A Method for Evaluating the Hemostatic Effect of Various Agents in Thrombocytopenic Rats and Mice

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Clinical studies have suggested that certain materials may be useful as platelet substitutes in thrombocytopenia. The following investigation was undertaken to devise an experimental model for initial evaluation of these agents. Thrombocytopenia was induced in rats and mice; following severance of the tips of their tails, the blood loss was measured under controlled conditions and compared with the blood loss in normal animals similarly tested. Various substances claimed to be of value in the treatment of thrombocytopenic states were evaluated. Only viable platelets were shown to be effective.

Methods

Fifteen Gm. Swiss mice and 50 Gm. Wistar rats were used. Thrombocytopenia was produced in the mice by 600 r total body irradiation (250 kv, filtered by \( \frac{3}{4} \) mm. Cu and 1 ml. Al at 100 cm.), and in rats by the intraperitoneal injection of a red cell-adsorbed rabbit antirat platelet serum. The mice were used 10 to 12 days postirradiation when random counts showed marked thrombocytopenia. The rats were injected with the antiserum, and all had overt purpura and extreme thrombocytopenia (platelet counts ranging from 0 to 10,000/cmm.) the following day when the experiment was performed.

The test materials included human lyophilized platelets, brain phospholipid extract, soya bean extract, fresh viable platelets obtained from rabbits or rats by differential centrifugation and lyophilized platelets prepared from the latter. Viable rabbit platelets were infused into the thrombocytopenic rats instead of rat platelets, since there was an excess of anti-rat platelet serum present in these animals, and only by using rabbit platelets could an effective platelet level be attained. Viable rat platelets were infused into the irradiated mice due to the difficulty in obtaining sufficient numbers of mouse platelets to raise the peripheral platelet count to an effective level.

Prior to injection the test materials were prepared as follows: (a) One unit of human lyophilized platelets (representing the platelet yield from 500 ml. of human plasma) was diluted with 20 ml. of isotonic saline, and 1 ml. of this was further diluted with 5 ml. of saline. The resultant solution had to be injected slowly into the test animal or death resulted. (b) One ml. of brain phospholipid was diluted with 5 ml. of physiologic saline. (c) Soya bean extract was prepared in concentrations of approximately 0.5, 2.5, 5.0 and 10.0 mg.

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†Prepared by ether extraction of acetone-dried human brain. A 1:60 dilution yielded a thromboplastin generation time of less than 10 seconds. Kindly supplied by Dr. Warren Bell, The University of Mississippi, University Hospital, Jackson, Miss.

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§The alcohol-insoluble fraction of commercial soya bean lecithin (Gliddex P) A concentration of 100 mg./ml yielded a thromboplastin generation time of less than 10 seconds. Kindly supplied by Dr. William J. Williams, Department of Medicine, Washington University, St. Louis, Mo.
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10.0 mg./ml. (d) Lyophilized rat platelets were prepared from an initial volume of 25 ml. of platelet-rich plasma. This material was diluted with 5 ml. of saline before use and had to be injected slowly as in (a).

Test animals were anesthetized with Nembutal given intraperitoneally, and injected intravenously with either 1 ml. of the appropriate test material for the rat and 0.2 ml. for the mouse, or the corresponding volume of isotonic saline. The injection was made into the tail vein except when a continuous infusion of the test substance was given, wherein a paw vein was utilized. When the injection was completed or, in those animals given a continuous infusion, after an equal "priming" volume had been administered through the infusion set, the animal's tail was severed approximately 1 mm. from its tip with a new razor blade and inserted 1 cm. beneath the fluid level in a calibrated tube containing 5 ml. of saline kept at 37 C. in a water bath. When the experimental animal used was a rat, the blood loss was read off the calibrated scale by measuring the increased volume in the tube at time intervals of 10 minutes, 30 minutes and finally when the animal died, or when the bleeding had ceased. In the mouse a hemoglobin determination was performed when the tail vein was cut. Quantitation was accomplished either by the above technic or calculated from hemoglobin recovered in the tube. The procedure employed herein is similar in principle to that described by Hengge (1955).2

RESULTS

The amount of blood lost in irradiated mice injected with normal saline as compared with normal mice similarly injected is shown in figure 1. The blood loss in a group of 50 Gm. albino rats made thrombocytopenic by anti-rat platelet serum is demonstrated in figure 2. There was a marked contrast between

Fig. 1.—Blood loss in normal mice (two tubes on the right) contrasted with that in thrombocytopenic mice (two tubes on the left).
Fig. 2.—Total blood loss in 50 Gm. rats. N = normal rats injected with 1.0 ml. of physiologic saline. \( T \) = thrombocytopenic rats similarly injected. \( S \) = thrombocytopenic rats injected with 1.0 ml. of soya bean extract, \( X < 0.5 \text{ mg./ml.} \), \( O = 2.5 \text{ mg./ml.} \), \( + = 5.0 \text{ mg./ml.} \), and \( * = 10 \text{ mg./ml.} \). \( B \) = thrombocytopenic rats injected with 1.0 ml. of brain phospholipid extract diluted 1:5 with physiologic saline. \( L_{H} \) = thrombocytopenic rats injected with 1.0 ml. of lyophilized human platelet solution (1 unit of platelet material weighing 446 mg. was mixed with 100 ml. of physiologic saline prior to injection). \( L_{P} \) = thrombocytopenic rats injected with 1 ml. of lyophilized platelet solution 24 hours before the experiment and at the time of the experiment. \( L_{H} \) = thrombocytopenic rats injected with lyophilized rat platelets. \( P \) = thrombocytopenic rats injected or infused with rabbit platelets. \( L_{N} \) = normal rats injected with lyophilized human platelet solution diluted as for \( L_{H} \).

the blood loss in the normal control group (\( N \)) and in the thrombocytopenic control group (\( T \)). The average total loss for the normal rats was 0.3 ml. as compared with a loss of 2.0 ml. in the thrombocytopenic group. The only infusion affecting the blood loss in these thrombocytopenic animals was that of intact platelets. No other material injected produced any reduction of blood loss, whether measured at 10 minutes, 30 minutes or at the termination of study. Exhibition of the test substance on both the previous day, as well as at the time of the experiment (fig. 2, \( L_{P} \)), the use of a continuous infusion of lyophilized human platelets and variations in concentration of the soya bean extract (fig. 2, \( S \)) did not materially affect the results. The results obtained with irradiated mice are shown in figure 3. Again, only administration of viable platelets corrected the blood loss, the platelet injections in this instance being made on the previous day.

The probability that the blood loss in the normal animals and the thrombocytopenic ones (with the exception of those receiving viable platelets) was
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Fig. 3.—Total blood loss in 15 Gm. mice. N = normal mice injected with 0.2 ml. of saline. T = thrombocytopenic mice injected with 0.2 ml. of saline. B = thrombocytopenic mice injected with 0.2 ml. of brain phospholipid extract. S = thrombocytopenic mice injected with 0.2 ml. of soya bean extract. LR = thrombocytopenic mice injected with 0.2 ml. of lyophilized rat platelets. P = thrombocytopenic mice injected with 0.2 ml. of rat platelets on the previous day.

Similar is less than 0.01. The loss in the normal animals was not significantly different from that in thrombocytopenic animals receiving platelets, nor was there any significant difference in the excessive loss incurred among the remaining thrombocytopenic groups, whether given "platelet substitutes" or only saline.

It is difficult or impossible directly to correlate effective dosages in pathologic states in man with those required in similar states experimentally in animals. In an attempt to obviate this, as high a dosage as possible compatible with the animal's survival was used, the amount given in most instances being calculated to be in excess of that given to patients. Recent work has shown that an excess of phospholipids may actually inhibit in vitro coagulation whereas a smaller quantity accelerates it. In an effort to exclude the former phenomenon, a group of normal rats was injected with the same dosage of lyophilized platelets as given to the thrombocytopenic animals; they lost the same amount of blood as the normal control group (fig. 2, L'). Furthermore, four thrombocytopenic rats were injected with one-fourth the concentration of phospholipid extract used, and another four rats with one-half the concentration of human lyophilized platelet solution, and again there were no differences in blood loss between animals receiving the same material in varying dosages. There
Fig. 4.—Percentage mortality occurring in the various groups of rats used in figure 2. N = normal rats injected with saline. T = thrombocytopenic rats injected with saline. S = thrombocytopenic rats injected with soya bean extract. B = thrombocytopenic rats injected with brain phospholipid extract. L_H = thrombocytopenic rats injected with lyophilized human platelets. P = thrombocytopenic rats injected with rabbit platelets.

was also no obvious correlation between the amount of blood lost and the amount of soya bean extract administered. (fig. 2, S). It seems unlikely that the inability to demonstrate correction of blood loss was due either to an excessive or suboptimal dosage. One group of four rats was injected with lyophilized human platelets on the day prior to the test and again on the same day in the usual manner, in an attempt to determine whether a longer exposure to the material might result in a correction of the bleeding; again there was no effect (fig. 2, L_H).

The ineffectiveness of the materials tested is also demonstrated in figure 4, which discloses the mortality in the thrombocytopenic animals ranging from 70 to 90 per cent unless platelets were administered. It should be emphasized that blood loss in thrombocytopenic animals receiving rabbit platelets remained excessive if the platelet count was low at the commencement of the experiment or if it fell off very rapidly. In one instance, for example, the initial count was 530,000/cu.mm., but fell to 20,000/cu.mm. 30 minutes later, in contrast to a successful platelet infusion where the initial count was 955,000/cu.mm. falling to 375,000/cu.mm. at the end of the experiment. The data are insufficient for estimating the exact level of platelets or the duration of time of such a level necessary to maintain adequate hemostasis in the rat and mouse, although it should be noted that the lowest blood loss recorded in the rats given platelets was obtained with continual infusion.
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DISCUSSION

This study was undertaken to devise an experimental method for testing the effectiveness of “platelet substitutes” and also as a screening procedure to evaluate various substances for which claims of hemostatic control in thrombocytopenic states had been made, with the hope that an effective preparation suitable for clinical trial could be thereby selected. To utilize an in vivo method was essential, due to the well known discrepancies which may occur between in vitro studies and the actual hemostatic status of the patient.4

The method described was adopted after the failure of an apparently logical approach to the problem; this is briefly outlined to forewarn others who might be attracted to a similar procedure. At the outset, an attempt was made to relate the severity of the thrombocytopenic bleeding to the amount of pulmonary hemorrhage induced in the rat or mouse when the whole animal was subjected to an acutely induced negative pressure sustained for one minute. The parameters studied were the negative pressure giving the LD50 and the quantity of blood extravasated into the lung when a negative pressure of 600 mm. of Hg was used. The amount of blood extravasated was measured by macroscopic gradation of the lung hemorrhages from + to ++++ and also by the amount of radioactivity present in the lungs of animals given a standard injection of Cr51-labeled homologous red cells prior to the experiment. These two methods of measurement showed excellent correlation, but there was little difference in the experimental and control groups; the approach was therefore abandoned. The reasons for the inadequacy of this experimental model are not apparent.

Although platelet substitutes are claimed to ameliorate severe bleeding episodes in patients with thrombocytopenia, no objective evidence for their value was adduced in the experimental model employed. It must be emphasized that the failure to correct the blood loss in thrombocytopenic animals when these various materials were administered merely means that unlike viable platelets, they were ineffective under these specific experimental conditions and it is still possible that they may be of value in certain clinical circumstances. The observations presented support the belief, however, that control of thrombocytopenic bleeding requires viable, circulating platelets.

SUMMARY

A simple method for measuring the control of blood loss in thrombocytopenic rats and mice is described.

The administration of lyophilized platelets, brain phospholipid extract and soya bean extract failed to correct the blood loss in the thrombocytopenic animal.

The results obtained with this method have failed to support the effectiveness of “platelet substitutes” in in vivo systems and reaffirm the requisites of viability and recirculation for platelet transfusions.

SUMMARIO IN INTERLINGUA

Es describite un simple metodo pro mesurar le efficacia de mesuras visante al rectification del perdita de sanguine in rattos e muses thrombocytopenic.
Le administration de lyophilisate plachettas, de extracto cerebro-phospholipidic, o de extracto de soja non sufficeva a corrigir le perdita de sanguine in animales thrombocytopenic.

Le resultatos obtenite per le presente metodo non corrobora le efficacia de "substitutos plachettal" in systemas in vivo; illos reaffirma le requirimento de transfusiones de plachettas viabile e recirculante.

REFERENCES


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