IMPORTANCE OF COMPOSITION OF THE INITIAL PROTEIN LAYER AND PLATELET SPREADING IN ACUTE SURFACE-INDUCED THROMBOSIS

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It is generally accepted that the initial adsorption of plasma proteins onto blood-contacting biomaterials profoundly affects the thrombogenicity of implanted biomaterials[1]. Based on the observation that albumin-coated surfaces minimize platelet adhesion while surfaces become more thrombogenic when precoated with fibrinogen[2,3], the relative propensity of a surface to adsorb albumin and fibrinogen has been used as a method for assessing thrombogenicity. A comparison of the total amount of each protein adsorbed in vitro, however, has not been conclusive in predicting a biomaterial's thrombogenicity. To test the importance of protein composition and surface concentration on thrombus formation, 3 polymer surfaces were precoated with albumin and fibrinogen under various conditions and tested in a canine ex vivo model. Sequential protein adsorption experiments were designed to evaluate the effect of the initial protein layer, rather than the total amount of protein on the surfaces, on platelet deposition and thrombus formation. Blood responses were analyzed in the canine ex vivo model using transient platelet deposition as an indicator of the surface thrombogenicity.

MATERIALS AND METHODS

Protein Preparation and Preadsorption on Polymer Shunts. Canine fibrinogen was purified from fresh citrated plasma by beta-alanine precipitation[4]. Fibronectin was removed from the fibrinogen using a gelatin-agarose column (Sigma). The purified fibrinogen showed clottability greater than 97%. Canine albumin (Sigma) was used as received. The concentration of each protein was calculated by measuring its absorbance at 280 nm using absorptivities of 1.506 cm^2/mg and 0.58 cm^2/mg for fibrinogen and albumin, respectively. Canine serum was prepared from nonanticoagulated whole blood by incubating for 3 hrs at 37° C. The clot was removed by centrifugation and the supernate was filtered through a 0.22 μm filter (Millipore).

The polymer shunts used in this study were plasticized polyvinyl chloride (PVC) (Tygon, Norton Plastics, 0.125 inch ID), polyethylene (PE) (Intramedic, 0.125 inch ID), and silicone rubber (SR) (Dow Corning, 0.132 inch ID). Shunts were cut into 70 inch lengths and washed with running distilled deionized water for 2 hrs. The PVC shunt was washed with 200 ml of 0.1% Ivory detergent before washing with distilled water. The washed shunts were filled with phosphate buffered saline (PB) at 4° C overnight. Protein adsorption on the shunts was carried out at room temperature before implanting to the animal. Bulk protein concentration used for preadsorption onto the shunts was 0.3 mg/ml for both albumin and fibrinogen. Canine serum was diluted 8 times with PBS and precoated on the PVC shunt for 2 hrs.

Sequential Protein Adsorption. The polymer shunts were precoated with either albumin or fibrinogen for a given time period and then flushed with PBS. Immediately after flushing, the same shunt was further exposed to the second protein. The protein adsorption time for the first protein was varied from 1 min to 1 hr and the total time for protein adsorption was 2 hrs at room temperature. The shunts were flushed with buffer, implanted into a dog, and the transient platelet deposition was measured. The surface concentration of each protein in the sequential protein adsorption experiments was measured using a combination of labeled and unlabeled proteins. Proteins were radiolabeled with ^{125}I using the Chloramine-T (Iodo-Beads, Pierce Chemical) method.

Measurement of Platelet Deposition on Shunt Surfaces. The measurement of platelet deposition on polymer shunts in the canine ex vivo model has been described previously[5]. Briefly, autologous platelets were labeled with ^{51}Cr and reinfused into the dog 15 hrs before the experiment. Platelet deposition was measured by counting radioactivity remaining on shunt surfaces after blood flow was stopped at predetermined time points and the shunt was flushed with modified Tyrode's solution. A small segment of the shunt was removed at each time point and prepared for scanning electron microscopy. Blood flow was resumed in the same shunt and blood exposure time counted cumulatively. The blood flow rate was 150-250 ml/min. SEM samples from the canine ex vivo experiments were fixed for 24 hrs in 2% glutaraldehyde solution in 0.1 M phosphate buffer, pH 7.4 at 4° C. The samples were dehydrated, critical point dried, coated with gold and examined with a JEOL JSM 35C scanning electron microscope (SEM) at an accelerating voltage of 10 to 20 kV.

RESULTS

Using the sequential protein adsorption technique, it was possible to maintain approximately the same surface concentrations of albumin and fibrinogen while varying the order of protein adsorption. Thus, the effect of the initial protein layer on the surface, rather than the total protein amount, on platelet deposition could be

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TABLE I. EFFECTS OF COMPETITIVE AND SEQUENTIAL PROTEIN ADSORPTION ON PLATELET DEPOSITION IN THE CANINE EX VIVO MODEL

Adsorption Condition*	Surface Protein Concentration ($\mu g/cm^2$) and Platelet Deposition (#/1,000 μm^2)								
	PVC			PE			SR		
	Albumin	Fibrinogen	Platelets+	Albumin	Fibrinogen	Platelets	Albumin	Fibrinogen	Platelets
Uncoated			320±170			760±210			90±40
A1'/F119#	0.20±0.01&	0.61±0.06		0.13±0.04	0.33±0.08	120	0.18±0.05	0.39±0.16	20
A3'/F117'	0.26±0.04	0.53±0.40	530	0.17±0.01	0.28±0.07	30	0.21±0.01	0.34±0.11	
A5'/F115'	0.28±0.05	0.79±0.20	575	0.18±0.02	0.25±0.06		0.19±0.03	0.38±0.08	30
A10'/F110'	0.32±0.05	0.41±0.20	40	0.20±0.04	0.23±0.15		0.23±0.02	0.07±0.06	
A60'/F60'	0.45±0.03	0.20±0.04	50	0.25±0.02	0.03±0.01		0.30±0.03	0.05±0.02	
A120'	0.57±0.07		50±10	0.32±0.05		70±20	0.46±0.07		< 50
serum 120'			60						
F1'/A119'	0.25±0.04	0.32±0.07		0.15±0.05	0.21±0.07	750	0.14±0.08	0.24±0.07	1,120
F3'/A117'	0.15±0.04	0.31±0.10	580	0.05±0.02	0.24±0.07		0.12±0.06	0.27±0.06	
F5'/A115'	0.16±0.06	0.32±0.10		0.06±0.05	0.27±0.07		0.12±0.04	0.31±0.08	
F10'/A110'	0.12±0.07	0.40±0.08		0.03±0.02	0.28±0.07	1,680	0.12±0.08	0.36±0.11	350
F60'/A60'	0.06±0.01	0.62±0.05	600	0.02±0.01	0.37±0.08		0.04±0.01	0.44±0.02	
F120'		0.79±0.09	2,000±500		0.43±0.04	1,940		0.50±0.06	580±130
F120'@		1.4	2,200						
A-F mix¢	0.30±0.07	0.73±0.08	340	0.17±0.03	0.26±0.03	150	0.28±0.03	0.33±0.03	30

*The bulk protein concentration was 0.3 mg/ml for both albumin and fibrinogen, except where noted.
+Maximum number of platelets deposited in the ex vivo shunt.
#A1'/F119' denotes adsorption of albumin for 1 min followed by fibrinogen for 119 mins.
&Mean ± SD (n=4).
@The bulk concentration was 1.5 mg/ml.
¢Mixture of albumin (0.3 mg/ml) and fibrinogen (0.3 mg/ml) for 120 mins.

analyzed. The surface concentrations of albumin and fibrinogen preadsorbed on 3 polymer surfaces under various conditions are listed in Table I. The maximum numbers of platelets deposited on each protein precoated shunt in the canine ex vivo experiment are also shown. When albumin was precoated first on PE and SR, the number of adhered platelets was always approximately 60/1,000 μm^2 and thrombi were not observed as examined by SEM. The surfaces behaved as if they were coated with albumin only, although the amount of adsorbed fibrinogen in many cases was higher than that of albumin. On PVC, however, the effect of fibrinogen as the second protein was significant and albumin had to be precoated for more than 10 mins to overcome the effect of fibrinogen. Precoating of the same surfaces with fibrinogen prior to albumin resulted in order of magnitude higher platelet deposition on all surfaces compared to albumin coated surfaces. Although the effect of the first protein layer on PVC in controlling the platelet deposition was not as decisive as on PE and SR, it appears that platelet deposition and thrombus formation is largely dependent on the nature of the initial protein layer.

When the polymer surfaces were coated with albumin and fibrinogen simultaneously, platelet deposition and thrombus formation was far less than that observed on surfaces which were coated with fibrinogen alone. The surface concentration of fibrinogen was not reduced significantly upon simultaneous adsorption with albumin (Table I). Thus, the total amount of adsorbed protein cannot account for the relatively nonthrombogenic nature of those surfaces. The data in Table I demonstrate that the extent of platelet deposition on polymer shunts is primarily determined by the composition in the initial protein layer rather than the total amount and type of protein on the surface.

Transient platelet deposition profiles on protein coated PVC and silicone rubber shunts are shown in Figure 1. When albumin or serum was precoated on polymer shunts, the number of platelets deposited on the surfaces did not exceed 90/1,000 μm^2 and no thrombi were observed on the SEM micrographs. The maximum platelet deposition on fibrinogen-precoated surfaces occurs after 30 to 45 mins of blood exposure. Figure 2 shows SEM micrographs of platelet and thrombus morphology on fibrinogen-coated silicone rubber. Comparison of the platelet deposition profile on the fibrinogen-coated silicone rubber in Figure 1 with micrographs of Figure 2 suggests that the increase in platelet deposition is primarily due to the formation of thrombi which tend to initiate on fully spread platelets. The correlation between platelet deposition, platelet spreading and thrombus formation was also observed on PE and PVC. The requirement of platelet spreading for thrombus formation was supported by the lack of platelet spreading on PVC coated with albumin or serum (Figures 3-A and B). As shown in Figure 2-C, the

thrombus formed on fibrinogen-coated PVC is anchored to the surface through fully spread platelets. As the thrombus ages on a surface, platelets retract and eventually embolize (Figures 2-C and 2-D) from the surface. In the SEM photographs, thrombi which appear about to embolize have very few platelets on their edges. Since thrombi appear to form after platelet spreading occurs, it is not likely that thrombus formation results from the attachment of platelet aggregates formed in the blood stream. It is also not likely that emboli reattach to the surface away from the embolization site.

In this study, a consistent observation has been that the initial platelet deposition up to about 10 mins is the same on all surfaces (< 90/1,000 μm^2) regardless of the surface and protein precoating (Figure 1). Even on albumin coated surfaces, the initial platelet deposition was not inhibited. As shown in Figures 3-A and B, PVC surfaces coated with serum are covered with platelets which still maintain their round shape, with some pseudopods, throughout the experimental time period of 2 hrs. On the other hand, platelets adhering on fibrinogen-coated PVC change their shape to the fully spread form (Figures 3-C and D). Whenever thrombi were formed on those surfaces, the number of deposited platelets was greater than 90/1,000 μm^2 and fully spread platelets were invariably observed.

The thrombi formed on fibrinogen-coated surfaces were large enough to be observed with the unaided eye. From visual as well as microscopic observations (Figures 2-C and 3-D), it was found that thrombus formation was not homogeneous over the entire surface. In addition, it was observed that thrombi were not formed on sites where a thrombus had previously embolized during the 2 hr experiment. The relationship between the short-term surface passivation after embolization and long-term surface thrombogenicity remains to be determined.

DISCUSSION

It has been suggested that precoating a surface with albumin may alter platelet-surface interactions by interfering with the deposition of fibrinogen from plasma[3]. However, this study demonstrates that albumin passivates the surface when it is precoated as the first protein, even in the presence of larger amounts of fibrinogen. Considering the observation that albumin and fibrinogen form a continuous film on various surfaces in the first few seconds of protein exposure[6], the dominant role of the first protein in platelet deposition may be expected since the second protein may adsorb more weakly and can be removed from the surface easily.

A rather surprising observation was that the effect of fibrinogen on platelet deposition was reduced considerably when fibrinogen was adsorbed competitively in the presence of albumin. Since the bulk concentration was 0.3 mg/ml for both albumin and fibrinogen, it might be expected that minimal platelet deposition would occur on surfaces exposed to higher ratios of albumin to fibrinogen, such as that found in the blood. This, however, does not occur (Table I). Despite a 10 times higher concentration of albumin than that of fibrinogen in the blood, previously uncoated biomaterials still produce thromboembolization. Thus, consideration of albumin and fibrinogen alone cannot explain surface-induced thrombosis. The role of other platelet adhesive proteins, such as von Willebrand factor[7], fibronectin[8], thrombospondin[8], or collagen[9], should be considered in surface-induced thrombosis.

In addition to platelet adherence, platelet spreading must be considered in surface-induced thrombosis. From comparisons of platelet deposition and platelet shape change by SEM, it was found that platelet spreading is an essential factor for thrombus formation. In most in vitro experiments where platelets are treated with anticoagulants and platelet activation is inhibited, the number of platelets adhering on polymer surfaces rarely exceeds 90/1,000 μm^2 [10-12]. The importance of platelet spreading on thrombus formation is also supported by Turitto et al[13] who have suggested that thrombus formation requires the transformation of platelets from the contact to the spread form. Since the spreading of a cell is an energy-requiring, active cell response and is not strictly analogous to the wetting of a surface by a fluid droplet[14], it is not surprising that broad correlations between thrombus formation and surface energy parameters are not readily found.

As thrombus formation is not homogeneous on the entire surface, i.e., the thrombus formation is a local event although spread platelets cover the whole surface (Figure 2-C), it appears that platelet spreading does not necessarily result in thrombus formation. The heterogeneity in the platelet adhesion, spreading rate, release of granular contents, and the ability of fully spread platelets to support the growth of thrombus results in patchwise formation of thrombus on the surface. Since the accumulation of platelets on a site can cause the failure of an artificial shunt and it is the formation of thrombi which results in the potential danger of using biomaterials, it may be more appropriate to compare surface thrombogenicities by measuring the size of thrombi produced by the surface rather than the average platelet deposition on the surface. The sequence of thromboembolization observed in this study is schematically described in Figure 4. The nature of the short-term surface passivation after embolization is currently under investigation.

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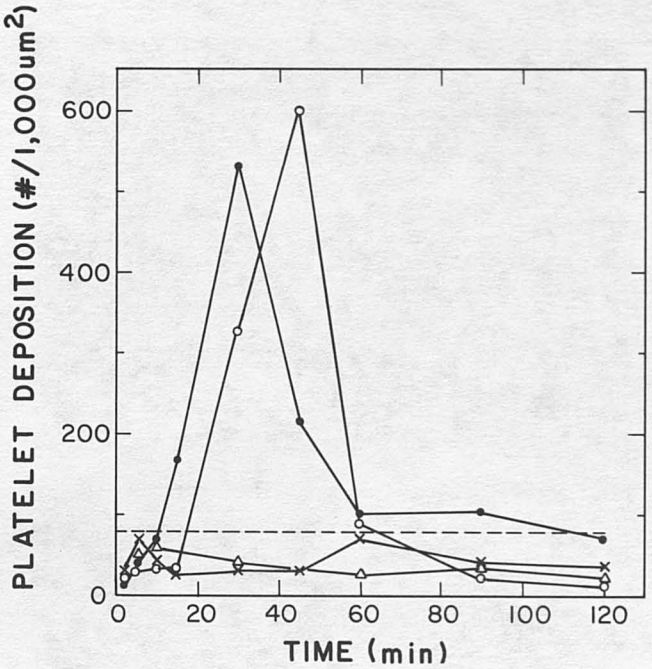

Figure 1. Transient platelet deposition profiles on protein-coated PVC and silicone rubber surfaces. PVC precoated with albumin for 2 hrs (x), PVC precoated with serum for 2 hrs (Δ), PVC precoated with albumin for 3 mins followed by fibrinogen for 117 mins (•), and silicone rubber precoated with fibrinogen for 2 hrs (o). Surface protein concentrations are listed in Table 1.

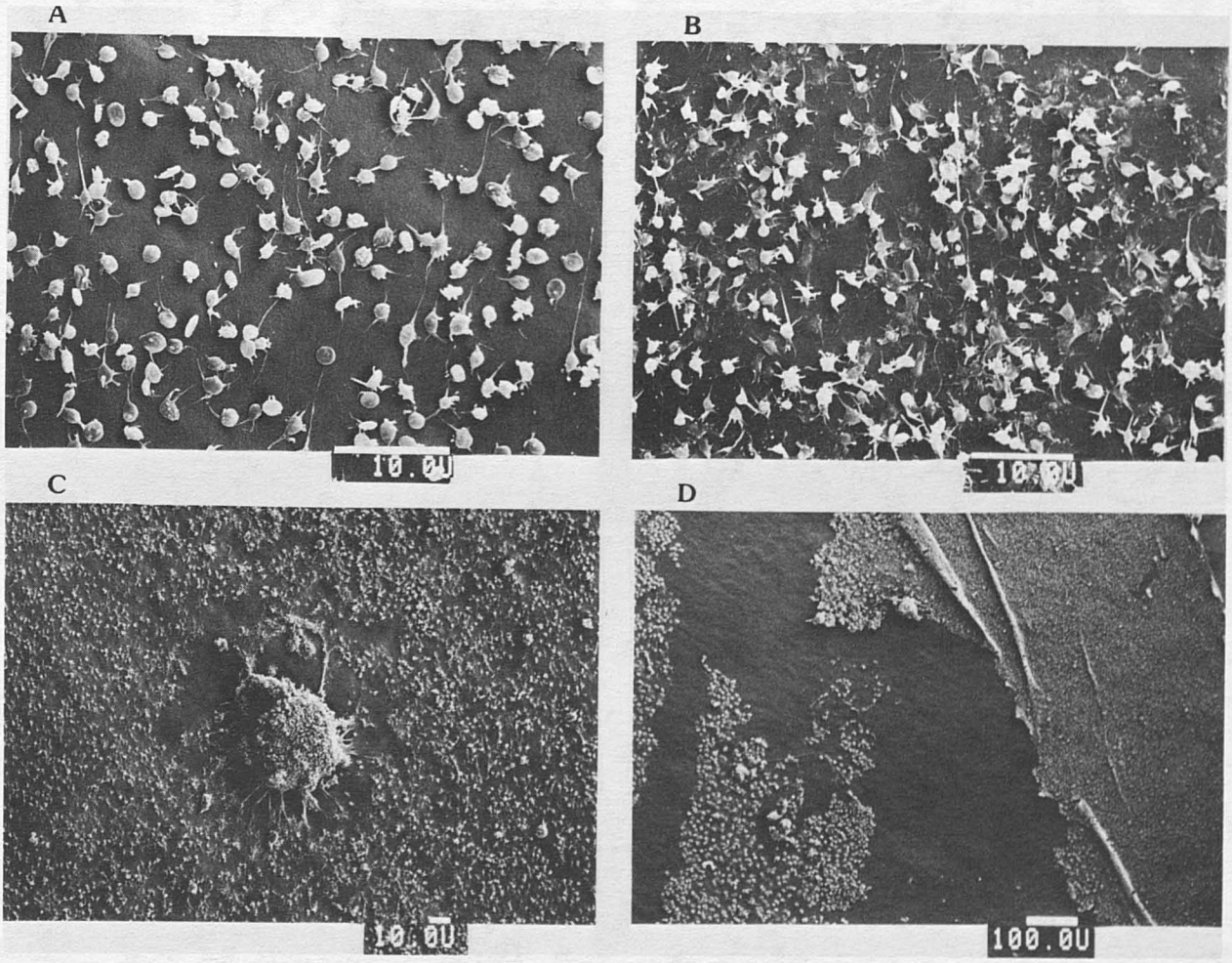

Figure 2. Sequence of thromboembolization on the fibrinogen-precoated silicone rubber surface. The surface was precoated with fibrinogen for 2 hrs at a bulk concentration of 0.3 mg/ml. Platelet deposition after blood exposure of (A) 15 mins, (B) 30 mins, (C) 45 mins, and (D) 60 mins.

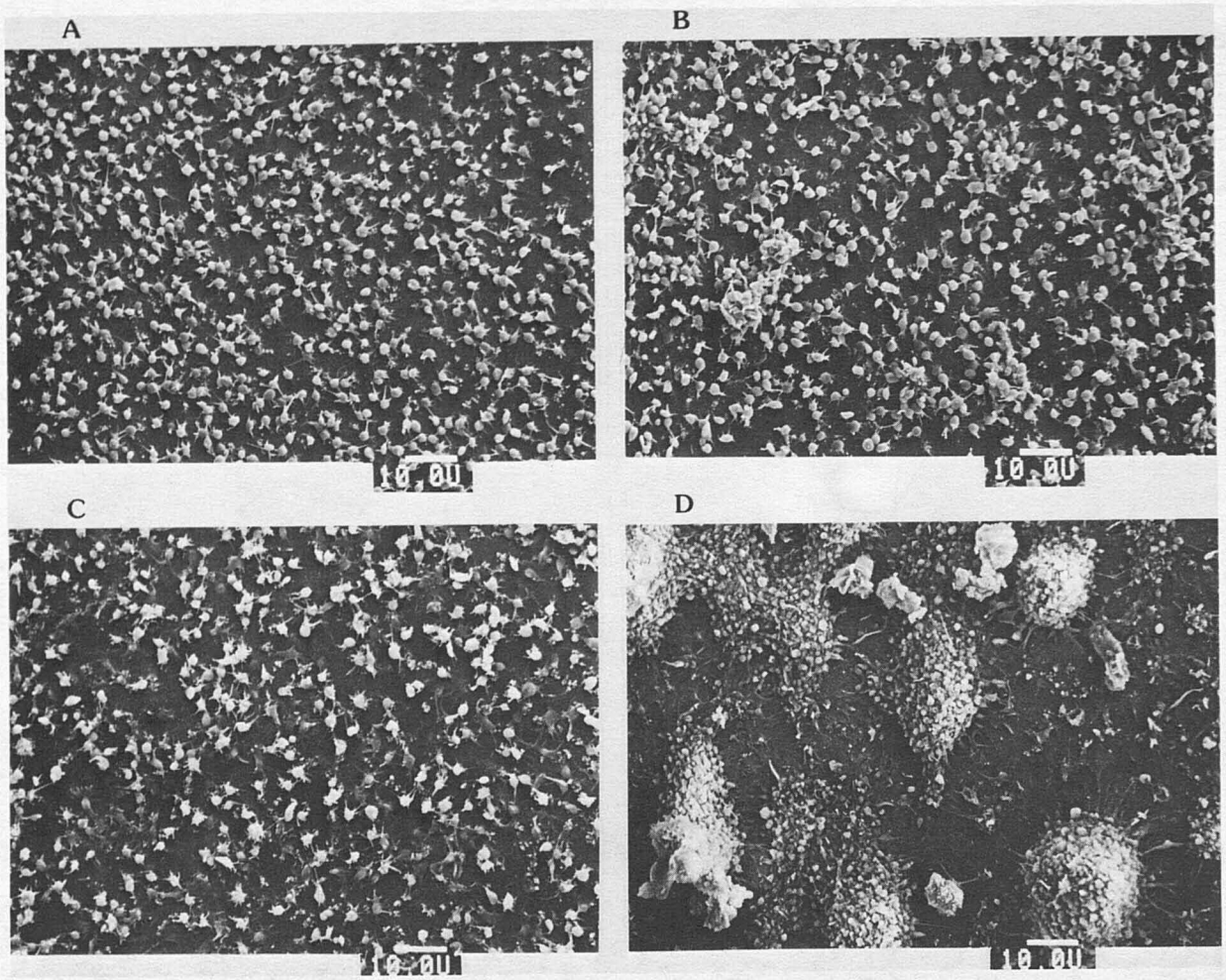

Figure 3. Scanning electron micrographs of platelet deposition on PVC precoated with serum (A,B) and fibrinogen (C,D). Platelet deposition after blood exposure of 5 mins (A,C) and 30 mins (B,D).

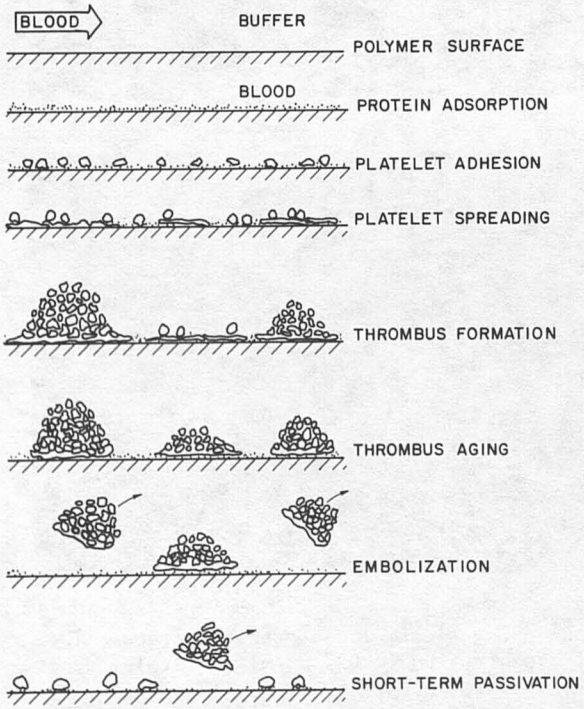

Figure 4. Schematic description of thromboembolization on polymer shunts exposed to the flowing blood in the canine ex vivo system.