the rate of platelet rise is actually a measure of the rate of new platelet formation. There is no evidence of platelet sequestration—if we reserve the term sequestration for those situations in which the platelets are temporarily trapped and then later released. The rate of return of fibrinogen was much quicker than the return of the platelet level. Fibrinogen returned to normal in one day. This experiment does not reveal whether this fibrinogen return was due to new production or to return of sequestered fibrinogen.

Experiment 4. The Effect on Fibrinogen Survival of an Infusion of a Large Amount of Thromboplastin

For this experiment, a 1:8 dilution of stock thromboplastin solution was used. The infusion was given at a rate of 2 ml. per minute. One day prior to the infusion radioactive fibrinogen was given to the dog. Figure 4 shows the results of this experiment. As in the previous experiments, the platelet count dropped, and the loss of fibrinogen occurred after the platelets disappeared. From 1½ to 2 hours, the thromboplastin infusion was stopped. The platelets showed a slight rise, and the fibrinogen levels showed a striking rise. The return of
fibrinogen radioactivity along with the return of fibrinogen levels indicates that up to this time, the fibrinogen had been sequestered, rather than destroyed. At 2 hours, the thromboplastin infusion was resumed and continued for another 1½ hours. This time, when the thromboplastin infusion was stopped, the fibrinogen levels and platelet levels remained low, as in experiment 3. The platelets returned to normal levels in 4 days, while the fibrinogen returned in 1 day. The radioactivity of the fibrinogen, however, did not return. This indicates that with the increased amount of thromboplastin the fibrinogen had been destroyed or used up in some way. It was no longer able to return to the circulation. The subsequent rise in fibrinogen levels was due to fibrinogen production rather than reappearance of sequestered fibrinogen.

Experiment 5. The Effect on Fibrinogen and Platelets of an Infusion of Inactivated Thromboplastin

The purpose of this experiment was to learn whether the effect of thromboplastin infusion was due to its role in coagulation or to the particulate nature of the material. The stock thromboplastin solution was heated to 100 C. for 90 minutes. The material was then diluted 1:12. This material still had some residual clotting activity, since it gave a prothrombin time of 37 seconds. The material was infused at a rate of 2 ml. per minute for 4 hours. Figure 5 shows the results of the experiment. Unlike the previous experiments, there was no significant drop in fibrinogen levels. The platelet counts did show a drop—to 65 per cent of the original levels. This platelet drop was less than that seen in the previous 4 experiments. It may have been due to the residual thromboplastin activity in the infused material, or to the particulate nature of the material.
PLATELET AND FIBRINOGEN SEQUESTRATION

DISCUSSION

The results clearly indicate that platelet and fibrinogen sequestration occurs in response to small doses of thromboplastin, and that platelet and fibrinogen destruction occurs in response to larger doses of thromboplastin. Bounameaux has furnished a clue to the etiology of this sequestration in his studies of the in vitro effect of thromboplastin on platelets. He found that thromboplastin causes an increase in platelet adhesiveness, due to its interaction with the adsorbed coagulants on the platelet surface. Roskam has long maintained that the platelet has on its surface a plasma atmosphere consisting of prothrombin, calcium, anti-hemophilic globulin, plasma Ac globulin and the other clotting factors. Bounameaux has shown that if calcium or prothrombin are removed from the adsorbed material on the platelet surface, thromboplastin will no longer cause platelet clumping.

We believe that intravenously administered thromboplastin initiates coagulation in the platelet surface. The platelets clump, and these clumps are actually visible in the counting chambers shortly after the thromboplastin infusion is started. The clumps may be filtered out in the capillary beds of the body; that occurrence could explain why the clumps disappear from the counting chamber after the first hour or more of thromboplastin infusion. The work with hypothermic dogs suggests that the filtering out process may occur in the sinusoidal beds of the liver and spleen, rather than in the capillary beds throughout the body. Wherever it occurs, this filtering out of the platelet clumps may be the cause of the platelet sequestration.

The clumping of platelets may then be associated with sufficient release of thrombin to result in partial polymerization of fibrinogen on the platelet surface. This mechanism could explain fibrinogen sequestration. If poly-
merization does not proceed too far, it may be reversible. If the process is far advanced, it may become irreversible and may also prevent the return of the platelet clumps to the circulation, thus resulting in irreversibility of the platelet sequestration.

The phenomenon of partial polymerization of the fibrinogen molecule is only a theory. However, it can explain many of the observed changes, including the difficulty in obtaining unclotted samples from the dogs during the time that the fibrinogen levels were rising in experiments 1 and 2. There was no difficulty in obtaining unclotted samples during the slow rise in fibrinogen levels in experiments 3 and 4.

In any experiment with intravenously administered thromboplastin, one must consider the possibility that the result obtained may be due to antibody reaction or to the particulate nature of the infused material. Although in our experiments human thromboplastin was injected into dogs, we feel that allergy plays no role in the results, since no animal received more than one exposure to the material. The particulate nature may be important, since it is well known that platelets will tend to clump around bacteria and other microscopic particles in vivo. We believe that centrifugation of the stock thromboplastin material removed all the large particles. Furthermore, the results of experiment 5 show that when the coagulation activity of the thromboplastin is lessened, the thrombocytopenic effect is lessened. Quick\textsuperscript{16} obtained decreases in platelet and fibrinogen levels in response to thrombin given intravenously—which cannot be due to a particle effect. In addition, Penick et al.\textsuperscript{17} showed that the thrombocytopenia and fibrinogenopenia of thromboplastin injected intravenously is lessened in hemophilic dogs on
dicoumarol therapy—again demonstrating that the effect is mediated through the coagulation process.

The rates of rise of platelets and fibrinogen in experiments 3 and 4 furnish us with a measure of rates of platelet and fibrinogen production under situations of stress. The return of platelet levels in four days is in good agreement with the results following induction of a massive intravascular clot. It would appear that the body can maintain a steady state at normal platelet levels if platelet survival should be shortened from the normal 7 to 12 day survival to a survival of 4 days. With a survival time less than 4 days, thrombocytopenia must occur.

The rate of production of fibrinogen appears to be much quicker than that of platelets, since fibrinogen levels can be restored within 24 hours. This rise in fibrinogen, however, may be due to the entrance into the circulation of fibrinogen from unlabeled extravascular pools. There is much evidence for the existence of such pools.

We believe that the transient platelet sequestration which occurs following platelet transfusion is due to thromboplastin activity produced during the phlebotomy from which the platelets were derived. In 1952, Stefanini and Chatterjea reported a transient drop in total platelet count which occurred in 31 of 34 recipients of a simple unit of blood, plasma or serum. With serum, the drop was greater and more prolonged than with plasma or whole blood. Here too, we believe the explanation lies in the thromboplastin present in the infused material. Krevans and Jackson have reported a severe and persistent thrombocytopenia following massive whole blood transfusions. We believe that these results are related to those of Stefanini and Chatterjea as our experiments 3 and 4 are related to our experiments 1 and 2.

**Summary**

1. Small amounts of thromboplastin intravenously cause platelet sequestration. This is followed by fibrinogen sequestration. Large doses of intravenously administered thromboplastin lead to irreversible destruction or utilization of platelets and fibrinogen.

2. The following theories are advanced: (a) This platelet sequestration may be due to platelet clumping caused by coagulation occurring on the platelet surface. Surface coagulation may be initiated by the action of thromboplastin or thrombin on the plasma proteins and calcium adsorbed on the platelet surface. The platelet clumps may be filtered out in the sinusoidal spaces of the body, especially in liver and spleen. (b) The coagulation on the platelet surface may lead to partial polymerization of the fibrinogen molecule. (c) As long as this polymerization is reversible, the entire process remains reversible. When polymerization proceeds far enough so as to become irreversible, the platelets and fibrinogen can no longer return to the circulation. (d) This same mechanism may explain other examples of platelet sequestration.

**Summario in Interlingua**

1. Micre quantitates de thromboplastina administrate per via intravenose causa un sequestration de plachettas. Isto es sequite per sequestration de
fibrinogeno. Grande quantitates de thromboplastina similemente administrate resulta in un irreversibile destruction o utilisation de plachettas e de fibrinogeno.

2. Le sequente theorias es presentate: (a) Iste sequestration de plachettas es causate possibilemente per unaggregation de plachettas que es effectuate per un coagulation occurrente al superficie del plachettas. Le coagulation superficial es possibilemente initiate per le action de thromboplastina o de thrombina super le proteinas del plasma e le calcium absorhite al superficie plachettal. Le aggregatos de plachettas es forsan retenite per filtration in Ic spatios sinusoide del corpore, specialmente le hepeate e le splen. (b) Le coagulation al superficie del plachettas resulta possibilemente in un polymerisation partial del molecule de fibrinogeno. (c) In tanto que iste polymerisation remane reversibile, le integre processo es reversibile. Quando le polymerisation progrede usque a un stadio dc irreversibilitate, il deveni impossibile pro le plachettas e le fibrinogeno de retornar al circulation. (d) Iste mesme mehcanismo explica possibilemente altere typos de sequestration de plachettas.

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PLATELET AND FIBRINOGEN SEQUESTRATION

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