

**Fresh, Disintegrated Platelets in Radiation Thrombocytopenia: Correction of Prothrombin Consumption without Correction of Bleeding.\* (25132)**

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Platelets have 2 major functions in the hemostatic mechanism. The first is a mechanical action of intact platelets, the platelet plug(1) being the visible result of this effect, and the second is a complex biochemical role in coagulation. Many factors are involved, but the prothrombin consumption test may be taken to reflect the more important of these factors. It is essential to separate these 2 effects both from a theoretical and a practical point of view. Theoretically it should be known whether a normal clotting system, including platelet factors is able to maintain hemostasis in absence of mechanical effects of platelets. Practically, it should be known whether thrombocytopenic patients may benefit from platelet substitutes. Much and conflicting work is going on in this field(2,3,4), but it is not known whether normalization of the clotting system by disintegrated platelets will stop bleeding in thrombocytopenia. The value of the irradiated thrombopenic dog or rat in evaluating factors concerned with hemostasis has been established(4,5). The concept of heparinemia as cause of radiation purpura has been adequately disproved(5). Transfusion of fresh platelets was first shown effective in preventing or stopping irradiation bleeding in the dog(6). The quantitative study of thrombopenic bleeding and its control was made possible by the thoracic duct cannulation technic(7) where outflow of red cells/unit time is used as index of capillary bleeding into the tissues. Irradiation, thoracic

duct cannulation and appropriate coagulation studies in dogs were combined to study the 2 roles of platelets in hemostasis. We report the failure of fresh disintegrated dog platelets to correct bleeding in irradiated thrombopenic dogs.

*Materials and methods.* The dogs were healthy mongrels of either sex, weighing 25-30 lb. They were anesthetized with Nembutal (Abbott Lab., Chicago, Ill.) and given 500 r total body irradiation by a 250-KVP General Electric X-ray machine operated at 30 ma and filtered with 0.5 mm Cu and 1 mm Al. Tissue dose rate was 27 r/minute at TSD of 110 cm. From seventh day 75 mg daily of a serotonin analogue (1-benzyl-2-methyl-5-methoxy-tryptamine-HCl from Merck, Rahway, N. J.) were given orally. After 9-11 days the animals had less than 2,500 platelets/cu mm and a generalized purpura. Under Nembutal anesthesia the thoracic duct was then cannulated(7), 5-ml volumes of lymph were collected in tubes containing 0.8 ml 3.13% sodium citrate, and number of red cells in each portion counted (8). The dog was maintained in fluid balance by continuous infusion of saline, 5% dextrose, and Ringer solution. If lymph flow exceeded 1 ml/minute, the lymph was infused back into the dog after removing aliquots for counting. Care was taken to maintain an even flow of lymph during experiment. Each experiment consisted of 3 periods. The first was a baseline period lasting at least 4 hours, during which output of red cells in lymph, platelet count, hematocrit, and prothrombin consumption were established. The second period started with intravenous infusion of fresh, disintegrated platelets, and the effect was again

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followed for 4-6 hours. The third period commenced with the transfusion of fresh intact platelets. Donor dogs were healthy mongrels of either sex, weighing 50-80 lb. Under Pentothal (Abbott) anesthesia, 400-600 ml blood were drawn from femoral artery. Blood was collected into chilled, siliconized 200-ml bottles, each containing 20 ml of 1% Na<sub>2</sub>EDTA in 0.7% saline. Following centrifugation at 800-900 rpm (160-200 g) for 30 minutes, the supernatant platelet-rich plasmas were pooled and centrifuged again at 2,500 rpm (1,550 g) for 30 minutes. The platelets were resuspended in saline to a concentration of 1-2 millions/cu mm, and either immediately transfused or disintegrated (for 7 minutes) in 10-kc magnetostrictive oscillator (Model DF-101, Raytheon Man. Co., Waltham, Mass.). Platelets were processed as quickly as possible at 4°C. Platelets in the recipients were counted in venous blood, using phase microscope(9). Blood for prothrombin consumption was collected in dry siliconized syringes with 20-gauge siliconized needles, either from a fresh venipuncture or from an indwelling polyethylene tube in the upper vena cava. The first ml of blood was discarded, and 1 ml was then quickly placed in each of 5 acid-cleaned, dry tubes measuring 10 x 70 mm. The first contained 0.2 ml of 3.13% sodium citrate; to others, citrate was added after incubation at 28°C for 15, 30, 45, and 60 minutes. The tubes were then centrifuged at 2,000 rpm (940 g) for 5 minutes, the supernatants were diluted 1/10 in citrate dilution fluid II (10, p. 12), and prothrombin assayed in the following test system at 37°C (see 11): 0.2 ml dog brain thromboplastin (10, p. 17), 0.2 ml oxalated, adsorbed and dialyzed ox plasma (10, p. 12), 0.2 ml dog serum (10, p. 44), 0.2 ml test material, and 0.2 ml CaCl<sub>2</sub> of optimal strength. The reagents were stored at -20°C, and the system was stable during the day. On double logarithmic paper there was, within the range 5-100%, a straight-line relationship between concentration of standard dog plasma and clotting time. The system is specific for prothrombin, and the results agree closely with results of a 2-stage system (unpublished, Stormorken and Hjort). Clot retraction was observed in freshly siliconized

tubes measuring 10 x 70 mm. Each tube contained 1 ml citrated platelet-free plasma from a thrombocytopenic dog. To this were added 0.2 ml platelet material, 0.2 ml CaCl<sub>2</sub> 50 mM, and 0.1 ml thrombin (Parke Davis Co., Detroit, Mich.), 100 NIH units/ml in 50% glycerol-saline. The tubes were incubated for 2 hours at 37°C.

*Results.* 1. *Effect of disintegration on platelets.* To test the effect of disintegration, dog platelets were suspended in plasma or saline and disintegrated for varying periods of time. After 3 minutes, less than 1% of platelets was still intact, and granules with a strong tendency to "agglutination" were the only remains. After 15 minutes, suspensions appeared completely empty in the phase microscope. After disintegration for 30 seconds, the platelets failed to bring about any clot retraction. Final concentration of "platelets" in the test system was 300,000 to 1 million/cu mm.

To test the effect of platelets on prothrombin consumption *in vitro*, blood was drawn from thrombocytopenic dogs having less than 2,500 platelets/cu mm. Aliquots of 1 ml were distributed in tubes containing 0.2 ml platelet material. Following incubation for 60 minutes at 28°C, citrate was added, and residual prothrombin determined. In this system disintegrated platelets were at least as active as intact platelets (Fig. 1), whether platelets were resuspended in plasma or saline. The platelet suspensions did not contain red cells on microscopical examination.

Disintegrated platelets are toxic(2,4). When large amounts are given intravenously over a short period of time, severe circulatory and respiratory effects are regularly observed (Fig. 2). We believe that these effects are caused mainly by serotonin, and they were markedly reduced when dogs were given a serotonin analogue (12) for 4 days before transfusions.

When platelet-rich plasma was treated in the oscillator, the plasma clotting system was also affected. For instance, thrombin time increased markedly (Fig. 3). To avoid this effect in *in vivo* experiments, we resuspended the platelets in saline after second centrifugation (see above).

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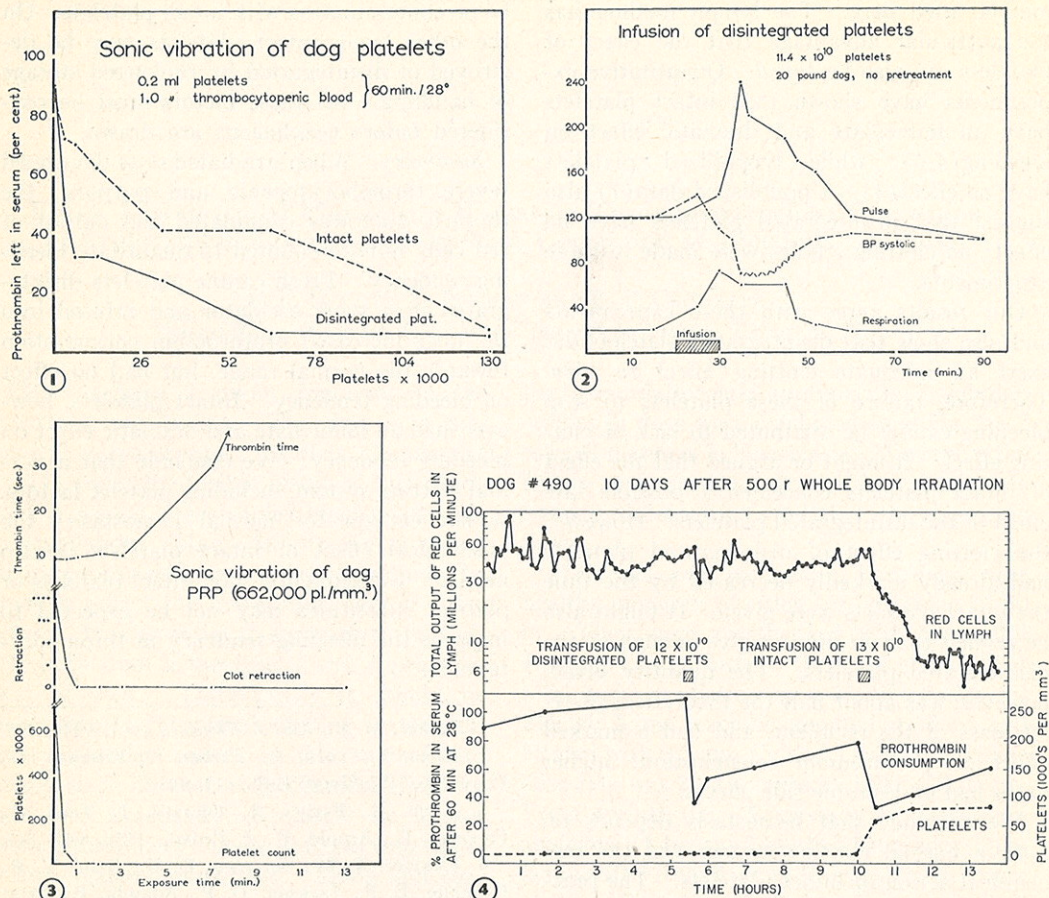


FIG. 1. Effect of platelets, intact or disintegrated, on prothrombin consumption *in vitro*.

FIG. 2. Toxic effects of disintegrated platelets.

FIG. 3. Effect of sonic vibration on platelet count, clot retraction and thrombin time.

FIG. 4. Transfusion experiment in a thrombocytopenic dog.

2. *In vivo* experiments. Fig. 4 gives results of a representative experiment. The disintegrated platelets had no effect on platelet count, nor on bleeding tendency as measured by output of red cells in lymph. However, they did temporarily normalize prothrombin consumption. Intact platelets, on the other hand, increased the platelet count to about 70% of calculated rise. Lymph started to clear during transfusion, and within 1 hour the output of red cells had dropped to about 12% of pre-transfusion value. The intact platelets also increased consumption of prothrombin, and to about the same extent as did the disintegrated platelets. Similar results were obtained in 3 other dogs.

*Discussion.* Disintegrated platelets were studied by Axelrod (13) and our findings agree with hers. Thus, disintegrated platelets may substitute completely for intact platelets in *in vitro* clotting systems.

Clinical observations in thrombocytopenic patients have been interpreted as indicating that platelets may have hemostatic effect, even if they are not intact (2,3). The bleeding tendency following irradiation is mainly due to thrombocytopenia, and is corrected by intact platelets (4-6). Bleeding tendency in such animals may be judged clinically, but definite conclusions require quantitative experiments. Thrombocytopenic bleeding tendency may be quantitated by bleeding time, from histologic sections of lymph nodes (4-6) or by the lymph

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method used here. The lymph method has the particular advantage that the effect of platelets can also be *timed*. Quantitative experiments have shown that intact platelets have an immediate and dramatic effect on bleeding(4-6), while lyophilized platelets have no effect(4). Unpublished data(7) also suggest that disintegrated platelets have no effect; no clotting studies were made in these experiments.

Our results agree with these experiments and also show that disintegrated platelets did exert an adequate clotting effect *in vivo*. Therefore, failure of these platelets to stop bleeding cannot be attributed to lack of clotting effect. It might be argued that the effect of intact platelets concealed a possible late effect of the disintegrated platelets. However, the clotting effect of disintegrated platelets had already markedly decreased by the time the intact platelets were given. It might also be argued that we did not give enough of the disintegrated platelets. The quantity given, however, was about half the theoretical platelet mass of the recipient, and had a marked effect on prothrombin consumption; higher doses had undesirable side effects.

We conclude that hemostasis depends on *both* a normal clotting system *and* the mechanical action of intact platelets. The practical consequence is that non-intact platelets or biochemical platelet substitutes may not be expected to improve the bleeding tendency in thrombocytopenia.

Some of the difficulties of the experimental technic should be mentioned. Lymph flow depends on many factors: exact position of cannula, position of dog, amount and kind of fluids given, anesthesia, and finally serotonin effect of platelets. It is essential to control all these factors and maintain an even flow. Sometimes a tiny vein may carry a constant amount of blood into the duct, thus ruining the experiment. A negative effect of some test material must therefore always be confirmed by demonstration of a positive effect of intact platelets in the same dog. Finally, it is not known how many intact platelets are necessary for a positive effect, but our experience suggests a low number. Therefore, if one tests disintegrated platelets, there must

be no contamination with intact platelets. On the other hand, intact platelets may be destroyed or disintegrated by prolonged storage or handling. All these factors must be considered before conclusions are drawn.

*Summary.* When irradiated dogs developed severe thrombocytopenia and purpura, the thoracic duct was cannulated and output of red cells/minute counted to quantitate bleeding tendency. Fresh canine platelets disintegrated in a sonic oscillator and infused into the dogs increased prothrombin consumption towards the normal range, but had no effect on bleeding tendency. Intact platelets, however, had an immediate and dramatic effect on bleeding tendency. We conclude that a normal clotting system, including platelet factors, is not enough for normal hemostasis; the mechanical effect of intact platelets is also needed. Consequently, non-intact platelets or platelet substitutes may not be expected to improve the bleeding tendency in thrombocytopenia.

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