



ARTICLE

Cell-Derived Microparticles Generated in Patients During Cardiopulmonary Bypass Are Highly Procoagulant

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ABSTRACT: *Background* Microparticles from platelets and other cells have been extensively studied and characterized in vitro. Although the level of platelet-derived microparticles is elevated in a variety of diseases, including cardiac surgery, virtually nothing is known about their functions in vivo. The aim of the present study was to investigate the procoagulant properties of microparticles generated in vivo. *Methods and Results* In 6 patients at the end of cardiopulmonary bypass, $14.8 \times 10^9/L$ (median; range, 9.7 to $27.4 \times 10^9/L$) platelet-derived microparticles were present in pericardial blood, whereas blood obtained from the systemic circulation contained $1.6 \times 10^9/L$ (median; range, 0.4 to $8.9 \times 10^9/L$) of such microparticles, as determined by flow cytometry. Microparticles stained positively for phosphatidylserine as determined with labeled annexin V. In contrast to systemic blood, pericardial blood contained

not only microparticles of platelet origin but also microparticles that originated from erythrocytes, monocytes, or granulocytes, and other hitherto unknown cellular sources. Plasma prepared from pericardial blood and to a lesser extent plasma from systemic blood obtained at the same time, stimulated formation of thrombin in vitro. This activity of pericardial plasma was lost after removal of its microparticles by high-speed centrifugation, whereas the corresponding microparticle pellet was strongly procoagulant. The generation of thrombin in vitro involved a tissue factor/factor VII-dependent and factor XII-independent pathway. *Conclusions* This study is the first to demonstrate that microparticles generated in vivo can stimulate coagulation.

Key Words: cardiopulmonary bypass ■ thrombin ■ microparticles

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Microparticles from platelets and other cells have been extensively studied and characterized in vitro. When isolated platelets are stimulated with a combination of the physiological agonists α -thrombin and collagen, the terminal complement proteins C5b-9 or the Ca^{2+} -ionophore A23187, they release large quantities of microparticles.^{1,2} Both activated platelets and microparticles express the aminophospholipid PS, thereby providing an essential procoagulant surface that supports the formation of activated clotting enzymes, ie, tenase- and prothrombinase complexes on membranes.³ Compared with activated platelets, microparticles contain a higher density of high-affinity binding sites for activated factor IX (IXa)⁴ and factor Va.² They have a continuous expression of high-affinity binding sites for factor VIII⁵ and support both factor Xa

activity⁶ and prothrombinase activity.^{2,7} In addition, inactivation of factor Va by activated protein C is enhanced in the presence of either activated platelets or microparticles, suggesting that microparticles may also have anticoagulant properties.¹ In vitro, procoagulant microparticles can not only be derived from platelets but also from other cells such as endothelial cells on interaction with complement, or monocytes stimulated with endotoxin.^{8,9} The presence of microparticles derived from other cells than platelets has, to the best of our knowledge, not been demonstrated in vivo. Increased numbers of platelet-derived microparticles in the circulation have been reported in patients undergoing cardiac surgery,¹⁰ plasmapheresis,¹¹ or coronary angiography,¹² as well as in patients suffering from diabetes,¹³ heparin-induced thrombocytopenia,¹⁴ infarctions,¹⁵ uremia,¹⁶ idiopathic thrombocytopenic purpura¹⁷ and diffuse intravascular coagulation.¹⁸ However, their functional activity in vivo remains unclear, which is especially due to the fact that the numbers of microparticles in these clinical conditions is low.

During CPB, blood becomes activated by extensive contact with the extracorporeal circuit of the heart-lung machine. This contact leads via activation of the factor XII-dependent contact-activation pathway to activation of the complement system as well as coagulation and fibrinolysis.¹⁹ Until recently, this so-called material-dependent activation of blood was thought to be the major if not the only cause of blood activation during heart surgery. Recently, however, several investigators showed that material-independent blood activation also occurs, especially in the operation field. Here, numerous blood vessels are cut or become damaged, and blood oozes into the pericardial cavity. Blood collected from this site (further designated as pericardial blood) has been in extensive contact with damaged tissues, and, as some studies show^{20,21} contains high

concentrations of tissue-type plasminogen activator and tissue factor, which trigger fibrinolysis and extrinsic coagulation, respectively.

In the present study we addressed the question whether pericardial blood, obtained from patients undergoing coronary bypass surgery, may contain cell-derived microparticles that support coagulation. Our results show that pericardial blood is indeed rich in platelet-derived microparticles, but also in erythrocyte- and monocyte- or granulocyte-derived microparticles and supports coagulation in vitro via a microparticle-associated tissue factor/factor VII-dependent pathway.

METHODS

Reagents and Assays

Reptilase was obtained from Boehringer Mannheim, thrombin substrate S2238 from Chromogenix AB, heparinase from Baxter Diagnostics, human albumin, and murine anti-GPIb (CLB-MB45) and murine normal serum from the Central Laboratory of the Netherlands Red Cross Bloodtransfusion Service (Amsterdam, The Netherlands). This MoAb was biotinylated as described earlier.²² PE-conjugated streptavidin, glycophorin A MoAb-FITC and anti-CD61-FITC MoAb were obtained from Dakopatts. IgG₁-FITC, IgG_{2b}-FITC, and anti-CD14 MoAb-PE were derived from Becton Dickinson, and the Ca²⁺-ionophore A23187 from Calbiochem. Artificial phospholipid vesicles containing 20% PS, 40% phosphatidylcholine, and 40% phosphatidylethanolamine were kindly provided by Dr M. van Wijnen (Department of Hematology, University Hospital Utrecht, Utrecht, The Netherlands). Kaolin was provided by B.L.B. Laboratoires du Bois de Boulogne (Puteaux, France), dynabeads M-280 streptavidin from Dynal A.S. (Oslo, Norway), FITC-labeled annexin V and biotin-labeled

annexin V from Nexins Research B.V. (Hoeven, The Netherlands), and factor VII–and factor XII–deficient human plasma from Biopool (Burlington, Ontario, Canada). OT-2, a MoAb directed against the active site of factor XII and XIIa, was prepared as described earlier.²³ Purified TFPI was a kind gift of Dr A.A. Creasey (Chiron Corp, Emeryville, Calif). All other chemicals were of the highest grade commercially available.

Clinical Studies

Six patients who underwent CPB for coronary bypass grafting were studied. These patients were connected to an extracorporeal circuit (ECC), which consisted of a Diceco D704 compac flow system oxygenator and S3 rollerpump. Polyvinyl chloride tubing was used throughout the circuit, except for the rollerpump, which was silicon rubber. The priming of the ECC contained Ringer's lactate (1.3 L), human albumin (200 mL 20% [wt/vol]), mannitol (100 mL 20% [wt/vol]), NaHCO₃ (50 mL 8.4% [wt/vol]), heparin (50 mg/L), and cefamandol (2g). Heparin (3 mg/kg) was given intravenously before cannulation of the aorta and repeated in a dose of 50 mg whenever the activated clotting time (Hemochron) became less than 480 seconds. Pump flows ranged from 2.0 to 2.4 L.m⁻².min.⁻¹ during moderate hypothermia (28 to 32°C). Myocardial protection was achieved with modified St Thomas solution infused in the aortic root, after clamping and external topical cooling. Systemic blood samples were obtained after anesthesia, but before skin incision¹; approximately 5 minutes after start CPB²; 10 minutes before release of the aortic clamp at the start of the last distal anastomosis³; and 5 to 10 minutes after release of the aortic clamp.⁴ Pericardial blood was sampled at time points 2, 3, and 4. The study protocol was approved by the Medical Ethical Committee of the Onze Lieve

Vrouwe Gasthuis. All patients had given informed consent to participate in the study.

Collection of Blood Samples

Systemic blood samples were drawn from the same central venous line. Pericardial blood was sampled directly from the pericardial cavity with a 10 mL plastic syringe. Blood was immediately put into plastic tubes containing 1/10th volume of 3.2% trisodiumcitrate. Cells were removed by centrifugation for 15 minutes at 1,550g at room temperature. Plasma samples were stored in aliquots at -70°C until tests were performed.

Flow Cytometric Analysis

Flow cytometry was performed as described by Shattil et al,¹⁰ with some modifications. The citrate-anticoagulated blood was added in 5- μ L aliquots to tubes containing 35 μ L of HEPES buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 1.0 mmol/L MgCl₂, 5.6 mmol/L glucose, 20 mmol/L HEPES, 1 mg/mL albumin, 3.3 mmol/L NaH₂PO₄, pH 7.4) and 5 μ L biotin-labeled anti-GPIb (5 μ g/mL final concentration). After 15 minutes of incubation at room temperature in the dark, 5 μ L of 10-fold diluted phycoerythrin-conjugated streptavidin was added. After another 15 minutes at room temperature in the dark, 2.5 mL HEPES-buffer containing 0.2% paraformaldehyde (wt/vol) was added. No changes in the expression of surface-antigens occurred within 48 hours after fixation if platelets were prepared according to this protocol. The samples were analyzed in a FACScan flow cytometer with PC-lysis software (Becton Dickinson). Both forward light scatter and sideward light scatter were set at logarithmic gain, and platelets or platelet-derived material was identified in whole blood by analyzing the phycoerythrine-glycoprotein Ib fluorescence at 585 nm. Regions were identified, corresponding to microparticles (R1), platelets (R2)

and complexes of platelets, platelets and leukocytes, and possibly platelet-derived microparticles and leukocytes (R3), respectively. Preliminary experiments performed in our laboratory confirmed that platelets stimulated in whole blood or in platelet rich plasma with the Ca^{2+} -ionophore A23187 (5 minutes incubation at 37°C , $2.5\ \mu\text{mol/L}$ fc.) shedded microparticles that were exclusively found in R1 and were highly positive for annexin-V-FITC. The absolute concentration of microparticles, ie, corrected for hemodilution in sample x, was calculated by using the following formula: $[(\text{platelet count in blood sample}_x / \% \text{ cells in R2}_x) \times (\% \text{ particles in R1}_x) \times (\text{hematocrit in first systemic sample} / \text{hematocrit in blood sample}_x)]$, in which the platelet count in the blood sample was determined on a SYSMEX 3000 (Toa Medical Electronics Co, Ltd), the % cells in R2 and R1 were the % of GPIb-positive events in R2 (platelets) and R1 (microparticles) as determined by flow cytometry, respectively.

Preparation of Microparticles In Vitro and Staining With Annexin V-FITC

Citrate-anticoagulated blood obtained from healthy volunteers was diluted 10-fold in HEPES-buffer (pH 7.4). Microparticles were generated by incubation with 0.001 volume of the Ca^{2+} -ionophore A23187 (final concentration, $2.5\ \mu\text{mol/L}$), dissolved in ethanol, for 30 minutes at room temperature. After centrifugation for 15 minutes at $700g$ at room temperature, plasma was removed and centrifuged for 1 hour at $13\ 000g$ at room temperature. The supernatant plasma was carefully removed by aspiration and the pellet was gently resuspended in 1 mL PBS (pH 7.4). This microparticle suspension was again centrifuged at $13\ 000g$ for 1 hour at room temperature. The pellet was finally resuspended in $1/8^{\text{th}}$ volume of apopbuffer, prepared according to the manufacturers instructions (Nexins

Research B.V). The microparticle fraction was diluted 20-fold in apopbuffer containing annexin-V-FITC (20 nmol/L fc.), and left in the dark for 15 minutes at room temperature before addition of 2 volumes of apopbuffer. Microparticles present in patient blood were isolated similarly, but A23187 was not added.

Identification of Microparticles by Flow Cytometry

Isolated microparticles (5 μ L) were diluted in apopbuffer (35 μ L), containing an additional 5 μ L 500-fold prediluted normal mouse serum. After incubation for 15 minutes at room temperature, isotype-matched control antibodies or cell-specific MoAbs, labeled with either FITC or PE, were added and the mixture was incubated in the dark for 15 minutes at room temperature. Subsequently, 250 μ L apopbuffer was added, containing annexin V-FITC (20 nmol/L fc.) or annexin V-PE (5 nmol/L fc.). Microparticles were washed once with PBS (pH 7.4) before flow cytometry.

Endogenous Thrombin Potential

The thrombin generation assay was performed as described by Kessels et al.²⁴ To prepare normal pooled plasma, blood was obtained from 40 healthy volunteers who had not taken any medication during the previous ten days. Plasma was prepared from citrate-anticoagulated blood by centrifugation at 1550g for 15 minutes at room temperature, removed, pooled, and stored in 1 mL aliquots at -70°C until use. After thawing, reptilase was added and the plasma incubated for 10 minutes at 37°C and, subsequently, for 10 minutes on melting ice. The fibrin clot was removed by rotating a plastic spatula. Patient plasma samples were not defibrinated, since only 20 μ L of this plasma was added to 240 μ L of normal pool plasma in all experiments and microparticles adhere to fibrin.²⁵ To prevent interference by heparin, which is administered to the patient

during cardiac surgery, patient plasma samples were deheparinized by treatment with heparinase for 30 minutes at room temperature. The heparinase treatment is known to successfully remove up to 10 U/mL heparin from the plasma without interference with the thrombin generation assay (see Reference 26²⁶ ; also our data, not shown). Where indicated, deheparinized patient plasma (800 μ L) was centrifuged at 13 000g for 1 hour at room temperature. After centrifugation, 700 μ L of the plasma was carefully removed (supernatant plasma) by aspiration, and the pellet was resuspended in the remaining plasma (100 μ L). For some experiments, the plasma was entirely removed, the pellet washed once in 1 mL PBS containing 0.1% (wt/vol) BSA (PBS-BSA), centrifuged as described above, and resuspended in 100 μ L PBS-BSA. In all experiments, 20 μ L of patient plasma or of the washed pellet suspension was added to 240 μ L normal pool plasma. This ratio was used to provide a sufficient amount of plasma coagulation factors. At $t=0$, thrombin generation was triggered by the addition of 60 μ L CaCl_2 (17 mmol/L fc.) to a prewarmed (37°C) mixture of plasma (240 μ L) and buffer A (60 μ L; buffer A: 50 mmol/L Tris-HCl, 100 mmol/L NaCl; pH 7.35). At fixed intervals after $t=0$, 10 μ L were removed from this mixture and added to prewarmed (37°C) buffer containing 4 mmol/L of the chromogenic substrate S2238 and 20 mmol/L EDTA. After 3 minutes, the conversion of S2238 was stopped by the addition of 300 μ L citric acid (1.0 mol/L) and the generation of p-Nitroaniline was determined on a spectrophotometer at $\lambda=405$ nm. The thrombin generation curve is characterized by a lagtime, a transient rise of thrombin amidolytic activity and a partial return to baseline level. This curve is the sum of both generation of the prothrombin activating enzyme complex and inactivation processes, ie, the binding of thrombin to for example antithrombin III and α_2 -macroglobulin. From this curve the velocity of

prothrombin conversion can be calculated independent of thrombin inactivation processes. In some of our present experiments, part of buffer A (60 μ L) in the mixture with normal pool plasma was replaced by artificial phospholipid vesicles (20 μ L) or kaolin (20 μ L).

Preparation of Dynabeads

Streptavidine-coated dynabeads (1.0 mg) were washed four times with PBS-BSA according to the instructions provided by the manufacturer, ie, incubation for 2 to 3 minutes in a dynal MPC-E magnetic particle concentrator. Finally, the pellet was resuspended in either 570 μ L PBS containing biotin-labeled annexin V (100 μ g/mL) or 570 μ L PBS. After rotation for 60 minutes at room temperature, the beads were washed five times with 1 mL PBS-BSA, and stored until use (all manipulations at room temperature). Before use, beads were washed twice with 1 mL Tris-buffer (50 mmol/L Tris-HCl, 100 mmol/L NaCl, 0.05% (wt/vol) albumin and 17 mmol/L CaCl_2 ; pH 7.35). Then the pelleted beads were added to resuspended pellets of washed pericardial microparticles in a total volume of 150 μ L Tris-buffer. Subsequently, the samples were rotated for 1 hour at room temperature to remove the beads. The supernatant was stored for a maximal 2 hours at room temperature until use.

Statistical Methods

Data were analyzed with SPSS for Windows, release 6. Differences were considered statistically significant at $P < .05$. For direct comparison of the number of microparticles in blood samples, Wilcoxon matched-pairs signed-rank test was used. Student's t test for paired samples was used to compare the means of the number of microparticles present in systemic and pericardial blood collected simultaneously.

RESULTS

Platelet-Derived Microparticles in the Systemic Circulation and in Pericardial Blood

To investigate the presence of platelet-derived microparticles in the circulation of patients undergoing coronary bypass surgery, blood was collected as described in “Methods” and analyzed by whole blood flow cytometry. Fig 1 shows representative dot blots of blood samples collected at the end of the operation and obtained from a single patient. Blood collected from the pericardial cavity (Fig 1B) contained substantial numbers of microparticles that expressed the platelet identification marker GPIb (region R1) and blood simultaneously collected from the systemic circulation contained considerably less (Fig 1A). To determine the concentration of microparticles generated during coronary bypass surgery, in the systemic circulation as well as in pericardial blood, blood from 5 additional patients undergoing coronary bypass surgery was analyzed. Fig 2 shows the concentration of platelet-derived microparticles, corrected for hemodilution. The concentration of these microparticles in the circulation significantly increased during CPB ($P = .027$; Table 1). The concentration of such microparticles was significantly higher in the pericardial blood samples at collection points 3 and 4 (each $P = .028$).

Staining of Microparticles Generated In Vitro and In Vivo With Annexin V

To compare microparticles generated in vitro and in vivo for their annexin V binding properties, microparticles were generated in blood from healthy volunteers by incubation with [A23187](#) (in vitro microparticles), and compared with microparticles from pericardial

blood (in vivo generated microparticles). Fig 2 shows that microparticles generated in vitro (upper panels) stained positively for annexin V-FITC (FL-1 fluorescence, Fig 2A), as did microparticles isolated from pericardial blood (Fig 2C). In the absence of annexin V, no FL-1 fluorescence was observed (Figs 2B and 2D). Microparticles isolated from systemic blood yielded similar results.

Identification of Microparticles in Systemic and Pericardial Blood

To provide direct evidence that (part of) the microparticles present in systemic and pericardial blood were platelet-derived, double labeling experiments, in which anti-GPIb-directed MoAb was used in combination with annexin V, were performed on isolated microparticles. In contrast to whole blood, however, microparticles were negative for GPIb. Since it is known that GPIb is highly susceptible to proteolysis,^{27,28} we considered the possibility that during isolation GPIb was lost from microparticles. Therefore, anti-GPIIIa (CD61) was used instead of anti-GPIb. GPIIIa is part of the integrin GPIIb/IIIa complex, which is present only on platelets and megakaryocytes. When isolated microparticles from systemic blood were incubated with anti-CD61-FITC, approximately 90% of the total microparticle fraction was found to be positive for CD61 (Fig 3B), in comparison to an FITC-labeled isotype control antibody (Fig 3A). On incubation with PE-labeled annexin V, about 90% of the microparticles bound this indicator for PS-exposure as shown by an increase in FL-2 fluorescence (Fig 3D), which was not observed in the absence of annexin V (Fig 3C). When neither antibody nor annexin V was added, all microparticles were in the lower left quadrant, indicating that no autofluorescence occurred (Fig 3E). In the presence of both anti-CD61 and annexin V, more than 90% of the microparticles bound

CD61 as well as annexin V (Fig 3F). When IgG₁-FITC was added in combination with annexin V-PE, no FL-1 fluorescence was observed and only a major increase in FL-2 fluorescence occurred (not shown).

About 25% of the microparticle fraction of pericardial blood bound anti-CD61-FITC (Fig 4B) when compared with control IgG₁-FITC (Fig 4A). The number of microparticles that bound annexin V-PE varied in different experiments from 44% to 58% and was lower than annexin V-FITC (Figs 4C and 4D; compare with Fig 2C). When microparticles were double-labeled with anti-CD61-FITC and annexin V-PE, almost all platelet microparticles, ie, positive for anti-CD61-FITC, bound annexin V-PE (Fig 4F; top right quadrant). Thus in contrast to systemic blood that predominantly contained platelet-derived microparticles, microparticles in pericardial blood were derived from other cell types as well. As shown in Figs 4I and 4J, pericardial blood also contained microparticles that bound anti-glycophorin A-FITC, indicating that these microparticles originated from erythrocytes. About 45% to 50% of the total microparticle population bound this antibody and in addition bound annexin V-FITC in double-labeling experiments (not shown). Thus, microparticles in pericardial blood are predominantly of platelets and erythrocyte origin. Preliminary data indicated that a small population of about 5% of the total microparticle fraction bound anti-CD14-PE, and, therefore, likely originated from monocytes or granulocytes (Fig 4G and 4H). Approximately 20% of the microparticles were of as yet unknown cellular source.

Thrombin Generation by the Total Microparticle Population

Upon addition of Ca²⁺-ions to pooled normal plasma only a modest amount of thrombin was generated after a lagtime of 5 to 6 minutes

(Fig 5A). Incubation of pooled normal plasma with both Ca^{2+} -ions and artificial phospholipid vesicles, which provide a negatively charged surface that facilitates the binding of coagulation factors, shortened the lagtime to 159 ± 30 seconds ($n=6$; $\text{mean} \pm \text{SD}$) and thrombin was generated ($\text{ETP} = 425 \pm 53$ nmol/L; $n=6$). In the presence of artificial phospholipid vesicles and kaolin, a trigger of the intrinsic factor XII-dependent coagulation pathway, a similar amount of thrombin was generated ($\text{ETP} = 443 \pm 25$ nmol/L), but with a shorter lagtime (66 ± 12 seconds). On addition of deheparinized patient plasma ($20 \mu\text{L}$) collected at study point 3 from the systemic circulation, the capacity of pooled normal plasma to generate thrombin slightly increased (Fig 5B). When a similar amount of pericardial plasma was added, the lagphase was shorter and the capacity to generate thrombin was increased. To demonstrate that the thrombin-generating activity of the patient plasma samples was due to the presence of microparticles, plasma samples were centrifuged for 1 hour after treatment with heparinase as described in "Methods." The microparticle-rich pellet was resuspended in microparticle-poor plasma (see "Methods"). Fig 5C shows the effect of the addition of either microparticle-poor plasma or the microparticle-enriched plasma to pooled normal plasma on the thrombin generation. Addition of the systemic microparticle-poor patient plasma did not support the generation of thrombin (Fig 5C). In contrast, addition of the microparticle-enriched systemic plasma caused a considerable increase in thrombin generation in normal plasma, although still a lagphase of 4 to 5 minutes occurred. When the microparticle-poor pericardial plasma was added, a minor increase in thrombin generation was observed compared with the control of pooled normal plasma (Fig 5D). Addition of an equal volume of the microparticle-enriched pericardial plasma induced a marked increase in the capacity to generate thrombin and the

lagphase shortened to less than 1 minute. To obtain further evidence for involvement of microparticles in thrombin generation, isolated microparticles (see “Methods”) were absorbed with uncoated- or annexin V–coated beads to remove PS-carrying microparticles. After removal of the beads, the remainder of the microparticle fraction was used to determine the ability to support thrombin generation. This experiment was performed twice with isolated pericardial microparticles, obtained from two different donors. When treated with uncoated beads, the amount of thrombin generated decreased from 347 (untreated control) to 294 nmol/L (donor 1) and from 346 to 246 nmol/L for donor 2. When treated with annexin V–coated beads, the ETP decreased more extensively to 137 and 86 nmol/L (donor 1) and to 129 and 146 nmol/L (donor 2).

Coagulation Pathway Involved in Thrombin Generation by Microparticles

Additional experiments were performed to determine whether the observed generation of thrombin in pooled normal plasma in the presence of pericardial pellet was due to stimulation of the intrinsic (factor XII) or extrinsic (factor VII) pathway of coagulation. We washed the pellet derived from the pericardial sample in buffer to remove the remaining plasma and thus exclude the presence of (activated) coagulation factors from that source. Typical results are presented in Figs 6B and 6C and the overall results in Table 2. When the washed pellet was added to factor XII–deficient plasma (n=6), no inhibition of thrombin generation or increase in lagtime was observed when compared with normal pool plasma (Table 2). Also, preincubation with MoAb OT-2, which functionally inhibits factor XII and factor XIIa, had no effects, whereas this antibody strongly delayed the kaolin-induced procoagulant activity of normal plasma

(not shown). These findings suggested that factor XII plays no major role under these conditions. On the other hand, when the pellet was added to factor VII–deficient plasma, the lagphase increased from 26 to 152 seconds, suggesting an involvement of the tissue factor/factor VII pathway. Further evidence for a role of factor VII was provided by the finding that no thrombin was generated when the pellet was added to pooled normal plasma in the presence of tissue factor pathway inhibitor (n=6; 0.4 $\mu\text{mol/L}$, final concentration).

DISCUSSION

CPB surgery induced a slight increase in the number of platelet-derived microparticles in systemic blood in all patients. However, the present study shows that cell-derived microparticles are not only present in the systemic circulation but also at higher levels in pericardial blood. Interestingly, the highest levels of microparticles in pericardial blood were found at sampling point 3, which is returned via the cardiectomy reservoir into the patients between sampling points 3 and 4. Thus, we propose that at least part of the observed increase in the number of platelet-derived microparticles in the systemic circulation reflects re-infusion of the pericardial blood. However, at present we cannot exclude the possibility that part of the platelet-derived microparticles in the systemic circulation are formed due to contact between blood and the artificial surface of the extracorporeal circuit, as has been shown to occur *in vitro*.²⁹ Conversely, the much higher concentration in pericardial blood virtually excludes the possibility that these microparticles originate from contact of blood with the extracorporeal circuit. More likely they were generated on blood contact with extravascular tissue in the pericardial cavity.

Preliminary flow cytometry experiments indicated that pericardial plasma not only contained microparticles of platelet and erythrocyte origin, but also microparticles derived from monocytes or granulocytes, which express CD14. Monocytes stimulated in vitro by endotoxin shed microparticles that express tissue factor and expose PS.⁸ Thus the presence of CD14-expressing microparticles in pericardial plasma may explain the presence of tissue factor in our experiments. Further characterization of these microparticles is needed and presently ongoing. About 20% of the microparticles in pericardial blood remain to be identified. The finding that the systemic circulation almost exclusively contains platelet-derived microparticles, suggests that erythrocyte- and CD14-positive microparticles may be rapidly cleared from the systemic circulation.

To determine the thrombin generating capacity of the various plasma samples and fractions, a thrombin generation assay was used. This assay has been used previously to determine the thrombin-generating capacity in plasma, platelet-rich plasma, and whole blood. The thrombin-generating capacity was shown to be increased in women using oral contraceptives, in patients with active venous thrombosis, in young stroke patients and in patients suffering from an antithrombin deficiency, whereas it is decreased in healthy volunteers using aspirin and is inhibited by activated protein C.^{24,30} Our data indicate that the in vivo generated microparticles support thrombin generation via a tissue factor/factor VII-mediated pathway, since (1) the microparticles generated thrombin much slower in factor VII-deficient plasma than in normal pool plasma, and (2) TFPI inhibited thrombin generation completely. In contrast to factor VII-deficient plasma, no inhibition was observed when the microparticle-enriched fraction was added to factor XII-deficient plasma, suggesting that thrombin generation under these conditions is factor

XII independent. This was confirmed with OT-2, a MoAb that inhibits factor XII and XIIa and which failed to affect microparticle-mediated thrombin generation. Recently, Chung and coworkers demonstrated that tissue factor is elevated on mononuclear cells in pericardial blood during CPB, accompanied by high levels of prothrombin fragment F_{1+2} and a high ratio of factor VIIa:factor VII.²¹ They concluded that expression of tissue factor, activation of the extrinsic coagulation pathway and thrombin formation occur predominantly in pericardial blood, and it was postulated that “the wound activated the extrinsic coagulation pathway during CPB by producing procoagulant cells and enzymes that enter the general circulation.” We agree with this statement but propose that this activation is at least partly microparticle-mediated.

It may be wondered whether the procoagulant activity is solely due to the expression of tissue factor or also partly dependent on the presence of PS and other negatively charged phospholipids on for instance the platelet-derived microparticles. When the microparticle-enriched fraction was preincubated with annexin V-coated beads, the thrombin-generating capacity was reduced when compared with uncoated beads but not completely inhibited. No additional inhibition was observed when up to 2.5 mg/mL annexin V-coated beads were added, indicating that the incomplete inhibition was not due to an insufficient amount of beads (not shown). Artificial phospholipid vesicles only bind annexin V when they express more than 5% PS.³¹ At first glance, the procoagulant activity therefore seems to be dependent on the presence of both tissue factor and the negatively charged phospholipids. However, we cannot exclude the possibility that preincubation with annexin V-coated beads removed the particles that not only expressed PS but also tissue factor. Further studies will therefore be necessary.

Despite the fact that the number of microparticles in the systemic circulation of the patients undergoing heart surgery increased gradually, severe thrombotic effects are not associated with this procedure. This is presumably related to the fact that patients undergoing heart surgery receive high doses of heparin in the systemic circulation (2 to 3 IU/mL), yielding concentrations in the pericardial blood of about 1 IU/mL.²⁰

The size of the microparticles cannot be estimated by flow cytometry, because the resolution of the size measurement is limited by the wavelength (488 nm) of the flow cytometer to particles of 0.6 µm and larger.

In summary, the present results demonstrate that procoagulant microparticles are generated during coronary bypass surgery, especially in pericardial blood, which support coagulation via a tissue factor/factor VII-mediated pathway. Thus pericardial blood may provide a unique tool to study functional properties of microparticles generated in vivo.

SELECTED ABBREVIATIONS AND ACRONYMS

BSA	=	bovine serum albumin
CPB	=	cardiopulmonary bypass
ETP	=	endogenous thrombin potential
MoAb	=	monoclonal antibody
PBS	=	phosphate-buffered saline
PS	=	phosphatidylserine
TFPI	=	tissue factor pathway inhibitor

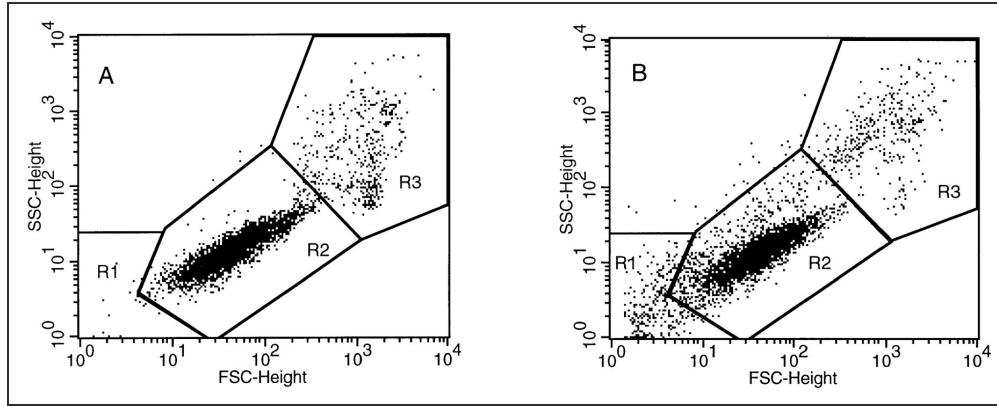


Figure 1. Representative dot plots of platelets and platelet-derived material in systemic (A) and pericardial blood (B). Blood was obtained from the systemic circulation and from the pericardial cavity at the end of the bypass procedure before release of the aortic clamp. These dot plots are typical examples of FSC/SSC plots obtained with blood samples from the same donor. Blood was analyzed by flow cytometry for GPIb-positive cells and particles as described in “Methods.” The regions R1, R2, and R3, therefore, represent platelet-derived microparticles (R1), platelets (R2), and platelet-platelet, or platelet–white blood cell aggregates and possibly platelet-derived microparticle-leukocyte complexes (R3). Identical results were obtained with the other five patients.

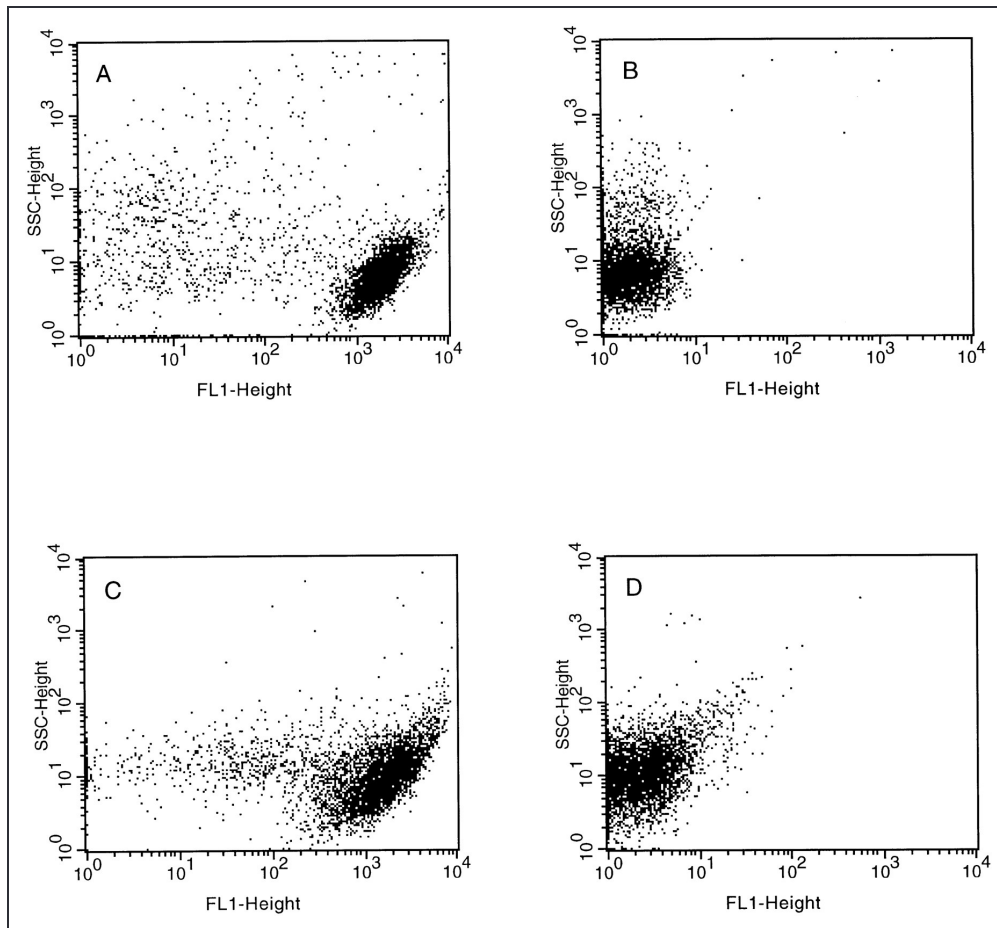


Figure 2. Exposure of PS on microparticles. Blood obtained from healthy volunteers was stimulated with the Ca^{2+} -ionophore A23187 (2.5 $\mu\text{mol/L}$) and microparticles were isolated and incubated with (A) or without (B) annexin V-FITC (20 nmol/L) in apopbuffer as described in “Methods.” Similarly, microparticles isolated from pericardial plasma at the study point 3 were incubated with (C) or without (D) FITC–annexin V. The dot plots shown are representative examples of SSC/FL-1 plots and were obtained within one experiment.

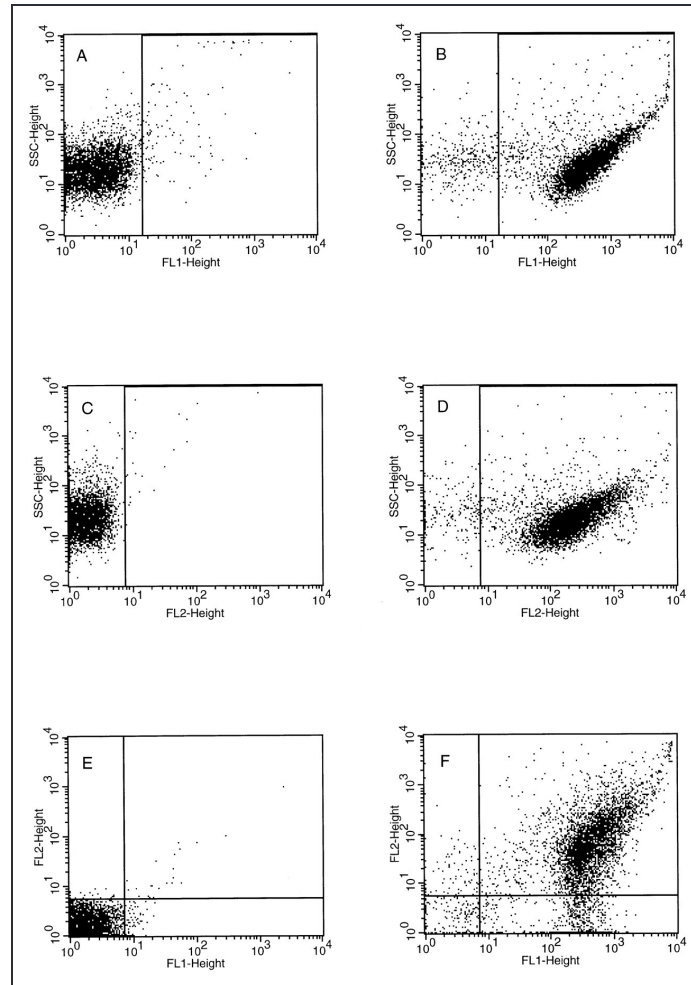


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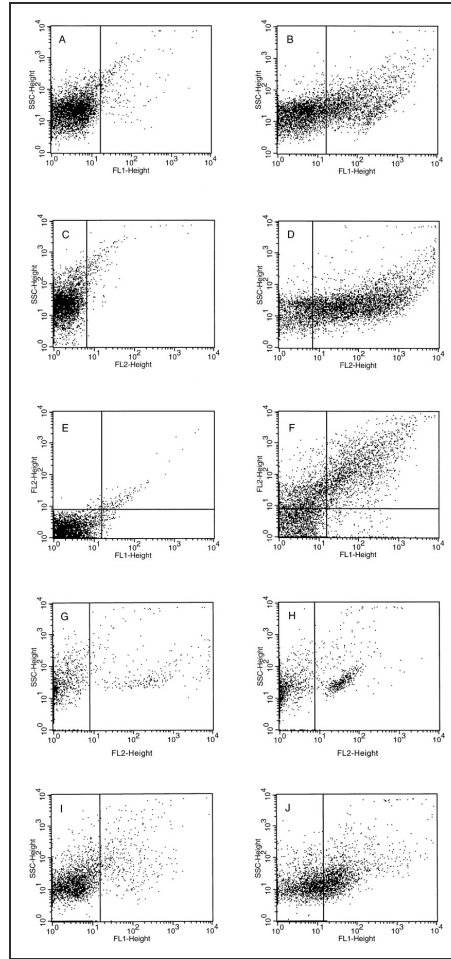


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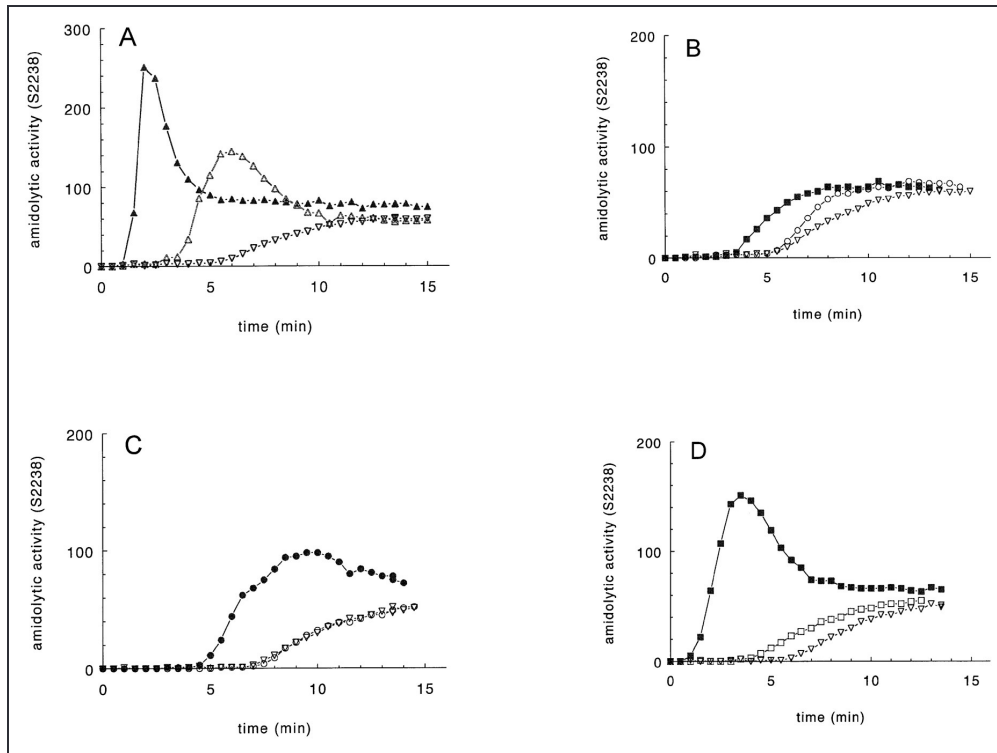


Figure 5. Thrombin generation in pooled normal plasma (A) or in pooled normal plasma supplemented with plasma samples obtained from patients undergoing heart surgery before (B) and after (C, D) high-speed centrifugation. A, Thrombin generation in pooled normal plasma on addition of Ca^{2+} -ions alone (∇ ; shown as control in A through D), or in the presence of artificial phospholipid vesicles (Δ), or artificial phospholipid vesicles and kaolin (\blacktriangle). B, Thrombin generation in normal pool plasma (240 μL), supplemented with deheparinized patient plasma (20 μL ; systemic plasma [\circ]; pericardial plasma [\blacksquare]). Systemic (C) and pericardial plasma (D) were subjected to high-speed centrifugation as described in "Methods." Subsequently, 20 μL of the high speed plasma samples (\circ , \square) and the resuspended pellet samples (\bullet , \blacksquare), prepared as described in "Methods," were added to pooled normal plasma. The tracings represent typical examples and were obtained from a single experiment. A total number of 3 experiments were performed.

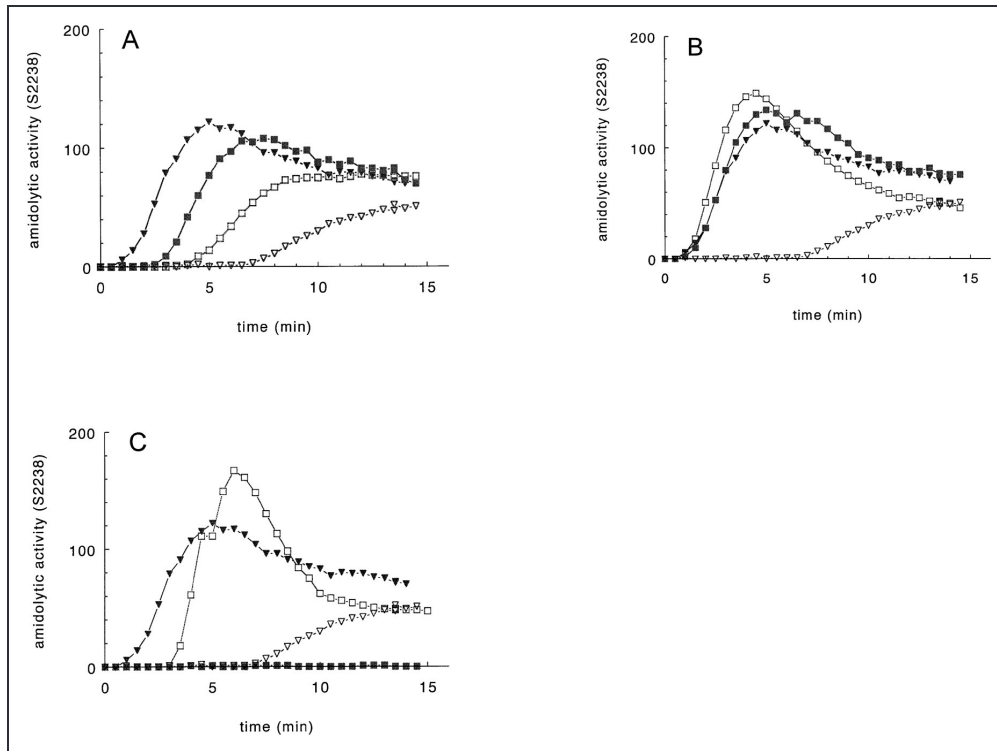


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- 1 All data are presented as median (range), n=6.
- 2 $P < .03$ (Wilcoxon matched-pairs signed-rank) when compared with the first systemic blood sample;
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- 4 $P < .03$ when compared with the corresponding systemic blood samples. Blood from 6 patients undergoing heart surgery was collected after anesthesia (1), 5 to 10 minutes after start of CPB (2), 10 minutes before (3), and after (4) release of the aortic clamp at the end of the bypass procedure. Blood was analyzed by flow cytometry as described in "Methods" for GPIIb-positive particles. The number of microparticles was calculated as described in "Methods."

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ETP indicates endogenous thrombin potential; TP, thrombin potential; (n=6).

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Sections

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 4. Flow Cytometric Analysis
 5. Preparation of Microparticles In Vitro and Staining With Annexin V-FITC
 6. Identification of Microparticles by Flow Cytometry
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3. Results
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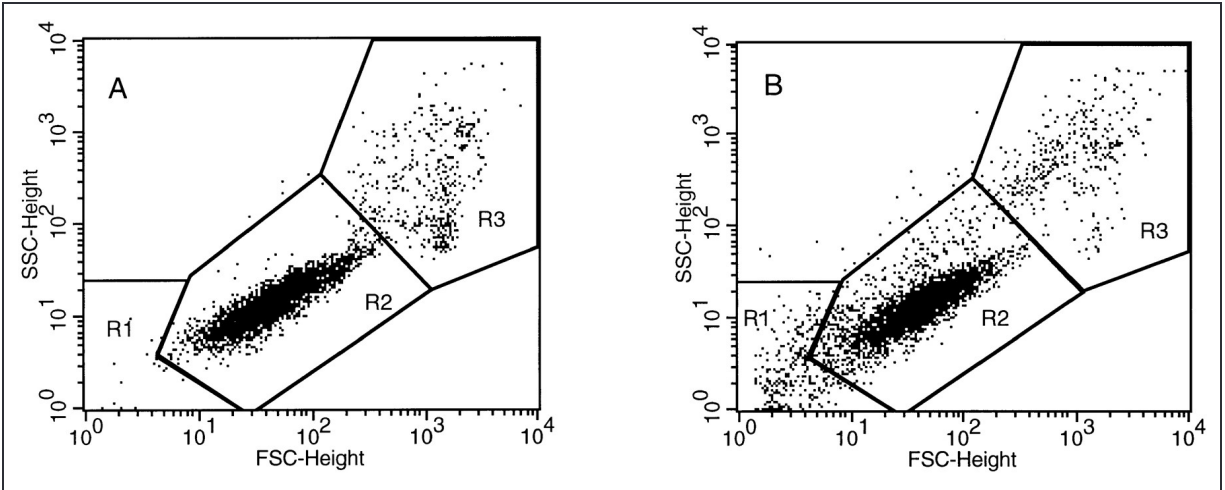


Figure 1. Representative dot plots of platelets and platelet-derived material in systemic (A) and pericardial blood (B). Blood was obtained from the systemic circulation and from the pericardial cavity at the end of the bypass procedure before release of the aortic clamp. These dot plots are typical examples of FSC/SSC plots obtained with blood samples from the same donor. Blood was analyzed by flow cytometry for GPIb-positive cells and particles as described in “Methods.” The regions R1, R2, and R3, therefore, represent platelet-derived microparticles (R1), platelets (R2), and platelet-platelet, or platelet–white blood cell aggregates and possibly platelet-derived microparticle-leukocyte complexes (R3). Identical results were obtained with the other five patients.

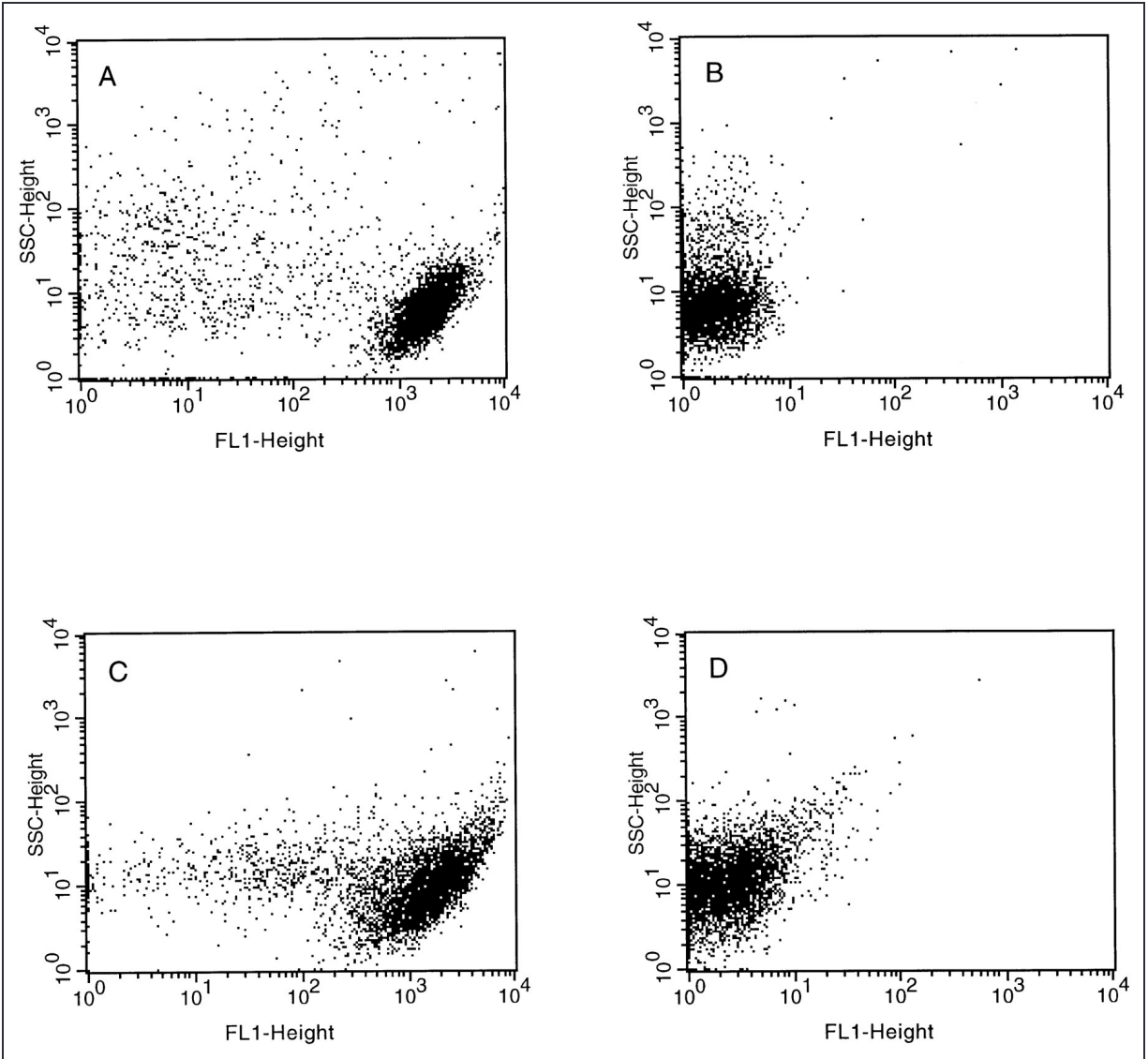


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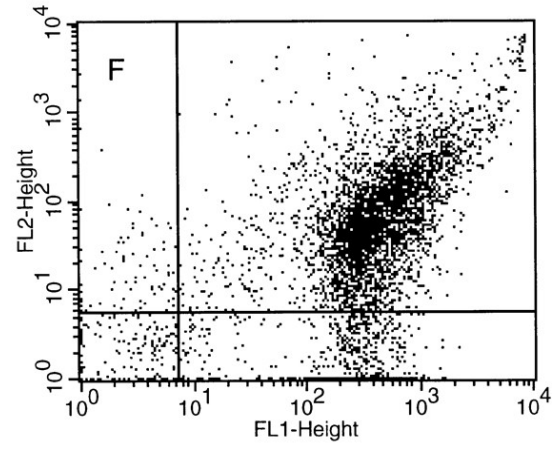
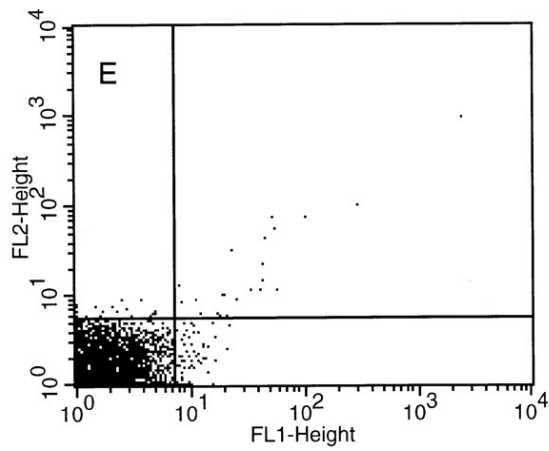
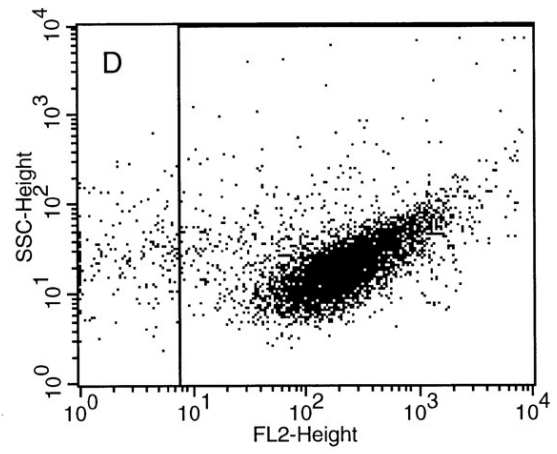
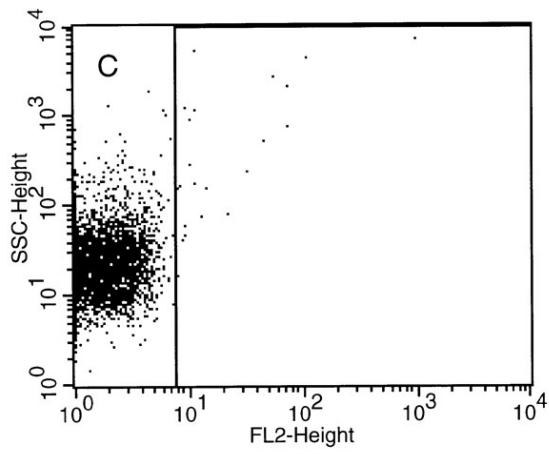
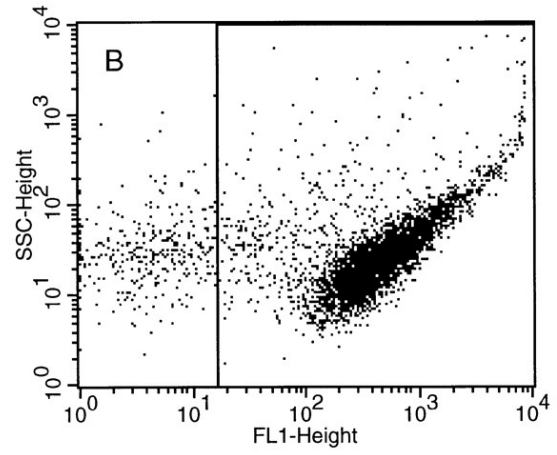
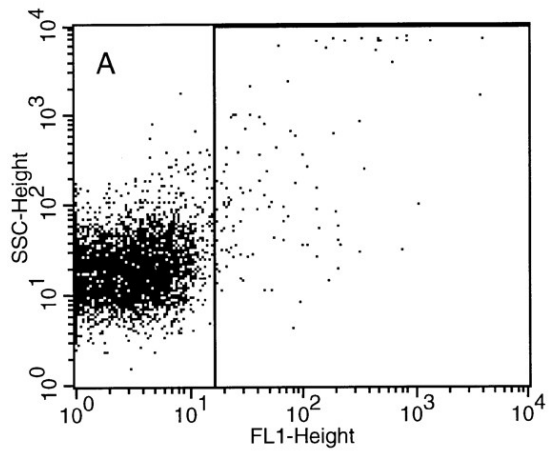
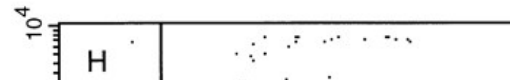
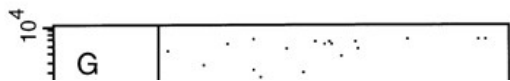
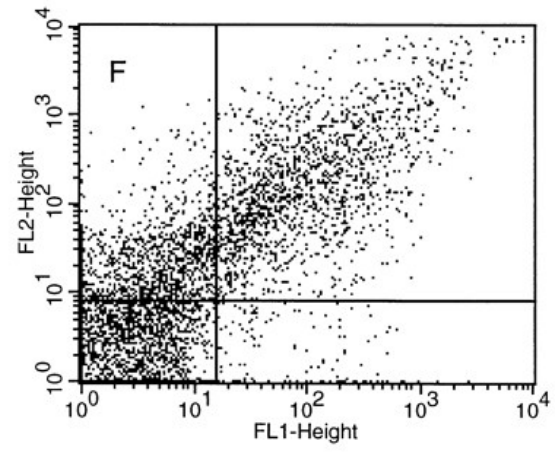
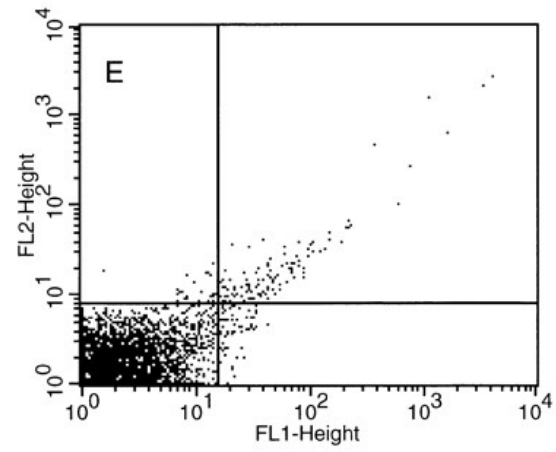
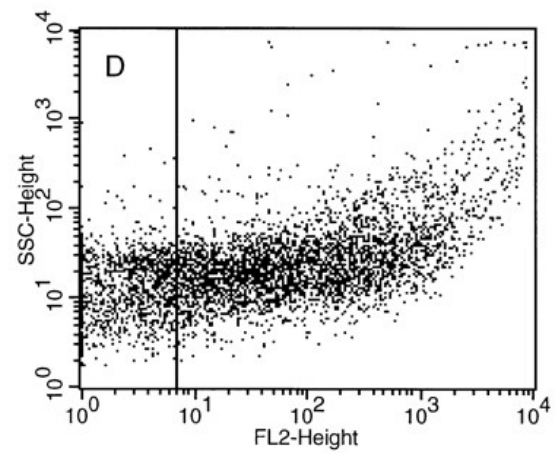
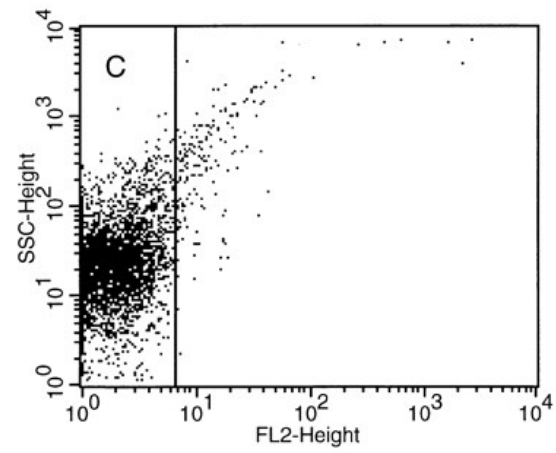
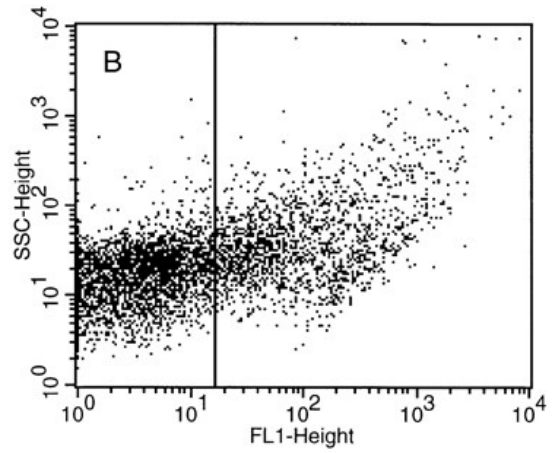
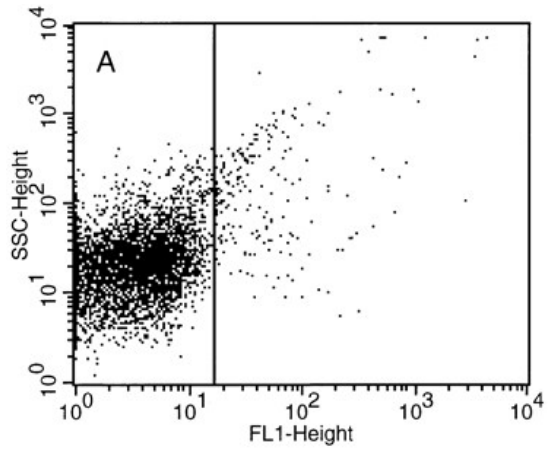


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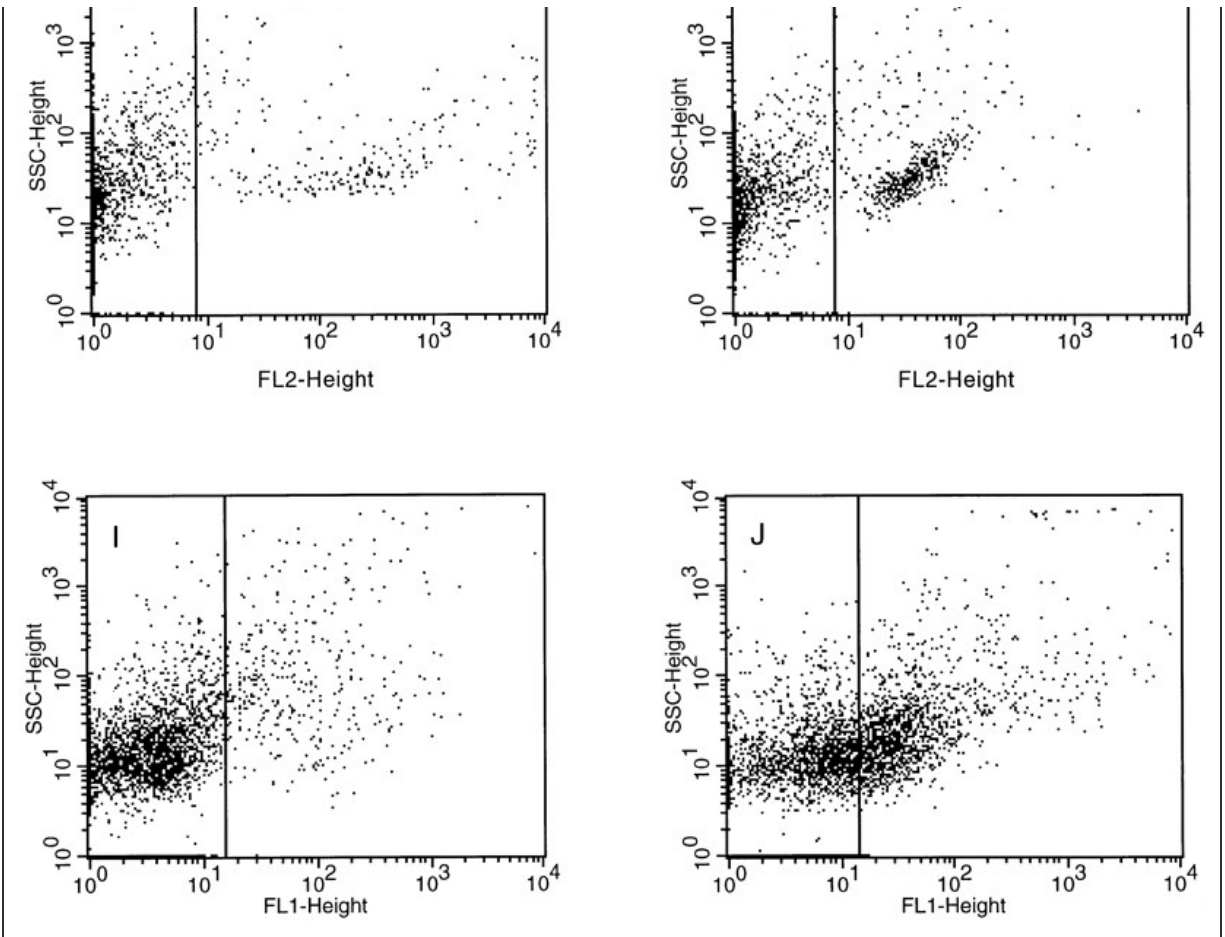


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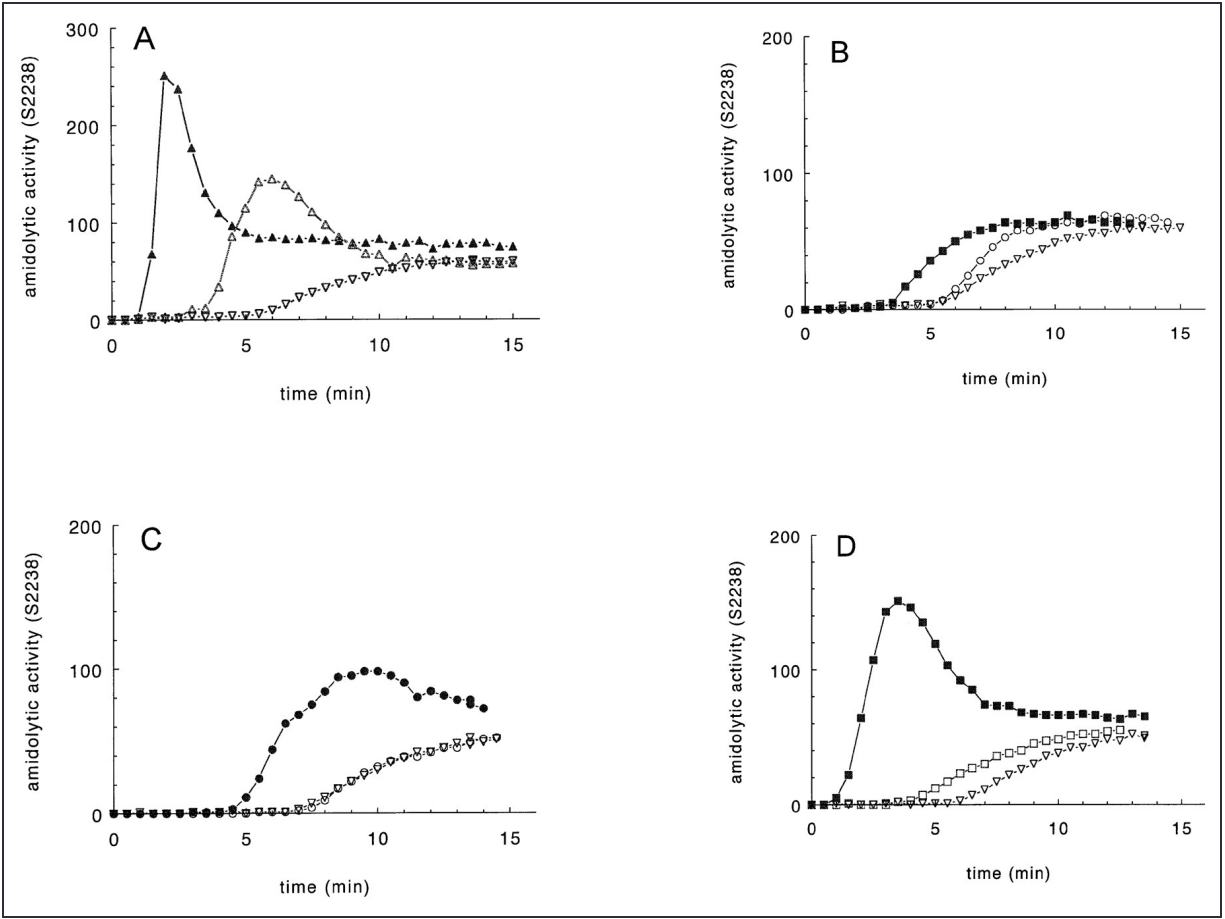


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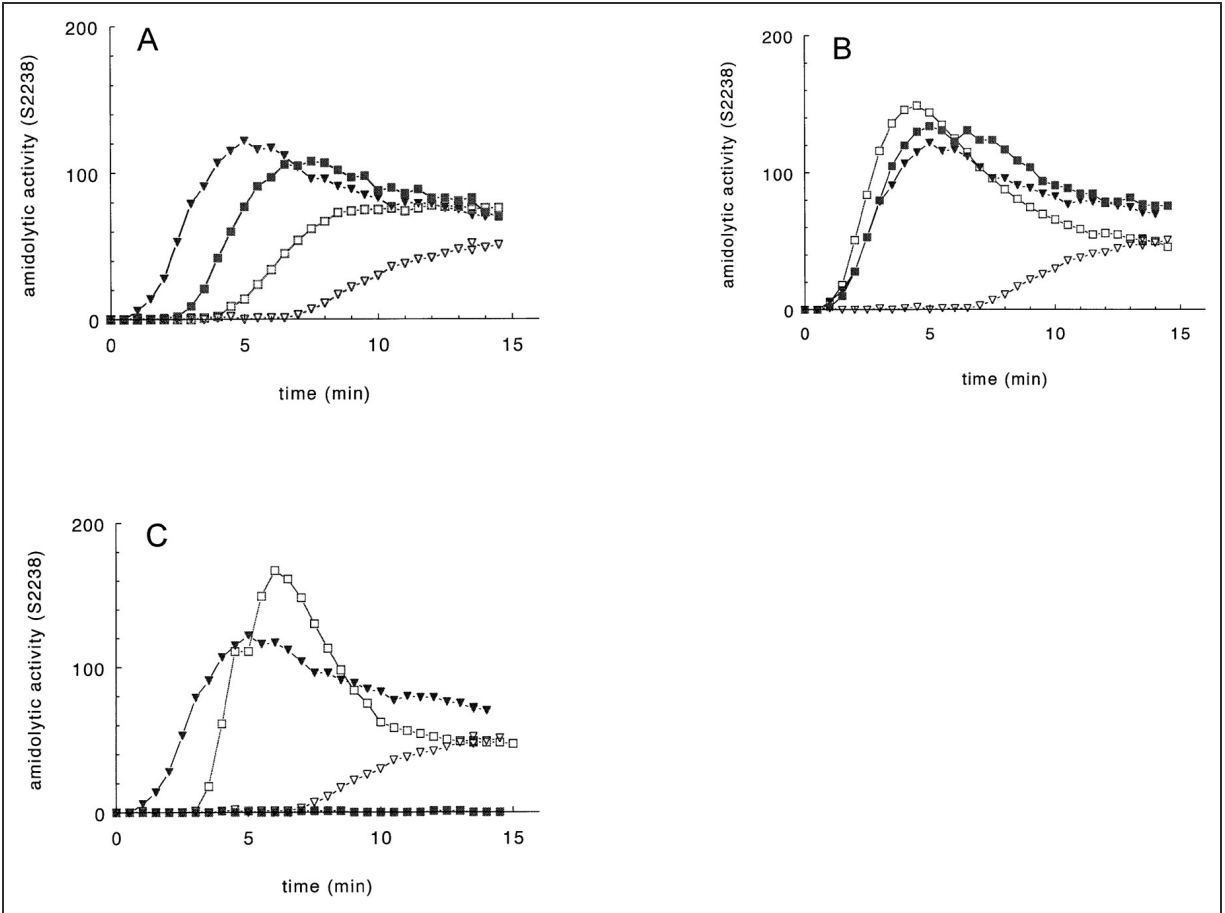


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CPB	=	cardiopulmonary bypass
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PBS	=	phosphate-buffered saline
PS	=	phosphatidylserine
TFPI	=	tissue factor pathway inhibitor

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