Mechanism of Abnormal Bleeding in Patients Undergoing Cardiopulmonary Bypass: Acquired Transient Platelet Dysfunction Associated With Selective α-Granule Release

By Laurence A. Harker, Thomas W. Malpass, Herman E. Branson, Eugene A. Hessel II, and Sherrill J. Slichter

The hemostatic alterations underlying the occasional bleeding diathesis associated with cardiopulmonary bypass surgery have been defined in 31 selected patients by simultaneously measuring the serial changes in concentration, function and kinetics of platelets, coagulation factors, and fibrinolytic components. The effects of uncomplicated bypass surgery were evaluated in 21 elective patients. Immediately upon bypass, the circulating levels of platelets, coagulation factors, and fibrinogen regularly fell about 50% due to dilution with nonblood prime in the oxygenator apparatus. However, none of these components was reduced below established hemostatic levels at any time during or following the procedure. Furthermore, the functional transformation of fibrinogen to fibrin was not measurably impaired. Heparin effectively blocked both fibrinolysis and fibrinogen consumption during bypass and was consistently neutralized with protamine without rebound at the end of bypass. Circulating platelets were uniformly activated in elective patients during bypass through a process that involved release and partial depletion of α-granules, but not release of dense granule constituents. Platelet activation was associated with transient marked impairment of function as evidenced by striking prolongation of the bleeding time and defective aggregation in vitro. In uncomplicated patients, platelet function largely normalized within 1 hr after bypass. Controlled studies in baboons demonstrated that platelet dysfunction was produced by either cardiopulmonary bypass under normothermic conditions or by hypothermia alone without bypass. In the baboon model, defective platelet function was also transient and characterized by selective depletion of platelet α-granule contents without release of dense granule contents. By contrast, in all of 10 patients in whom bypass surgery was complicated by substantial abnormal bleeding, there was persistent bleeding time prolongation to greater than 25 min that persisted for hours after bypass despite platelet counts above 100,000/μL. With platelet transfusions, the bleeding time shortened rapidly, followed by cessation of bleeding. From these studies we conclude that cardiopulmonary bypass patients demonstrate abnormal clinical bleeding when there is a persistence of platelet dysfunction manifested as a bleeding time of greater than 20 min after protamine administration. Such bleeding patients with a prolonged bleeding time should receive platelet transfusions even when the platelet count is greater than 100,000/μL.

Despite the technical improvements in procedures and equipment, patients undergoing heart surgery and pump oxygenator bypass are still threatened by the possibility of serious bleeding.1-6 Such life-threatening hemorrhage is related to both the surgical damage to blood vessels and acquired defects in hemostasis. The mechanism and course of the occasional hemorrhagic diathesis produced by cardiopulmonary bypass is difficult to characterize because of the complexity of the hemostatic process, the technical and logistical difficulties imposed by the operative procedures, and the many associated uncontrolled variables. For example, measurements of coagulation factors during bypass are often not meaningful because the heparin that is administered interferes with accurate coagulation factor assays. Furthermore, in vivo platelet function in these patients has not been systematically investigated with template bleeding times or assays of plasma for released platelet constituents.4,7-12 Postoperative studies of bypass patients indicate that the levels of platelets and coagulation factors (especially factor V) are lower than prebypass levels,1,3,8,13-15 but the extent of that fall is not necessarily related to the likelihood of abnormal bleeding. Enhanced fibrinolytic activity also occurs, but it similarly has uncertain hemostatic significance because it occurs frequently and resolves rapidly.1,3,8,13,14,16 While inadequate heparin neutralization and protamine effects are also implicated in some episodes of postoperative bleeding, most patients with severe bleeding have other hemostatic defects.1,3,14,15

Reductions in the concentrations of platelets and coagulation factors have become less profound as bypass apparatus has been revised to decrease thrombogenesis. Therefore, the continued occurrence of severe bleeding requires evaluation of the functional as well as the quantitative integrity of the hemostatic components. For example, abnormalities of platelet aggregation and glass bead retention have been reported in pump oxygenator patients, but the data are nonquantitative and of uncertain significance.4,7,10,12,17-19 The interpretation of such tests is complicated by other variables that might alter hemostasis during
extracorporeal bypass, such as the effects of anesthetics or pharmacologic agents used during and after the procedure, the kind of priming solution (blood or nonblood), the nature of transfused materials, hemodilution, hypothermia, and the type of oxygenator (bubble or membrane).^{3,10,20-22}

The present study was designed to document quantitatively and functionally the components of the hemostatic mechanism during bubble oxygenator perfusion in patients undergoing cardiac surgery. For this purpose, coagulation and fibrinolytic assays were performed after removal of heparin and protamine from the samples, and platelet behavior was assessed in vivo during bypass using measurements of platelet kinetics, template bleeding times, platelet factor 4 (PF4) and β-thromboglobulin (β-TG) in plasma, serum, and urine. In addition, controlled experiments were carried out in baboons to determine independently the effects of bubble oxygenation and hypothermia.

MATERIALS AND METHODS

Patient Studies

The study was conducted on 31 patients, 26 male and 5 female. Twenty-one of these patients, (designated as group 1) were studied serially at 18 predetermined times before, during, and after bypass procedures. No attempt was made to modify established operative protocols. Ten patients (group 2) had had severe bleeding during surgery, requiring >10 U of transfused blood. They were sampled several times during the immediate postoperative period. Comparisons were made between patients in groups 1 and 2 at 2–4 and 6–8 hr after heparin neutralization. The mean age of the combined patients was 55 yr. Twenty-nine patients underwent coronary artery graft procedures, one patient had atrial septal repair, and the other had ventricular septal repair.

Preoperative medication included analgesics (fentanyl, meperidine, or morphine), tranquilizers (hydroxyzine, promethazine, diazepam), and myoneural or parasympathetic depressants (atropine, pancuronium, scopolamine). Mannitol and furosemide were either included in the perfusate or administered during bypass. Anesthetic agents included enflurane, nitrous oxide, and thiopental. The patients received all available residual oxygenator perfusate times before, during, and after bypass.

Commonly, pump primes included, in addition to heparin, antibiotics (cefazolin in 12 patients or methicillin in 9 patients) and steroids (methylprednisolone or dexamethasone).

The dilution produced by the nonblood priming solution was measured as an index derived as the mean of both the reduction in hematocrit and the decrease in albumin concentration compared with the baseline values.

Animal Studies

Twelve baboons (Papio cynocephalus) weighing 12–18 kg were also studied. Observations on six animals were performed during cardiopulmonary bypass without antibiotics, steroids, hypothermia, or associated operative procedures, other than vascular access. The animals were on no medication before bypass. Anesthesia was induced with 10 mg/kg ketamine (Parke-Davis, Detroit, Mich.) and maintained with halothane after endotracheal incubation. Vascular access was obtained through the femoral artery and vein and internal jugular vein using medical grade Tygon Bardic catheters (Norton Plastics, Akron, Ohio). Blood pressure monitoring and blood sampling were done through an internal carotid catheter. A roller head cardiopulmonary bypass apparatus was used for extracorporeal circulation, with the pump being primed initially with 1500 ml lactated Ringer's solution. The animals received an additional 1500–2000 ml of that solution during the period of bypass. Disposable hard shell infant-sized bubble oxygenators (Bentley Temptrol, Irvine, Calif.) were used. The animals were anticoagulated with beef lung heparin (Upjohn) 300 U/kg initially, then one-half of that dose every hour. Extracorporeal circulation was maintained for 3 hr in each animal. At the end of the procedure, heparin was neutralized with protamine sulfate (Eli Lilly) given in a ratio of 1.5 mg protamine sulfate/100 U heparin.

Six other animals were studied during hypothermia of 20°C without cardiopulmonary bypass. They were anesthetized initially with ketamine and maintained with halothane and ether. The animals were then packed in ice until rectal and esophageal temperature reached 20°C, at which time they were rewarmed to body temperature. All six animals experienced several episodes of bradycardia and ventricular fibrillation that required electrical counter-shock and various drugs, including atropine, lidocaine, and epinephrine. All survived without residual ill effects.

Laboratory Procedures

Coagulation tests. Samples were obtained at predetermined times (Table 1). No significant difference was noted between venous or arterial samples drawn either from the patient or the pump oxygenator during the period of extracorporeal circulation. Heparin and protamine sulfate were removed from the citrated plasma samples (1/10 volume of 3.8% trisodium citrate) by ECTEOA cellulose and carboxymethyl-cellulose columns (BioRad Laboratories, Richmond, Calif.). Samples not assayed immediately (i.e., within 4 hr) were centrifuged at 20,000 g for 30 min to remove the platelets, processed over the columns, and stored at −80°C for subsequent assaying. The results obtained with these samples were found to be equivalent to those obtained with samples processed immediately (data not shown). The coagulation screening tests (prothrombin time, partial thromboplastin time, and thrombin time) and specific clotting assays were performed by standard methods. Fibrinogen was determined both by the kinetic assay of Claus^23 and the total clottable protein method described by Jacobsen.^24 The concentration of fibrinogen in 100 normal subjects was 285 ± 24 mg/dl. The factor X assay of Yin and Wessler was utilized to determine plasma heparin levels. Fibrinolytic tests. A caseinolytic assay for plasminogen was
employed, and activity reported in terms of CTA (Committee on Thrombolytic Agents) U/ml. The average value in 65 normal subjects was 2.6 ± 0.5 CTA U/ml.28 Plasma fibrinogen-fibrin-related antigen (FFRA) was assayed by means of the tanned red cell hemagglutination inhibition method.29 In 30 normal subjects the results were <0.1 g/ml.

**Platelet counting.** Platelets were counted with an electronic particle counter on blood collected in EDTA.28 They averaged 250,000 ± 40,000/µl in 100 normal human subjects.

**Bleeding times.** Standardized template bleeding times were performed on either the volar surface of the forearm or on the medial or lateral aspect of the leg just above the knee (in the supine position only).31 The bleeding times in the arm and leg were found to be equivalent in 15 normal subjects (coefficient of correlation, 0.989).

**Platelet aggregation.** Platelet aggregation induced by adenosine diphosphate (ADP) (Sigma, St. Louis, Mo.), or collagen (Hormon, Munich, West Germany) was studied using platelet-rich plasma anticoagulated with 3.2% trisodium citrate, stored capped at 4°C within 30 mm of collection. One milliliter of this platelet-free plasma was removed and stored at −80°C. Assay for PF4 was performed by competitive radioimmunoassay using 125I-PF4 and rabbit anti-human PF4.31 β-TG was performed using a commercially available assay (Amersham, Arlington Heights, Ill.). The mean plasma concentration of PF4 in 105 normal subjects in this laboratory was 1.7 ng/ml ± 0.7 (range, 0.7–3.3) and 11.7 ng/ml ± 6.6 (range, 8–28) for β-TG.

The mobilizable platelet content of PF4 and β-TG was calculated by measuring the concentration of PF4 and β-TG in serum divided by the platelet count and mean platelet volume in the whole blood sample; the results were expressed as nanograms of PF4 or β-TG per fl platelet volume (×10^4). Serum was prepared by drawing the blood in glass tubes and allowing it to stand overnight at room temperature. In a series of studies different methods of serum preparation were analyzed by measuring PF4 and β-TG in the supernatant of platelet-rich plasma (PRP) with a wide range of platelet counts after induction of release by the following methods: (1) clotting citrated PRP using CaCl2 and bovine thrombin (20 U/ml PRP); (2) lysis of platelets in citrated PRP with 1% Triton-X (100 µl/ml PRP); and (3) lysis of platelets in citrated PRP by 6 cycles of freezing and thawing. After the induction of platelet release, all samples were allowed to stand overnight at room temperature in glass tubes before centrifugation at 1000 g for 10 min and removal of supernatant. Clotted PRP produced higher mean serum values than by lysis of platelets with Triton-X (7811 ± 688 versus 3916 ± 1726 ng PF4/ml, respectively; p<0.025) and produced similar values to platelet lysis by repeated freezing and thawing (7455 ± 236 ng PF4/ml; p>0.1).

![Table 1. Coagulation Factor Changes in Cardiopulmonary Bypass Patients](http://www.bloodjournal.org/)

<table>
<thead>
<tr>
<th>Time of Samples</th>
<th>Fibrogen (mg/dl)</th>
<th>Factor II (%) Activity</th>
<th>Factor V (%) Activity</th>
<th>Factor VII (%) Activity</th>
<th>Factor VIII (%) Activity</th>
<th>Factor IX (%) Activity</th>
<th>Factor X (%) Activity</th>
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<tr>
<td>Group 1 patients (n = 21)</td>
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<tr>
<td>B*</td>
<td>313 ± 22</td>
<td>94 ± 6.8</td>
<td>105 ± 8.3</td>
<td>114 ± 8.7</td>
<td>113 ± 9.8</td>
<td>106 ± 7.2</td>
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<td>PH</td>
<td>334 ± 49</td>
<td>91 ± 8.0</td>
<td>96 ± 7.5</td>
<td>126 ± 13.0</td>
<td>127 ± 18</td>
<td>88 ± 5.1</td>
<td>91 ± 8.0</td>
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<td>H</td>
<td>285 ± 72</td>
<td>72 ± 6.1</td>
<td>59 ± 5.7</td>
<td>109 ± 11</td>
<td>111 ± 13</td>
<td>92 ± 9.3</td>
<td>81 ± 8.0</td>
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<td>BP</td>
<td>132 ± 14</td>
<td>44 ± 5.9</td>
<td>28 ± 3.3</td>
<td>58 ± 5.9</td>
<td>89 ± 14</td>
<td>60 ± 4.9</td>
<td>47 ± 4.8</td>
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<td>0.5</td>
<td>157 ± 16</td>
<td>43 ± 6.1</td>
<td>29 ± 4.0</td>
<td>64 ± 15</td>
<td>105 ± 12</td>
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<td>43 ± 5.8</td>
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<td>112 ± 15</td>
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<td>41 ± 7.7</td>
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<td>47 ± 7.0</td>
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<td>39 ± 3.0</td>
<td>99 ± 47</td>
<td>53 ± 3.6</td>
<td>64 ± 16</td>
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<td>3.0</td>
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<td>24 ± 9.0</td>
<td>39 ± 4.5</td>
<td>53 ± 16</td>
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<td>P</td>
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<td>44 ± 4.3</td>
<td>51 ± 8.8</td>
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<td>85 ± 12.7</td>
<td>115 ± 9.6</td>
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<td>105 ± 5.9</td>
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<td>74 ± 7.0</td>
<td>86 ± 8.0</td>
<td>83 ± 11</td>
<td>145 ± 32</td>
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<td>25–48</td>
<td>423 ± 20</td>
<td>73 ± 4.1</td>
<td>103 ± 4.4</td>
<td>106 ± 19</td>
<td>167 ± 14</td>
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<td>49–72</td>
<td>436 ± 28</td>
<td>86 ± 6.5</td>
<td>132 ± 17</td>
<td>123 ± 22</td>
<td>233 ± 56</td>
<td>123 ± 12</td>
<td>109 ± 9.8</td>
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<tr>
<td>73–96</td>
<td>459 ± 25</td>
<td>92 ± 5.9</td>
<td>129 ± 15</td>
<td>139 ± 24</td>
<td>200 ± 27</td>
<td>123 ± 13</td>
<td>95 ± 8.3</td>
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<td>Group 2 patients (n = 10)</td>
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<td>2–4</td>
<td>154 ± 11</td>
<td>54 ± 7.5</td>
<td>22 ± 9.7</td>
<td>70 ± 6.5</td>
<td>68 ± 13</td>
<td>91 ± 5.5</td>
<td>70 ± 10</td>
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<td>6–8</td>
<td>192 ± 27</td>
<td>65 ± 4.7</td>
<td>38 ± 4.7</td>
<td>63 ± 5.8</td>
<td>57 ± 11</td>
<td>72 ± 14</td>
<td>58 ± 11</td>
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</tbody>
</table>

*Prebypass: Baseline (B)—24 hr before extracorporeal circulation; preheparin (PH)—variable period before heparin administration including sternotomy and administration of preoperative medications; heparin (H)—period (10 min) after heparin loading dose. Bypass: Immediate bypass perfusion (BP)—sampling interval (10 min) after start of perfusion by pump prime; bypass (0.5, 1.0, 1.5, 2.0, etc.)—sampling period in 0.5-hr increments from immediate bypass. Postbypass: Immediate postbypass (P)—20 min after cessation of bypass and after the total neutralizing dose of protamine; postbypass (2–4, 6–8, etc.)—sampling intervals defined in hours after neutralization of heparin by protamine.

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The urinary excretion of PF4 was determined in duplicate on random collections using 1-mI volume for the test and corrected for the variable effects of urine on the assay by measuring the recovery of 1 ng of added PF4 as an internal standard and dividing by the creatinine concentration in the aliquot of urine. The results were expressed as nanograms PF4 per milligram creatinine.

Platelet content of ATP and ADP. Platelet ATP and ADP were measured directly by Dr. Brian Savage of the Puget Sound Blood Center using high performance liquid chromatography39 preceding and following cardiopulmonary bypass in four patients and two bypass aspheres. The levels of ATP and ADP are 3.69 ± 0.23 and 2.17 ± 0.21 μMole/1011 platelets in 16 normal human subjects (ATP/ADP = 1.70 ± 0.13), and 3.4 ± 0.2 and 3.5 ± 0.3 μMole/1011 platelets in 6 normal baboons. (ATP/ADP = 0.97 ± 0.03).

Isotope studies. 14C-serotonin and 35Cr-autologous platelets and 125I-homologous fibrinogen were labeled according to established techniques. 14C-serotonin platelet kinetics were included in these studies to assess platelet dense granule release independent of ATP/ADP measurements and morphometric analysis of dense bodies per platelet. We assessed the possibility that serotonin from platelet dense bodies might be preferentially released compared with 35Cr by normalizing the ratio of 14C-serotonin to 35Cr-platelet activity (each expressed as percent of baseline) during kinetic studies. In an extensive study in vivo, 14 it has been shown that platelet 14C-serotonin was selectively released by reserpine and ionophore, but that 4C-serotonin and 35Cr disappeared at equivalent rates following thrombin infusion, collagen infusion, and ongoing thrombotic consumption. In contrast to in vitro studies, there was no reuptake of released 14C-serotonin into circulating platelets in vivo.

Measurements using platelets doubly labeled with 35Cr and 14C-serotonin were carried out during and following bypass in 6 patients with stable blood volumes not requiring transfusions. Rapid dilution at the time of bypass precluded meaningful turnover calculations during that period. Following bypass, however, the platelet count varied little, particularly compared to the short platelet survival time produced by the surgical trauma, therefore, turnover estimations were calculated. The platelet survival time was analyzed by computer fitting to a gamma function. In 35 normal human subjects the mean value was 9.5 ± 0.6 days; the mean immediate recovery was 65% ± 5%; and platelet turnover averaged 35,000 ± 4600 platelets/μl/day. Mean 35Cr-platelet survival in normal baboons in our laboratory was 5.5 ± 0.3 days, with a mean immediate recovery of 85% ± 5% and platelet turnover of 59,000 ± 7000 platelets/μl/day. The mean fibrinogen survival (mean time in circulation) and turnover in 35 normal human subjects, as derived from the least squares computed disappearance half-time divided by the natural log of 2, were 5.1 ± 0.2 days and 0.57 ± 0.05 mg/ml/day, respectively.

Platelet granule number. Platelet pellets from platelet-rich plasma derived from 20 ml of whole blood drawn into ACD-ASA-PGE, anticoagulant-inhibitor, were fixed in glutaraldehyde and post-fixed with osmic acid. The samples were then dehydrated, embedded in Epon (Shell Chemical Co., Houston, Texas), thin sectioned on an ultramicrotome, and stained with uranyl acetate and lead citrate. Photomicrographs were prepared from 10 random sections for each pellet. The number of α-granules and dense granules per platelet was counted in a blinded manner for each photomicrograph containing 100–150 platelets in cross-section. Morphometric analysis of platelet granule number was performed baseline, immediately after bypass, and 24 hr after bypass in 2 patients.

RESULTS

Patients in Group 1

Prebypass Period

Prior to heparin administration (PH), the mean concentrations of platelets, fibrinogen, clotting factors II, V, VII, VIII, IX, X, and plasminogen of these patients were essentially normal (99.2% ± 4.1% of baseline; Tables 1 and 2; Figs. 1–3). The bleeding

| Table 2. Changes in Platelet Number and Function in Cardiopulmonary Bypass Patients |
|-----------------------------------------------|----------------|----------------|----------------|----------------|
| Time of Samples (conc. x 10^11/μl) | Platelet Count (ng/ml) | Plasma PF4 (ng/ml) | Plasma β-TG (ng/ml) | Bleeding Time (min) |
| Group 1 patients (n = 21) |
| B* | 225 ± 22 | 6.4 ± 6.3 | 22.6 ± 3.9 | 4.8 ± 0.1 |
| PH | 208 ± 31 | 25.8 ± 6.3 | 37.5 ± 5.3 | 5.4 ± 0.4 |
| H | 185 ± 30 | 306.0 ± 236.4 | 55.2 ± 11.3 | 6.7 ± 0.4 |
| BP | 151 ± 17 | 173.1 ± 116.9 | 184.4 ± 35.1 | 19.0 ± 0.8 |
| 0.5 | 135 ± 14 | 210.4 ± 94.3 | 312.0 ± 150.1 | 22.0 ± 0.6 |
| 1.0 | 117 ± 20 | 423.0 ± 173.7 | 748.2 ± 248.7 | 25.0 ± 1.0 |
| 2.0 | 112 ± 23 | 561.0 ± 146.0 | 706.0 ± 288.0 | >30 |
| 2.5 | 113 ± 29 | 614.0 ± 197.0 | 756.0 ± 274.0 | >30 |
| P | 116 ± 18 | 226.3 ± 101.1 | 680.0 ± 376.0 | 15.2 ± 0.6 |
| 2–4 | 132 ± 16 | 89.0 ± 35.8 | 306.5 ± 177.4 | 8.9 ± 0.6 |
| 6–8 | 130 ± 24 | — | — | 8.3 ± 1.8 |
| 19–24 | 114 ± 17 | 9.3 ± 2.9 | 27.8 ± 4.9 | — |
| 25–48 | 106 ± 17 | 20.7 ± 13.0 | 33.5 ± 6.2 | — |
| Group 2 patients (n = 10) |
| 2–4 | 110 ± 15 | — | — | 23.0 ± 7.5 |
| 6–8 | 118 ± 40 | — | — | 29.0 ± 0.7 |

*Prebypass: Baseline (B)—24 hr before extracorporeal circulation; preheparin (PH)—variable period before heparin administration including sternotomy and administration of preoperative medications; heparin (H)—period (10 min) after heparin loading dose. Bypass: Immediate bypass perfusion (BP)—sampling interval (10 min) after start of perfusion by pump prime; bypass (0.5, 1.0, 1.5, 2.0, etc.)—sampling period in 0.5-hr increments from immediate bypass.

Postbypass: Immediate postbypass (P)—20 min after cessation of bypass and after the total neutralizing dose of protamine; postbypass (2–4, 6–8, etc.)—sampling intervals defined in hours after neutralization of heparin by protamine.
Changes in coagulation during cardiopulmonary bubble oxygenator bypass. Nonblood prime in the extracorporeal oxygenator bypass dilutes the red cells and albumin to about half (solid circles). Circulating fibrinogen levels (open circles) are similarly decreased by dilution as are the prothrombin complex factors (II, VII, IX, and X; Table 1). Coagulation factor V (open triangles) is reduced in excess of dilution and factor VIII (closed triangles) levels are relatively unaffected by dilution (variances about the mean given in Table 1). Clotting factor levels return to baseline values or above within 12 hr after bypass. Horizontal solid bar indicates period of bypass. Timing of observations is detailed in Table 1. Error bars represent ±1 SE.

Times were also normal (4.8 min ± 0.5; Table 2) as were the in vitro platelet aggregation tests (ED<sub>50</sub> for ADP was 0.9 μM ± 0.1 and 1.0 μg/ml ± 0.1 for collagen), and platelet ATP and ADP contents (3.39 ± 0.98 and 1.96 ± 0.80, respectively, with ATP/ADP of 1.82 ± 0.33).

Baseline plasma levels of PF4 and β-TG were elevated compared with asymptomatic control subjects, perhaps related to the presence of atherosclerotic vascular disease in these patients<sup>41</sup> (PF4, 6.4 ng/ml ± 0.7 compared with 1.7 ng/ml ± 0.7; β-TG 22.6 ng/ml ± 8.8 compared with 11.7 ng/ml ± 6.6; p < 0.0005 and p < 0.01, respectively; Table 2, Fig. 2). Baseline platelet content of PF4 was 1.32 ± 0.15 x 10<sup>8</sup> ng/fl prebypass compared with 1.36 ± 0.24 x 10<sup>-8</sup> in normal subjects, and the baseline number of α-granules and dense granules per platelet cross-section was 5.6 ± 3.5 and 0.04 ± 0.05, respectively. Urine excretion of PF4 was 0.189 ng/mg creatinine ± 0.12 in patients prebypass compared with 0.178 ng/mg creatinine ± 0.15 in normal subjects. The preheparin levels for PF4 and β-TG were further increased above baseline (p < 0.025 and p < 0.05, respectively; Table 2, Fig. 2).

After heparin administration (labeled H, ranging 30–90 min into surgery), the dilution index was reduced to 0.88% ± 0.04% of baseline, presumably due to dilution with nonblood infusions during the early operative period. As shown in Tables 1 and 2 and Figs. 1–3, the concentrations of platelets, fibrinogen, coagulation factors II, VII, IX, X, and plasminogen were also decreased, directly reflecting dilution indicated by the overall correlation coefficient with the dilution times were also normal (4.8 min ± 0.5; Table 2)}
index of 0.907. Only factor V was discordantly decreased compared with the dilution index (59% ± 6% of baseline; p < 0.005), while factor VIII was moderately increased (111% ± 12% of baseline; p < 0.05). FFRA values became transiently increased during this early operative period (Fig. 3), apparently reflecting activation of the fibrinolytic system in response to surgical tissue injury and local fibrin deposition.

Heparin levels averaged 3.5 ± 0.6 U/ml 10 min after bolus infusion (Fig. 3). The bleeding time (Table 2; Fig. 2) was not significantly prolonged from baseline values by systemic heparin (5.4 ± 0.7 min with heparin compared with 4.8 ± 0.6 without; p > 0.05). ED₅₀ for ADP and collagen-induced aggregation measurements were unchanged following heparinization, i.e., 0.9 μM ± 0.1 and 1 μg/ml ± 0.1, respectively, p > 0.5. Following the administration of heparin, there was an acute transient peak in plasma PF4 (Table 2, Fig. 2) that was not observed in plasma levels of β-TG.

³¹Cr-platelet and ¹²⁵I-fibrinogen activities did not differ from the dilution index (Fig. 4, p > 0.1), and the normalized ratio of ¹³⁵C-serotonin and ³¹Cr-platelets was not decreased (1.0 ± 0.07 compared with 1.1 ± 0.09, p > 0.1).

**Period of Bypass**

After bypass perfusion (BP) was started, the dilution index fell by half due to the addition of the electrolyte priming volume to the circulation (dilution index was 0.47 ± 0.05, compared with 1.0 baseline and 0.88 ± 0.3 prebypass, post heparin). Since a similar decrease occurred in the concentrations of platelets, fibrinogen, factors II, VII, IX, and X, and plasminogen, initial reduction in these hemostatic factors was attributable to dilution by the nonblood prime (Figs. 1–4; Tables 1 and 2) (correlation coefficient between the concentrations of these hemostatic factors and the dilution index was >0.894). Interestingly, the reduction in factor V was greater than that predicted by dilution alone (Fig. 1; Table 1; p < 0.01), perhaps related to its interaction with activated platelets, biomaterials, oxygen bubbles, or surgically damaged tissues. Conversely, factor VIII remained in the normal range despite dilution, demonstrating the rapidity with which factor VIII was mobilized. The FFRA level was reduced to about half (Fig. 3). In two additional patients, cardiopulmonary bypass was performed using fresh frozen plasma as the priming solution (2250 ml each). Although the reduction in coagulation factors and plasminogen in these two patients was significantly less than the average reduction observed in patients undergoing nonblood prime, the pattern of change was similar (19% and 22% reduction compared with 35%, p < 0.05).

Throughout the period of bypass, the levels of platelets, soluble coagulation factors, and plasminogen showed little additional decrease beyond those initially produced by dilution (Figs. 1–3). Subsequent variabilities in replacement volume from patient to patient invalidated using the dilution index after bypass (Fig. 1).

With the onset of extracorporeal circulation, the mean template bleeding time increased from 5.4 min ± 0.07 to 19 min ± 0.8 (p < 0.002), despite maintenance of the platelet count at 151,000 platelets/μl ± 17,000 (Table 2). Thereafter, the bleeding time progressively increased, the extent being directly related to the duration of bypass; this relationship was mathematically represented by the equation y = 0.11x + 6.7. By 2 hr of bypass, no patient had a bleeding time of less than 30 min (the limit of sensitivity of this test), although the mean platelet count had not changed significantly (112,000 platelets/μl ± 23,000; p > 0.1). No correlation was found between the platelet count or fibrinogen level and time of bypass (coefficients were 0.351 and 0.271; p > 0.1).

The two patients receiving fresh frozen plasma for oxygenator priming showed the same bleeding time prolongation of >30 min as those receiving nonblood priming.

Plasma PF4 and β-TG levels increased progressively throughout bypass, in parallel with the changes in bleeding time (Fig. 2, Table 2).
Mean rectal temperature averaged 30.7°C ± 1.03 at 30 min into bypass and 28.3°C ± 0.75 at 90 min. In one patient who was normothermic during bypass, the platelet concentration dropped from (B) 232,000/μl to (BP) 121,000/μl, and the bleeding time increased to >30 min during bypass. One of the hypothermic patients received 10 platelet concentrates during bypass after his bleeding time had risen to >30 min. Despite normalization of the platelet count by the transfused platelets, the bleeding time was not measurably affected during hypothermia.

Aggregometry revealed greatly reduced responsiveness of platelets to ADP and collagen that began immediately after the onset of bypass and persisted for 3 hr after bypass. For example, the ED50 for ADP increased from 0.9 μM ± 0.1 baseline to 11 μM ± 3 at 0.5 hr into bypass, 17 μM ± 4 at 2 hr into bypass, and 5 μM ± 2 at 3 hr postbypass (p < 0.01). Aggregation induced by collagen was similarly impaired (data not shown).

The initial decrease in 51Cr-platelet and 125I-fibrinogen activities primarily reflected dilution. An additional increase in 51Cr-platelet removal was observed during bypass (from 60% ± 7% of baseline activity to 45% ± 5%; Fig. 4; p < 0.05). The change in 125I-fibrinogen during bypass did not differ significantly from that predicted by dilution (p > 0.1), presumably due to the heparin blockade of activation of coagulation factors. The normalized ratios of platelet 14C-serotonin:51Cr did not decrease during the bypass period or during the first postoperative day, suggesting that platelet dense granules were not undergoing release in vivo. Studies in vitro using 14C-serotonin-labeled platelets from the patients showed that thrombin induced release of 14C-serotonin as readily as before infusion (data not shown).

The period of bypass was associated with dilutional decreases in both plasminogen and FFRA levels, which remained reduced through bypass. The absence of fibrin degradation products during the bypass phase appears to be a consequence of heparin-mediated blockade of fibrin formation as implied by the reciprocal relationship between heparin and FFRA levels during surgery (Fig. 3).

**Postbypass Period**

Discontinuance of bypass and completion of protamine neutralization of heparin (sampling interval P, 20 min postbypass) in group I patients produced no significant change in the concentrations of coagulation factors, plasminogen, or platelets (Figs. 1–3). However, the bleeding time decreased to 15.2 min ± 0.6 (p < 0.02). Similar observations in bleeding time were found in the normothermic patient (shortening of bleeding time from >30 min to 13 min at a platelet count of 145,000/μl), and the two patients primed with fresh frozen plasma (bleeding time from >30 to 14 and 16 min). In the patient receiving platelet concentrates the bleeding time shortened similarly from >30 min to 10 min. Normalization of the bleeding time regularly occurred within 2–4 hr after protamine was given (Table 2).

Within 20 min after protamine neutralization of heparin when the plasma levels of PF4 and β-TG were markedly elevated, the circulating platelets were partially depleted of α-granules, i.e., the mean number of α-granules per platelet cross-section was 3.3 ± 3.1 compared with 5.6 ± 3.5 baseline, p < 0.005. As expected, the platelet content of PF4 was similarly decreased to 0.96 ± 0.16 x 10^-6 ng/μl platelet volume (compared with 1.32 ± 0.15 x 10^-6 prebypass, p < 0.001). Moreover, the urinary excretion of PF4 was greatly enhanced, i.e., 29.8 ng PF4/mg creatinine ± 18.9 compared with 0.189 ± 0.12 baseline, p < 0.001. No significant change was observed in the number of dense bodies per platelet cross-section (p > 0.4), although a modest change could not be excluded due to the low number of dense granules in normal platelets.

Platelet ATP and ADP contents did not change significantly in four patients during bypass. The levels of platelet ATP and ADP at the end of bypass were 2.99 ± 0.75 and 1.82 ± 0.59 μmole/10^11 platelets, respectively, with ATP/ADP of 1.65 ± 0.14, compared with 3.39 ± 0.98 and 1.96 ± 0.80 μmole/10^11 platelets and ATP/ADP 1.82 ± 0.33 baseline; p > 0.10 using paired t statistics.

Three hours after protamine administration when heparin levels had fallen to undetectable values, β-TG and PF4 had decreased from peak levels by 86% and 60%, respectively, and the concentrations of fibrinogen, factors II, V, VII, VIII, IX, X had increased by about a third (Tables 1 and 2; Figs. 1 and 2; p < 0.01).

At 12 hr postbypass, fibrinogen and factors V, VII, VIII, and IX were at basal values; at 24 hr, factors II and X and plasminogen were basal, as well as plasma PF4 and β-TG. Although the platelet count was persistently about half normal for several days, the bleeding time returned completely to normal within 12 hr (Table 2). At 24 hr after bypass, the platelet α-granule content remained reduced (3.8 ± 3.5 α-granules per platelet compared with 5.6 ± 3.5 baseline, p < 0.025).

51Cr-platelet removal was accelerated after heparin neutralization (Fig. 4; p < 0.001). Since the platelet
count remained relatively steady compared with the degree of shortening in platelet survival, calculations of platelet turnover during the first postoperative day were made and they averaged 117,000 platelets/$\mu$l/day ± 27,000 compared with 35,000 ± 5000 in control subjects (Fig. 4; $p < 0.01$). Fibrinogen turnover was not calculated because of the rapidly changing plasma fibrinogen concentration during this period.

FFRA values became progressively abnormal following neutralization of heparin, reaching a peak value of 3.2 $\mu$g/ml at 4 days (Fig. 3; $p < 0.001$). The kinetic and total clottable protein measurements of fibrinogen in these patients were equivalent throughout the study (correlation coefficient 0.937), suggesting that there was no functional kinetic defect in the rate at which fibrinogen was transformed into fibrin.

Patients in Group 2

The patients with pathologic bleeding demonstrated changes in coagulation and fibrinolytic factor levels similar to those of group 1 patients except for factor V, which was somewhat lower (Tables 1 and 2; Figs. 1–3; $p < 0.002$). At 6–8 hr postbypass, these group 2 patients also had significantly lower levels of factors VIII and VII compared with group 1 patients ($p < 0.01$). While the platelet counts at 2–4 hr postbypass were comparable (open circles) in both groups, difference was further exaggerated 6–8 hr postbypass in group 2 patients (23 min ± 7.5 compared with 8.9 min ± 0.1, $p < 0.01$). The difference was further exaggerated 6–8 hr postbypass (29 min ± 0.7 compared with 8.3 min ± 1.8, $p < 0.001$). This striking increase in bleeding time in group 2 patients correlated with measurements of blood loss (correlation coefficient 0.773, $p < 0.01$).

However, in 6 of these patients, the bleeding time shortened significantly (from >30 min to 14 min ± 4) within half an hour after the transfusion of platelets sufficient to raise the platelet count 71,000/$\mu$l ± 23,000.

Baboon Studies

Since a number of potentially important mechanisms underlying the genesis of platelet dysfunction could not be independently controlled in human subjects, we examined in 12 baboons the effects of bubble oxygenator bypass and hypothermia on bleeding time, platelet release in vivo, platelet kinetics, and platelet aggregation ex vivo.

The bleeding time was prolonged from the baseline value of 3.3 ± 0.4 to greater than 25 min within an hour of bubble oxygenator bypass in the absence of drugs, hypothermia, priming variables, or surgery (except for vascular access) (Fig. 5A). A similar effect on bleeding time was noted in animals undergoing hypothermia to 20°C in the absence of extracorporeal oxygenation, heparinization, or surgery (except for vascular access) (Fig. 5B). The bleeding time lengthened despite maintenance of the platelet count above 150,000 platelets/$\mu$l in both groups of animals. As observed in the human subjects, the bleeding time normalized rapidly following cessation of extracorporeal oxygenation or hypothermia (Fig. 5A and B). Plasma PF4 and $\beta$-TG increased rapidly after the onset of bubble oxygenation. These values also fell rapidly following discontinuance of extracorporeal oxygenation. One additional animal was studied during extracorporeal bypass with a pediatric bubble oxygenator rather than the smaller infant-size apparatus. In this animal, peak PF4 levels were 20-fold higher than the mean peak PF4 level in the animals studied with the smaller bubble oxygenators.

In the baboons undergoing cardiopulmonary bypass, collagen-induced platelet aggregation was impaired during and after bypass as shown by a shift in the ED$_{50}$ from a mean of 0.8 $\mu$g/ml to 2.6 $\mu$g/ml collagen ($p < 0.025$). In two animals, the platelet
ATP and ADP contents did not change significantly during bypass. ATP values were 3.9 and 3.5 μmole/10^{11} platelets basal, and 3.7 and 3.3 μmole/10^{11} platelets postbypass. Platelet ADP contents were 3.5 and 3.4 μmole/10^{11} platelets baseline, and 3.3 and 3.1 μmole/10^{11} platelets following bypass. ATP/ADP ratios were 1.11 and 1.03 baseline and 1.12 and 1.10 after bypass oxygenation.

In the baboons subjected to hypothermia alone, the bleeding time lengthened before any measurable elevations in plasma PF4 and β-TG had occurred. PF4 and β-TG did not become significantly elevated until rewarming was begun; p < 0.05 at 25°C rewarming (Fig. 5B). No significant changes in platelet aggregation were demonstrated in the hypothermic baboons when comparing baseline ED_{50} values with results obtained at 25°C (rewarming temperature) using ADP or collagen. Simultaneous labeling of platelets with {\textsuperscript{51}}Cr and {\textsuperscript{14}}C-serotonin in hypothermic baboons showed no preferential loss of {\textsuperscript{14}}C-serotonin compared with {\textsuperscript{51}}Cr-platelet activity (the normalized ratio of {\textsuperscript{14}}C-serotonin to {\textsuperscript{51}}Cr remained unchanged throughout). Whereas, significant {\textsuperscript{51}}Cr-platelet destruction occurred during cardiopulmonary bypass, amounting to 19% ± 3% during the 3-hr period of bypass (p < 0.01), platelets were not destroyed during hypothermia alone. The temporary drop in platelet count observed with hypothermia was due to transient sequestration.

**DISCUSSION**

Multiple complex changes occur in the hemostatic mechanism during cardiopulmonary bypass.\textsuperscript{1-4,7-16,19,20} The present study was designed to assess the relative importance of the disturbances in each component of hemostasis to clarify the pathogenesis of abnormal bleeding associated with cardiopulmonary bypass surgery. Particular emphasis was given to in vivo measurements of function during extracorporeal bubble oxygenation.

In spite of the predictable decreases in coagulation factors associated with bypass, no patients in this study as well as other studies\textsuperscript{1,3,13-15} showed a fall to levels thought to produce abnormal bleeding, at least with respect to a single factor (i.e., levels above 30% are considered to be adequate for all factors except factor V, which shows adequate hemostasis at levels of 10%–15%).\textsuperscript{42} In addition, the kinetic and total clottable fibrinogen measurements in all patients were equivalent, which suggests that fibrinogen dysfunction did not contribute to abnormal hemostasis. Moreover, since fibrinolytic products only appeared postoperatively after heparin was neutralized, it seems unlikely that activation of the fibrinolytic system contributed to the development of abnormal bleeding during cardiopulmonary bypass. Finally, at no time after protamine administration was there evidence of significant residual heparin activity, heparin rebound, or protamine-related coagulopathy in group 1 or group 2 patients. Consequently, these and previous studies indicate that abnormal bleeding following cardiopulmonary bypass is generally not due to: (1) reduction in the levels and function of coagulation factors; (2) heparinization with the potential for subsequent inadequate neutralization or protamine excess; (3) qualitative defects in the polymerization of fibrin; and (4) increased fibrinolytic activity and the secondary effects of fibrin-fibrinogen degradation products on coagulation. Therefore, we evaluated changes in the capacity of these patients to form platelet hemostatic plugs.

During the prebypass period no significant quantitative or qualitative variation in platelets was observed, and heparin (3.5 U/ml) had no discernable impact on the template bleeding time (Table 2, Fig. 2). This finding is in accord with the capacity of this test to assess the overall competence of platelet plug formation independent of coagulation changes. However, heparin-induced prolongation of the bleeding time has been observed occasionally.\textsuperscript{43} As expected, heparinization produced a transient peak in plasma PF4 but not β-TG.\textsuperscript{44}

With the onset of bypass and persisting throughout its course, the usual relationship between the platelet concentration and the standardized template bleeding time became increasingly disparate (Table 2, Fig. 2). While the platelet count fell in concert with the dilution index (Table 2, Fig. 2) the mean was never less than 100,000/μl, the level above which the bleeding time normally remains at 5.0 min ± 1.5.\textsuperscript{31} The immediate prolongation of bleeding time by more than 12 min that followed the initiation of bypass (from 6.7 ± 0.7 to 19 ± 0.8 min between H and BP) suggested that hypothermia and possibly the constituents in the nonblood priming solution contributed to this initial change. The bleeding time progressively lengthened to >30 min by 2 hr of bypass. The prolongation in bleeding time was associated with a striking rise in both plasma and urine PF4 and β-TG, and a reciprocal depletion of platelet α-granules and their contents. A direct relationship was observed among the length of the bleeding time, rise in plasma PF4 and β-TG, and the duration of bypass. The controlled studies in baboons undergoing bypass similarly showed parallel increases in bleeding time and plasma and urine levels of platelet-specific proteins (PF4 and β-TG) together with the reciprocal decrease in the α-granules and their constituents. We therefore
concluded that the progressive platelet dysfunction was a consequence of platelet activation induced by the cardiopulmonary oxygenator apparatus and hypothermia. The loss of α-granules and the release of PF4 and β-TG were not accompanied by measurable loss of dense granules or their contents during bypass as shown by: (1) the constant levels of platelet ATP and ADP; (2) the unchanging ratio of platelet 14C-serotonin; and (3) the constant number of platelet dense granules.

The defect in platelet function was rapidly reversible, i.e., the bleeding time shortened significantly within 20 min after bypass was ended and additional improvement occurred during the subsequent several hours. During this period, PF4 and β-TG also disappeared at rates consistent with their reported T50 removal rates of about 15 and 100 min, respectively (Fig. 2). It is of interest that normalization of the bleeding time occurred without reconstitution of the lost α-granules and their constituents.

Thus, platelet dysfunction paralleled the depletion of platelet-specific proteins from platelets and their appearance in plasma, while normalization of platelet function paralleled the clearance of these platelet proteins from plasma. However, the rapid return of function without reformation of the lost α-granules following bypass indicated that the platelet dysfunction and selective α-granule release were independent consequences of oxygenator-induced platelet activation and were not necessarily causally related. This defect in platelet function has some similarities to the in vitro refractory state induced by ADP. While the nature of this dysfunction is not known, it may be due to a transient depletion of some functional platelet component, generation of some as yet unidentified labile platelet inhibitor or a reversible membrane abnormality. In some preliminary studies, we have found that the infusion of platelet releasates into baboons does not produce a prolongation in bleeding time (unpublished observation), implying that platelet dysfunction is not mediated by a stable inhibitor released from platelets.

This process of cumulative but transient platelet dysfunction differs from the reported examples of acquired platelet dense granule storage pool deficiency (δSPD) by its reversibility and selective release and loss of only α-granules. Presumably, the selective release of α-granules during bypass reflects the facility with which they undergo release compared with dense granules. Interestingly, the reported examples of acquired δSPD have been associated with autoantibody formation, suggesting some immune-related mechanism for δ-granule release in vivo.

In 10 group 2 patients the mean template bleeding time correlated with the amount of clinical bleeding as assessed by the volumes of transfused whole blood (correlation coefficient 0.775; \( p < 0.01 \)). Bivariate analysis of simple correlation coefficients relating the blood volume transfused and duration of perfusion to the bleeding time showed that both variables correlated significantly (0.710, \( p < 0.01 \) and 0.750, \( p < 0.01 \), respectively) and were comparable in effect. The estimated blood loss correlated well with the quantity of whole blood transfused (correlation coefficient 0.952, \( p < 0.001 \)). Prolonged bleeding times (>20 min) beyond 20 min postprotamine neutralization was a characteristic of all group 2 patients. The persistence of platelet dysfunction in these patients may have been related to either severe and continuing activation or some additional intrinsic platelet defect. Platelet transfusions successfully shortened the bleeding time in these patients and were followed by a cessation of clinical bleeding.

From these studies we conclude that the severity of postoperative hemorrhage in bypass patients is probably due in large part to defective platelet plug formation. The degree of impairment in platelet function is directly proportional to the duration of bubble oxygenator bypass and is also probably related to the level of hypothermia. The process of platelet dysfunction is rapidly reversible in the usual bypass patient, but produces abnormal bleeding in those patients with a persistent functional platelet defect. Under the conditions of this study the standardized template bleeding time was shown to predict those patients at risk of serious postoperative bleeding. While occasional bypass patients with bleeding have platelet counts below 50,000/μl and are thereby routinely given platelet transfusions, the present data emphasize that platelet transfusions are also required by bleeding patients whose bleeding time is prolonged beyond 20 min after protamine neutralization even when the platelet count exceeds 100,000/μl. We therefore propose that the bleeding time is a better indicator of the need for platelet transfusion than the platelet count itself and that both measurements are needed in bypass patients who fail to stop bleeding after protamine neutralization.

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Mechanism of abnormal bleeding in patients undergoing cardiopulmonary bypass: acquired transient platelet dysfunction associated with selective alpha-granule release

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