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Activation of Prothrombin by Platelets Plus Globulin.*

J. H. MILSTONE.† (Introduced by M. C. Winternitz.)

From Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.

The clot-promoting effect of platelet suspensions is attributed to the platelets themselves, rather than to accompanying blood components. The mode of this platelet action still remains to be clarified. It is often stated that platelets plus calcium ions activate prothrombin. But it is obvious that the facts are not that simple; for platelets, calcium and prothrombin coexist in the circulating blood. It seems that at least one other factor must be involved. The following experiments differ from previous two-stage tests in that a globulin co-factor has been removed from both the platelets and the prothrombin, and then restored to the system for the prothrombin activation tests.

Materials and Methods. In general, the materials and methods were those which have been described,[1] except that the buffered saline contained 9 g NaCl, 200 ml 0.1 M sodium diethylbarbiturate and 144 ml 0.1 M HCl per liter of solution.

Twenty-five ml rabbit blood was drawn from the heart into a syringe containing 25

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ml cold acid-citrate (37.5 g 2 $Na_3C_6H_5O_7$. 11 H_2O plus 1.05 g $H_3C_6H_5O_7 \cdot H_2O$ per liter of solution, pH 6.3). The platelets were separated by 3 cycles of differential centrifugation during which they were washed twice with cold buffered saline, pH 7.4. The centrifugations were carried out in 9½ x 1½ cm lusteroid tubes. The 3 slow centrifugations were at 1100 r.p.m. in an International, Size 1, Type C, centrifuge at room temperature; whereas the 3 alternate fast runs were made at 3400 r.p.m. in a Servall, Type SP angle centrifuge kept in a refrigerator at 2-4°C. After the first slow 20-minute run, the turbid plasma was pipetted off and the red cells were discarded. A 30-minute fast run served to sediment the platelets out of the plasma, along with some red and white cells. The sediment was suspended in 20 ml cold buffered saline with the aid of a glass pipette, and the suspension was centrifuged slowly for 10 minutes to remove most of the remaining red cells, along with some platelets. The turbid supernatant was subjected to a 20-minute fast centrifugation, and the resulting sediment was resuspended in 20 ml cold buffered saline. The ensuing fifth and sixth runs were like the third and fourth. The final platelet sediment was suspended in 2.5 ml buffered saline. Microscopic examination revealed one red cell per several hundred platelets. No white cells were found. Wright stains showed platelets with the characteristic reddish purple granules, but the blue hyalomere was observed only occasionally.

All biologic materials except the platelets were bovine. The crude globulin was prepared by adding calcium chloride to a plasma euglobulin solution, removing the resultant fibrin and allowing the solution to age in the refrigerator. Its thrombin was just detectable in the dilutions used. The factor or factors significant for the experiments that follow, although of unknown nature, will be referred to as "globulin."

All activation experiments were performed in glass, at room temperature, in the presence of calcium chloride. The molarity of the latter was usually in the neighborhood of 0.0025 M.

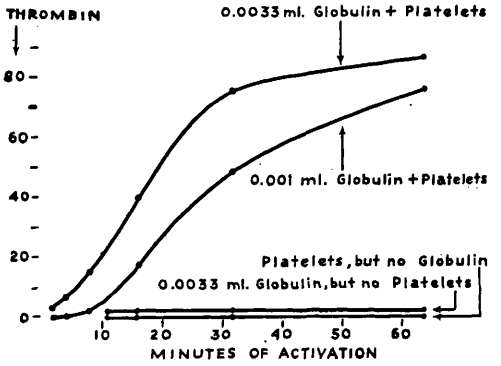

FIG. 1.

Activation of prothrombin by platelets, calcium, and globulin. 0.3 ml buffer, 0.4 ml prothrombin, 0.1 ml platelets, 0.1 ml Ca 0.0275 M, and 0.1 ml globulin dilution (1/30 or 1/100). Ca added 15 seconds before globulin. Activation timed from the addition of globulin. The height of the "Globulin but no Platelets" curve reflects the amount of thrombin in the globulin preparation.

Experimental. The platelets were studied microscopically as they aged in buffered saline without added anticoagulant. When suspensions were kept at room temperature in a sealed hanging drop preparation, the platelets lost their refractility slowly over a period of several hours; concomitantly they became swollen and less distinct in outline. These changes were only a little faster, if at all, when calcium chloride was added. Rapid disruption of twice-washed platelets in the presence of calcium and in contact with glass was not observed.

Although the behavior of different platelet suspensions varied, activation of prothrombin by fresh, twice-washed platelets was always delayed. Production of thrombin was much faster when a small amount of globulin was included. In the two-stage tests of Fig. 1, platelets plus globulin activated prothrombin whereas neither the platelet suspension nor the globulin was efficient alone. On prolonged incubation, some activation occurred with platelets, little, if any, with globulin. Platelets plus globulin did not yield thrombin unless prothrombin was added. The quantity of platelets used for 1 ml activation mixture was derived from 1 ml whole blood. The quantity of globulin used for the highest curve of Fig. 1 was derived from about 0.005 ml blood, and represented less than one-hundredth the amount of globulin used as throm-

bokinase in a previous study.[1] In these estimates, no allowance was made for large losses during preparation.

The effectiveness of the platelet-globulin system was much reduced by heating either component at 70°C for 10 minutes. A suspension of kaolin could not be substituted for the platelet suspension, although the same kaolin suspension was able to hasten the production of thrombin in a cruder test system.

When a platelet sediment was suspended in distilled water, it was at once less turbid than a similar sediment suspended in buffered saline. The water-clear extract obtained with distilled water behaved like the suspension of Fig. 1, although not all of the original activity was recovered in the extract. Merely setting the platelet factor free was not enough to enable it to activate prothrombin promptly. The presence of the globulin was still necessary.

Varying the amount of platelets and of globulin independently showed that the rate of thrombin formation was determined by both factors but was strictly proportional to neither. That the effects of the two components were not simply additive was further indicated by the fact that much faster activation was obtained with platelets and globulin than was obtained with eight times as much platelet suspension without globulin. When the quantity of platelets was raised above a certain level, activation was retarded.

Supplementary tests with dog platelets showed considerable quantitative differences, but the basic platelet-globulin relationship was maintained. Future work with all materials from the same species is contemplated.

Three-stage tests[1] were used to follow the thrombokinase activity of platelet-globulin mixtures. Although some results were suggestive, they were generally too erratic to permit the conclusion that thrombokinase activity was being developed progressively. Rapid inactivation of thrombokinase was sometimes apparent. This complication may have contributed to the capricious results obtained with the three-stage analyses, and also to the equivocal quantitative relationships observed in the two-stage tests.

Discussion. It has been shown that a suspension of washed platelets which induced very slow activation of prothrombin was not therefore of low potency. Such a suspension displayed considerable activity when tested in conjunction with a small quantity of a globulin preparation. The latter preparation, in turn, showed little if any thrombokinase activity, in the dilutions used, when tested without platelets.

Conceivably, platelets might contain prothrombokinase as suggested by Collingwood and MacMahon,[2] or a platelet enzyme might activate the prothrombokinase ("thromboplastinogen") of plasma, as suggested by Quick.[3] The present experiments do not decide between these hypotheses, nor do they eliminate other possibilities.

Lenggenhager[4,5] believed that the effect of platelets on blood coagulation was entirely due to their particulate nature, and consequent surface activity, and reported that kaolin particles had similar effects. The function of the platelets as defined by the present system was probably due to a substance derived from the platelets, and not to such surface effects, because the platelet suspensions could be replaced by platelet extracts but not by kaolin. Some possibility remains that the substance was adsorbed on the platelets or associated with inconspicuous accompanying particles, and although not completely removed by two washes, it was set free by the treatment with distilled water. Finally, the questions of particulate nature and surface activity recur at another level, when it is considered that the coagulant substance in the platelet extracts might have been in the form of particulates of macromolecular dimensions.

1 Milstone, J. H., *J. Gen. Physiol.*, 1948, **31**, 301.

2 Collingwood, B. J., and MacMahon, M. T., *J. Physiol.*, 1912, **45**, 119.

3 Quick, A. J., *Am. J. Med. Sc.*, 1947, **214**, 272.

4 Lenggenhager, K., *Klin. Woch.*, 1936, **15**, 1835.

5 Lenggenhager, K., *Helv. Med. Acta*, 1940, **7**, 262.

The early work of Wright and Minot[6] showed that a factor associated with the serum globulins was important in bringing about the metamorphosis and disintegration of platelets in the presence of calcium. This would account for the relative stability of washed platelets. It will be of interest to inquire how the globulin factor as detected by platelet metamorphosis is related to the substance or substances which function as co-factor for platelets in the activation of prothrombin.

6 Wright, J. H., and Minot, G. R., *J. Exp. Med.*, 1917, **26**, 395.

Summary. 1. Twice-washed platelets were relatively stable in the presence of calcium ions and in contact with glass.

2. In the presence of calcium, washed rabbit platelets plus a small amount of bovine globulin activated bovine prothrombin. Activation was much slower with platelets alone, and no activation was detected with the globulin alone.

3. Water-clear platelet extracts could be substituted for platelet suspensions in this test system. In such case, the presence of the globulin was still necessary for prompt activation of prothrombin.

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The Rickettsiostatic Action of Crystalline Penicillin Fractions in Embryonate Eggs.*

DONALD GREIFF AND HENRY PINKERTON.

From the Departments of Biology and Pathology, St. Louis University.

In 1944, we found that crude commercial penicillin strikingly inhibited the growth of typhus rickettsiae in the yolk sac,[1] and markedly reduced or even completely prevented the mortality from typhus infection in mice.[2] In fertile eggs, the total dosage used was 975 units per egg, given in 3 doses on the 2nd, 4th and 6th, or on the 3rd, 5th and 7th days after rickettsial injection. Doses of 100 units had little or no effect.[3] In mice, the most impressive results were obtained with daily doses ranging from 640 to 1100 units, starting 6-48 hours after injection with lethal doses of rickettsiae. (The unit age content of these samples of penicillin was determined by assay against *Staphylococcus aureus*.)

The rickettsiostatic and chemotherapeutic action of penicillin in eggs and mice was found in the same year to compare favorably with that of para-aminobenzoic acid.[2,3] Uniformly good rickettsiostasis by penicillin was noted in at least 5 different experiments.

In 1945 and 1946, the samples of penicillin which we obtained were usually completely ineffective against rickettsial infection in doses of 1000 *S. aureus* units per egg. Similar observations, indicating variability in the therapeutic action of commercial penicillin against bacterial and spirochetal infections, in spite of standardization *in vitro* in terms of bacteriostatic units, have been reported by others.[4] We considered the possibility that the satisfactory rickettsiostatic action of the earlier samples might have been caused by some impurity other than penicillin. The realization that commercial penicillin has contained varying amounts of several chemical types of penicillin, including penicillins G, K,

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1 Greiff, D., and Pinkerton, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 116.

2 Moragues, V., Pinkerton, H., and Greiff, D., *J. Exp. Med.*, 1944, **79**, 431.

3 Greiff, D., Pinkerton, H., and Moragues, V., *J. Exp. Med.*, 1944, **80**, 561.

4 *J.A.M.A.*, 1946, **131**, 271.