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Abnormal Platelet Response to Thromboplastin Infusion in Rabbits Deficient in the Sixth Component of Complement[1] (38182)

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Intravascular coagulation has been induced in experimental animals by the infusion of a variety of substances including thromboplastin (tissue factor), thrombin, foreign proteins, snake venoms, ellagic acid, etc. Wide variations in the consumption of platelets and coagulation factors have been found, depending on the nature of the experimental system employed (1). The mechanism of platelet participation in some of these models has been partially clarified using *in vitro* systems. Inulin, endotoxin, immunoglobulin aggregates and staphylococcal protein A have been found to accelerate the clotting of normal rabbit blood; however, no accelerating effect has been demonstrated with rabbits genetically deficient in the sixth component of complement (C6) (2). Studies of the platelet release reaction using zymosan, inulin, endotoxin and antigen-antibody complexes also demonstrate a complement requirement (3). Collagen, thrombin, and kaolin, in contrast, appear to have a direct effect and do not seem to require complement in order to induce the platelet release reaction (3).

The present study was undertaken to ascertain whether complement plays a role in the platelet response associated with intravenous infusion of thromboplastin in rabbits. The responses in peripheral blood platelet count, levels of fibrinogen and coagulation factors V, VII, and X, and split products of fibrinogen and fibrin were studied as thromboplastin was slowly infused into normal rabbits as well as into rabbits genetically deficient in C6. Part of this work has been published in abstract form (4, 5).

Materials and Methods. Thromboplastin preparation. Frozen brains from normal adult rabbits (Pel-Freez Biologicals, Inc., Robers, Ark.) were thawed and acetone-dried according to the method of Quick (6) and the thromboplastin powder was stored at −70°. A single preparation was used throughout these studies. Immediately before an experiment, 300 mg of brain powder was emulsified with 10 ml of a sterile, pyrogen free, 0.15 *M* NaCl solution, incubated at 45° for 6 min, then rapidly cooled and centrifuged at 1750*g* and 4° for 10 min. The supernatant was used as the thromboplastin preparation, which throughout the studies yielded a prothrombin time of 12.1–12.9 sec with control human plasma (Ortho Diagnostics). Prothrombin times with normal rabbit plasma anticoagulated with 0.1 vol of 3.8% Na citrate ranged from 9.2 to 10.4 sec. Before use in infusion experiments, the thromboplastin was diluted 1:10 with the 0.15 *M* NaCl solution. The prothrombin times of this preparation with normal rabbit plasma ranged from 16.1 to 19.2 sec.

Infusion experiments. They were carried out in normal adult albino rabbits (mean weight, 3.6 kg) and in C6 deficient rabbits (7) (mean weight, 3.8 kg) obtained from Rancho de Conejo, Vista, Calif. Both fe-

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moral veins were exposed under local anesthesia with 1 ml of 2% xylocaine (Astra Pharmaceutical Products, Inc., Worcester, Mass.). Forty-five milliliters of the 1:10 dilution of thromboplastin was infused at a constant rate over a 3 hr period (0.25 ml/min) into the right femoral vein with a 19 gauge siliconized butterfly infusion set needle (Abbott Laboratories, North Chicago, Ill.). Blood samples were drawn from the left femoral vein with a 20 gauge siliconized needle and a plastic syringe. Samples were obtained immediately before infusion and at various intervals during the next 5 hr. Serum was obtained after blood was clotted in glass for 1 hr at room temperature. Plasma for coagulation factor analyses was prepared using plastic tubes and pipets and 3.8% Na citrate as the anticoagulant. Platelet-free plasma was obtained using a Spinco ultracentrifuge and an SW-50 rotor at 17,000 rpm and 20° for 30 min. EDTA plasma was used for hematocrit, blood smears, platelet counts and fibrinogen determinations. Plasma and serum samples were stored in plastic tubes at −70° until assayed.

Platelet, coagulation and complement assays. Platelet counts were obtained with a Coulter counter method (8). Plasma fibrinogen concentrations were measured by a standard procedure (9). Factor V was determined using human factor V deficient substrate and normal pooled rabbit plasma for control curves (10). Factors VII and X were assayed using human plasma congenitally deficient in each specific factor and normal pooled rabbit plasma was used to obtain control curves (10). Split products of fibrinogen and fibrin were measured by a staphylococcal clumping technique (11) using serum from blood collected in soybean trypsin inhibitor. Total complement levels were assayed by a standard 50% hemolysis test (12).

Reconstitution of the complement system of C6 deficient rabbits. Three C6 deficient rabbits were given a source of C6 intravenously and 10 min later a blood sample was drawn to evaluate the degree of reconstitution of the complement system. Immediately thereafter, the thromboplastin infusion was started. One rabbit received 5.5 ml of platelet-free plasma prepared from normal rabbit blood collected in sodium citrate. Another rabbit received 2 mg of partially purified rabbit C6, and a third rabbit was given 2 mg of human C6 (13). These treatments resulted in reconstitution ranging from 8 to 20% of the normal rabbit total complement hemolytic activity.

Results. The percent changes in platelet number produced by the administration of thromboplastin to normal and C6 deficient rabbits are illustrated in Fig. 1. In most normal rabbits there was a decrease in platelet number 1 hr after initiation of the infusion. At 2 hr, all counts fell below preinfusion values. At 3 hr, when the infusion was stopped, the platelet count of several rabbits showed a tendency toward recovery and in 2 animals exceeded the preinfusion values. In the 5 animals studied for 5 hr, the platelet number varied over a wide range. In marked contrast to the effect on normal rabbits, the administration of thromboplastin to C6 deficient rabbits generally resulted in small increments in platelet number during the infusion period. Two hr after discontinuation of the infusion, the platelets remained above preinfused levels in 2 of the 4 C6 deficient animals. Normal rabbits infused with saline had only minor changes in platlet number as follows (mean values of 5 experiments): 2% increase at 1 hr, 2% decrease at 2 hr, 8% increase at 3 hr, and 9% increase at 5 hr. Mild increments in platelet number were also obtained in 1 C6 deficient rabbit infused with saline. No platelet clumping was observed in blood smears from any group of animals. The preinfusion platelet count (mean ± S.E.) in the normal rabbits was 398,600 per mm^3 ± 41,000 (N = 11). In the C6 deficient group the mean platelet number was 371,600 per mm^3 ± 29,500 (N = 7).

The effect of reconstitution of the complement system in C6 deficient rabbits upon the platelet response to thromboplastin administration is shown in Fig. 2. For purposes of comparison, the mean changes in platelet number caused by administration of thromboplastin in unreconstituted C6 defi-

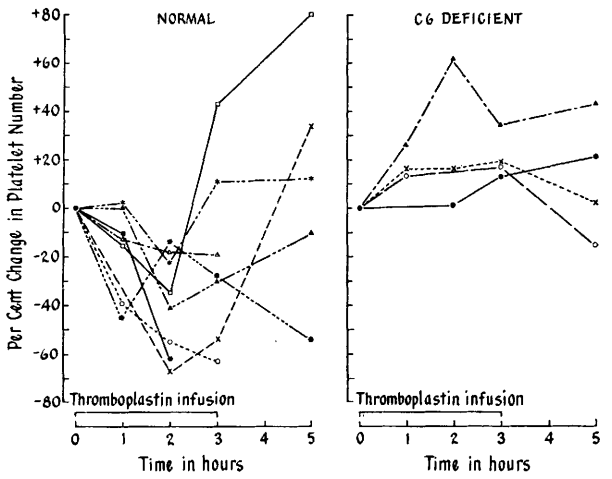

FIG. 1. The platelet response of normal and C6 deficient rabbits to infusion with thromboplastin. In the group of normal rabbits, the 5 hr sample was not obtained in 2 animals, and 1 animal died after drawing the 2 hr sample.

cient and in normal rabbits are also indicated in Fig. 2. The results demonstrated that the administration of a small amount of rabbit plasma or purified human or rabbit C6 to C6 deficient rabbits resulted in complete restoration of the ability to develop thrombocytopenia after infusion with thromboplastin. The reconstituted rabbits had severe and prolonged thrombocytopenia, a behavior that was observed in about one-third of the thromboplastin infused normal rabbits (Fig. 1). No modification in platelet number occurred in one C6 deficient rabbit infused with purified human C6 and saline.

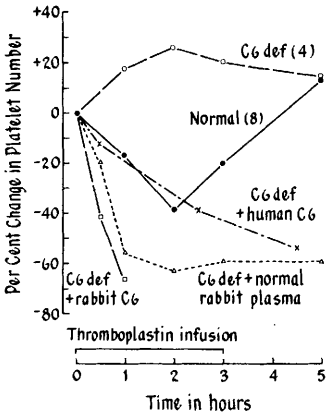

FIG. 2. The platelet response to the infusion with thromboplastin after reconstitution of the complement system in C6 deficient rabbits. The response of each reconstituted animal is represented in the graph. The mean change in platelet number obtained in thromboplastin infused normal and unreconstituted C6 deficient animals is also given (in parenthesis, number of experiments).

The plasma concentration of coagulation factors in untreated C6 deficient rabbits was previously reported as not significantly different from that of normal rabbits (14). Similar results were obtained in the present study for factors V, VII, and X. The concentration of fibrinogen in C6 deficient rabbits was found to be somewhat higher than in normal controls, but the difference was not statistically significant. The mean fibrinogen concentration of 8 C6 deficient rabbits was 398 mg% ($\pm$23.1, S.E.) and that of 13 normal rabbits was 322 mg% ($\pm$42.0, S.E.; $P > 0.1$).

The experimental manipulation resulted in a steady fall in fibrinogen level in all animals studied, which, after termination of the infusion period, attained an average value 20% below the preinfusion level. Since this change also occurred in the saline infused controls it was considered not significant. The changes in fibrinogen occurred in parallel with a progressive decrease in hematocrit values that took place over the 5 hr of the experiment. Split products of fibrinogen and fibrin were not significantly

increased over preinfusion levels in any group with two exceptions. One was a normal rabbit infused with thromboplastin that had a 54% reduction in platelet number 2 hr after termination of the infusion, as well as a significant reduction in factor V activity and a level of split products of 80 μg/ml compared to a preinfusion level of 1.7 μg/ml. The other exception was a C6 deficient rabbit infused with thromboplastin that had a significant reduction in factor V and 1593 μg/ml split products 2 hr after termination of the infusion (preinfusion level, 6.6 μg/ml). No thrombocytopenia was observed in this rabbit. All other experimental animals had no evidence of fibrinolysis or consumption of factor V. None of the animals developed consumption of factors VII or X.

Serum complement levels were measured in normal animals infused with thromboplastin or saline. As indicated in Fig. 3, there was a reduction in total complement activity in the thromboplastin infused rabbits as compared to the saline infused controls, which was statistically significant at hours 2, 3, and 5 ($P < 0.05$).

Normal and C6 deficient rabbits infused with saline as well as C6 deficient rabbits infused with thromboplastin survived the procedure and remained healthy during a

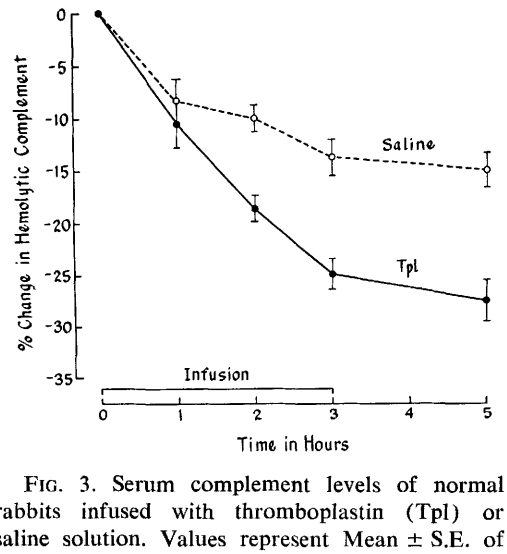

FIG. 3. Serum complement levels of normal rabbits infused with thromboplastin (Tpl) or saline solution. Values represent Mean ± S.E. of 8 thromboplastin infused and 5 saline infused animals.

postinfusion 30-day observation period. One of the thromboplastin infused normal rabbits died during the experiment. Macroscopic and histologic examination revealed no fibrin deposits or other lesions in the tissues of this animal, including the lungs, as well as in 2 other thromboplastin infused normal rabbits that were sacrificed immediately after drawing the 5 hr blood sample. No thrombosis was seen in the iliac vein, inferior vena cava or heart of these animals. The other thromboplastin infused normal rabbits remained healthy during a postinfusion 30-day observation period. No lesions could be demonstrated in a thromboplastin infused C6 deficient rabbit reconstituted with purified rabbit C6 that died 2 hr after the beginning of the infusion or in a rabbit reconstituted with human C6 that died shortly after drawing the 5 hr blood sample.

Discussion. The thrombocytopenia induced in rabbits by the administration of thromboplastin under the conditions described in this study occurred only if there was an intact complement system. The administration of thromboplastin usually caused no significant changes in coagulation factors I, V, VII, or X, and in most cases there was no development of split products of fibrinogen or fibrin. In contrast, thromboplastin caused significant *in vivo* consumption of complement, suggesting that activation of the complement system might have occurred. Because there was little alteration in levels of clotting factors, the thrombocytopenic response of normal rabbits to thromboplastin appears to have resulted directly from the activation of the complement system. Alternatively, thromboplastin could have generated small amounts of thrombin by the extrinsic pathway, which in turn acted upon the platelets through the participation of the complement system. If this occurred, there was minimal clotting factor utilization. Another possibility, under the conditions of these experiments, is that the formation of thrombin by the administration of thromboplastin is complement dependent. The thromboplastin as prepared for this study probably did not contain endotoxin. In rabbits it has been shown that factor V levels are reduced during endo-

toxin-induced intravascular clotting (15). This was not observed in the present study.

The requirement for an interaction of complement with platelets for efficient clotting has been documented in a rabbit system using plasma from C6 deficient rabbits (16). Similarly, the platelet release reaction is complement dependent when release is triggered by inulin, endotoxin, zymosan, aggregated gamma globulin, and antigen–antibody complexes (3). Reconstitution of the complement system in C6 deficient rabbit plasma with purified C6 restored to normal the platelet release reaction as well as the accelerating effect of platelets on clotting, thus demonstrating that the lack of platelet participation in clotting is not due to an intrinsic platelet abnormality in C6 deficient rabbits (3, 16). It is of interest that the *in vivo* thrombocytopenic effect of endotoxin is also complement dependent (17–19).

The *in vitro* effect of thromboplastin in clotting is best illustrated by the one-stage prothrombin time test. The generation of thrombin through the extrinsic coagulation pathway results in clot formation in a few seconds. During this process fibrinogen and factors II and V are consumed in human plasma. This potent *in vitro* clot promoting property of thromboplastin has been implicated in a wide variety of clinical states with thrombosis. The significance of the potential participation of thromboplastin in thrombosis is emphasized by the widespread distribution of this procoagulant in vessel walls and other tissues (20). Experimentally, thromboplastin infusion into animals leads to a wide variety of changes depending on the mode of injection, quantity infused and animal model used. Unlike the *in vitro* effect, a lack of complete defibrination and a lack of major changes in coagulation factors have been noted by other investigators when rabbits are infused (1). Indeed, paradoxic changes in platelets and fibrinogen have been observed in dogs subjected to prolonged infusions with small doses of thromboplastin (21).

The lack of a thrombocytopenic response following thromboplastin infusion into C6 deficient rabbits suggests that complement may play an important role in thrombogenesis. We propose that the complement system is a sensitive mechanism capable of responding to quantities of thromboplastin that are insufficient to trigger thrombin generation by the extrinsic coagulation pathway. This may lead to platelet participation in hemostasis independently of the plasma coagulation mechanism, a concept that requires further investigation. At present, however, it is not possible to apply this concept to human systems since one human with C6 deficiency did not have an abnormal prothrombin consumption (22), in contrast to the findings in the C6 deficient rabbit model (14).

Summary. The slow infusion into normal rabbits of a low dose of thromboplastin (tissue factor), generally insufficient to cause thrombosis or consumption of coagulation factors, produced thrombocytopenia and a reduction in total serum complement activity. In contrast, the administration of thromboplastin to rabbits genetically deficient in C6 resulted in small increments in platelet number. Reconstitution of the complement system of C6 deficient rabbits with purified C6 restored the capacity to develop thrombocytopenia following thromboplastin administration. These studies demonstrate that thromboplastin can act on platelets through the participation of the complement system and that C6 is essential for this reaction.

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