Antigenic Relationship Between Blood Platelets and Vascular Endothelium

By Francis S. Morrison and Mario G. Baldini

Sensitization of animals with allogeneic blood platelets induces accelerated rejection of allogeneic skin as well as renal grafts. In the latter instance the histologic lesions of the rejected graft were described as primarily vascular. These as well as earlier observations have suggested that an antigenic relationship exists between blood platelets and vascular endothelium. The experiments to be reported here were undertaken with the objective of directly testing this possibility. Repeated injections of antigen derived from allogeneic aortic endothelium sensitized dogs to allogeneic blood platelets. This was demonstrated by a sharp reduction in the survival of allogeneic platelets in the circulation of the sensitized recipients.

Methods

Throughout these studies, normal male and female mongrels, weighing 6.9 to 10.4 Kg., were used after they had spent a routine disinfestation and equilibration period in the animal farm.

Platelet Survival in the Dog

For each experiment, 300 grams of blood were collected from the femoral artery of the dog, into a plastic bag containing the anticoagulant. The bag was kept in motion during collection, and care was taken to insure rapid and continuous flow in the tubing. The blood remaining in the tubing was discarded. Immediately after separation of the platelet-rich plasma (PRP) by differential centrifugation (200 g. for 40 minutes at 40 C.) the erythrocytes were returned to the donor animal.

Platelet survival was determined using an adaptation of the technic previously described for human platelets. No surface active agent was used for resuspension of the platelet button, nor was ascorbic acid utilized as a reducing agent for excess chromate. The platelet button was resuspended in 4 ml plasma and 4 ml isotonic saline, 350 microcuries of $^{51}$ chromium were added and the suspension was incubated at room temperature for 30


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Francis S. Morrison, M.D.: Formerly, Research Fellow and Trainee in Hematology, Blood Research Laboratory, New England Medical Center, Boston, Mass. (Dr. W. Dameshek, Director): Presently, Instructor in Medicine, Tufts University School of Medicine; Clinical Instructor in Medicine, Boston University School of Medicine; Research Medical Officer, Naval Blood Research Laboratory, Chelsea, Mass.

Mario G. Baldini, M.D.: Formerly, Associate Director, Blood Research Laboratory, New England Medical Center: Presently, Director, Division of Hematologic Research, The Memorial Hospital, Pawtucket, R. I. and Brown University Institute for Health Sciences, Providence, R. I.


† Chromotope sodium diagnostic, specific activity about 50 mc., per mg., 500 mc. per ml., Squibb, New York, New York.
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minutes. The excess chromate was removed by washing the platelets with approximately 150 ml. of the autologous platelet-poor plasma (PPP). The platelets were finally resuspended in 20 ml. of the autologous PPP and re-infused into the dorsal anterior leg vein of the animal being studied. The entire procedure required less than two hours from the time of blood collection. Two aliquots (0.1 ml.) of the injected platelet concentrate were saved and washed, once with 10 ml. of 1 per cent ammonium oxalate and once with 10 ml. of 0.02 per cent Triton * in isotonic saline. These samples were counted for radioactivity in a well type scintillation counter,† and the average value was used to determine the amount of platelet radioactivity infused into the animal. After infusion of the platelet concentrate, 9 ml. samples of blood were withdrawn, at predetermined intervals, from the jugular vein into a syringe containing 1 ml. of EDTA. Routinely, blood samples were taken 30 minutes and two hours after infusion and once daily thereafter. When platelet radioactivity of the two hour sample was not lower than in the 30 minute sample, a third sample was obtained at four hours. Platelet counts were done in duplicate using phase contrast microscopy on each whole blood sample and on the PRP separated from each sample. The platelet button was then prepared and the radioactivity counted. Radioactivity values of the platelet buttons were expressed as counts per 10⁶ platelets and from this was derived the amount of platelet radioactivity in one ml. of blood. The total circulating platelet radioactivity was subsequently derived by multiplying by the blood volume.

The blood volume was determined using autologous ⁵¹ chromium tagged erythrocytes at the conclusion of the platelet survival study.

The total circulating platelet radioactivity observed was expressed as a percentage of the infused platelet radioactivity. These percentages, i.e., the platelet recovery values, were then used to plot the platelet survival curves. To facilitate comparison, the platelet survival time was taken as the time at which 10 per cent of the peak value of the survival curve was reached.

Various anticoagulants were studied in these experiments in an attempt to compare their effect on the platelet survival:
1. disodium EDTA was used in the dose of 50 ml. for 300 ml. of blood;
2. the specially prepared ACD buffer proposed by Aster and Jandl for human studies † was also used in a quantity proportional to the quantity of blood (55 ml. per 300 ml.). This was freshly prepared for each experiment and buffered the PRP at pH 6.5. Other ACD solutions were also utilized with various citrate and dextrose concentrations and pH values. The most satisfactory solution consisted of 0.07 M citric acid and 0.08 M trisodium citrate in 2.5 per cent dextrose. Fifty ml. of this solution was placed in the plastic bag prior to the blood collection. Osmolarity of this solution, measured with the Fiske Osmometer, was 450 milliosmoles. The pH of the PRP using this anticoagulant solution ranged from 6.1 to 6.4.

After the optimal anticoagulant had been determined, eight survival studies were done using allogeneic platelets in dogs not previously used.

The effect of repeated infusions of allogeneic platelets was then investigated. Dogs were paired, one being the donor, the other the recipient. Nine such pairs were studied by infusing the platelets obtained from 300 ml. of blood from the donor animal into the recipient once a week. At various intervals the platelets were labelled with ⁵¹ chromium before infusion and the survival in the circulation of the recipient dog was determined.

Preparation of Aortic Antigen

Aortas were obtained from healthy mongrel dogs. Each aorta was cut longitudinally to expose the endothelial surface and all blood was removed by copious lavage with isotonic

* Triton WR-1339, Winthrop Laboratories, 1450 Broadway, New York, N. Y.
† Iodide crystal scintillation detector, Baird Atomic, Cambridge, Mass.
† DISodium ethylene diamine tetracetate dihydrate 1.5 Gm. in 100 ml. 0.7 per cent sodium chloride solution.
Fig. 1.—Platelet survival curves in dogs using EDTA as the anticoagulant. Actual recovery values are plotted as a function of time (left). The same values are plotted (right) as a percentage of the peak value. The latter method allows better definition of survival time and the appearance of the regression curve.

saline. Gentle scraping with a scalpel yielded a pasty white material on the blade. On histologic examination, this material was found to consist of endothelial cells and a considerable quantity of elastic fibers. The material was suspended in isotonic saline, subjected to two passes in a tissue homogenizer and then strained through a tantalum gauze. The residue on the gauze was primarily elastic tissue which was not broken up in the homogenizer. The filtrates from several aortas were pooled, concentrated by dialysis against PVP, and stored at -20°C until the time of injection. The protein concentrations of these suspensions were estimated by the Kjeldahl technic and averaged 30-50 mg. per cent.

Sensitization of the Dogs

Dogs were injected at weekly intervals with one of the following preparations: a) prepared aortic endothelium (PAE), b) PAE emulsified in an equal volume of complete Freund's adjuvant (CFA), or c) CFA alone. A total of five weekly injections was given each dog. Each injection consisted of 8 cc. divided among four subcutaneous sites on the back. A small amount was also injected intradermally at each site.

Approximately one week after the last sensitizing injection, the survival of collected platelets from a donor dog was determined. One week later this study was repeated using platelets from another donor animal. After another week, the survival of autologous platelets was determined in each animal.

Serologic Tests for Platelet Antibody

Serum was obtained from the sensitized dogs after the first and immediately preceding the second allogeneic platelet survival study.

The serum was tested for antiplatelet activity. Agglutination tests were done according to the method of Dausset et al. Clot retraction in the presence of the sensitized serum was also tested. The clot retraction method used was that of Hartman and Conley, as described by Ozge et al. The effect of the sera on the ability of normal platelets to take
Fig. 2.—Platelet survival curves in dogs using buffered ACD as recommended for human studies.4

up 14C-5-hydroxytryptamine (serotonin uptake test) was also studied using the method described by Bridges et al.7

RESULTS

Survival of Autologous Platelets

When EDTA was used as the anticoagulant, a large and variable portion of the injected radioactivity temporarily disappeared from the circulation after infusion (Fig. 1). The initial recovery varied from 8 per cent to 61 per cent. In seven of the experiments, the highest recovery value found was in the four hour sample while in one experiment, the two hour sample was highest. The peak recovery varied between 24 per cent and 72 per cent of the injected platelet radioactivity with an average of 52 per cent. The survival time was 5 to 6.5 days and averaged 5.9 days. The shape of the curve was rectilinear.

When the buffered ACD solution, suggested for human platelets,4 was used, the highest recovery value was in the 30 minute sample in each experiment (Fig. 2). The two hour sample always yielded results so near to the initial sample that the values were averaged for charting. The recovery varied from 38 per cent to 100 per cent with an average of 68 per cent. The survival time was 4.5 to 6.5 days with an average of 5.9 days. The curves in these experiments had an early rapid decline which gave the curves the appearance of exponential regression. Many modifications of the citrate, citric acid and dextrose concentrations were tried. Heparin was also evaluated in an effort to improve the viability curve. These results were reported elsewhere.8 The final solution employed (see Methods) resulted in the curves shown in Figure 3. The recovery values ranged from 52 per cent to 103 per cent with an average of 75 per cent. The survival time was five to seven days and averaged
6.2 days. The shape of the survival curves was nearly rectilinear in all experiments. Again, there was no evidence of initial temporary sequestration. This anticoagulant solution was used for all subsequent experiments reported here.

Survival of Allogeneic Platelets

Figure 4 shows the results of eight survival studies of allogeneic platelets in normal, nonsensitized dogs. The maximum recovery ranged from 57 to 79 per cent and the mean value was 69 per cent. The survival time ranged from 5.8 to 6.9 days with a mean value of 6.3 days. These curves compare well with the normal autologous platelet survival curves (shaded area). Therefore, no difference was demonstrated between survival of autologous and allogeneic platelets.

The survival of allogeneic platelets was also determined in eight dogs which had previously received five weekly injections of CFA. The maximum recovery value ranged from 49 to 78 per cent with a mean of 57.2 per cent. The mean survival time was 5.6 days while the range was 5.1 to 6.1 days. These experiments demonstrated that the injection of CFA alone could not produce reduction of allogeneic platelet survival.

When allogeneic platelets were infused at weekly intervals it was found that after four to 12 such infusions the survival of the allogeneic platelets was invariably affected. With repeated infusions the survival time could be sharply reduced (less than 24 hours in four dogs). In no experiment was sensitization induced by less than four infusions of allogeneic platelets. These findings are similar to previously reported studies. Survival of autologous platelets as well as the platelet number in the recipient dogs always remained normal.
Platelet Survival in Dogs Sensitized with Aortic Endothelium

Figure 5 shows the results of 18 allogeneic platelet survival studies performed in nine dogs. Each dog was sensitized by five weekly injections of PAE plus CFA. It is readily apparent that the maximum recovery value was markedly reduced. The mean value was 35 per cent with a range from 0 to 92 per cent. The mean survival time was shortened to only 18.5 hours. The sharp initial fall in all of the curves indicated a very rapid clearance from the circulation of a large proportion of the infused platelets. The two studies which resulted in measurable platelet radioactivity still remaining in the circulation on the third day after transfusion were both done in the same animal and suggested a lesser degree of sensitization in this particular dog.
Figure 6 illustrates eight platelet survival studies done on four dogs sensitized by five weekly injections of PAE alone. A definite alteration in all parameters of the survival curve (maximum recovery, survival time and shape of the curve) is again evident. The mean value of maximum recovery was 47 per cent. The mean survival time was 3.8 days. The shape of the curves again suggested rapid random destruction of a large number of the infused platelets.

In Figure 7 are seen the results of 12 autologous platelet survival studies done after the allogeneic platelet survival experiments were completed. Four of the dogs were those which had been sensitized to PAE alone and the other eight dogs had been sensitized with PAE plus CFA. The maximum
recovery ranged from 65 to 89 percent with a mean value of 72 per cent. The survival time ranged from 4.5 to 5.9 days with a mean of 5.3 days. These values and the appearance of the curves were not different from the results obtained in normal, nonsensitized dogs (shaded area in Fig. 7).

Survival of Allogeneic Erythrocytes

After completion of the platelet studies, the survival of allogeneic erythrocytes was determined in three of the dogs sensitized to PAE plus CFA. The T½ of the 51chromium labelled red cell in these dogs was found to be 24, 22 and 31 days respectively and these are normal values.10

Transfer of Plasma from a Sensitized to a Normal Dog.

In two experiments, 200 ml. of plasma were collected from dogs which had been sensitized with PAE-CFA emulsion. Sensitization had been demonstrated by very short survival of donor platelets infused into these dogs before as well as after collecting the plasma. Infusion of 200 ml. of this plasma into normal dogs (7 and 10.1 Kg. body weight respectively) produced no detectable reduction in the platelet count from five minutes to 48 hours after infusion. No purpura or other manifestation of vascular injury was observed.

Serologic Tests for Platelet Antibody.

Serologic tests were done using serum from the dogs sensitized with PAE plus CFA. In the agglutination studies no difference was observed between the test sera and the normal control sera. Clot retraction in the presence of sera from sensitized animals was always normal. No inhibition of serotonin uptake could be demonstrated. This last test has been found to be one of the most sensitive in detecting the presence of platelet isoantibody in humans.7

Discussion

The dog has been a useful experimental animal for platelet survival studies. Although similarities between human and dog platelets have been demonstrated, there are significant differences. It is apparent that EDTA is damaging to dog platelets as well as human platelets.3,4,8,11 Clumping of dog and human platelet concentrates prepared in ACD can be avoided by a reduction in pH, effected by additional ACD. However, the most satisfactory pH and citrate concentration appear to differ between the two species. The final ACD solution utilized for dog platelets resulted in reproducible, almost rectilinear survival curves with a mean recovery value of 75 per cent. These findings demonstrate that in dogs, as in humans, the long recognized “lesion of collection” is not entirely the result of centrifugation or other manipulation outside the circulation but largely an effect of the anticoagulant (i.e., EDTA). The manner in which EDTA produces this drastic change in platelet survival is not known.4 The fact that with ACD the mean recovery value was not 100 per cent suggests that splenic pooling, similar to that found in humans,12,13 exists in the dog as well.

The consistent finding of a sharply reduced survival of allogeneic platelets
in dogs previously sensitized to a preparation of washed allogeneic aortic endothelium is interpreted as demonstration that platelets and endothelium have antigen in common.

It had previously been shown that in rabbits sensitized by one or more skin grafts the survival of allogeneic platelets was impaired.\textsuperscript{14} Bosch et al.\textsuperscript{15} demonstrated that in humans the survival of donor platelets is shortened after rejection of a skin allograft. It is doubtful that the few platelets contained in skin grafts could account for the results in those experiments but the possibility could not be unequivocally excluded. The present experiments preclude this possibility since the antigen used was derived from washed aortic endothelium. One cannot exclude the possibility that some blood platelets may physiologically become an integral part of the endothelial vascular wall, but free blood platelets were not present in the antigenic preparation utilized.

The reverse experiment, namely sensitization with allogeneic platelets prior to placing a graft also has been done. In rabbits sensitized only to platelets, skin grafts were rejected in an accelerated fashion.\textsuperscript{1} Although Wilson found that rat platelets did not induce accelerated rejection of skin grafts from the platelet donor,\textsuperscript{16} Dausset et al. were able to induce accelerated rejection of human skin grafts by prior sensitization with donor platelets injected intradermally.\textsuperscript{17} Furthermore, sensitization of dogs with a small number of allogeneic platelets resulted in accelerated rejection of renal transplants.\textsuperscript{2} Vascular damage was the prominent histologic finding in the rejected kidneys. The present experiments suggest an explanation for the histologic lesion. Obviously, there are various means by which an allograft may be damaged and rejected. A reaction to the vascular endothelium may be only one of these means. However, the demonstration that platelets have antigen in common with endothelium provides an experimental basis for understanding the accelerated rejection of an allograft after platelet sensitization.

Many investigators have studied platelet and endothelial antigenicity in xenogeneic systems. Bedson, in 1922\textsuperscript{18} produced thrombocytopenic purpura in guinea pigs using antiplatelet serum prepared in the rabbit. He observed that lowering the platelet level to a similar degree using agar gell produced no purpura. He deduced the antiplatelet serum was probably damaging vessels as well as platelets. Tocantins\textsuperscript{19} used rabbit and goat anti-dog platelet serum to confirm and extend these observations. Katsura\textsuperscript{20} found that the anti-dog platelet serum fixed complement when reacted with endothelial cells. He also found that a sub-purpurigenic dose of anti-endothelial serum combined with a subpurpurigenic dose of anti-platelet serum would produce severe purpura. The anti-endothelial serum in higher dosage caused purpura but no thrombocytopenia despite in vitro agglutination of platelets. Clark and Jacobs,\textsuperscript{21} using rabbit anti-dog-endothelium serum produced severe purpura in dogs while platelet counts remained normal and in vitro tests for antibodies were uniformly negative.

The failure to demonstrate antibody by serologic tests or by transfusion of plasma from a sensitized dog to a normal dog may be due to very low concentration of the antibody. Although in the xenogeneic systems it has
usually been possible to demonstrate platelet antibody in vitro this has not been the rule in allogeneic studies. Potent platelet isoantibody may be present when no in vitro test can detect it. It is because of this fact that it is necessary to study viability of infused allogeneic platelets in order to investigate possible sensitization.

The results of the studies presented here provide experimental basis for the not uncommon clinical observation that purpura is more frequently seen and is more severe in immunologic types of thrombocytopenia than in thrombocytopenia of similar degree due to other causes (e.g., production deficit). The antigenic relationship between vascular endothelium and blood platelets underscores the need for further work on the immunologic characterization of platelet antigenic determinants and their relationship to antigens in other body tissues. Delineation of these determinants may become more urgent as progress is made in tissue transplantation.

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