Sequential Protein Adsorption and Thrombus Deposition on Polymeric Biomaterials[1]

WILLIAM G. PITT, KINAM PARK,[2] AND STUART L. COOPER[3]

Department of Chemical Engineering, University of Wisconsin-Madison, Madison, Wisconsin 53706

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A canine *ex vivo* arteriovenous shunt model was employed to investigate the effect of preadsorbed blood plasma proteins upon platelet deposition. Albumin and fibrinogen were singly, sequentially, or competitively adsorbed on polyvinyl chloride (PVC), polyethylene (PE), and crosslinked silicone rubber (SR) tubing. Results indicate that platelet deposition and thrombus formation are strongly influenced by the sequence of protein adsorption. The platelet response appears to be determined by the first protein which is preadsorbed to the surface. This response does not appear to correlate well with the total amount of preadsorbed albumin or fibrinogen. To clarify how the sequence of protein adsorption affected the blood response, the sequential adsorption phenomenon was studied using ^{125}I-labeled proteins, Fourier transform infrared spectroscopy, and immunogold particle labeling techniques. It was observed that in the sequential adsorption of albumin followed by fibrinogen, there is a linear correlation between the surface concentrations of fibrinogen and albumin on PVC. On PE and SR, a linear correlation between the fibrinogen and albumin concentrations exists only below a monolayer coverage of albumin. On SR, the initial fibrinogen adsorption rate correlates linearly with the submonolayer concentration of adsorbed albumin. When fibrinogen adsorption is followed by albumin adsorption, no linear correlations in protein adsorption are observed.

INTRODUCTION

During the past several years, much research effort has been directed toward understanding the interaction of blood with synthetic polymeric biomaterials in hopes of designing a material that is compatible with the cardiovascular system. Despite these efforts, an adequate understanding of blood–materials interactions has evaded researchers primarily because the blood response to a material is very complex and difficult to study. A commonly observed blood response is the rapid formation of thrombi at the biomaterial–blood interface. Since most thrombi are primarily composed of platelets and blood plasma proteins, research emphasis has been placed on understanding the interactions between the proteins, platelets and the biomaterial surface. It is generally accepted that one of the first events which occur during blood contact is the adsorption of plasma proteins at the polymer–blood interface. This protein adsorption is usually followed by platelet adhesion. Subsequent events such as platelet activation and aggregation, fibrin polymerization, thrombus formation, and embolization appear to depend on many variables such as polymer composition (1), polymer mechanical properties (2), and blood flow conditions (3).

In addition, many plasma proteins adsorbed at the polymer–blood interface have been found to influence platelet activation and thrombogenesis. Fibrinogen, fibronectin, γ-globulins, thrombospondin, von Willebrand factor, and others, enhance thrombus formation when precoated onto a polymer surface prior to blood contact (4–6). Other proteins,

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[2] Present address: Department of Physical Pharmacy, Purdue University, West Lafayette, Ind. 47907.

[3] To whom correspondence should be addressed.

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such as albumin and transferrin, have the opposite effect and passivate the surface (6). Most of the research on the role of plasma proteins in thrombosis at the biomaterial surface has focused on the correlation between the amount of a particular protein adsorbed and the thrombogenic response. Several studies have indicated that platelet adhesion and aggregation is a function of the amount of fibrinogen on a surface (7–10).

Other efforts have been directed toward correlating surface thrombogenicity, or the ability of a surface to form thrombi, with *in vitro* platelet adhesion (11). Although this may appear to be a more direct measurement of surface–blood interactions, the hemodynamic and hematological conditions used in many of these tests are very different from *in vivo* conditions. These differences may introduce numerous artifacts which make data interpretation difficult. For example, the number of adhering platelets is reported to vary widely with the type of anticoagulant used, the species of animal providing the platelets, the presence of plasma proteins, and other experimental conditions. Other artifacts are observed in the morphological response of platelets upon contact with artificial surfaces. For example, *in vivo* or *ex vivo* studies invariably reveal spreading of adherent platelets which is often followed by mural thrombus formation. In contrast, *in vitro* studies often reveal only a partial surface coverage by single rounded platelets which are apparently inhibited from complete activation by the presence of anticoagulants (12). The morphological changes of adherent platelets *in vitro* are usually arrested after about 10 min of surface contact, which is the point at which observable differences in thrombus formation on different surfaces begin to occur in *ex vivo* arteriovenous shunts (1, 2, 4–6). These types of artifacts raise doubts as to whether *in vitro* platelet–protein interaction in an anticoagulated system can be a good predictor of actual *in vivo* events.

In this laboratory we have developed an acute *ex vivo* canine arteriovenous shunt model which eliminates many of the artifacts created by the nonphysiological hematology and hemodynamics encountered in anticoagulated *in vitro* studies. This canine model has been successful in providing continuous quantitative monitoring of radiolabeled platelet deposition on the surface of a biomaterial from 0 to 120 min of blood contact (4–6). In this paper, we report the acute blood response to three prevalent blood contacting polymers in an *ex vivo* canine experiment. These polymers have been precoated with two abundant plasma proteins in an effort to clarify the role of the adsorbed protein layer in blood–surface interactions. We realize that the acute blood–protein interaction alone does not establish the long-term efficacy of a polymeric biomaterial since the chronic response may be quite different. It is hoped, however, that understanding the acute blood response may help to elucidate mechanisms of artificial surface-induced thrombosis which will facilitate our understanding and prediction of long-term blood response to polymers.

Our goal in this research was to study the role of adsorbed plasma proteins upon platelet deposition by varying the protein concentration and surface distribution at the blood–polymer interface. This was done by singly, sequentially, or competitively preadsorbing the proteins onto the polymer shunts prior to blood contact in the canine model. Since the platelet deposition appeared to be influenced by the sequence of protein preadsorption, the surface concentration and distribution of these preadsorbed proteins were extensively studied in separate experiments using radiolabeled proteins, Fourier transform infrared spectroscopy, and immunogold labeling techniques. Fourier transform infrared spectroscopy coupled with attenuated total reflectance optics (FTIR/ATR) provides continuous and noninterrupted observation of protein adsorption kinetics on polymer surfaces. Immunogold labeling is a new technique employed to reveal the distribution of adsorbed protein on a polymer surface. It is also useful in identifying the presence and location of adherent protein on surfaces after exposure to blood.

MATERIALS AND METHODS

Protein Preparation

Canine fibrinogen was prepared from fresh citrated plasma by the β-alanine precipitation method (13) followed by fibronectin removal on a gelatin–agarose column (Bio-Rad, Richmond, Calif.) which was equilibrated with divalent cation free phosphate-buffered saline (PBS, at pH 7.4). Bovine fibrinogen (Type I-S, Sigma Chemical, St. Louis, Mo.) was purified by the method of Laki (14) and dialyzed for 2 days against PBS. Both canine and bovine purified fibrinogen had a clottability of at least 97% as measured by the method of Coller (15). The fibrinogen preparations were stored as concentrates at −70°C. Canine albumin was prepared in PBS from fraction V powder (Sigma) without further purification. Protein solutions were prepared from frozen concentrate or powder within 24 h of use and stored at 4°C.

Fibrinogen and albumin were radiolabeled with ^{125}iodine (New England Nuclear, Boston, Mass.) using the chloramine-T method (Iodo-Beads, Pierce Chemical, Rockford, Ill.). Free iodide was removed from the labeled protein on a gel column (Bio-Gel P30, Bio-Rad) equilibrated with PBS.

Protein Adsorption

The polymer tubings used in this study were plasticized polyvinyl chloride ($\frac{1}{8}$-in.-i.d. Tygon, Norton Plastics, Akron, Oh.), polyethylene ($\frac{1}{8}$-in.-i.d., Intramedic, Parsippany, N.J.), and silicone rubber (Medical Grade Silastic, 0.132-in-i.d., Dow Corning, Midland, Mich.). The tubings were cut into 70-in. lengths for the *ex vivo* surgery and washed with flowing distilled deionized water for 2 h. Prior to this wash, the polyvinyl chloride tubing (PVC) was washed at room temperature with 500 ml of 0.1% Ivory detergent. Contact angle measurements on PVC following the wash indicated that this wash removes the detergent (16). The washed shunts were filled with PBS and stored at 4°C overnight. Some of the shunts were subjected to protein adsorption 2 h prior to surgery as described below.

For the protein adsorption studies, the polymer tubings were washed as above, exposed to PBS at 4°C overnight, and subjected to single, sequential, or competitive protein adsorption. Single protein adsorption involved exposing the tubing to either 0.3 mg/ml canine albumin or fibrinogen for various lengths of time up to 2 h. Sequential protein adsorption was accomplished by exposing the polymer tubing surfaces to a single protein solution, displacing the solution with PBS, exposing the surface to a second protein solution, and again displacing the solution with PBS. The total time of exposure to both protein solutions was 2 h, with the first protein exposure varying from 1 to 60 min (see Table I). The protein solutions, canine albumin or canine fibrinogen, had a concentration of 0.3 mg/ml. Competitive adsorption of albumin and fibrinogen involved a 2-h exposure to a mixture of 0.3 mg/ml canine albumin and 0.3 mg/ml canine fibrinogen. All protein adsorption was carried out at room temperature. Protein adsorption was terminated by displacing the protein solution with PBS. The tubing was either implanted as an *ex vivo* shunt, or it was cut into small sections for radiometric quantitation or for immunogold labeling of the adsorbed proteins. In sequential and competitive adsorption, the surface concentration of both proteins was determined by counting the radioactivity in a Beckman gamma counter following two identical adsorptions, one with only fibrinogen labeled and the other with only albumin labeled.

Desorption of protein while the protein solution was displaced with PBS was not investigated. However, in sequential adsorption experiments, the amount of the first adsorbed protein desorbed or displaced during adsorption of the second protein was calculated from the single and sequential surface concentration data. Since the single adsorption experiments were identical to the first step of the sequential adsorptions, the single adsorption data showed how much protein adsorbed during the first

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TABLE I

Surface Concentrations for Sequential and Competitive Adsorption of Canine Albumin and Fibrinogen

First protein adsorbed		Second protein adsorbed		Surface concentration ($\mu g/cm^2$)[a]					
				PVC		PE		SR	
Type	Adsorption time (min)	Type	Adsorption time (min)	Albumin	Fibrinogen	Albumin	Fibrinogen	Albumin	Fibrinogen
Albumin (0.3 mg/ml)	1	Fibrinogen (0.3 mg/ml)	119	0.20 ± 0.01	0.61 ± 0.06	0.13 ± 0.04	0.33 ± 0.08	0.18 ± 0.05	0.39 ± 0.16
	3		117	0.26 ± 0.04	0.53 ± 0.40	0.17 ± 0.01	0.28 ± 0.07	0.21 ± 0.01	0.34 ± 0.11
	5		115	0.28 ± 0.05	0.79 ± 0.20	0.18 ± 0.02	0.25 ± 0.06	0.19 ± 0.03	0.38 ± 0.08
	10		110	0.32 ± 0.05	0.41 ± 0.20	0.20 ± 0.04	0.23 ± 0.15	0.23 ± 0.02	0.07 ± 0.06
	30		90	0.41 ± 0.01	0.34 ± 0.20	0.25 ± 0.01	0.07 ± 0.01	0.32 ± 0.04	0.05 ± 0.01
	60		60	0.45 ± 0.03	0.20 ± 0.04	0.25 ± 0.02	0.03 ± 0.01	0.30 ± 0.03	0.05 ± 0.02
	120		0	0.57 ± 0.07	—	0.32 ± 0.05	—	0.46 ± 0.07	—
Fibrinogen (0.3 mg/ml)	1	Albumin (0.3 mg/ml)	119	0.25 ± 0.04	0.32 ± 0.07	0.15 ± 0.05	0.21 ± 0.07	0.14 ± 0.08	0.24 ± 0.07
	3		117	0.15 ± 0.04	0.31 ± 0.10	0.05 ± 0.02	0.24 ± 0.07	0.12 ± 0.06	0.27 ± 0.06
	5		115	0.16 ± 0.06	0.32 ± 0.10	0.06 ± 0.05	0.27 ± 0.07	0.12 ± 0.04	0.31 ± 0.08
	10		110	0.12 ± 0.07	0.40 ± 0.08	0.03 ± 0.02	0.28 ± 0.07	0.12 ± 0.08	0.36 ± 0.11
	30		90	0.10 ± 0.01	0.60 ± 0.02	0.06 ± 0.05	0.37 ± 0.08	0.06 ± 0.01	0.43 ± 0.03
	60		60	0.06 ± 0.01	0.62 ± 0.05	0.02 ± 0.01	0.37 ± 0.04	0.04 ± 0.01	0.44 ± 0.02
	120		0	—	0.79 ± 0.09	—	0.43 ± 0.04	—	0.50 ± 0.06
Competitive adsorption for 120 min (0.3 mg/ml albumin and 0.3 mg/ml fibrinogen)				0.30 ± 0.07	0.73 ± 0.08	0.17 ± 0.03	0.26 ± 0.03	0.28 ± 0.03	0.33 ± 0.03

[a] Mean ± SD ($n = 4$).

part of sequential adsorption. If any of this first protein was removed from the surface during the second protein adsorption and rinse, then the final surface concentration of this first adsorbed protein was less than the single adsorption surface concentration.

FTIR/ATR Studies

FTIR/ATR studies of protein adsorption on silicone rubber (SR) from a nonflowing solution were performed using a polycarbonate flow cell described elsewhere (17). One wall of the cell was a polymer-coated germanium (Ge) internal reflection element (50 × 20 × 3 mm) with a 45° aperture angle (Harrick Scientific, Ossining, N.Y.). To apply the polymer coating, the Ge element was polished twice with 0.2 μm alumina/water paste, rinsed with distilled deionized water, rinsed with ethanol, and then spin-coated with a 0.10 wt% dispersion of Medical Grade Silastic (Q7-2213, Dow Corning, Midland, Mich.) in 1,1,1-trichloroethane (99%, Aldrich Chem., Milwaukee, Wisc.). The SR film was cured in a convection oven for 1 h at 60°C, after which a 700-Torr vacuum was applied for at least 2 h to remove residual solvent and to minimize contamination of the surface. The FTIR/ATR spectrum of the film was collected at four-wavenumber resolution using a Nicolet 170SX FTIR (Nicolet Instruments, Madison, Wisc.). An ATR spectrum of the silicone rubber tubing was also collected for comparison.

For each experiment, a freshly coated Ge element was removed from vacuum, immediately assembled in the flow cell, and mounted in the sample chamber of the FTIR. Following a dry air purge of the sample chamber, a spectrum of the polymer-coated Ge crystal (background spectrum) was collected. PBS was then pumped through the cell with a peristaltic pump for at least 10 min. The flow was stopped and a reference spectrum of the PBS in the cell was collected. The PBS was then rapidly (100 ml/min) displaced with 15 ml (12 cell volumes) of 0.3 mg/ml canine albumin injected via a disposable syringe, and sample spectra were collected. At 1 to 10 min of contact, the albumin solution was displaced with 50 ml of PBS, and a spectrum was collected. Then the PBS was displaced with 15 ml of 0.5 mg/ml bovine fibrinogen. The use of bovine instead of canine fibrinogen was an economic necessity because FTIR experiments consume large amounts of protein. Spectra were collected during at least 45 min of solution contact, after which the fibrinogen solution was displaced with PBS and the experiment terminated.

All protein adsorption spectra were collected at eight wavenumber resolution. Both background and reference spectra consisted of 1000 coadded scans while the sample spectra were collected as coadditions ranging from 5 to 600 scans. The protein spectra were obtained by subtracting the buffer spectrum from the sample spectra. The relative amount of protein at the surface was determined using the absorbance of the 1550-cm^{-1} peak. The absorbance due to soluble protein sampled by the evanescent wave beyond the adsorbing surface was calculated by the method of Fink (18) and subtracted from the total signal. FTIR experiments were performed at a room temperature of 19°C.

Immunogold Bead Labeling

For this study, colloidal gold particles with an average diameter of 18 nm were prepared since they are easily viewed by SEM and are approximately the same size as albumin (14 × 4.4 nm) and fibrinogen (9 × 45 nm) molecules. Details of the gold bead preparation are described by Loftus and Albrecht (19). Briefly, 0.5 ml of 4% $HAuCl_4$ solution was added to 200 ml of deionized distilled water and brought to a boil. Then 4 ml of freshly prepared 1% trisodium citrate were rapidly mixed into the boiling solution. The mixture was refluxed for 30 min. The formation of the monodisperse colloidal particles was indicated by a color change from dark blue to red. The colloidal solution was cooled and the pH was adjusted to 7.4 by addition of 0.2 N K_2CO_3.

The solution was filtered through a 0.45-μm microporous filter (Millex-HA, Millipore, Bedford, Mass.) and stored at 4°C. The gold particle concentration was determined by the absorbance at 525 nm using Horisberger's finding that $A_{525nm} = 1.0$ corresponds to 7.7×10^{11} particles/ml (20).

Rabbit antiserum against human albumin (Calbiochem-Behring, La Jolla, Calif.) and goat antiserum against human fibrinogen (Sigma) were eluted through a diethylaminoethyl cellulose column (DEAE-Sephacel, Sigma) equilibrated with 0.05 *M* Tris buffer (pH 8.5). Antibodies against these human proteins were found to bind to canine proteins and were used because of their availability. The antibody concentration was measured using a protein assay solution (Bio-Rad) and adjusted to 150 μg/ml with deionized distilled water (DDW). This solution was then dialyzed against DDW for 2 h and filtered through a 0.2-μm-pore filter (Nucleopore, Pleasanton, CA).

One milliliter of the protein solution (150 μg/ml) was added to ten milliliters (about 10^{12} gold particles/ml) of the filtered gold solution. After 5 min, 0.5 ml of freshly prepared and filtered (0.45 μm Millex-HA, Millipore) 1% polyethylene glycol (MW = 20,000, Sigma) was added to prevent flocculation of the protein-coated gold particles. The antibody-labeled gold particles (immunogold) were centrifuged in polycarbonate tubes in an angle rotor (Beckman, Irvine, Calif.) at 10,000 rpm for 30 min. The supernate was discarded, and the concentrated immunogold beads were resuspended in 2 ml of 0.1 *M* phosphate buffer (pH 7.4) and stored at 4°C for up to 1 week.

The specificity of each preparation of immunogold particles was tested by observing the immunogold adhesion to fibrinogen-coated, albumin-coated, and bare polymer surfaces. Specific binding was observed with the occasional exception of antialbumin gold beads which sometimes bound to fibrinogen-coated surfaces. In such cases, the data were discarded.

Protein coated polymer surfaces were prepared as previously described and exposed to immunogold particles as described in Fig. 1. Fifteen-millimeter-long segments of PVC, PE, and SR were connected in series and exposed to protein solutions as described above. Following the displacement of the protein solution with PBS, 3-mm-long segments were cut from the tubing sections. The solution capillarity prevented the PBS from flowing from the lumen of this short segment until it was briefly blotted on tissue paper. The segment was immediately placed on a flat polystyrene surface and filled with the immunogold solution. Again, the capillary pressure kept the solution from flowing from the lumen. After 30 min of immunogold labeling, the segments were gently rinsed in PBS and then stored overnight in 2% glutaraldehyde solution at 4°C. The samples were dehydrated in a graded ethanol series and dried by the critical point method using molecular sieve-dried CO_2 as the transitional fluid. Samples were sputter-coated with 10 nm of Au or Au–Pd and examined on a JEOL JMS 35C scanning elec-

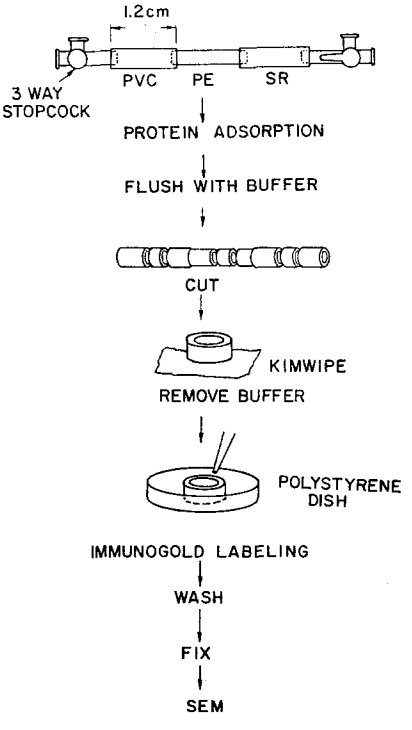

FIG. 1. Immunogold particle labeling procedure.

tron microscope (SEM) at 20 kV accelerating voltage.

Samples excised from the *ex vivo* shunt were fixed in 1.5% glutaraldehyde before exposure to the immunogold using the procedure described above.

Canine Model

The canine *ex vivo* shunt model and surgical procedures, as well as the counting procedures for surface adherent platelets have been described previously (21). Briefly, platelets are obtained from adult mongrel dogs weighing 20–35 kg and radiolabeled with ^{51}Cr by the method of Abrahamsen (22). Radiolabeled platelets are reinfused into the dog 15 h before the experiment. The experiment begins by anesthetizing the animal with sodium thiamylal. The femoral artery and vein are exposed and clamped, and the tubing is implanted as a femoral A-V shunt as Fig. 2 illustrates. The middle of the shunt is wrapped around a lead-shielded NaI solid crystal detector. Blood displaces the buffer upon removal of the clamps on the femoral artery and vein. Platelet deposition is measured and tubing samples removed for electron microscopy at time points of 2, 5, 10, 15, 30, 45, 60, 90, and 120 min of blood exposure in the following manner. The blood flow is stopped by clamping the vessels, and the blood in the shunt is displaced with 50 ml of modified Tyrode's solution via a branch artery. Then the platelet deposition is quantified by counting the ^{51}Cr activity in the shunt in contact with the solid crystal detector. Following the counting, the clamps are released and blood flow continued. The blood flow rate, continuously monitored with an electromagnetic flow transducer (SP2202, Statham Instruments, Oxnard, Calif.), was 150–250 ml/min.

At each time point, a 2-cm section of the tubing was excised distal to the detector and immediately fixed in 1.5% glutaraldehyde and prepared for scanning electron microscopy as described by Ihlenfeld *et al.* (21) with the exception that the microscopy was done at 12 kV. Some portions of the fixed tubing were prepared for immunogold bead labeling. Although some of the *ex vivo* experiments were done in triplicate for a preadsorbed protein surface, most surfaces in this preliminary study were examined in only single experiments.

RESULTS

Protein Adsorption

The surface concentrations of sequentially and competitively adsorbed albumin and fibrinogen are presented in Table I. The adsorption times for the first and second sequentially adsorbed protein are given on the left and the protein surface concentration (in $\mu g/cm^2$) are listed on the right. The data on competitively adsorbed protein are presented at the bottom of the table. Entries are the mean ± standard deviation for four separate determinations.

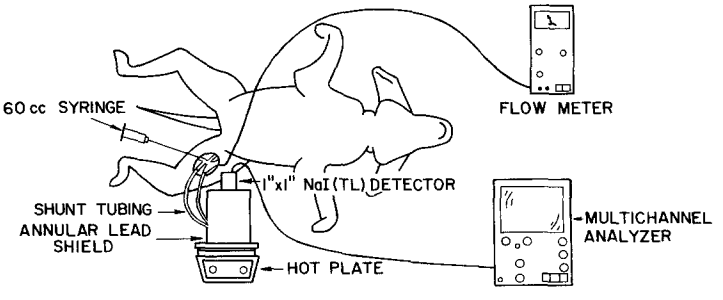

FIG. 2. *Ex vivo* A-V shunt experiment. The shunt is wrapped around the NaI detector which counts the radiolabeled platelets on the tubing surface.

TABLE II

Single Protein Adsorption and Displacement of the First Adsorbed Protein by the Second Protein in Sequential Adsorption

Protein adsorbed	Adsorption time (min)	Single adsorption surface concentration ($\mu g/cm^2$)[a]		Amount of first adsorbed protein displaced ($\mu g/cm^2$)	
		PVC	PE	PVC	PE
Albumin (0.3 mg/ml)	1	0.24 ± 0.03	0.20 ± 0.05	0.04	0.07
	3	0.31 ± 0.02	0.23 ± 0.02	0.05	0.06
	5	0.36 ± 0.03	0.25 ± 0.03	0.08	0.07
	10	0.40 ± 0.03	0.27 ± 0.02	0.08	0.07
	30	0.47 ± 0.03	0.30 ± 0.03	0.06	0.05
	60	0.52 ± 0.05	0.31 ± 0.02	0.07	0.06
	120	0.56 ± 0.07	0.32 ± 0.05	—	—
Fibrinogen (0.3 mg/ml)	1	0.32 ± 0.02	0.29 ± 0.03	0.00	0.08
	3	0.38 ± 0.02	0.32 ± 0.03	0.07	0.08
	5	0.40 ± 0.02	0.33 ± 0.04	0.08	0.06
	10	0.45 ± 0.03	0.35 ± 0.03	0.05	0.07
	30	0.55 ± 0.04	0.39 ± 0.05	—	0.02
	60	0.66 ± 0.05	0.40 ± 0.03	0.04	0.03
	120	0.78 ± 0.09	0.43 ± 0.04	—	—

[a] Mean ± SD (n = 4).

The data for single protein adsorption on PVC and PE is presented in Table II (mean ± SD, n = 4). Table II also presents the amount of the first adsorbed protein which was displaced or desorbed from the surface during adsorption of the second protein in the sequential adsorption experiments. As discussed previously, this protein desorption was determined by subtracting the final surface concentration of the sequential adsorptions from the surface concentration of the single adsorption experiments. In nearly all experiments, some of the first protein was displaced which indicates that a small fraction of the first adsorbed protein is reversibly bound. It is also possible that some reversibly bound protein is rapidly desorbed while the first or second protein solution is displaced by the PBS buffer. This work does not attempt to measure this type of rapid reversible desorption into the buffer, but other researchers have found this type of desorption to be very small in nonflowing systems (23).

To facilitate the display of these tabulated data, the concentration of protein remaining bound to the surface at the completion of sequential adsorption (Table I) is plotted in Figs. 3 and 4 with the amount of the first protein adsorbed plotted on the abscissa and the amount of the second protein adsorbed plotted on the ordinate.

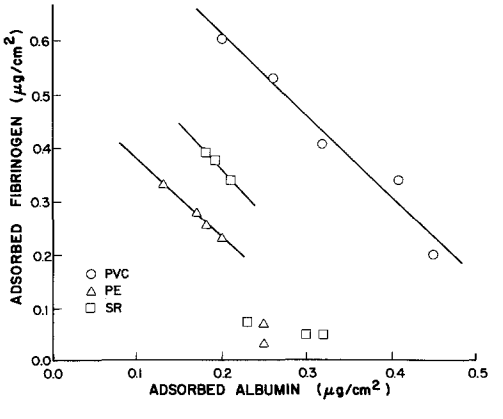

FIG. 3. Cross plot of sequential adsorption of 0.3 mg/ml canine albumin followed by 0.3 mg/ml canine fibrinogen on PVC (○), PE (△), and SR (□). The data are the averages of four separate determinations. Solid lines are a least-square fit of the data. The adsorption times are given in Table I.

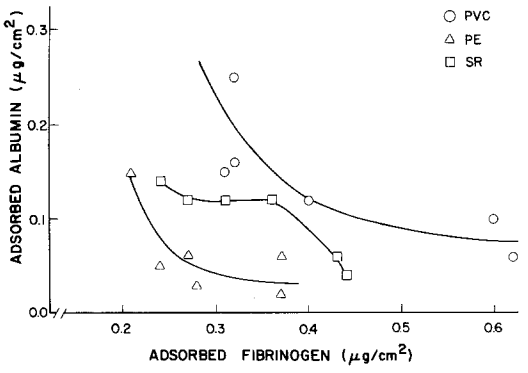

FIG. 4. Cross plot of sequential adsorption of 0.3 mg/ml canine fibrinogen followed by 0.3 mg/ml canine albumin on PVC (○), PE (△), and SR (□). The data are the averages of four separate determinations. The adsorption times are given in Table I.

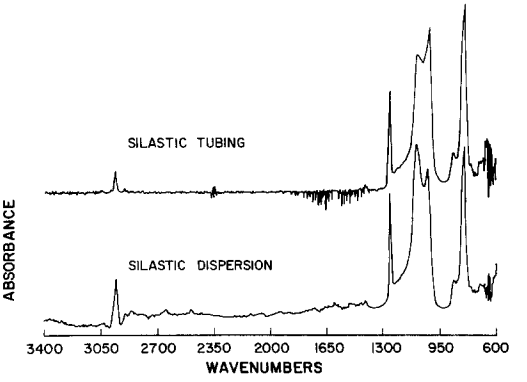

FIG. 5. FTIR/ATR spectra of silicone rubber tubing (Silastic) pressed against a Ge element (top), and silicone rubber dispersion coated and cured on a Ge element (bottom).

When albumin was the first protein adsorbed, Fig. 3 indicates that on PVC the amount of adsorbed fibrinogen is linearly related to the albumin surface concentration. On polyethylene and silicone rubber, this linearity is observed up to an albumin surface concentration of about 0.22 $\mu g/cm^2$. For higher albumin surface concentrations, the fibrinogen surface concentration appears to be small and independent of albumin concentration. When fibrinogen is the first adsorbed protein, there are no linear regions in the data (Fig. 4).

FTIR/ATR Results

The infrared spectra of the silicone rubber tubing and spin-cast film are shown in Fig. 5. The peaks at 1085 and 1020 cm^{-1} absorb differently in the tubing and film. These absorbances are due to Si—O vibrations. The ratio of the 2900-cm^{-1} peak (C—H stretching) to the 1260-cm^{-1} peak (Si—CH_3 vibration) is larger in the film than in the tubing. These differences indicate that the SR film has a slightly different chemical structure than the tubing. The effect of these differences upon the surface characteristics and protein adsorption is unknown. Surface characterization of both materials using contact angle techniques is currently being done. Until a reason for the differences in the two polymer spectra can be established, their protein adsorption properties should be compared with caution.

Figure 6 shows some typical plots of protein adsorption (measured by the 1550-cm^{-1} peak) versus time for the sequential adsorption of albumin followed by fibrinogen on the spin-coated SR. Although each experiment was at least 45 min, this figure shows only the first 25 min of the experiment. The arrows indicate the time at which the albumin was flushed from the cell. The sharp increase in absorbance following flushing indicates introduction of the fibrinogen solution. The initial fibrinogen adsorption rates, defined as the time derivative

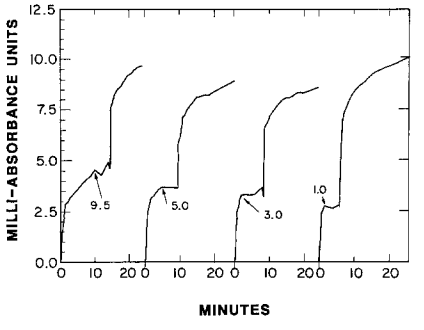

FIG. 6. Height (milliabsorbance units) of the 1550-cm^{-1} peak during the sequential adsorption of albumin followed by fibrinogen. The arrows indicate the time at which the albumin solution was displaced with buffer solution.

of the 1550-cm^{-1} absorbance (the slopes at these sharp increases), are plotted in Fig. 7. This plot, with an ordinate of adsorption rate, is qualitatively similar to Fig. 3 which has an ordinate of adsorbed fibrinogen surface concentration. Below a certain albumin surface concentration, there is a linear correlation in the fibrinogen initial adsorption rate with decreasing albumin on the surface. Above that concentration, the initial adsorption rate appears to be independent of albumin concentration. The end of the linear region appears to be near 3.5 milliabsorbance units (mAU) of surface albumin. Preliminary radiolabeling studies in the FTIR flow cell indicate that 1 mAU corresponds roughly to 0.07 or 0.03 $\mu g/cm^2$ of adsorbed albumin or fibrinogen, respectively. Thus the break in the fibrinogen adsorption rate occurs roughly near 0.24 $\mu g/cm^2$ of adsorbed albumin which is at approximately the same albumin surface coverage as the break in the plot of the amount of adsorbed fibrinogen versus adsorbed albumin (Fig. 3).

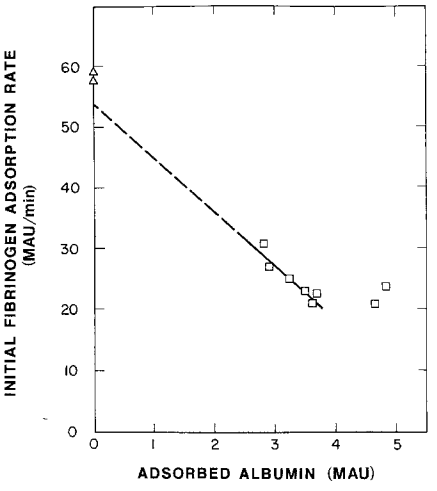

FIG. 7. Initial fibrinogen adsorption rate (mAU/min) versus surface albumin concentration (mAU) for the sequential adsorption of albumin followed by fibrinogen (□) and for single adsorption of fibrinogen (△). The line is the least-square fit of the six points between 2 and 4 mAU of albumin.

Immunogold Studies

Although the efficiency of immunogold labeling is less than unity (not every protein binds one gold particle), previous studies have shown that the immunogold particles bind only where adsorbed protein is present. Thus, the distribution of bound immunogold reveals the general distribution of the antigenic proteins adsorbed to the surface.

Figure 8A shows the distribution of antifibrinogen gold beads on polyethylene which has been preadsorbed with fibrinogen for 2 h. A similar uniform distribution was observed on PVC and SR coated with fibrinogen only. No antifibrinogen gold beads were observed on albumin coated polymers or bare polymer control surfaces, indicating the absence of nonspecific binding of antifibrinogen immunogold. Neither were antialbumin beads observed on bare control surfaces. Occasionally antialbumin beads were found on fibrinogen-coated surfaces; when this occurred all the data from that experiment were discarded.

When antialbumin gold beads did adsorb specifically, the distribution was always nonuniform. For example, the distribution of antialbumin gold beads on albumin adsorbed on PE for 2 h (Fig. 8B) shows a nonrandom distribution in which the gold particles form strings of beads. This pattern does not seem to be an artifact of SEM preparation since the same preparation technique produces uniform distributions of antifibrinogen immunogold bound to adsorbed fibrinogen.

After the specificity of immunogold binding was established, the immunogold suspension was applied to surfaces sequentially or competitively adsorbed with protein. Figure 9 shows the distribution of antifibrinogen (A) and antialbumin (B) gold beads on PE following competitive adsorption of fibrinogen and albumin. Again, the distribution of antifibrinogen particles appeared homogeneous while antialbumin particles were not uniformly distributed. In all sequential adsorption experiments, the same type of distribution was observed: antifibrinogen markers were uniformly distributed while antialbumin markers were

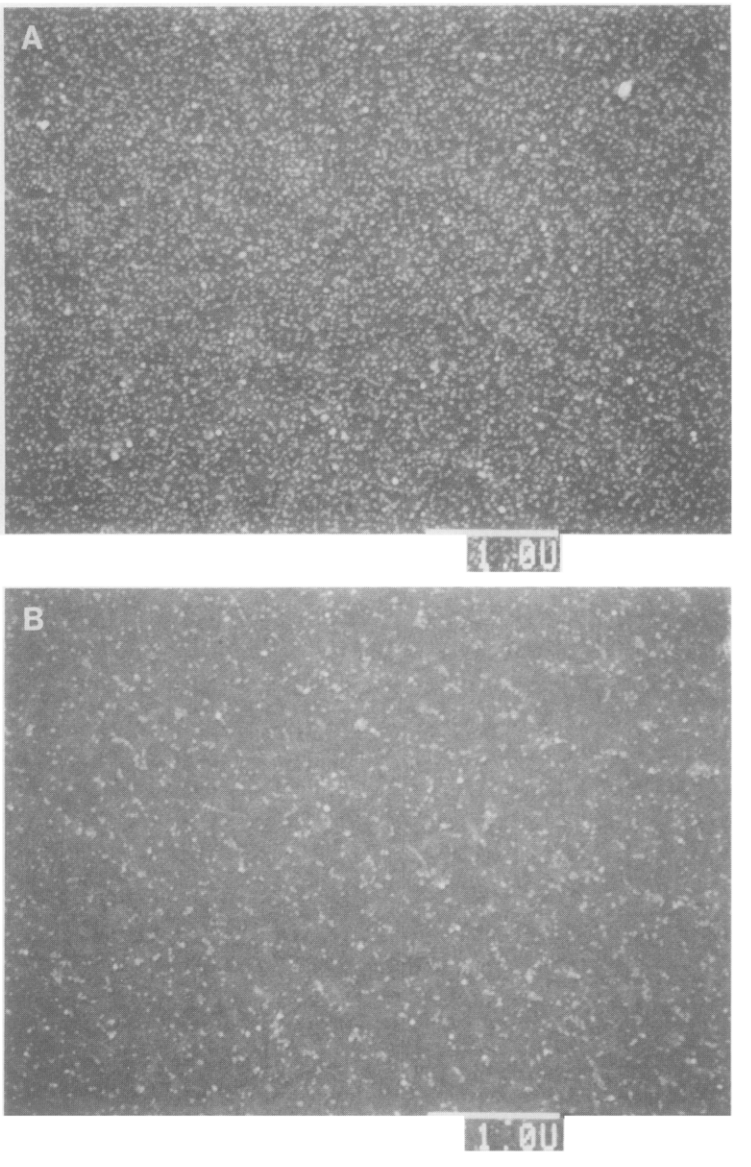

FIG. 8. Distribution of immunogold particles on polyethylene. (A) Antifibrinogen immunogold on PE adsorbed with fibrinogen for 2 h. (B) Antialbumin immunogold on PE adsorbed with albumin for 2 h.

found in strings and patches. The uniformity of antifibrinogen and the irregularity of antialbumin beads showed no observable correlation with surface concentration. This result suggests that this immunogold method may not be as sensitive in measuring the amount of protein sequentially adsorbed as are the radiolabelling and FTIR/ATR techniques.

Canine Model

The data generated in the *ex vivo* canine model is extensive and have been presented in part elsewhere (24) and is summarized in Table III. These values of platelet deposition are the maximum number of surface-bound platelets measured during the two hours of

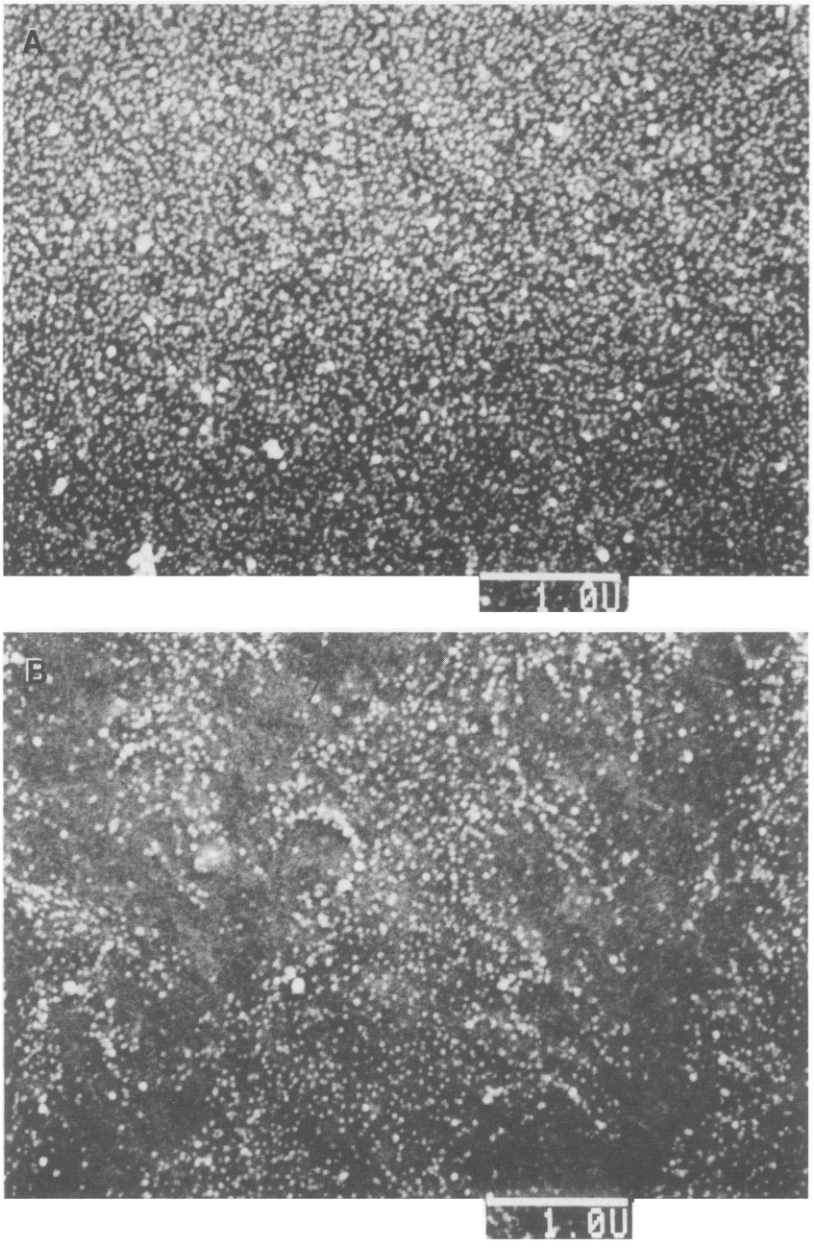

FIG. 9. SEM micrographs of the immunogold distribution on PE subjected to competitive adsorption of 0.3 mg/ml albumin and 0.3 mg/ml fibrinogen. (A) Antifibrinogen immunogold. (B) Antialbumin immunogold.

blood exposure. This maximum always occurred between 5 and 45 min of blood exposure.

Figure 10 shows an example of the transient platelet deposition profiles on silicone rubber preadsorbed with fibrinogen and albumin sequentially or competitively. This platelet deposition data is from single-shunt *ex vivo* experiments for reasons discussed previously. In this figure, the surface fibrinogen concentra-

TABLE III

Maximum Level of Platelet Deposition on Polymers Exposed to Sequential and Competitive Protein Adsorption

First protein adsorbed		Second protein adsorbed		Platelet deposition[a] (per 1000 μm^2)		
Type	Adsorption time (min)	Type	Adsorption time (min)	PVC	PE	SR
Albumin	1	Fibrinogen	119	—	120	20
(0.3 mg/ml)	3	(0.3 mg/ml)	117	530[b]	30	—
	5		115	575	—	30
	10		110	40	—	—
	30		90	—	—	—
	60		60	50	—	—
	120		0	50 ± 10[c]	70 ± 20	<50
Fibrinogen	1	Albumin	119	—	750	1120
(0.3 mg/ml)	3	(0.3 mg/ml)	117	580	—	—
	5		115	—	—	—
	10		110	—	1680	350
	30		90	—	—	—
	60		60	600	—	—
	120		0	2000 ± 500	1940	580 ± 130
Competitive adsorption for 120 min (0.3 mg/ml albumin and 0.3 mg/ml fibrinogen)				340	150	30
No preadsorbed protein (bare polymer surface)				320 ± 170	760 ± 210	90 ± 40

[a] Maximum in platelet versus time adsorption profile.
[b] Single determination.
[c] Mean ± SD.

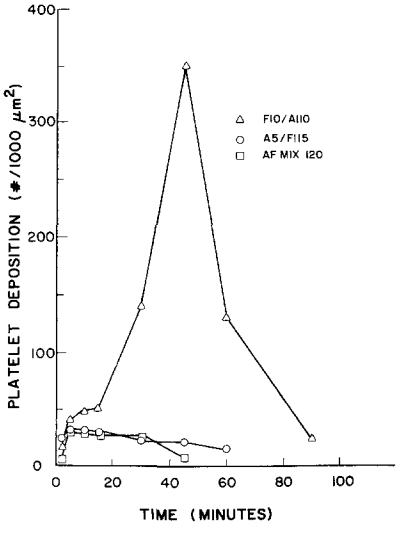

FIG. 10. Transient platelet deposition on silicone rubber tubing preadsorbed with fibrinogen 10 min followed by albumin 110 min (△), albumin 5 min followed by fibrinogen 115 min (○), and competitive adsorption of albumin and fibrinogen for 120 min (□).

tions are 0.33, 0.36, and 0.38 $\mu g/cm^2$, respectively, for competitive adsorption, fibrinogen followed by albumin adsorption, and albumin followed by fibrinogen adsorption. On the surface sequentially preadsorbed with fibrinogen followed by albumin, the peak in platelet deposition is indicative of thrombus formation followed by embolization (1, 2).

It is interesting that even though the protein concentrations are nearly the same on all three surfaces (see Table I), extensive platelet deposition occurred when fibrinogen was the first protein adsorbed. This is a general observation as Table III indicates. Platelet aggregation occurred in all sequential adsorption experiments in which fibrinogen was the first protein adsorbed, even if the fibrinogen adsorption was only for 1 min. (In this communication, we define platelet aggregation on a surface as a surface concentration greater than 100 platelets/1000 μm^2 since a nonspread platelet cov-

ers 9–10 μm^2.) When albumin was the first protein adsorbed, aggregation was observed on PE and PVC only when the subsequent fibrinogen adsorption time was at least 119 and 115 min respectively.

Scanning Electron Microscopy

Figure 11 shows a SEM sequence of platelet activation, thrombus formation and embolization on silicone rubber tubing which was preadsorbed with fibrinogen for 2 h and then exposed to nonanticoagulated blood in the *ex vivo* shunt. Figure 11A shows the platelet morphology at 10 min of blood contact. Most platelets are rounded with pseudopod extensions. A relatively small number have started spreading on the surface. At 30 min of blood contact, Fig. 11B shows platelets with various degrees of pseudopod extension deposited on top of fully spread platelets. Figure 11C shows a massive platelet aggregate (thrombus) at 45 min of blood exposure. There appears to be a film of material covering the surface of the polymer which is attached to the edges of the thrombus. Figure 11D shows the surface at 90 min of blood contact. Single rounded platelets with a few pseudopods are deposited on a film of material.

The SEM data on the other surfaces have been presented elsewhere (24). Briefly, the fibrinogen coated surfaces and the surfaces sequentially adsorbed with fibrinogen as the first protein displayed a sequence of platelet morphology and aggregation similar to Fig. 11, although there were differences in the size and number of thrombi at the point of maximum platelet deposition. Those surfaces which were sequentially adsorbed with fibrinogen followed by albumin had less thrombus formation than the surfaces exposed to fibrinogen for 2 h.

When only albumin was preadsorbed or was the first sequentially adsorbed protein, no large platelet aggregates were observed at any time point except in the case of the PVC where the albumin adsorption was followed by at least 115 min of fibrinogen adsorption. With this exception, the SEM of the surfaces showed single platelets which had a few pseudopod extensions.

When the segments excised from the *ex vivo* shunt were stained with immunogold, antifibrinogen beads were observed near adhered platelets. Figure 12A shows antifibrinogen markers between spread platelets on PE sequentially exposed to fibrinogen for 10 min, then exposed to albumin for 110 min, and finally exposed to flowing blood for 90 min. The presence of antifibrinogen markers in the same region as the spread platelets suggests that adsorbed fibrinogen is associated with platelet adhesion and spreading. On this same surface, the density of antialbumin beads is very low (Fig. 12B). Although the number of antifibrinogen and antialbumin gold beads appear to be proportional to the surface concentration of the proteins before exposure to the blood (0.28 $\mu g/cm^2$ fibrinogen and 0.03 $\mu g/cm^2$ albumin), it is unknown whether the proteins labeled are those deposited in the sequential adsorption or proteins deposited from the blood. During 90 min of blood exposure, exchange or replacement with blood proteins is likely to have occurred.

DISCUSSION

Since the *ex vivo* results indicate that the sequence of protein adsorption has a strong influence on platelet deposition, one of our first objectives is to understand the sequential adsorption phenomenon and how it may influence the blood response to a surface. In this study of sequential adsorption, we have investigated how the presence of the first protein adsorbed to the surface affects the subsequent adsorption of the second protein. It has been shown that the concentration of the first protein sequentially adsorbed influences both the amount (Figs. 3 and 4) and the initial adsorption rate (Fig. 7) of the second protein sequentially adsorbed. When albumin is the first protein adsorbed, the amount of fibrinogen adsorbed and the initial fibrinogen adsorption rate correlate linearly with the amount of adsorbed albumin. It is interesting that on PE

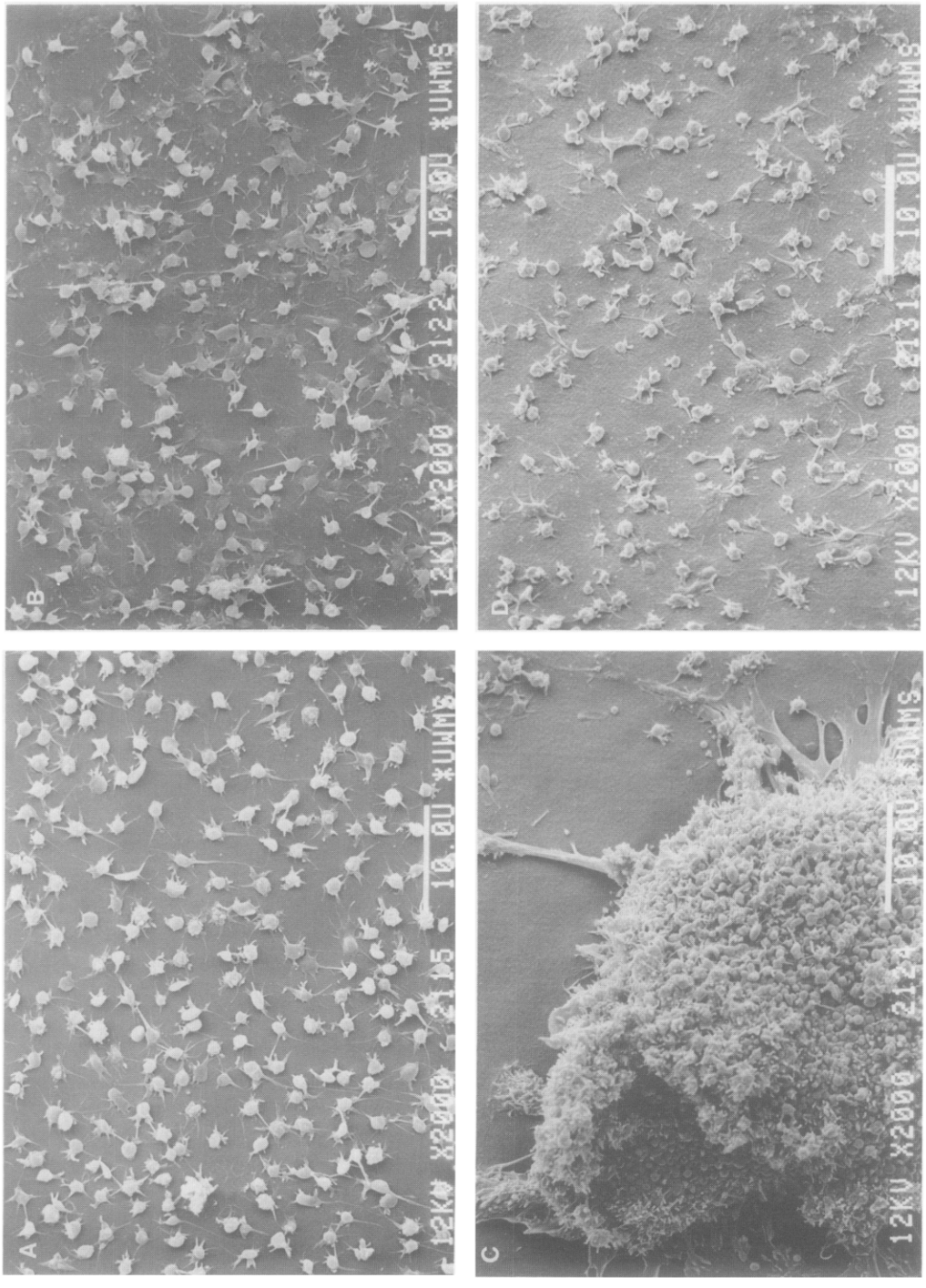

FIG. 11. SEM micrographs of platelet deposition on silicone rubber preadsorbed with fibrinogen for 2 h. (A) 10 min, (B) 30 min, (C) 45 min, (D) 90 min.

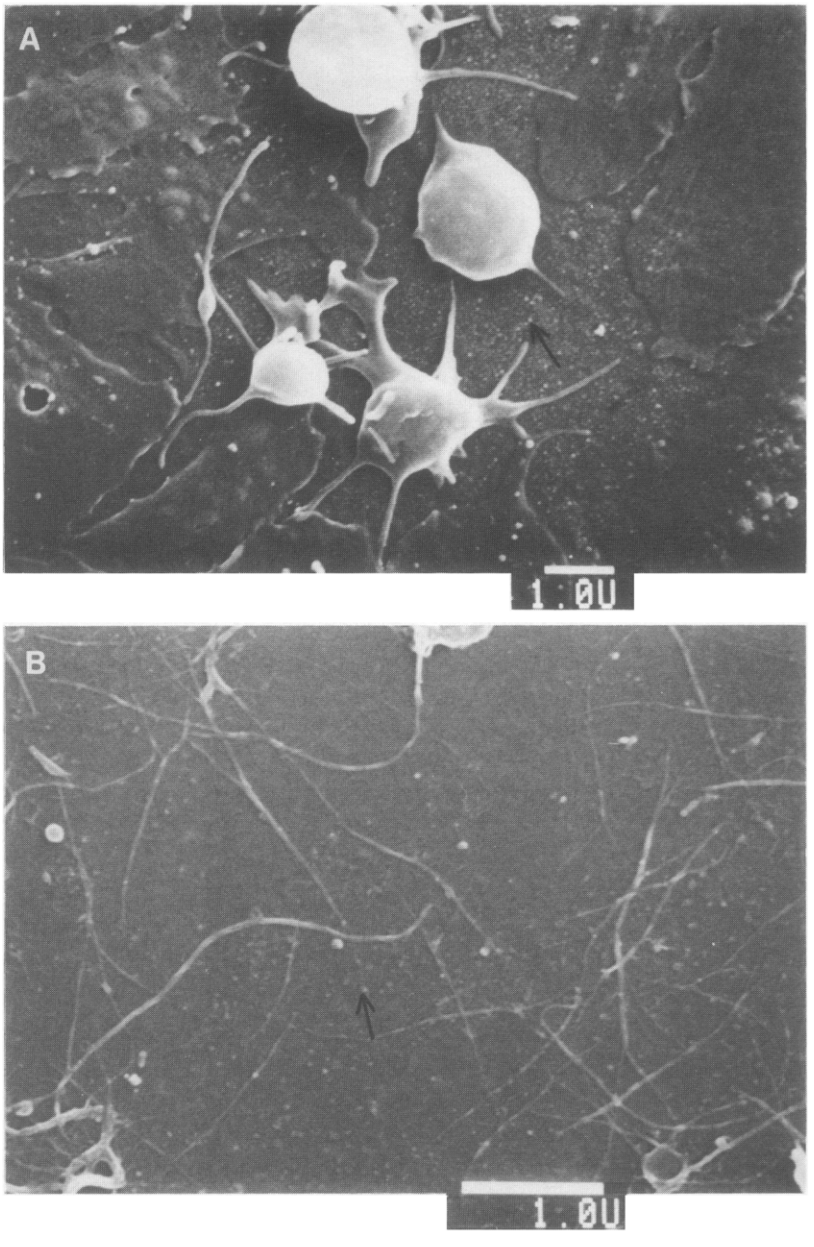

FIG. 12. SEM micrographs of the immunogold on a PE shunt exposed to blood for 90 min. The shunt was preadsorbed sequentially with fibrinogen for 10 min and then with albumin for 110 min. (A) Antifibrinogen immunogold between spread platelets. (B) The same surface exposed to antialbumin immunogold shows very few gold beads. The arrows point to the immunogold particles.

and SR these correlations only exist up to near 0.22 $\mu g/cm^2$ of adsorbed albumin which is very near the calculated value of 0.18 $\mu g/cm^2$ for a close-packed monolayer of side-on adsorbed albumin. Although this correlation may be fortuitous, it does suggest that on PE and SR, the amount of fibrinogen adsorption is linearly related to the area of bare polymer exposed in

a partial monolayer of albumin. It is unknown why a similar break in linearity on PVC is not observed. It is doubtful that the "monolayer coverage" on PVC occurs at higher albumin surface concentrations because adsorption isotherms obtained in separate studies indicate that the mass of an albumin monolayer on PVC is nearly the same as that on SR and PE (16).

The initial fibrinogen adsorption rate data is also interesting because a linear regression analysis of the data points in the linear region extrapolates to an ordinate intercept of 54.0 ± 4.4 mAU/min (mean ± SD). This extrapolated value is very near the experimentally observed initial fibrinogen adsorption rate of 58.3 ± 0.78 mAU/min on SR with no preadsorbed albumin (Fig. 7) which suggests that this linearity of initial fibrinogen adsorption rate continues to the limit of zero adsorbed albumin. Again assuming the existence of a partial monolayer of albumin, this linearity suggests that the initial fibrinogen adsorption rate is directly proportional to the area of bare silicone rubber exposed to the fibrinogen solution. In terms of adsorption kinetics, this proportionality indicates that the fibrinogen adsorption rate is first-order with respect to the bare polymer surface area. This is consistent with the results of sequential protein adsorption by Vroman *et al.* (25) who have suggested that albumin films may simply have holes or that loosely bound albumin molecules allow space to be filled in by other species like fibrinogen. This hypothesis is currently being investigated by collecting FTIR/ATR fibrinogen adsorption data at very low albumin coverages.

When fibrinogen adsorption is followed by albumin adsorption, no linear correlations are observed. The reason for this is unknown, but could possibly be due to very rapid adsorption of fibrinogen which may leave little, if any, bare polymer surface exposed to the albumin solution. This is reasonable considering the fact that fibrinogen is a major component of protein layers adsorbed from plasma and from fibrinogen–albumin mixtures (26, 27). Previous work indicates that a monolayer of fibrinogen is adsorbed on these surfaces in 3 or 4 min at this bulk concentration (0.3 mg/ml) (16). Another possibility is that the orientation or conformation of the fibrinogen is changing with time which may make the subsequent albumin adsorption difficult to analyze. A conformational change with time has also been suggested by Horbett who observed that adsorbed fibrinogen becomes less easily eluted the longer it has resided on the surface (28).

Immunogold Labeling

The very different distribution of antifibrinogen and antialbumin particles is readily apparent. Since these differences do not appear to be an SEM artifact, we assume that the difference in immunogold distribution reflects differences in the adsorption characteristics of fibrinogen and albumin. The nonuniformity of the antialbumin gold beads could be caused by several factors: (1) the albumin is adsorbing nonhomogeneously in islands or patches; (2) as the albumin molecules adsorb, their orientation or conformation is not uniform, and thus the availability or efficiency of the antigenic sites to bind the immunogold is not uniform; or (3) albumin may polymerize in the bulk solution before adsorption and polymerized albumin may be labeled with immunogold markers while single albumin molecules may not. The first, of these possible factors, patchwise adsorption of albumin on PE, SR, or PVC, has not been observed previously to our knowledge; however, Rudee and Price recently have shown evidence of nonuniform albumin adsorption on polystyrene and polycarbonate (29). The second possible factor, variations in the orientation or conformation of the adsorbed albumin, may also produce heterogeneous antibody labeling. In an analogous example, heterogeneity in protein orientation has been implicated in decreased antibody binding to adsorbed fibronectin (30). Similarly, antibody binding to albumin could also show an albumin orientation dependence, although we are unaware of any previous re-

ports of this nature. Unfortunately, the immunogold technique cannot reveal whether the protein distribution or orientation is heterogeneous, and other methods must be used to address this question.

Heterogeneities in the polymer surface itself may be a cause of the nonuniform albumin adsorption. In synthetic polymers, one can expect surface heterogeneities ranging from Angstrom to micron size caused by surface roughness, impurities, residual mechanical stresses, plasticizers, etc. If surface heterogeneities affect protein adsorption, one could expect the albumin and fibrinogen to adsorb similarly. The observation of a more uniform distribution of antifibrinogen immunogold may be an effect of the greater affinity of fibrinogen for the surface. High surface affinity causes a more uniform protein adsorption since the stronger bond between the protein and the surface inhibits surface diffusion and reversible adsorption which are necessary for patchwise adsorption or island formation. It is apparent here that further work is required to substantiate these hypotheses.

Protein Influence on Platelet Deposition

The fundamental purpose of this research was to understand in more detail how adsorbed proteins influence *ex vivo* platelet deposition and thrombus formation. Although there may be a difference in the protein adsorption behavior between human and canine blood, our data using a canine *ex vivo* model clearly indicate that the total amount of fibrinogen on a surface does not correlate well with the platelet response. More importantly, it seems that, in sequential adsorption, the first protein to contact the surface dominates the platelet response. (The notable exception involves very short albumin adsorption times and long fibrinogen adsorption times on PVC and PE.) Although a small fraction of the first preadsorbed protein is displaced by the second protein, it is the tightly bound first protein which remains on the surface to interact with the whole blood in the *ex vivo* experiment.

In competitive adsorption, the surfaces appear to be dominated mainly by an albumin-type passivation response, but they do evoke a small amount of platelet aggregation on PVC and PE. Since in competitive adsorption the albumin diffuses to the surface faster than fibrinogen, albumin has a greater probability of contacting a given surface site first. Thus, the competitive adsorption data also supports the postulate that the first preadsorbed protein influences the platelet response much more than proteins which adsorb later.

The importance of the first tightly adsorbed protein leads one to hypothesize the following simplified model for platelet deposition on protein-coated polymers. As discussed previously it is assumed that the polymer surface is heterogeneous on the submicrometer scale. The source of the heterogeneities may be due to impurities, fabrication stresses, plasticizers, fillers, surface roughness, etc. These polymer heterogeneities produce a heterogeneous interfacial energy distribution. As protein molecules randomly diffuse to, and strike the surface, they bind much more tightly at the high energy sites than at the more reversible lower energy binding sites.

As larger elements such as platelets reach the protein-coated surface, they will bind tightly to platelet adhesive proteins such as fibrinogen. A platelet which encounters a nonadhesive protein, such as albumin, or one which binds to adhesive proteins which are not tightly bound to the surface may desorb from the surface which precludes platelet deposition and thrombus formation. However, a platelet which binds to an adhesive protein which has adsorbed at a high energy site will become bound to the surface and become activated, serving as a nucleation site for further aggregation and thrombogenesis.

The data presented above correspond well to this model. In the event that proteins are sequentially preadsorbed, the first protein to contact the surface will irreversibly occupy the high energy sites and control the platelet response. In the case of competitive adsorption, the high energy sites will be occupied by a dis-

tribution of proteins depending upon the diffusivities and concentrations of the proteins; thus the platelet response will be a mixture of the response to the individual proteins, as seen in our experiments. Blood contact on a bare polymer would be a complicated example of hundreds of proteins competitively adsorbing.

This simplified and preliminary model for the interaction between polymers, proteins, and platelets is not expected to explain all observations of blood–biomaterial interactions. The model does provide a basis for the interpretation of the nonanticoagulated blood response to sequentially and competitively protein precoated polymer tubing. Further research remains to be done to fully test the hypothesis described above.

SUMMARY

Proteins adsorbed at the blood–polymer interface play a critical role in platelet deposition and thrombus formation. Although past research generally has focused on the surface concentrations of the proteins adsorbed on polymers, the sequence of protein adsorption appears to play a much greater role in controlling the thrombogenic response in these studies. In particular, the first protein adsorbed in sequential adsorption seems to dominate the response observed in a nonanticoagulated canine *ex vivo* shunt experiment. This behavior is attributed to the first adsorbed protein binding tightly to high energy surface sites and thereby affecting the blood response.

The mechanisms controlling the blood response to sequentially adsorbed proteins may be probed by investigating the protein adsorption process in detail. The use of radiolabeled proteins, FTIR/ATR spectroscopy, and immunogold labeling provides additional information on sequential protein adsorption. In the sequential adsorption of albumin followed by fibrinogen on PVC, the surface concentration of fibrinogen is linearly correlated with the amount of preadsorbed albumin. On SR and PE, this linearity is also observed up to a surface concentration corresponding to a monolayer of side-on adsorbed albumin. On SR, the initial fibrinogen adsorption rate correlates linearly with the amount of adsorbed albumin in the submonolayer regime.

In the sequential adsorption of fibrinogen followed by albumin, no linear correlations are observed between the two proteins adsorbed on the surface.

Immunogold labeling of proteins adsorbed singly, sequentially, or competitively on PVC, SR, and PE reveals that albumin adsorption appears to be nonuniform while fibrinogen adsorbs much more uniformly. Further research must be pursued to determine if the inhomogeneities in albumin adsorption are due to patchwise adsorption or to a distribution of orientations among homogeneously adsorbed albumin.

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REFERENCES

1. Lelah, M. D., Pierce, J. A., Lambrecht, L. K., and Cooper, S. L., *J. Colloid Interface Sci.* **104,** 422 (1985).
2. Lelah, M. D., Grasel, T. G., Pierce, J. A., Lambrecht, L. K., and Cooper, S. L., *Trans. Amer. Soc. Artif. Intern. Organs* **30,** 411 (1984).
3. Turitto, V. T., Weiss, H. J., and Baumgartner, H. R., *J. Rheol. (N.Y.)* **23,** 735 (1979).
4. Barber, T. A., Lambrecht, L. K., Mosher, D. F., and Cooper, S. L., *Scanning Electron Microsc. 1979* **III**, 881 (1979).
5. Young, B. R., Lambrecht, L. K., and Cooper, S. L., *in* "Biomaterials: Interfacial Phenomena and Applications" (S. L. Cooper and N. A. Peppas, Eds.), p. 317. Amer. Chem. Soc., Washington, D.C., 1982.
6. Young, B. R., Lambrecht, L. K., Albrecht, R. M., Mosher, D. F., and Cooper, S. L., *Trans. Amer. Soc. Artif. Intern. Organs* **29,** 442 (1983).
7. Roohk, H. V., Pick, J., Hill, R., Hung, E., and Bartlett, R. H., *Trans. Amer. Soc. Artif. Intern. Organs* **22,** 1 (1976).
8. Zucker, M. B., and Vroman, L., *Proc. Soc. Exp. Biol. Med.* **131,** 318 (1969).
9. Vroman, L., *in* "Biocompatible Polymers, Metals, and Composites" (M. Szycher, Ed.), pp. 81–88. Technomic Pub. Co. Inc., Lancaster, 1983.

10. Packham, M. A., Evans, G., Glynn, M. F., and Mustard, J. F., *J. Lab. Clin. Med.* **73,** 686 (1969).
11. Lyman, D. J., *in* "Structural Order in Polymers" (F. Ciardelli and P. Giusti, Eds.), p. 205. Pergamon, New York, 1981.
12. O'Rourke, S. T., Folts, J. D., and Albrecht, R. M., *Scanning Electron Microsc. 1984* **III,** 1337 (1984).
13. Jakobsen, E., and Kierulf, P., *Thromb. Res.* **3,** 145 (1973).
14. Laki, K., *Arch. Biochem. Biophys.* **32,** 317 (1951).
15. Coller, B. S., *Blood* **53,** 325 (1979).
16. Young, B. R., "Protein Adsorption on Polymeric Biomaterials and Its Role in Thrombogenesis," Doctoral thesis, pp. 261–277. Univ. of Wisconsin-Madison, 1984.
17. Bellissimo, J. A., and Cooper, S. L., *Trans. Amer. Soc. Artif. Intern. Organs* **30,** 359 (1984).
18. Fink, D. J., and Gendreau, R. M., *Anal. Biochem.* **139,** 140 (1984).
19. Loftus, J. C., and Albrecht, R. M., *Scanning Electron Microsc. 1983* **IV,** 1995 (1983).
20. Horisberger, M., and Rosset, J., *J. Histochem. Cytochem.* **25,** 295 (1977).
21. Ihlenfeld, J. V., Mathis, T. R., Riddle, L. M., and Cooper, S. L., *Thromb. Res.* **14,** 953 (1979).
22. Abrahamsen, A. F., *Scand. J. Haemat.* **5,** 53 (1968).
23. Brash, J. L., Uniyal, S., and Samak, Q., *Trans. Amer. Soc. Artif. Intern. Organs* **20,** 69 (1974).
24. Park, K., Mosher, D., and Cooper, S. L., *J. Biomed. Mater. Res.*, in press.
25. Vroman, L., Adams, A. L., and Klings, M., *Fed. Proc.* **30,** 1494 (1971).
26. Horbett, T. A., and Hoffman, A. S., *in* "Applied Chemistry at Protein Interfaces" (R. E. Baier, Ed.), p. 230. Amer. Chem. Soc., Washington, D.C., 1975.
27. Vroman, L., Adams, A. L., Klings, M., and Fischer, G., *in* "Applied Chemistry at Protein Interfaces" (R. E. Baier, Ed.), p. 255. Amer. Chem. Soc., Washington, D.C., 1975.
28. Bohnert, J. L., and Horbett, T. A., *J. Colloid Interface Sci.* **111,** 361 (1986).
29. Rudee, M. L., and Price, T. M., *J. Biomed. Mater. Res.* **19,** 57 (1985).
30. Grinnell, F., and Feld, M. K., *J. Biol. Chem.* **257,** 4888 (1982).