Acute surface-induced thrombosis in the canine *ex vivo* model: Importance of protein composition of the initial monolayer and platelet activation

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The initial events occurring at the blood–polymer interface were examined using a canine *ex vivo* arteriovenous shunt model. Thrombogenic (fibrinogen) and nonthrombogenic (albumin) proteins were preadsorbed on poly(vinyl chloride), polyethylene, and silicone rubber shunt surfaces, and the blood responses were analyzed using the platelet deposition profile as an indicator of surface thrombogenicity. The distributions of preadsorbed protein molecules on the various polymer surfaces were studied using an immunogold bead (colloidal gold particles coated with antibodies) staining technique and shown to be homogeneous. A sequential protein adsorption technique was developed to probe the nature of competitive protein adsorption and to observe the effect of surface protein concentration on thrombogenicity. The thrombogenicity of a surface was determined by the composition of the initial protein layer rather than the total concentration of protein on the polymer surfaces. The composition of this layer determines the extent of platelet activation and the adhesive strength between platelets and the polymer surface.

INTRODUCTION

Surface-induced thrombosis is a major complication in the development of blood-contacting prosthetic devices. Typically, this phenomenon has been studied by measuring the number of platelets that adhere to a test material. A traditional premise is that the protein molecules that are adsorbed on artificial surfaces upon exposure to blood control surface thrombogenicity.[1,2] When a biomaterial is exposed to anticoagulated blood or to an anticoagulated platelet suspension, under controlled flow conditions or in a static system, only 20–30% of the available surface area is covered by single platelets[3] and the number of adhered platelets is always less than 90/1000 μm^2.[4–8] *In vivo* or *ex vivo*, adhesion of single platelets invariably occurs on artificial materials and is often followed by the appearance of gradually enlarging masses of attached platelets, which apparently develop by accretion.[9] It is the formation of thrombi that distinguishes thrombogenic

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from thromboresistant surfaces. It was suggested by Turitto et al.[3] that surface coverage with an incomplete layer of platelets is due to inhibited platelet spreading and that maximal attachment and thrombus formation necessitates the transformation of platelets from the contact to the spread form. Thus, it appears that the measurement of platelet transformation and thrombus formation is necessary to accurately determine surface thrombogenicity.

The canine *ex vivo* arteriovenous (A-V) shunt model developed in this laboratory allows continuous quantitative monitoring of thromboembolization with nonanticoagulated blood under the influence of shear flow. The presence of flow is especially important in studying thrombus formation and embolization.[10] The flow field not only provides a continuous supply of platelets to form thrombi, but it also assists in thrombus removal from the surface. It is thus expected that the adhesive strength between thrombi and a polymer surface determines in part the size of thrombi released from the surface. The adhesive strength may well be related to the plasma protein layer that forms before platelet adhesion. In the present study, we examined the effects of competitive protein adsorption and the distribution of protein in the initial protein layer on platelet spreading and the extent of artificial surface-induced thrombogenesis.

MATERIALS AND METHODS

Protein preparation

Canine fibrinogen, prepared from fresh citrated plasma by beta-alanine precipitation[11] and commercially obtained human fibrinogen,* purified by the method of Laki,[12] were further purified to remove fibronectin by using a gelatin-agarose column,† which was equilibrated with phosphate-buffered saline (PBS, pH 7.4) without divalent cations. The purified fibrinogen showed clottability of at least 97% as measured by the method of Coller.[13] Crystalline human and canine albumin‡ were used as received. The absorptivities used for 0.1% protein solutions at 280 nm were 0.58 for albumin[14] and 1.506 for fibrinogen.[15] Human fibrinogen and albumin were used as controls for canine proteins in immuno-gold bead labeling studies where antibodies against human proteins were used. Canine serum was prepared from 30 mL of nonanticoagulated whole blood, which was clotted for 3 h at 37°C. The clot was removed by centrifugation and the supernate was filtered through a 0.22-μm filter.§ The filtered serum was diluted 8 times with PBS and used to precoat a polymer shunt.

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†Bio-Rad, Richmond, California.
‡Sigma, St. Louis, Missouri.
§Millipore, Bedford, Massachusetts.

Preparation of radiolabeled species

Fibrinogen and albumin were radiolabeled by ^{125}I[‖] using the Chloramine-T (Iodo-Beads[¶]) method. Free iodide was separated from labeled protein on a gel column (Bio-Gel P30, 0.7 × 30-cm Econo Column[**]) equilibrated with PBS.

Preadsorption of proteins on polymer shunts

The polymer shunts used were plasticized polyvinyl chloride (PVC) (Tygon[††], 0.125 in. i.d.), polyethylene (PE)[‡‡] (0.125 in. i.d.), and silicone rubber (SR)[§§] (0.132 in. i.d.). Shunts were cut into 70-in. lengths and washed with running distilled deionized water for 2 h. The PVC shunt was first washed at room temperature with 200 mL of 0.1% Ivory detergent and then washed like the other tubings. The washed shunts were filled with PBS and kept at 4°C overnight. The PBS was displaced by 30 mL of protein solution, which remained in the shunt at room temperature for 2 h before implantation. Bulk protein concentrations used for preadsorption were 0.3 mg/mL for both fibrinogen and albumin. For competitive protein adsorption, a mixture of albumin and fibrinogen at a concentration of 0.3 mg/mL for each protein was used. Canine serum was also precoated as described above. At the end of the protein adsorption period, the bulk protein solution was removed by flushing with 50 mL of PBS, and the shunt filled with the buffer was implanted.

Sequential protein adsorption

Polymer shunt surfaces were sequentially preadsorbed with albumin and fibrinogen. The polymer shunts were preadsorbed with one protein, either fibrinogen or albumin, for a given time period and then flushed with PBS. The same shunt was then further exposed to the second protein. The protein adsorption time for the first protein was varied from 1 min to 1 h (Table I). The total time for protein adsorption was 2 h at room temperature. The amount of each protein adsorbed to the shunt surface was measured by using radiolabeled protein. The shunt was flushed with buffer, implanted in a dog, and the transient platelet deposition was measured as described below.

[‖]New England Nuclear, Boston, Massachusetts.
[¶]Pierce Chemical, Rockford, Illinois.
[**]Bio-Rad, Richmond, California.
[††]Norton Plastics, Akron, Ohio.
[‡‡]Intramedic, Parsippany, New Jersey.
[§§]Dow Corning, Midland, Michigan.

TABLE I
Surface Concentrations for Sequential Adsorption of Canine Albumin and Fibrinogen

First protein adsorbed		Second protein adsorbed		Surface concentration ($\mu g/cm^2$)[a]			
				Albumin		Fibrinogen	
Type	Adsorption time (min)	Type	Adsorption time (min)	PVC	PE	PVC	PE
Albumin (0.3 mg/mL)	1	Fibrinogen (0.3 mg/mL)	119	0.20 ± 0.01	0.13 ± 0.04	0.61 ± 0.06	0.33 ± 0.08
	3		117	0.26 ± 0.04	0.17 ± 0.01	0.53 ± 0.40	0.28 ± 0.07
	5		115	0.28 ± 0.05	0.18 ± 0.02	0.79 ± 0.20	0.25 ± 0.06
	10		110	0.32 ± 0.05	0.20 ± 0.04	0.41 ± 0.20	0.23 ± 0.15
	30		90	0.41 ± 0.01	0.25 ± 0.01	0.34 ± 0.20	0.07 ± 0.01
	60		60	0.45 ± 0.03	0.25 ± 0.02	0.20 ± 0.04	0.03 ± 0.01
	120		0	0.57 ± 0.07	0.32 ± 0.05	—	—
Fibrinogen (0.3 mg/mL)	1	Albumin (0.3 mg/mL)	119	0.25 ± 0.04	0.15 ± 0.05	0.32 ± 0.07	0.21 ± 0.07
	3		117	0.15 ± 0.04	0.05 ± 0.02	0.31 ± 0.10	0.24 ± 0.07
	5		115	0.16 ± 0.06	0.06 ± 0.05	0.32 ± 0.10	0.27 ± 0.07
	10		110	0.12 ± 0.07	0.03 ± 0.02	0.40 ± 0.08	0.28 ± 0.07
	30		90	0.10 ± 0.01	0.06 ± 0.05	0.60 ± 0.02	0.37 ± 0.08
	60		60	0.06 ± 0.01	0.02 ± 0.01	0.62 ± 0.05	0.37 ± 0.04
	120		0	—	—	0.79 ± 0.09	0.43 ± 0.04

[a]Mean ± S.D. ($n = 4$).

Measurement of platelet deposition on shunt surfaces

The canine *ex vivo* A-V shunt model and the procedure for platelet counting on shunt surfaces have been described previously.[16] Briefly, platelets were obtained from adult mongrel dogs weighing 20–35 kg and radiolabeled with ^{51}Cr by the method of Abrahamsen.[17] Radiolabeled platelets were reinfused into the dog 15 h before the experiment. The tubing was implanted as a femoral A-V shunt with the middle of the shunt wrapped around a solid crystal detector and blood flow was started. For the measurement of platelet deposition, blood flow was stopped by clamping the artery with a vascular clamp. At the same time, buffer was introduced via a branch artery at a flow rate of 60 mL/min to remove the blood in the shunt. Platelet deposition was quantitated by measuring the ^{51}Cr radioactivity on the shunt surface after flushing the shunt. A small segment of the shunt was removed at each time point and prepared for scanning electron microscopy. Platelet deposition was measured and samples were taken after blood exposure for 2, 5, 10, 15, 30, 45, 60, 90, and 120 min of blood contact. The blood flow rate was 150–250 mL/min.

Visual observation of thrombus formation on shunt surfaces

When polymer shunts were preadsorbed with fibrinogen, at certain time points the thrombi that were formed were large enough to be observed with the unaided eye. White thrombi were observed when the shunt was flushed with buffer solution, and the sites of thrombi formation were marked on the outside of the tubings with a felt-tip pen. Blood flow was resumed and the shunt was flushed again with the buffer. No thrombi were seen on the sites marked with the pen, and new thrombi formed on other sites; 35-mm photographs of these observations were taken.

Ex vivo protein desorption study

Radiolabeled fibrinogen or albumin was preadsorbed onto polymer surfaces as described above and desorption from the shunt was measured after implantation in a dog. The shunt was wrapped around a solid crystal detector and the decrease in radioactivity during blood flow was continuously monitored by counting for 20 s every minute. Desorption of precoated protein was also measured *in vitro*. Desorption was monitored continuously by counting the radioactivity remaining on the shunt surface during flushing with PBS at a flow rate of 100 mL/min. In other experiments the effect of a proteolytic enzyme on protein desorption was evaluated by adding plasmin* to the flushing buffer at a final concentration of 0.0015 U/mL. The *in vitro* experiments were carried out at room temperature.

*Sigma, St. Louis, Missouri.

Immuno-gold bead labeling of the protein layer

Colloidal gold particles with an average diameter of 18 or 50 nm and antibody–gold complex (immuno-gold) were prepared as described by Loftus and Albrecht.[18] Immuno-gold particles coated with antibodies against human albumin or fibrinogen were introduced to the human or canine protein-covered polymer shunt surfaces. For the labeling of polymer shunts, which were exposed to flowing blood *ex vivo*, the shunts were prefixed with 0.05% glutaraldehyde in PBS for 15 min at room temperature and treated with 1% glycine solution for 10 min. The prefixation was necessary to preserve platelet shape during immuno-gold bead staining. After 30 min at room temperature, the immuno-gold beads were flushed with PBS and fixed with 2% glutaraldehyde. Preliminary experiments showed that antibodies against human proteins react with canine proteins. The glutaraldehyde fixed samples were further processed for scanning electron microscopic examination.

Scanning electron microscopy

Samples from the canine *ex vivo* experiments and gold bead experiments were fixed for 24 h in 2% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4, at 4°C. Samples were then serially dehydrated in ethanol and critical point dried using CO_2 as the transitional fluid. Samples were coated with gold and examined with a JEOL JSM 35C scanning electron microscope (SEM) at the accelerating voltage of 10–20 kV.

RESULTS

Effect of preadsorbed fibrinogen on platelet deposition

Platelet deposition profiles on the PVC shunt and the shunt preadsorbed with fibrinogen or serum are shown in Figure 1. Preadsorption of fibrinogen resulted in peak platelet deposition that is an order of magnitude higher than that observed on the PVC control surface. The difference in the peak platelet deposition results from formation of larger thrombi on the fibrinogen-precoated PVC. However, no significant difference in platelet deposition was observed during the first 5 min of blood exposure. The time for maximum platelet deposition varied depending on the nature and the quantity of preadsorbed protein. Figure 2 shows morphologic stages of platelet adhesion, spreading, and mural thrombus formation on the fibrinogen-coated PVC shunt. After 5 min of blood exposure, the polymer surface is covered with spread platelets and unactivated spherical platelets are attached to these spread platelets (Fig. 2a). The spread platelets continue spreading even though the hyalomeres of platelets are in contact with those of other platelets. The lack of contact inhibition between platelets results in the over-

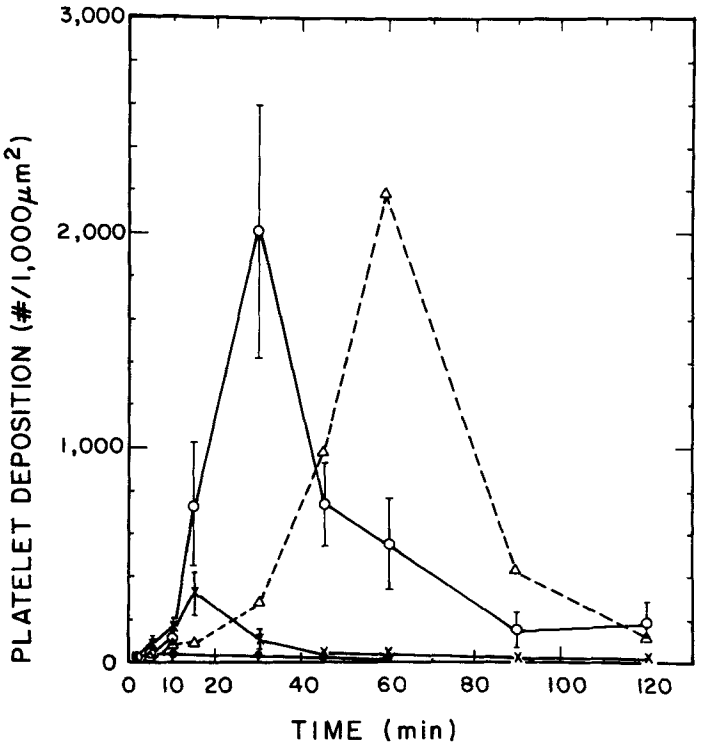

Figure 1. Transient platelet deposition (average ± S.E.M.) on PVC (x, $n = 5$) and PVC shunts preadsorbed with serum (●, $n = 2$), and fibrinogen at surface concentrations of 0.79 ± 0.09 $\mu g/cm^2$ (○, $n = 4$) and 1.5 $\mu g/cm^2$ (△, $n = 1$).

lapping of spread platelets and at 10 min the periphery of the upper platelets tends to ruffle (Fig. 2b). After 15 min, the distinction between overlapping platelets becomes unclear (Fig. 2c). The overlapped platelets eventually fuse to form a sheet and serve as a base for thrombus formation (Fig. 2d). Under the flow conditions used in this study, all thrombi appeared to be white from visual as well as microscopic observations.

On serum-coated PVC, the number of platelets was consistently less than 100/1000 μm^2 (Fig. 1), and SEM micrographs show only single, round platelets with some pseudopods (Fig. 3). No spread platelets and no thrombi were observed throughout the experimental time period. It is important to note that the initial platelet deposition on the serum-precoated surface is not prevented. Instead, it appears that the transformation of deposited platelets to their fully spread form is inhibited by the precoated serum proteins. Thus, it appears that platelet spreading is necessary for thrombus formation. The albumin-like passivation by serum proteins is expected, since albumin is by far the most abundant protein in serum.

The effect of the amount of preadsorbed fibrinogen on platelet deposition was examined by varying its surface concentration. A surface concentration of 1.5 $\mu g/cm^2$ was obtained when a PVC shunt was precoated with fibrino-

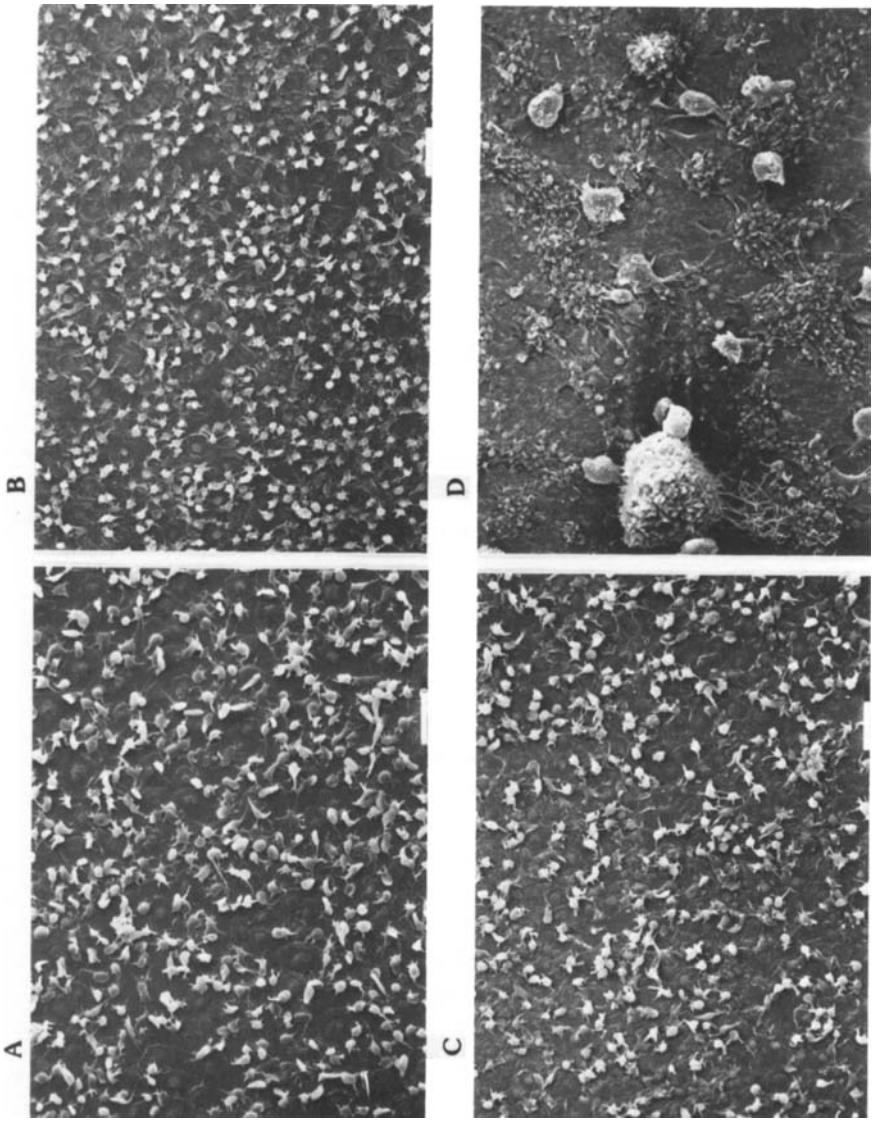

Figure 2. Scanning electron micrographs of platelet deposition on PVC preadsorbed with fibrinogen at a surface concentration of 0.79 ± 0.09 $\mu g/cm^2$. Platelet deposition after blood exposure of (a) 5 min, (b) 10 min, (c) 15 min, and (d) 30 min.

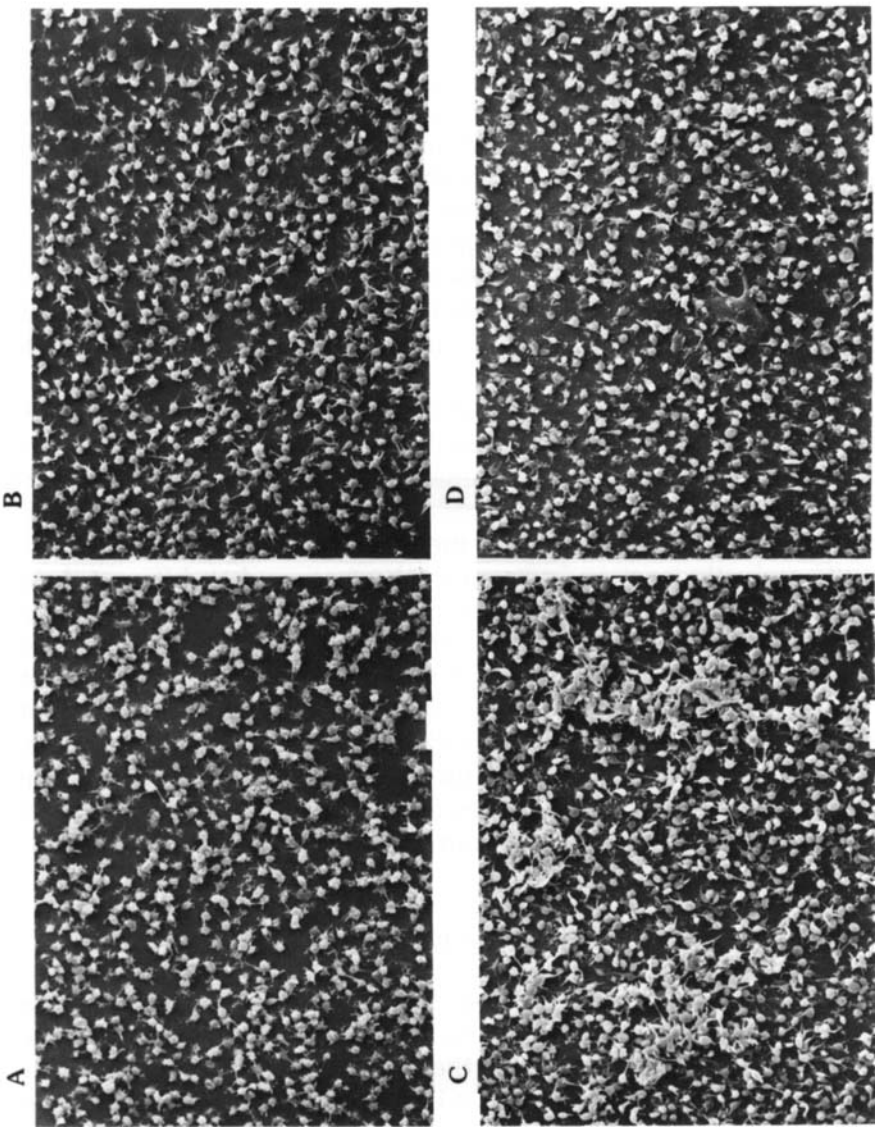

Figure 3. Scanning electron micrographs of platelet deposition on PVC pre-adsorbed with serum. Platelet deposition after blood exposure of (a) 2 min, (b) 15 min, (c) 30 min, and (d) 90 min.

gen solution containing 1.4 mg/mL of protein for 2 h at room temperature. As shown in Figure 1, the maximum number of platelets deposited on the shunt did not change. A comparison of the platelet deposition profile with that on PVC precoated with 0.3 mg/mL of fibrinogen solution (surface concentration of 0.79 ± 0.09 $\mu g/cm^2$, Table I) indicates that there is essentially no change in the overall shape of the platelet deposition curve, but the time for maximum platelet deposition is significantly delayed on the PVC shunt with the higher concentration of preadsorbed fibrinogen. SEM micrographs show that platelet spreading does not occur until 30 min of blood exposure. Thus, it appears that a larger amount of preadsorbed fibrinogen is not necessarily more thrombogenic. We are tempted to speculate that fibrinogen adsorbed in the multilayer regime is nonthrombogenic. However, one cannot rule out the possibility that fibrinogen might adsorb nonuniformly. One could well have bare surface or multilayer adsorption at any bulk phase protein concentration. The loosely attached fibrinogen molecules in the multilayer regime may not bind platelets to the surface nor do they appear to activate platelets to the fully spread state, a step that is a prerequisite to thrombus formation.

Platelet deposition profiles on polyethylene and silicone rubber shunts precoated with fibrinogen were compared. The results shown in Figure 4 demonstrate that platelet deposition profiles are influenced by the nature of polymer surfaces, even when the surfaces are coated with the same protein. The maximum platelet depositions on the three fibrinogen-precoated surfaces of PVC, polyethylene, and silicone rubber occurred at different times of blood exposure. On the fibrinogen-precoated polyethylene surface, platelet deposition continued to increase throughout the experimental time period (Fig. 4a). On silicone rubber, precoated fibrinogen increased platelet deposition compared with that of a control surface (Fig. 4b), but the maximum platelet deposition was much less than that on PVC or polyethylene precoated with fibrinogen. It is noted that the number of deposited platelets is less on the fibrinogen-precoated surfaces for blood exposure times of up to 10 min for polyethylene and up to 15 min for silicone rubber.

Distribution of precoated protein molecules on polymer surfaces

Thrombi formed on fibrinogen-coated PVC are shown in Figure 5. The figure clearly shows the competitive nature of thrombus formation and embolization. No thrombus formed on the site where a thrombus had embolized and new thrombi formed on sites that were previously bare (arrows in Fig. 5). The same phenomenon was observed on polyethylene and silicone rubber. As observed by other investigators,[3,19] thrombus initiation does not occur uniformly over a surface. This observation leads to the question as to whether patchwise thrombus formation is due to a heterogeneous protein distribution.

Visual observations of protein distribution were achieved using an immuno-gold bead labeling technique. In this technique, antibody is at-

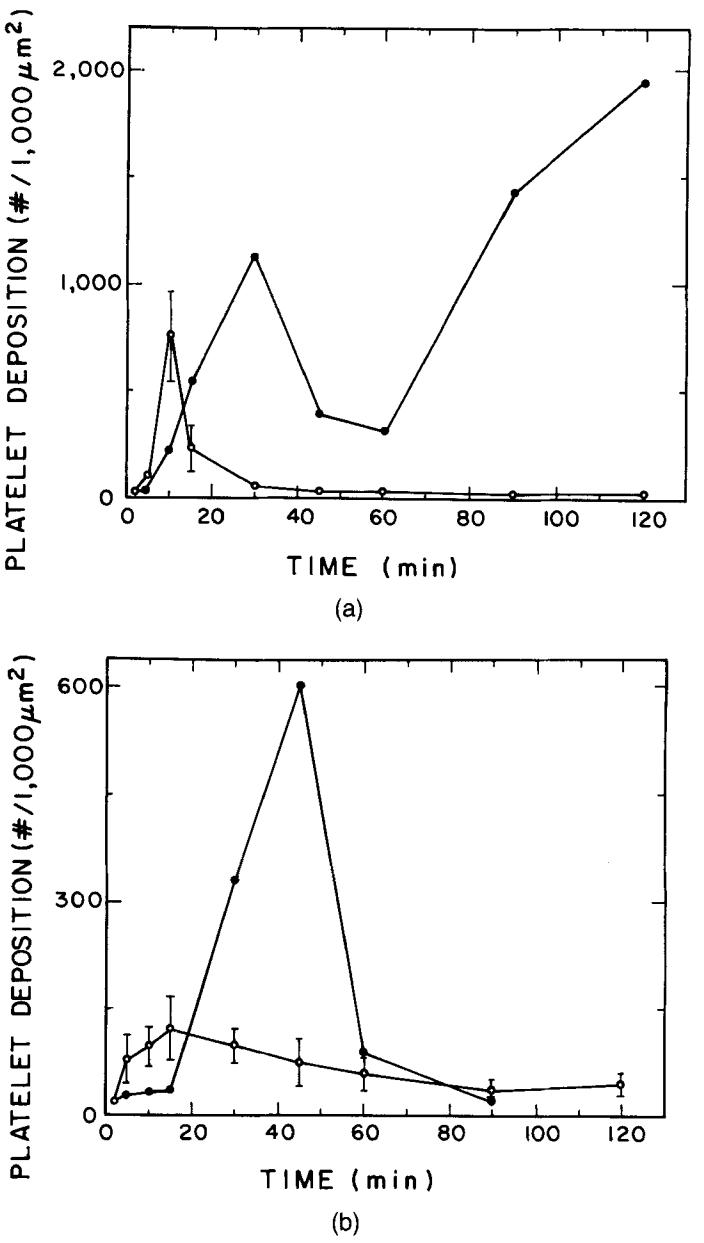

Figure 4. Transient platelet deposition on polyethylene and silicone rubber. (a) Control polyethylene (○, $n=3$) and fibrinogen-preadsorbed polyethylene (●, $n=1$). (b) Control silicone rubber (○, $n=5$) and fibrinogen-preadsorbed silicone rubber (●, $n=1$).

tached to rather bulky, high-density gold beads. The immuno-gold beads adhere to protein molecules only when their antigenic sites are exposed. In addition, if the protein molecules are not strongly adsorbed onto a surface, the protein–immuno-gold bead complex is easily washed off.

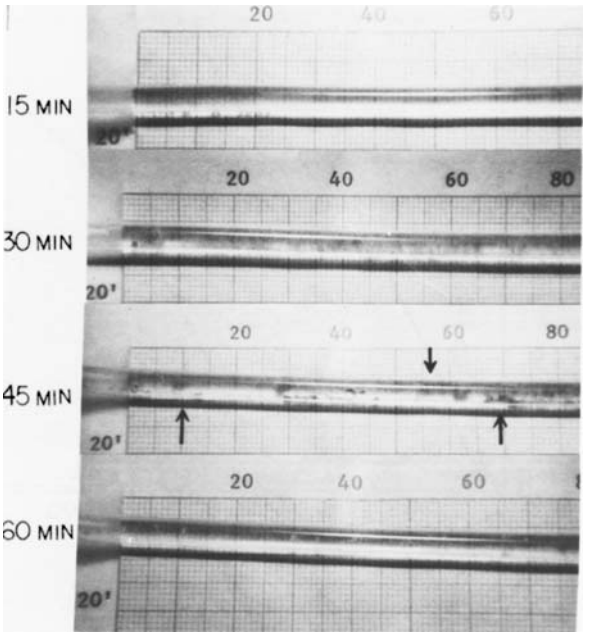

Figure 5. Visual observations of platelet deposition from 15 to 60 min of blood exposure on the same PVC shunt preadsorbed with fibrinogen at a surface concentration of 0.79 ± 0.09 $\mu g/cm^2$. The shunt was flushed to remove blood at each time point. Arrows indicate new thrombi formed on sites that were previously bare.

The distribution of antifibrinogen-gold beads on fibrinogen-precoated PVC and polyethylene is shown in Figure 6A and B. SEM micrographs show a homogeneous coverage of antifibrinogen-coated gold beads, which implies uniform coverage of fibrinogen on the surfaces. No antifibrinogen-gold beads were observed on the control surfaces and the surfaces coated with albumin. The surface coverage by immuno-gold beads is not complete. The incomplete coverage by gold particles is most likely due to the nature of the nonoverlapping adhesion of single noninteracting particles.[20] Other possibilities are that some protein molecules have conformations that do not favor the interaction with the immuno-gold beads or that the distribution of protein molecules is patchwise on a smaller scale than that of the beads. The distribution of immuno-gold beads on fibrinogen-coated silicone rubber was also homogeneous, but the protein layer on this surface tended to peel off, as shown in Figure 6c. Since the distribution of preadsorbed fibrinogen was observed to be homogeneous, patchwise formation of thrombi requires further study. Antifibrinogen-gold beads were also used to label a polyethylene surface that was sequentially preadsorbed with fibrinogen and albumin and implanted in the canine *ex vivo* model (Fig. 6d). The surface was labeled with immuno-gold beads after 90 min of blood exposure. Although it is not known whether the observed fibrinogen molecules are the remainder of the

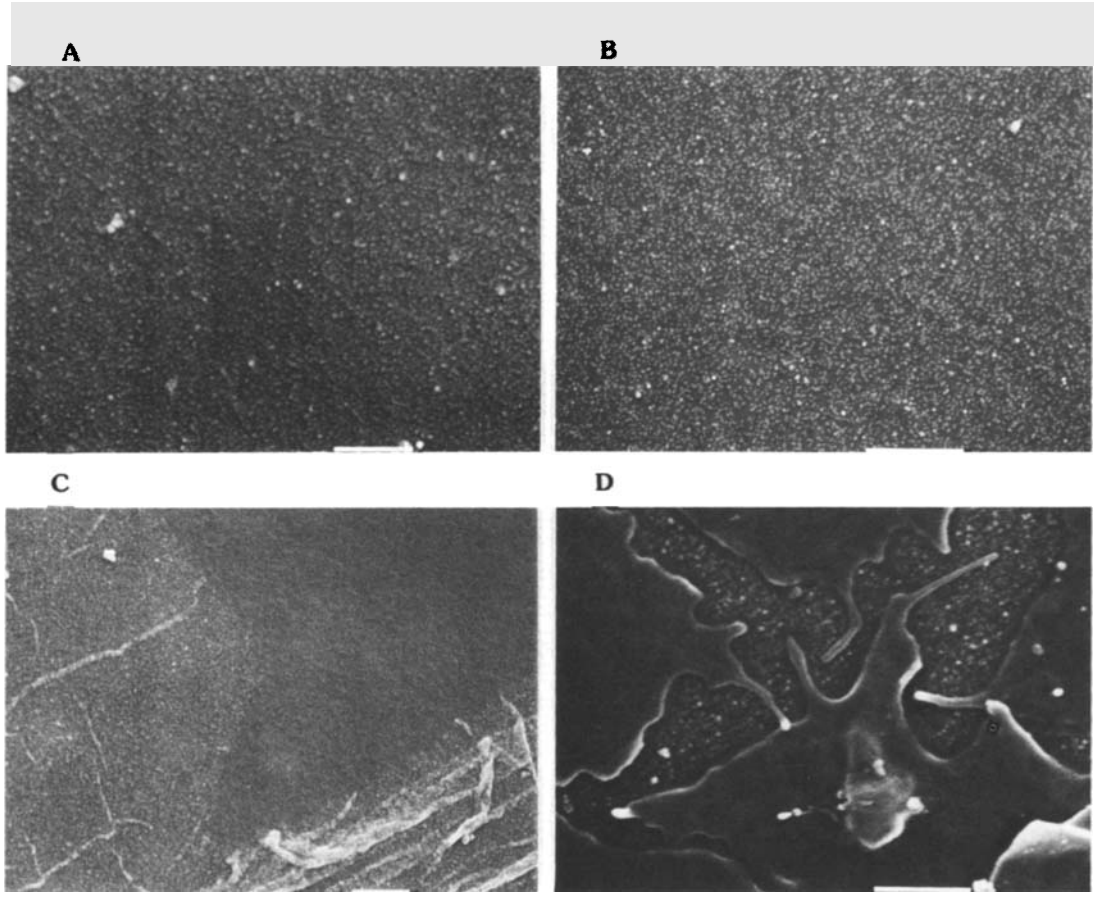

Figure 6. Scanning electron micrographs of antifibrinogen-gold bead distribution *in vitro* on (a) PVC, (b) polyethylene, and (c) silicone rubber, all of which were preadsorbed with fibrinogen for 2 h at room temperature. Fibrinogen surface concentrations are listed in Table I. Figure D shows the distribution of immuno-gold beads on polyethylene preadsorbed with fibrinogen for 1 min followed by albumin for 119 min. The surface was labeled with immuno-gold beads after 90 min of blood exposure in the canine *ex vivo* system.

preadsorbed fibrinogen or of newly adsorbed plasma fibrinogen, the presence of this tightly bound fibrinogen on the surface suggests a latent thrombogenicity and the potential for subsequent thrombus formation (see results of sequential protein preadsorption).

Desorption kinetics of preadsorbed proteins *ex vivo*

The kinetics of desorption of preadsorbed fibrinogen and albumin were measured to relate the amount of the protein on the surface to platelet deposition. Figure 7 shows the desorption kinetics of preadsorbed fibrinogen and albumin on the PVC shunt. Albumin and fibrinogen show distinct desorption profiles. About 30% of the albumin desorbs rapidly during the first minute of blood exposure (the initial surface concentration is 0.57 $\mu g/cm^2$). Such a dramatic decrease is not observed *in vitro* even in the presence of plasmin (Fig. 8). It is likely that loosely adsorbed albumin molecules are displaced by plasma proteins. Figure 9 shows that the albumin-precoated PVC shunt remains thromboresistant for a time period of 2 h of blood exposure.

The desorption of fibrinogen in the first minute of blood exposure is minimal. Desorption occurs most rapidly between 10 and 40 min of blood exposure. More than 60% of the preadsorbed fibrinogen was removed before maximum platelet deposition occurred at about 30 min of blood exposure. Fibrinogen desorption continued throughout the experimental time period, but the desorption rate decreased dramatically after about 50 min of blood exposure. Comparison of the results shown in Figures 1, 5, and 7 suggests

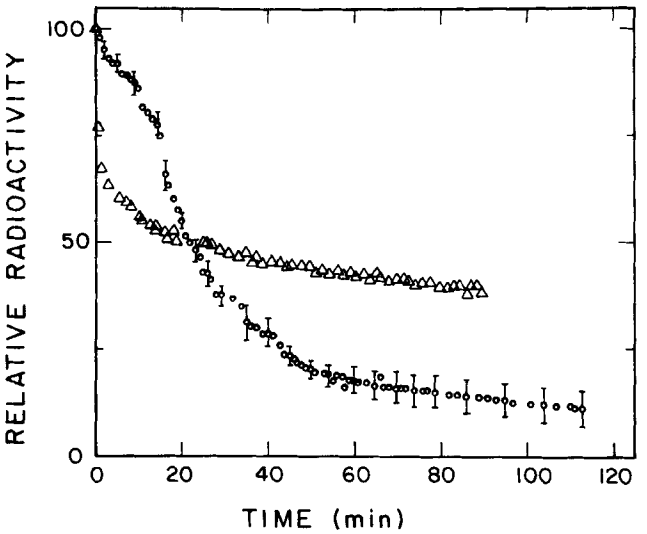

Figure 7. *Ex vivo* desorption of preadsorbed fibrinogen (○, $n = 3$) and albumin (△, $n = 1$) from PVC. The surface concentration was 0.79 ± 0.09 $\mu g/cm^2$ for fibrinogen and 0.57 ± 0.07 $\mu g/cm^2$ for albumin.

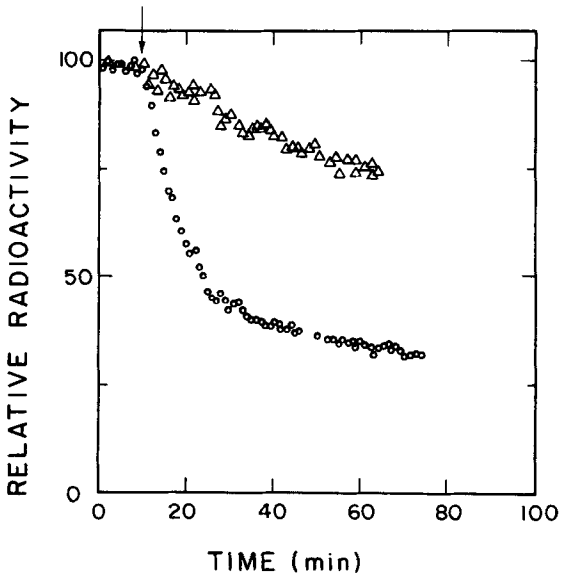

Figure 8. *In vitro* desorption of preadsorbed fibrinogen (○) and albumin (△) from PVC shunts in the presence of plasmin. The surface concentration was 0.57 ± 0.07 $\mu g/cm^2$ for albumin and 0.79 ± 0.09 $\mu g/cm^2$ for fibrinogen. Plasmin was added to flushing buffer at the time indicated by the arrow at a concentration of 0.0015 U/mL. The buffer flow rate was 100 mL/min.

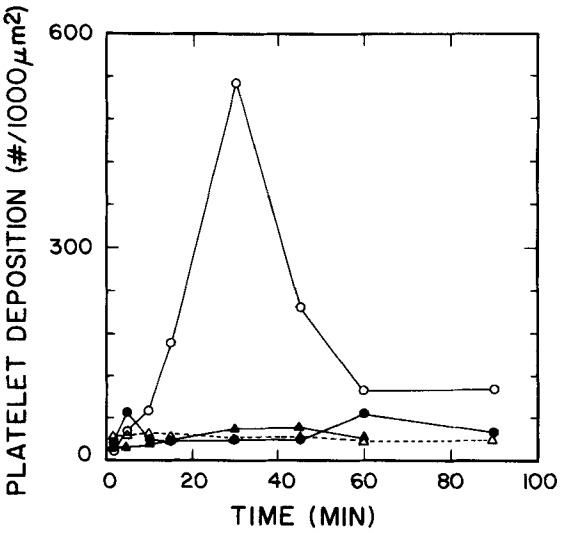

Figure 9. Transient platelet deposition on PVC preadsorbed sequentially with albumin and fibrinogen. Albumin preadsorption for 3 min was followed by fibrinogen for 117 min (○); albumin preadsorption for 10 min followed by fibrinogen for 110 min (△); albumin preadsorption for 1 h followed by fibrinogen for 1 h (▲); albumin preadsorption for 2 h (●). Surface concentrations of albumin and fibrinogen are listed in Table I.

that preadsorbed fibrinogen molecules are released rapidly while thrombus formation and embolization are actively occurring, i.e., fibrinogen desorption is accompanied by the embolization of thrombi that are formed on fibrinogen molecules. The concept that the release of preadsorbed fibrinogen is not due to simple desorption is supported by *in vitro* experiments. Continuous flushing of the fibrinogen preadsorbed on PVC with PBS at a flow rate of 100 mL/min for 30 min did not remove a significant amount of the protein from the surface. A rapid release was observed, however, when plasmin was added to the flushing buffer, as shown in Figure 8. *In vitro* desorption study supports the notion that fibrinogen release in *ex vivo* experiments is associated with embolization of thrombi, which may be caused by either shear removal or enzymatic mechanisms. Displacement of adsorbed fibrinogen molecules by blood proteins is a possibility, but it is not likely a dominating factor. Such a displacement cannot alone account for the high rate of desorption of preadsorbed fibrinogen, which is observed between 10 and 40 min of blood exposure. If the displacement by plasma proteins is responsible, the rapid release should occur during the first few minutes of blood exposure as observed with albumin.

Results of sequential protein preadsorption

Although the effect of preadsorbed protein on platelet deposition is relatively well documented, it is still not clear what specific features of protein adsorption determine the blood response. Since the precoating of fibrinogen on polymer surfaces results in platelet deposition that is an order of magnitude greater than on control surfaces and since albumin passivates the same surfaces, the competitive adsorption of these proteins onto polymer surfaces might be expected to markedly affect their thrombogenicity. The importance of the quantity of protein adsorbed and the adsorption kinetics on thrombus formation was studied by sequentially preadsorbing both albumin and fibrinogen on PVC and polyethylene shunts.

On PVC, when albumin was preadsorbed for more than 10 min (surface concentration of 0.32 ± 0.05 $\mu g/cm^2$, Table I) before fibrinogen adsorption, no thrombi were formed on the surface regardless of the amount of fibrinogen on the surface (Fig. 9). When albumin was preadsorbed for less than 10 min and then followed by fibrinogen adsorption, thrombi formed on the surface (Fig. 9). When PVC was preadsorbed with fibrinogen followed by albumin, the surface becomes more thrombogenic than the uncoated control PVC. Three minutes of fibrinogen adsorption followed by 117 min of albumin adsorption resulted in the same extent of platelet deposition as 1 h of fibrinogen preadsorption followed by 1 h of albumin adsorption (Fig. 10), although the amount of fibrinogen on the surface was much less to begin with on the former surface, as shown in Table I. However, the peak platelet deposition was delayed when fibrinogen was preadsorbed for only 3 min. Competitive preadsorption of a fibrinogen–albumin mixture onto a PVC

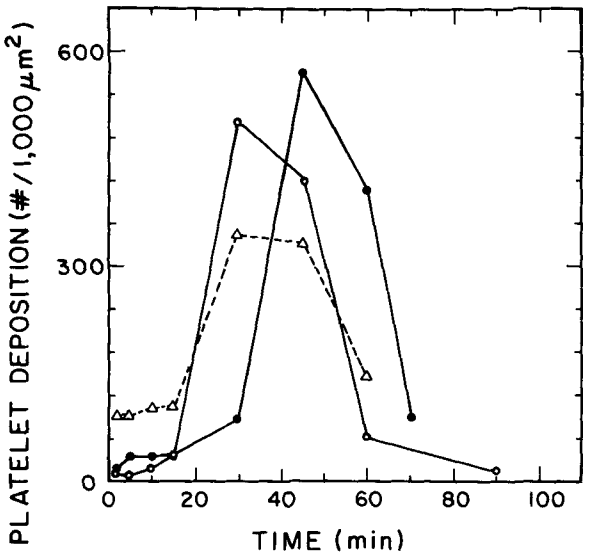

Figure 10. Transient platelet deposition on PVC preadsorbed sequentially with fibrinogen and albumin. Fibrinogen preadsorption for 3 min followed by albumin for 117 min (●); fibrinogen preadsorption for 60 min followed by albumin for 60 min (○); competitive adsorption from an albumin–fibrinogen mixture for 2 hr (△). Surface concentrations of each protein are shown in Table I and Figure 11.

shunt resulted in a platelet deposition profile similar to that of sequentially adsorbing fibrinogen for 1 h and albumin for 1 h. *In vitro* study of the protein adsorption kinetics (Fig. 11) indicates that the kinetics of fibrinogen adsorption from an albumin–fibrinogen mixture is not significantly different from that occurring in a simple fibrinogen solution. On the other hand, adsorption of albumin from the albumin–fibrinogen mixture was reduced dramatically compared with that from a simple albumin solution. This suggests that either fibrinogen has a higher binding affinity or that fibrinogen adsorbs faster than albumin or both, so that fibrinogen molecules form the initial layer. Comparison of the results of sequential protein adsorption (Table I) with those of competitive adsorption from an albumin–fibrinogen mixture (Fig. 11) suggests that fibrinogen has a higher binding affinity. Albumin adsorption is significantly affected by only a 1 min preadsorption of fibrinogen. On the other hand, the effect of preadsorbed albumin on fibrinogen adsorption was noticeable only after 10 min of albumin preadsorption.

On the polyethylene surface, fibrinogen and albumin could be sequentially preadsorbed in such a way that the surface concentrations of albumin and fibrinogen remained the same even when the order of preadsorption was reversed. These experiments involved the adsorption of fibrinogen for 1 min followed by 119 min of albumin and the adsorption of albumin for 3 min followed by 117 min of fibrinogen (Table I). The surface concentration of each protein is the same, even though the order of preadsorption is reversed. As

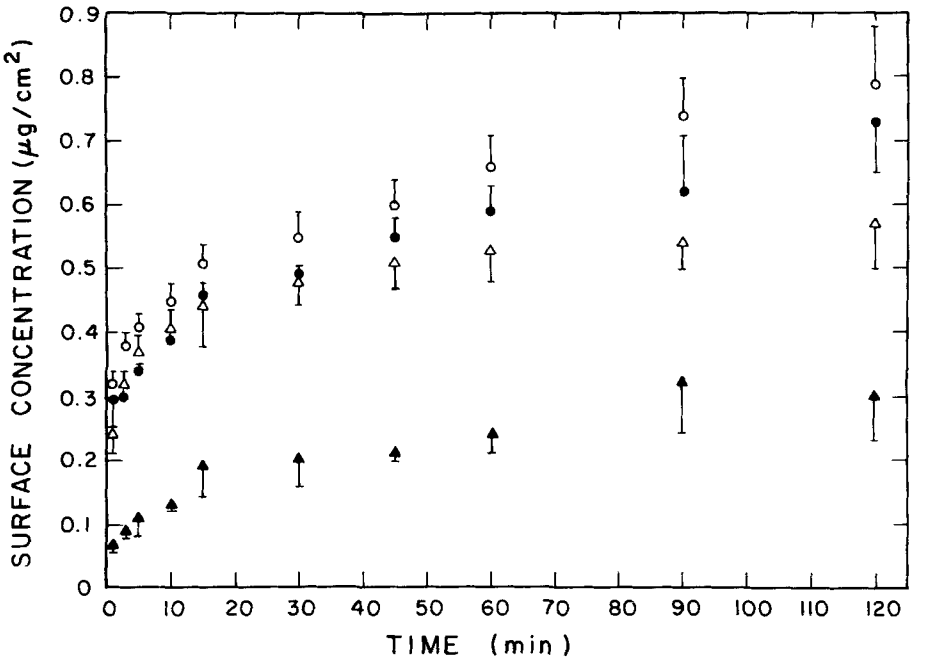

Figure 11. *In vitro* adsorption kinetics of albumin and fibrinogen on PVC (average ± S.D., $n = 4$). Fibrinogen (○) and albumin (△) adsorption from single protein solutions (bulk concentration of 0.3 mg/mL); fibrinogen (●) and albumin (▲) from a fibrinogen–albumin mixture (0.3 mg/mL each).

shown in Figure 12, the first protein adsorbed determines the thrombogenic response of the polyethylene. On polyethylene precoated by competitive adsorption of both proteins, the platelet deposition was similar to that of an albumin-coated surface. The *in vitro* protein adsorption study (Fig. 13) shows that fibrinogen adsorption on the polyethylene surface is significantly inhibited by the presence of albumin, although the amount of fibrinogen present is still greater than that of albumin. The adsorption of fibrinogen onto polyethylene is much lower than that onto PVC under the same conditions.

These experiments indicate that the total quantity of albumin and fibrinogen on the surface is not predictive of the thrombogenic response of the system. It appears that platelet deposition largely depends on the nature of the initial protein layer rather than the absolute surface concentration. The initial protein layer in this study is defined as the layer of protein molecules that occupy the surface first regardless of the fraction of the surface coverage.

DISCUSSION

The nature of platelet adhesion to a polymer surface is influenced by molecular processes at the blood–material interface. The composition of the

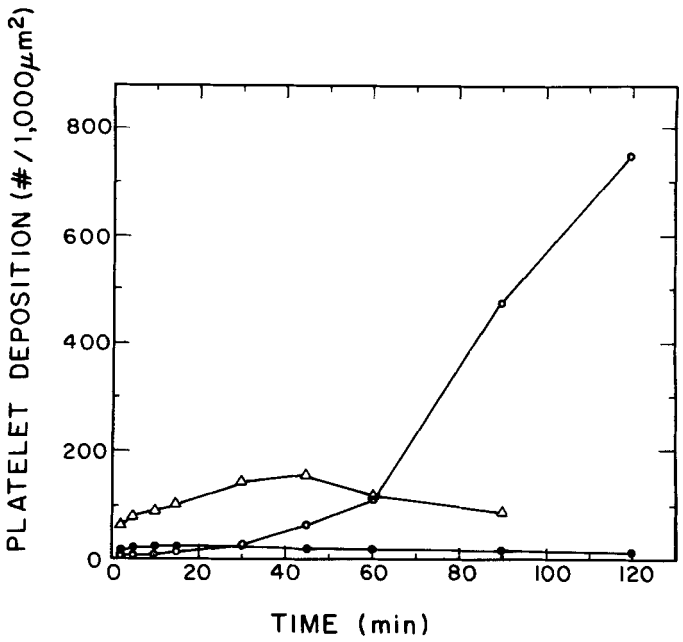

Figure 12. Transient platelet deposition on polyethylene preadsorbed sequentially with fibrinogen and albumin. Fibrinogen preadsorption for 1 min followed by albumin for 119 min (○); albumin preadsorption for 3 min followed by fibrinogen for 117 min (●); competitive adsorption from an albumin–fibrinogen mixture for 10 min (△). Surface concentrations of each protein are shown in Table I and Figure 13.

adsorbed protein layer adsorbed on a polymer surface determines this molecular-level interaction. One distinct feature of platelet adhesion on surfaces precoated with surface-passivating substances, such as albumin, serum, chitosan,[21] or heparin,[21,22] is that all the platelets remain as single, round platelets and no thrombi are formed as observed by SEM. It has been shown that the thromboresistant nature of heparin-coated artificial surfaces is not related to the anticoagulant effect of heparin.[22,23] Salzman and Merrill[24] suggested that only spread platelets form a stable base for the accretion of a platelet thrombus. In addition, Baumgartner and Muggli[25] demonstrated the presence of spread platelets beneath mural thrombi by transmission electron microscopy. Their suggestion is supported by our finding that platelets transform to the fully spread form and large thrombi are formed on fibrinogen-coated PVC (Fig. 2) while no spread platelets are observed on the serum-coated PVC (Fig. 3). Since platelet spreading appears to be the first step in thrombus formation, it is not likely that platelet aggregates, which are formed in the bulk blood, will adhere to the surface.

The lack of spreading of the adherent platelets on surfaces precoated with nonthrombogenic substances is thought to occur for at least two reasons. First, adsorption of platelet-adhesive proteins is restricted due to the preadsorbed layer of nonthrombogenic protein such that the concentration of

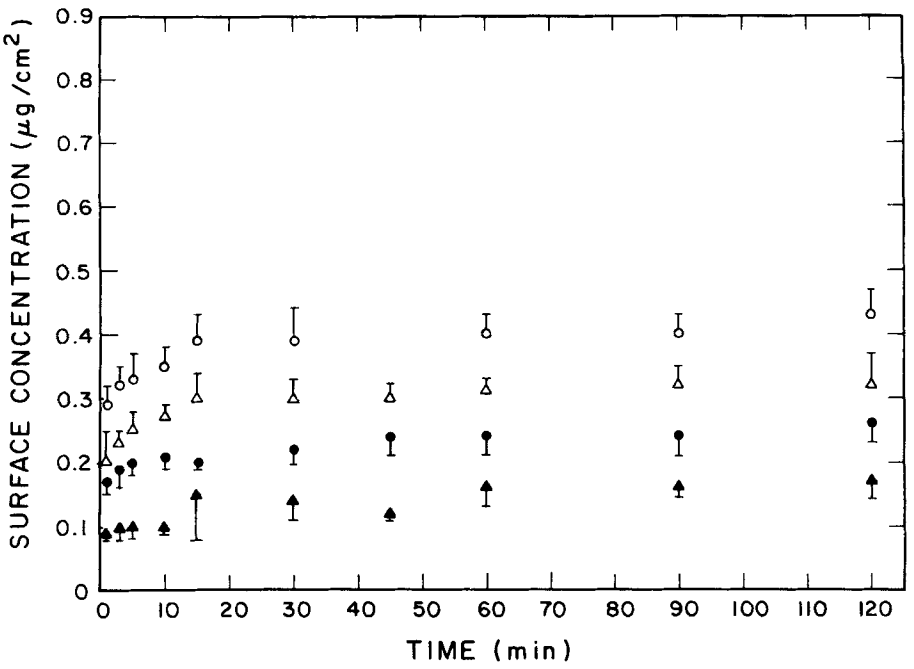

Figure 13. *In vitro* adsorption kinetics of albumin and fibrinogen on polyethylene (average ± S.D., $n = 4$). Fibrinogen (○) and albumin (△) adsorption from single protein solutions (bulk concentration of 0.3 mg/mL); fibrinogen (●) and albumin (▲) from a fibrinogen–albumin mixture (0.3 mg/mL each).

platelet-adhesive protein is not sufficient to cause platelet spreading. The surface concentration of adhesive protein is important, since cell spreading is known to be an all-or-none response that depends on a critical threshold of platelet-adhesive protein for its initiation.[26] Second, although the surface concentration of platelet-adhesive proteins may be above a critical threshold, the conformation and two-dimensional array in which they adsorb may not favor multiple, simultaneous interactions with sites on the platelet surface.[27] This may be particularly significant regarding high concentrations of platelet-adhesive proteins that happen to adsorb in the multilayer regime. The conformational changes that occur in the monolayer regime are likely to cause stable platelet adhesion and activation.

Unactivated platelets do not express fibrinogen binding sites on their membrane, and no fibrinogen associates with these platelets.[28] In addition, no Factor VIII-von Willebrand factor binds to unactivated platelets in suspension unless the antibiotic ristocetin is introduced.[29] It has been suggested that platelets are stimulated prior to surface contact through more than one pathway.[6] Since 20–40% of carefully collected canine and human platelets are partially activated,[30] the above suggestion is plausible. The adhesion of partially activated platelets to a fibrinogen-coated polymer surface occurs even in the presence of plasma fibrinogen. Binding of fibrinogen and other platelet-adhesive proteins to a polymer surface may induce conformational

changes, which could result in a higher affinity for platelets while there is little or no interaction between partially activated platelets and protein molecules in solution.[31]

On certain surfaces, such as polyethylene preadsorbed with albumin for 3 min followed by fibrinogen for 117 min, platelet spreading occurs as observed by SEM without evidence of thrombus formation (Fig. 12). It may be that the spread platelets on the polyethylene surface can be easily peeled from the surface, i.e., fibrinogen molecules adhere tightly enough to result in platelet spreading but not tightly enough to hold growing platelet aggregates in the shear field. This results in the embolization of small platelet aggregates and no large thrombi are observed on the surface. Thus, even though platelet spreading appears to be the first step leading to thrombus formation, platelet spreading does not necessarily cause thrombus formation.

It is expected that a polymer surface affects the conformation of adsorbed fibrinogen and other platelet-adhesive protein molecules and thereby influences subsequent platelet adhesion. Assuming that the conformation and distribution of adsorbed fibrinogen molecules on the surface favors platelet spreading and thrombus formation, the adhesive strength between the polymer surface and the adsorbed protein molecules should determine the size of the platelet aggregates. As the platelet aggregates on a polymer surface grow larger, the formed thrombi may cause the failure of a polymer biomaterial implant. In addition, large emboli can result in transient ischemic attacks or strokes while emboli of smaller size induce no clinically detectable effect. Thus, the *in vivo* formation of large adherent thrombi is considered to be more deleterious than the formation of many smaller thrombi that embolize rather easily from a surface. The adhesive strength of the protein–surface interaction may explain why excess fibrinogen on the surface is not thrombogenic. Fibrinogen molecules in the multilayer regime may not have much of a change in their conformation and therefore do not possess a high binding affinity to platelets. Adsorbed multilayer fibrinogen may not attach to the surface of the platelet or to the protein in the monolayer regime tightly enough to withstand the shear force produced by the flowing blood.

Kaeble and Moacanin[32] suggested that polymer surfaces with a combination of high dispersion with low polar component forces promote more tightly bound and retained plasma proteins. If fibrinogen and other platelet-adhesive proteins are adsorbed on such surfaces, the surfaces become very thrombogenic. However, it is not known in detail how surface energetics influences which protein will adsorb. In addition, as shown in this report, platelet spreading is an important factor in determining surface thrombogenicity. Cell spreading appears to be dependent only on the polar surface free energy.[33] The strength of plasma protein adsorption and cell spreading appear to depend on different components of the surface tension. Thus, the measurement of the polar and dispersion components of the surface tension does not in a straightforward fashion serve as a predictor of surface thrombogenicity.

As the sequential protein adsorption experiments demonstrate, the initial protein layer is more important than the total amount of protein on the surface. It also appears that to be thrombogenic, platelet-adhesive proteins have to bind to the polymer surface tightly enough to mediate adhesion of spread platelets and platelet aggregates. Fibrinogen was examined in this study as a model platelet-adhesive protein, but the adsorption of other platelet-adhesive proteins in blood, such as von Willebrand factor,[5,34] fibronectin,[27] collagen,[35] or thrombospondin,[27] to a polymer surface may also have to be considered to determine more accurately the thrombogenicity of a given material.

Platelet deposition on surfaces in the acute canine *ex vivo* system is a result of two different but competitive and continuous phenomena, thrombus formation and embolization. As shown in Figure 5, once a thrombus embolizes, no thrombus reforms at the same site. The reason that platelets are not activated where a thrombus was previously formed is not clear, but this phenomenon explains why the surface becomes passivated after the peak in platelet deposition is observed (Fig. 1). The length of time of this surface passivation is not yet known. Although the surface becomes thromboresistant in the acute system, ultimately thrombi will reform causing the failure of chronic shunts. The relationship between the performance of a material in the acute *ex vivo* test and long-term patency remains to be determined. The question also arises of why thrombus formation is not homogeneous even when the surface is completely coated with a single protein. Whether this results from the molecular scale heterogeneity of the adsorbed protein, from heterogeneities in the presumably homogeneous polymer surface, or from a nonuniform interaction of blood with the surface remains to be determined.

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