Fibrin(ogen) Mediates Acute Inflammatory Responses to Biomaterials

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Summary

Although "biocompatible" polymeric elastomers are generally nontoxic, nonimmunogenic, and chemically inert, implants made of these materials may trigger acute and chronic inflammatory responses. Early interactions between implants and inflammatory cells are probably mediated by a layer of host proteins on the material surface. To evaluate the importance of this protein layer, we studied acute inflammatory responses of mice to samples of polyester terephthalate film (PET) that were implanted intraperitoneally for short periods. Material preincubated with albumin is "passivated," accumulating very few adherent neutrophils or macrophages, whereas uncoated or plasma-coated PET attracts large numbers of phagocytes. Neither IgG adsorption nor surface complement activation is necessary for this acute inflammation; phagocyte accumulation on uncoated implants is normal in hypogammaglobulinemic mice and in severely hypocomplementemic mice. Rather, spontaneous adsorption of fibrinogen appears to be critical: (*a*) PET coated with serum or hypofibrinogenemic plasma attracts as few phagocytes as does albumin-coated material; (*b*) in contrast, PET preincubated with serum or hypofibrinogenemic plasma containing physiologic amounts of fibrinogen elicits "normal" phagocyte recruitment; (*c*) most importantly, hypofibrinogenemic mice do not mount an inflammatory response to implanted PET unless the material is coated with fibrinogen or the animals are injected with fibrinogen before implantation. Thus, spontaneous adsorption of fibrinogen appears to initiate the acute inflammatory response to an implanted polymer, suggesting an interesting nexus between two major iatrogenic effects of biomaterials: clotting and inflammation.

Implanted biomaterials frequently trigger inflammatory responses accompanied by an accumulation of phagocytic cells (especially macrophages and neutrophils [PMN]) on and adjacent to the implant surface (1–5). These inflammatory responses may, in turn, presage serious iatrogenic consequences, such as the "hardening" and degradation of mammary implants (6, 7), stress cracking of pacemaker leads (8–10), and fibrous thickening surrounding many types of implants (11–21).

The acute and chronic inflammatory responses to these implants are puzzling in view of the inert and nontoxic nature of commonly used polymeric biomaterials. Because protein adsorption is much more rapid than the migration of cells to foreign surfaces, inflammatory cells most likely respond not to the material surface itself but to a chaotic layer of spontaneously adsorbed, partially "denatured" host proteins (22–25). In an earlier attempt to understand the processes involved in these responses (26), we employed films of polyethylene terephthalate (PET).[1] The knitted form of this material, Dacron®, has been used extensively in vascular grafts and is known to provoke inflammatory responses (1, 20, 27, 28). When untreated disks of PET film are implanted intraperitoneally in mice, large numbers of phagocytes (especially macrophages and PMN) are attracted to the implant surfaces. However, as previously shown by a number of other investigators (e.g., 1, 29), if the material is preincubated with albumin, the surface attracts <10% as many phagocytes.

Surprisingly, PET preincubated with human or mouse plasma attracts large numbers of phagocytes, despite the fact that albumin is the major protein in plasma and the most abundant surface protein on many biomaterials after contact with blood (30). These observations suggested that unknown plasma components that spontaneously adsorb to material surfaces might be important in the subsequent recruitment of inflammatory cells. We have tentatively ruled out two possibilities: surface activation of complement components and adsorbed IgG. Untreated PET implanted in mice with either severe combined immunodeficiency (with almost undetectable levels of IgG) or complement deficiency (induced by injection of cobra venom factor) had near-normal recruitment of phagocytic cells to the implant surface (26).

However, in the course of these earlier experiments, we

[1] *Abbreviations used in this paper:* EPO, eosinophil peroxidase; MPO, myeloperoxidase; NSE, nonspecific esterase; PET, polyethylene terephthalate.

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observed that material preincubated with serum attracts as few phagocytes as does material coated with albumin. This suggested that one or more elements present in plasma, but absent in serum, were responsible for the proinflammatory effects of implanted materials. For a number of reasons, we suspected that this element might be fibrinogen, which is quantitatively lost from the solution phase upon clotting. We now show that adsorbed fibrinogen is the primary component of plasma responsible for acute inflammatory responses (i.e., phagocyte recruitment) to implanted materials and that severely hypofibrinogenemic animals do not mount an inflammatory response to an implant unless the surface is previously coated with, or has spontaneously adsorbed, exogenous fibrinogen. These observations suggest an interesting connection between inflammation and coagulation, perhaps the two major iatrogenic effects of biomaterials.

Materials and Methods

Materials. Human albumin (Albumar-5) was purchased from Armour Pharmaceutical Co. (Kankakee, IL). Human fibrinogen (Type I), mouse fibrinogen (Fraction I), bovine thrombin, 3-amino-1,2,4-triazole, horseradish peroxidase, guaiacol (*o*-methoxyphenol), hydrogen peroxide (30% solution), eserine (physostigmine), *o*-nitrophenyl butyrate, dimethyl sulfoxide, β-nicotinamide adenine dinucleotide, reduced form (β-NADH, from yeast), sodium pyruvate, goat anti-human fibrinogen, rabbit anti-goat IgG (whole molecule) with peroxidase label, *o*-phenylene diamine (OPD), Triton X-100 (octyl phenoxy polyethoxyethanol) and ancrod (from venom of *Agkistrodon rhodostoma*) were obtained from Sigma Chemical Co. (St. Louis, MO). Human ^{125}I-fibrinogen was purchased from ICN Chemical and Radioisotope Division (Costa Mesa, CA). Polyethylene terephthalate (PET) Mylar® film (type A, 0.005 mm thick), was obtained from Cadillac Plastic and Chemical Company (Birmingham, MI).

Preparation of PET Disks. Disks of 1.2 cm diameter were cut from PET film. The disks were stirred for 24 h in 70% ethanol, with frequent changes of ethanol, in order to remove dust and sterilize the surface, and stored in 100% ethanol. Before use, the disks were hydrated by immersion in sterile, pyrogen-free saline for at least 1 h. Protein-coated plastic disks were produced by incubating hydrated PET disks with solutions of albumin (25 mg/ml), fibrinogen (1.5 mg/ml), human or murine plasma (1:3 dilution), human or murine serum (1:3 dilution), or PBS (100 mM, pH 7.3, as control) at room temperature in a rotary shaker (100 rpm) for 4 h under sterile conditions. Human plasma was obtained from venous blood drawn from normal human volunteers after informed consent, and was minimally heparinized (3 U/ml) to avoid nonspecific effects of excess heparin on inflammatory responses. Murine plasma was obtained from blood drawn from anesthetized mice by axial incision into citrate anticoagulant. For both murine and human serum, nonanticoagulated blood was drawn as above and held at 4°C for 2 h (to minimize the depletion of fibronectin during fibrin clot formation) before centrifugation.

Unless otherwise noted, all protein solutions (in physiologic concentrations), plasma and serum were diluted 1:3 with PBS before addition of material. The coated disks then were rinsed with PBS before implantation. Incubation for 4 h produces a layer of protein (by calculation, roughly equivalent to a monolayer) that survives rinsing in isotonic solutions and ~60% of which is resistant to removal by powerful detergents such as SDS (31).

Measurement of the Amounts of Surface Fibrinogen. The amounts of pure fibrinogen that spontaneously adsorb to PET were determined radiometrically. Disks were incubated with a mixture of nonisotopic and ^{125}I-labeled human fibrinogen (final specific activity = ~4 μCi/mg) in a concentration of 15 μg/ml dissolved in PBS for up to 8 h while being rotated at 50 rpm. The disks were then thoroughly rinsed with 50 mM PBS and, in some cases, were then incubated for an additional 1 h with 1% SDS solution (also with rotary mixing at 50 rpm) in order to remove elutable (nondenatured) fibrinogen (31). The difference between the surface-associated radioactivity on disks rinsed with PBS vs. 1% SDS was taken as a measure of the proportion of total surface fibrinogen that was nondenatured (23, 32).

An ELISA procedure was developed to measure the surface-bound fibrinogen. Standards were prepared by incubating Dacron® disks of varying surface area with human fibrinogen (15 μg/ml in PBS) for 4 h, rotated at 50 rpm at room temperature. The fibrinogen-coated disks were then removed from the protein solution, rinsed thrice with large volumes of PBS and placed in 24-well tissue culture plates (Costar Corp., Cambridge, MA). The protein-free surfaces on these disks were blocked with 1 ml of 1% BSA solution in PBS for 1 h (rotated at 50 rpm). The specimens were removed and rinsed with PBS 3×, placed in new 24-well tissue culture plates and incubated with 1.0 ml of a 1/1,000 dilution of goat anti-human fibrinogen in BSA for 1 h, rotated at 50 rpm. The disks were then incubated with 1.0 ml of a 1/1,000 dilution of horseradish peroxidase-labeled mouse anti-goat IgG (peroxidase activity 4 U/ml). These specimens were removed from the solution with forceps and rinsed 3× with PBS, placed in new 24-well plates, and immersed in 1 ml of chromogenic peroxidase substrate (*o*-phenylenediamine, 0.4 mg/ml, H_2O_2, 0.012% in a buffer composed of 0.24 M citric acid and 0.05 M Na_2HPO_4, pH 5.0). After 40 min, the reaction was stopped by addition of 100 μl of 8 N sulfuric acid and the product was measured at 450 nm (33).

The results of ELISA determinations were standardized by comparison of disks of varying surface area incubated with nonisotopic or a mixture of nonisotopic and radiolabeled human fibrinogen for 4 h as described above (both at a final concentration of 15 μg/ml). The surface fibrinogen was analyzed by scintillation counting and by immunoreactivity (ELISA). There was an excellent linear relationship between the amount of surface fibrinogen determined radiometrically and by ELISA (r = 0.998).

Implantation of PET Disks. As an in vivo model for assessing inflammatory cell responses to biomaterials, experimental intraperitoneal implants of variously prepared sterile samples of PET were performed using BALB/c mice (male, 20-g body wt) (Harlan Sprague Dawley, Inc., Indianapolis, IN). A number of earlier investigators have used intraperitoneal implants to study biomaterial-induced tissue reactions. In this location, reactions elicited by the polymer are clearly defined, with minimal participation of the coagulation system and minimal contact with the interstitium of normal tissues (15, 26, 34–36). Since the degree of inflammatory response to implanted biomaterials is affected by numerous factors such as host age and time of day, precisely age-matched mice were used in each experiment. Note that appropriate control groups were included in each experiment and, although absolute numbers of surface-adherent phagocytes do vary between experiments, the proportionate differences are relatively constant.

Mice were anesthetized with ether, a ~1.5-cm midabdominal longitudinal incision was made, and 1.2-cm diameter PET disks were implanted intraperitoneally (3 disks/mouse; two lateral and one central). The incision was closed with standard 4-0 silk sutures. Explanation was performed at 16 h (earlier found to be the time of maximal phagocyte accumulation [26]). The PET disks

were carefully removed from the peritoneal cavity and washed with PBS. The disks were then incubated with 0.5 ml of 0.5% (vol/vol) Triton X-100 for 1 h (to release cytosolic and granular contents of adherent cells). The Triton solution was then assayed for peroxidase (both myeloperoxidase [MPO] and eosinophil peroxidase [EPO]) and nonspecific esterase (NSE). In many cases, lactate dehydrogenase activity was also assayed as an independent estimate of the total number of surface-adherent cells. The results (not shown) were always consistent with the preponderance of adherent cells being PMN and macrophages.

Measurement of Enzyme Activities. Peroxidase activities associated with explants were determined as a measure of surface-associated PMN and eosinophils. MPO (largely from PMN although monocytes may have small amounts) and EPO (from eosinophils), were measured by a guaiacol reaction (37). To distinguish MPO from EPO, 1 mM (final concentration) 3-amino-1,2,4-triazole was used. This preferentially inhibits EPO activity while causing only minor inhibition of MPO activity (38, 39). The results from numerous experiments showed that >95% of material-associated peroxidase activity was MPO. Control studies on purified PMN from BALB/c mice indicated that the MPO activity of mouse peripheral PMN is about 23 nU/cell.

NSE is relatively restricted to monocytes/macrophages (40), and the activity of this enzyme was used as a measure of the number of adherent macrophages. The activity of NSE was determined by following the rate of hydrolysis of *o*-nitrophenyl butyrate (41) in the presence of eserine (10 mM, final concentration), which will eliminate possible interference by cholinesterase (42). Enzyme assays on nonelicited mouse peritoneal macrophages (obtained by peritoneal lavage) indicated that the NSE activity of mouse peritoneal resident macrophages is ~11 nU/cell.

Because these enzyme assays were used as primary indicators of the numbers of phagocytes associated with explant surfaces, control experiments were carried out to ensure that the two parameters correlated. Duplicate plasma-coated samples of material were incubated with phagocytes (either purified PMN or resident peritoneal macrophages). One of each set was then fixed, stained, and adherent cells enumerated microscopically. The enzyme activity (MPO and NSE) of adherent phagocytes was measured on the duplicate sample. The results indicated that measured enzyme activity was a reliable indicator of surface-adherent phagocytes (r = 0.90 and 0.96; n = 8 for NSE and MPO).

Induction of Hypofibrinogenemia in Mice. Mice were made profoundly hypofibrinogenemic by injection of ancrod in order to test the possible importance of fibrinogen, spontaneously adsorbed in vivo, as a mediator of phagocyte accumulation. Male BALB/c mice were pretreated with four sequential injections of ancrod via tail vein to deplete plasma fibrinogen. The first injection was with a dose of 0.04 U in 0.1 ml saline (injection of higher initial doses was found to be lethal). 12 h later, a second injection of 0.2 U was given. At 24 and 36 h, the dose was increased to 1 U in 0.1 ml of saline. 2 h after the fourth injection, blood was drawn from some animals by axial incision in order to check fibrinogen levels (as described below). No fibrinogen could be detected in blood from ancrod-treated mice whereas results on normal mice were in good agreement with published values for mice 2–6-mo-old (1–2 mg/ml) (43, 44).

Measurement of Fibrinogen Concentration in Plasma. For fibrinogen determinations on small plasma samples, we employed an assay based on the turbidity produced when thrombin acts on fibrinogen producing fibrin polymers that scatter light (45). As a standard for this assay, we employed purified human fibrinogen, diluted with barbitone-saline buffer (0.1 M barbital sodium in 150 mM NaCl, pH 7.2) in different proportions. The OD of the fibrinogen solution was determined at 470 nm and 50 μl of calcium/thrombin solution was admixed by inversion for 10–15 s. After 30 min, the OD of the mixed solution again was read at 470 nm. Citrated murine plasma, from whole blood obtained by axial incision, was diluted 1:3 (for ancrod-treated mice) or 1:6 (for normal mice) with barbitone-saline buffer and the fibrinogen concentration was measured with the same procedure. The change in OD was directly proportional to the fibrinogen content of the sample. When individual (pooled) samples were assayed by both this turbidometric method and a gravimetric technique (46), nearly identical results were obtained.

Determinations of Endotoxin Contamination. Subsamples of Dacron® disks, both untreated and preincubated with various protein preparations, were assayed for endotoxin contamination by the chromogenic Limulus Amebocyte Lysate (LAL) test (Whittaker Bioproducts, Inc., Walkersville, MA). In no case was significant surface-associated endotoxin found (i.e., no sample contained >0.01 ng endotoxin/cm² of material surface).

Results

Divergent Inflammatory Responses to Implanted PET Disks Coated with Serum vs. Plasma. Substantial numbers of PMN and macrophages were present on both uncoated PET disks and disks preincubated with human plasma following 16 h intraperitoneal implantation in BALB/c mice. In contrast, PET implants preincubated with human albumin attracted relatively few phagocytes after similar implantation (see Fig. 1). These results indicate that plasma component(s) aside from the predominant protein, albumin, are important in mediating the attraction of phagocytes to implant surfaces. A clue to the possible nature of this component was provided by the observation that PET implants preincubated with human serum, like material precoated with albumin, caused minimal accumulation of phagocytes (Fig. 1).

In the foregoing experiments, for reasons of economy, human plasma and serum were used to precoat PET samples. To ensure that some heterologous foreign protein reaction was not occurring, similar experiments were carried out using serum and plasma from mice. The results (data not shown) were similar to those for material precoated with human plasma, serum, and albumin. Therefore, it appears that the accumulation of phagocytes on material precoated with plasma, but not serum, is independent of the species of origin of the plasma and serum used. The observation that material preincubated with serum appears "passivated" as if pretreated with pure albumin suggested that one or more proteins lost in the process of coagulation might be critical. For a number of reasons, we suspected that fibrinogen might be of greatest importance.

Fibrinogen-supplemented Serum and Pure Fibrinogen Restore "Normal" Inflammatory Cell Recruitment. To more directly assess the possible importance of surface fibrinogen in triggering inflammatory responses to implanted materials, PET disks were preincubated with human fibrinogen (1.5 mg/ml in PBS), with citrated human plasma, with human serum to which an equivalent amount of citrate buffer had been added after clotting, and with fibrinogen-reconstituted re-

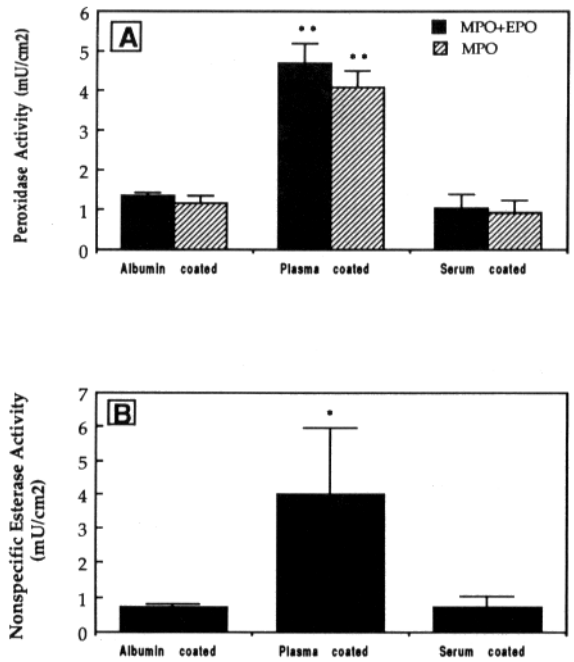

Figure 1. Phagocyte accumulation on the surfaces of PET disks preincubated with albumin, plasma, or serum and then implanted in BALB/c mice for 16 h. (*A*) Peroxidase activity as an estimate of the numbers of PMN. Values shown represent total (i.e., eosinophil and myeloperoxidase activity) (*solid bars*) and myeloperoxidase activity (assayed in the presence of 3-amino-1,2,4-triazole to inhibit eosinophil peroxidase activity) (*cross-hatched bars*). Vertical lines denote ± 1 SD (n = 6 in all cases). (Significance vs. human serum-coated disks: $**p < 0.01$.) As estimated by MPO activity, numbers of surface-associated PMN were ~203,000 ± 22,000/cm² on human plasma-coated disks, 45,000 ± 15,000/cm² on human serum-coated disks, and 58,000 ± 4,000/cm² on human albumin-coated disks. (*B*) NSE activity as an estimate of the numbers of monocyte/macrophages. Vertical lines denote ± 1 SD (n = 6 in all cases). (Significance vs. human serum-coated disks: $*p < 0.05$.) Estimated surface macrophage numbers were 363,000 ± 181,000 macrophages/cm² on human plasma-coated disks, 64,000 ± 29,000 macrophages/cm² on human serum-coated disks, and 64,000 ± 9,000 macrophages/cm² on human albumin-coated disks.

citrated serum (containing 1.5 mg/ml supplemental human fibrinogen). All serum and plasma samples were diluted 1:3 with PBS. After 16 h implantation, it was found that, as before, plasma-coated disks attracted large numbers of PMN and macrophages whereas serum-coated disks did not (Table 1). Importantly, PET disks preincubated with serum samples to which approximately physiologic amounts of fibrinogen had been restored attracted at least as many phagocytes as did material treated with human plasma. Furthermore, material preincubated with purified fibrinogen accumulated even greater numbers of adherent PMN and macrophages (Table 1).

Implanted Material Preincubated with Afibrinogenemic Plasma Fails to Attract Phagocytic Cells. Although the major difference between plasma and serum is the presence or absence of fibrinogen, there are several minor plasma components, such as coagulation factors, which are activated or modified by clotting. To control for possible effects caused by these minor elements, plasma from an afibrinogenemic human patient (plasma fibrinogen concentration by nephelometric analysis <0.10 mg/ml) was employed. Afibrinogenemic plasma, afibrinogenemic plasma to which 2.7 mg/ml (final concentration) human fibrinogen was added, and normal human plasma were incubated with PET disks as described above. There was almost no detectable fibrinogen adsorbed to PET disks after preincubation of the disks with afibrinogenemic plasma, although disks incubated with fibrinogen-repleted afibrinogenemic plasma had the expected amounts of surface fibrinogen, similar to those after incubations with normal plasma (Table 2).

As expected, disks preincubated with normal plasma attracted a large number of adherent phagocytes. However, those disks incubated with afibrinogenemic plasma attracted only small numbers of phagocytes (Fig. 2). The decrement in inflammatory responses to disks preincubated with afibrinogenemic plasma was almost certainly due specifically to the lack of fibrinogen; fibrinogen-repleted afibrinogenemic plasma prompted a normal degree of inflammatory response (Fig. 2).

The Amount and State of Fibrinogen Adsorbed on PET Surfaces. If adsorbed fibrinogen is an important mediator of acute inflammatory responses to implanted polymeric biomaterials, it is necessary that significant amounts of this protein should adsorb spontaneously to PET even in the presence of competitive proteins. As shown in Fig. 3, the binding of human fibrinogen to PET surfaces reaches saturation within ~30 min. More than 65% of the surface-adsorbed fibrinogen becomes irreversibly bound (resistant to SDS elution) to the PET surface after 4 h. The surface concentration of fibrinogen on disks incubated with varying protein sources was also estimated by ELISA as described under Materials and Methods. PET disks incubated with human plasma for 4 h had large amounts of adsorbed fibrin(ogen), approximately half as much as disks incubated with purified fibrinogen (Table 2). This supports earlier conclusions that fibrinogen adsorbs readily and in large amounts to PET surfaces (22, 30, 47). By contrast, material incubated with serum or with hypofibrinogenemic plasma had almost undetectable amounts of surface-adsorbed fibrin(ogen) (Table 2). Due to the fact that implanted disks were covered by a large number of cells, the amount of fibrin(ogen) adsorbed to uncoated material after 16 h implantation could not be measured.

Inflammatory Responses to PET Implants in Afibrinogenemic Mice. The foregoing results supply strong, albeit indirect, support for a central role for fibrinogen in inflammatory responses to PET. However, most of these experiments were carried out with material preincubated with various plasma, serum or protein preparations. In contrast, the protein layer found on biomedical implants arises from spontaneous adsorption of host proteins from plasma or interstitial fluids. We therefore carried out experiments in mice treated with ancrod in order to directly assess the inflammatory responses to uncoated PET samples. Intravenous infusion of ancrod into humans and animals at appropriate doses is generally well tolerated and results in severe hypofibrinogenemia, hypoplasminogenemia, elevation in fibrinogen-fibrin degradation products, and reduction in blood viscosity (44, 48–50).

Injection of mice with ancrod for 2 d before implantation caused almost total loss of fibrinogen (plasma fibrinogen levels falling from a control level of 1.25 ± 0.07 mg/ml to unde-

Table 1. *Phagocyte Accumulation on the Surfaces of Variously Treated PET Disks after Implantation for 16 h in BALB/c Mice*

Sample coating	n	Peroxidase activity	PMN	NSE activity	Monocytes/macrophages
		mU/cm^2	$\times 10^4/cm^2$ calculated	mU/cm^2	$\times 10^4/cm^2$ calculated
Plasma	6	3.03 ± 0.49‡	13.2 ± 2.1‡	5.99 ± 1.68‡	54.5 ± 15.3‡
Serum	6	0.18 ± 0.18	0.9 ± 0.9	0.62 ± 0.17	5.6 ± 1.5
Serum + fibrinogen (final concentration 2.7 mg/ml)	6	4.07 ± 1.40‡	17.7 ± 6.1‡	3.76 ± 1.86‡	34.2 ± 16.9‡
Fibrinogen (1.5 mg/ml)	3	5.54 ± 1.63‡	24.1 ± 7.1‡	9.00 ± 2.38‡	81.8 ± 21.6‡

* Phagocyte numbers calculated on the basis of measured activities of 23 nU/cell (PMN myeloperoxidase) and 11 nU/cell (monocyte/macrophage nonspecific esterase).
‡ Differs from serum at $p < 0.01$.

tectable; $n = 5$). After implantation of PET disks for 16 h, normal mice responded with a rapid increase in fibrinogen, to levels approximately twice the baseline values (2.93 ± 0.09 mg/ml; $n = 5$). In ancrod-treated animals the fibrinogen levels increased from undetectable to 1.42 ± 0.3 mg/ml ($n = 4$) at 16 h after implantation of the PET samples. (However, for at least 3–4 h after initial implantation, fibrinogen levels in these animals remained undetectable.)

As expected, albumin-coated disks attracted only small numbers of PMN and macrophages in both afibrinogenemic and normal mice (Fig. 4). Most importantly, large numbers

Table 2. *Surface-adsorbed Fibrinogen on PET Samples*

Sample	Number	Fibrinogen
1. Control*	5	0 (±4)
2. Albumin‡	5	0 (±0)
3. Fibrinogen§	3	183 (±24)
4. Plasma‖	5	82 (±14)
5. Serum¶	5	2 (±4)
6. Hypofibrinogenemic plasma**	5	3 (±1.9)
7. Fibrinogen repleted hypofibrinogenemic plasma‡‡	5	102 (±9)

* All incubations carried out for 4 h at 37°C. Controls incubated with PBS.
‡ Incubated with human albumin (15 mg/ml).
§ Incubated with purified human fibrinogen (15 μg/ml).
‖ Incubated with heparinized (3 U/ml) human plasma diluted 1:9 in PBS.
¶ Incubated with human serum, heparinized (3 U/ml) after clotting, diluted 1:9 in PBS.
** Incubated with heparinized hypofibrinogenemic plasma diluted 1:9 in PBS.
‡‡ Incubated with heparinized hypofibrinogenemic plasma repleted with purified human fibrinogen to 2.7 mg/ml (final concentration), diluted 1:9 in PBS.

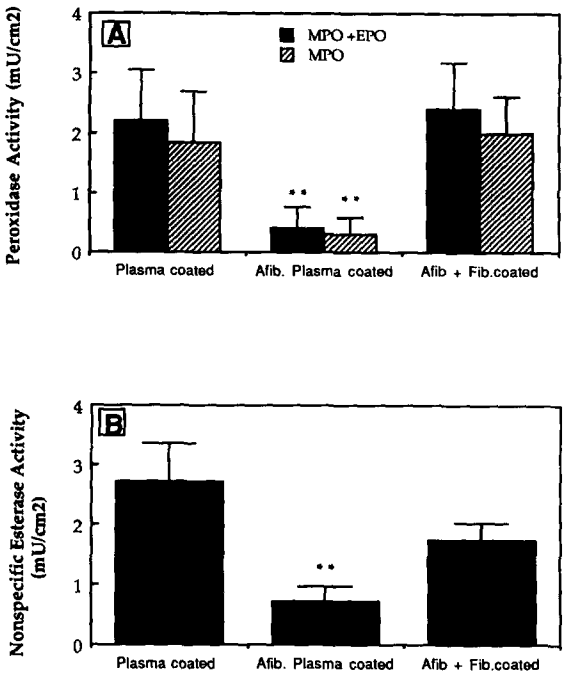

Figure 2. Phagocyte accumulation on the surfaces of PET disks preincubated with normal plasma, afibrinogenemic plasma, or afibrinogenemic plasma reconstituted with physiologic concentrations of fibrinogen (final concentration 2.7 mg/ml). (*A*) Peroxidase activity as an estimate of the numbers of PMN. Values shown represent total (i.e., eosinophil and myeloperoxidase activity (*solid bars*) and myeloperoxidase activity (assayed in the presence of 3-amino-1,2,4-triazole to inhibit eosinophil peroxidase activity) (*cross-hatched bars*). Vertical lines denote ± 1 SD ($n = 6$ in all cases). (Significance vs. human serum-coated disks: $**p < 0.01$.). Estimated surface concentration of PMN: 96,000 ± 37,000/cm² on normal human plasma-coated disks, 17,000 ± 16,000/cm² on afibrinogenemic plasma-coated disks, and 104,000 ± 34,000/cm² on fibrinogen-repleted afibrinogenemic plasma-coated disks. (*B*) NSE activity as an estimate of the numbers of monocyte/macrophages. Vertical lines denote ± 1 SD. ($n = 6$ in all cases). (Significance vs. normal human plasma-coated disks: $**p < 0.01$.) Estimated numbers of surface macrophages were 247,000 ± 58,000/cm² on normal human plasma-coated disks, 66,000 ± 22,000/cm² on afibrinogenemic plasma-coated disks and 159,000 ± 25,000/cm² on fibrinogen-repleted afibrinogenemic plasma-coated disks.

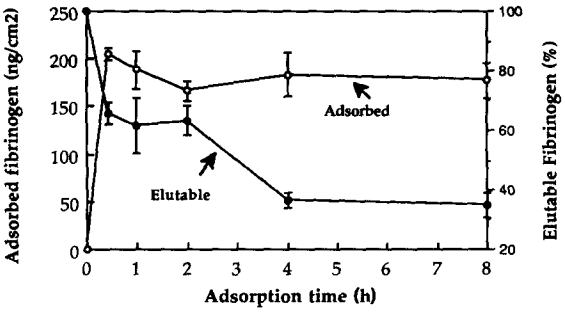

Figure 3. Time course of ^{125}I-human fibrinogen (15 μg/ml) adsorption to and elutibility from PET disks. Proportions of adsorbed elutable albumin were determined using a 1% SDS wash as described in Materials and Methods. Vertical lines denote ± 1 SD (n = 3 in all cases).

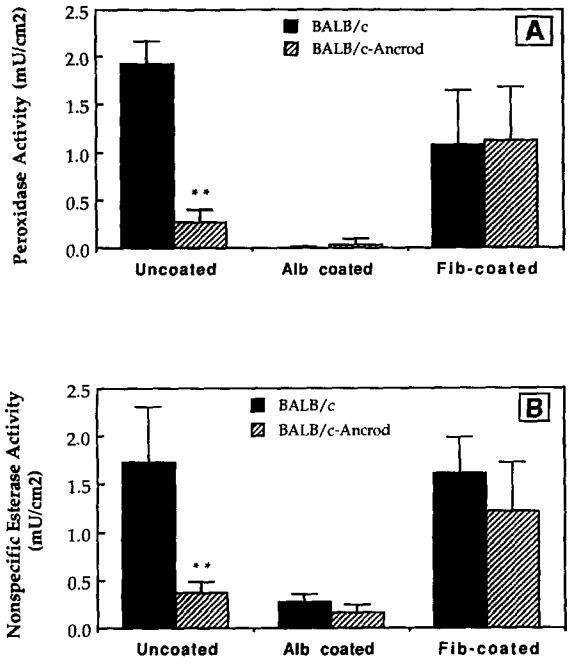

Figure 4. Phagocyte accumulation on the surfaces of PET disks implanted in control (*solid bars*) and ancrod-treated BALB/c (*cross-hatched bars*) mice. Disks were variously preincubated with mouse fibrinogen (1 mg/ml), albumin (25 mg/ml), or buffer (PBS) (n = 6 in all cases). (Significance vs. the same protein-coated implants in control mice; $**p < 0.01$.) (*A*) Peroxidase activity as an estimate of the number of PMN. Estimated surface concentration of PMN: 100 ± 500/cm^2 on albumin-coated disks in control mice vs. 2,000 ± 2,000 PMN/cm^2 on the albumin-coated disks in ancrod-treated mice. On fibrinogen-coated disks: 46,000 ± 25,000/cm^2 in control mice vs. 49,000 ± 25,000/cm^2 in ancrod-treated mice. On uncoated disks: 84,000 ± 32,000/cm^2 in control mice vs. 12,000 ± 6,000/cm^2 in ancrod-treated mice. (*B*) NSE activity as an estimate of the numbers of monocyte/macrophages. Vertical lines denote ± 1 SD. (Significance vs. the same protein-coated implants in control mice; $**p < 0.01$.) Calculated macrophage numbers on albumin-coated disks were ~25,000 ± 8,000/cm^2 in control mice and 15,000 ± 7,000/cm^2 in ancrod-treated mice. On mouse fibrinogen-coated disks, the calculated macrophage numbers were 147,000 ± 33,000/cm^2 in control mice and 111,000 ± 46,000/cm^2 in ancrod-treated mice. On uncoated disks, there is a large difference between control mice (158,000 ± 51,000 macrophages/cm^2) and ancrod-treated mice (34,000 ± 10,000 macrophages/cm^2).

of implant-associated phagocytes were found only on untreated PET implants in normal control mice; afibrinogenemic mice had practically no response to uncoated disks. This was almost certainly due to an in vivo deficit in fibrinogen because the inflammatory response of afibrinogenemic animals to PET disks preincubated with pure fibrinogen was normal (Fig. 4).

The recruitment of phagocytes to implant surfaces in ancrod-treated mice could also be normalized by correction of the hypofibrinogenemia. In some animals, after ancrod treatment, 2 mg of mouse fibrinogen (1.5 mg/ml in pyrogen-free sterile saline) or 2 mg of human serum albumin (also 1.5 mg/ml) was injected intraperitoneally 1 h after the last ancrod injection. Uncoated PET disks were then implanted intraperitoneally 15 min after protein injection. As expected, uncoated disks accumulated only small numbers of PMN and macrophages in albumin-repleted hypofibrinogenemic mice (PMN, 40,000 ± 33,000/cm^2; macrophages, 101,000 ± 136,000/cm^2). However, phagocyte recruitment was approximately normal in hypofibrinogenemic mice injected with supplemental fibrinogen (PMN, 188,000 ± 63,000; macrophages, 354,000 ± 136,000/cm^2) (n = 5 in all cases). These results indicate that the effect of ancrod in eliminating the inflammatory responses to implanted PET is due to fibrinogen depletion per se.

Discussion

Many problems associated with implanted biomaterials, such as surface cracking on pace-maker leads, degradation of breast implants, and fibrosis surrounding many types of implants likely involve phagocyte interactions with the biomaterial surfaces (7, 8, 10–13, 20, 21, 51–54). However, the mechanisms by which biomaterials might trigger phagocyte accumulation are unclear. These responses are particularly mystifying in view of the inert and nontoxic nature of most types of implanted polymeric elastomers.

Clearly, interactions between the implant surface and the host must be of predominant importance, and these events are likely initiated by surface–protein adsorption. Implantable biomaterials are spontaneously covered by a layer of host proteins within seconds after contact with body fluids (55–59). These adsorbed proteins gradually change conformation and become irreversibly adsorbed (24, 31, 32, 60–62). Because this accumulation of protein occurs so rapidly, it almost certainly precedes the arrival of cells on implant surfaces. Hence, host cells likely interact with protein-coated material rather than directly with the material (23, 47). Therefore, we and many other investigators have assumed that the nature of the surface layer of protein is crucial for determining the biocompatibility of different biomaterials (23, 26, 47, 56, 63).

In an effort to dissect the likely mechanism(s)of phagocyte recruitment to implant surfaces, we have employed polyethylene terephthalate (Mylar®) as a model polymer. We chose this material because Dacron®, the woven form of PET, has been used for vascular prostheses for over 30 yr, and is particularly thrombogenic and pro-inflammatory (1, 20, 21, 27, 28). Albumin, fibrinogen and IgG are detected in greatest amounts on Dacron surfaces following incubation with whole

blood (30, 47). However, the most abundant plasma protein, albumin, is clearly not responsible for the recruitment of inflammatory cells to implant surfaces. Many previous studies show that albumin coating "passivates" biomaterial surfaces, blunting both proinflammatory and thrombogenic responses (1, 26, 29, 64). Furthermore, we have recently found that neither surface-bound IgG nor complement activation is a necessary element in inflammatory responses to biomaterials such as PET (26).

The possible identity of the critical "proinflammatory" plasma component was indicated by our observation that whereas material preincubated with plasma does trigger phagocyte recruitment, PET incubated with serum does not, suggesting that surface adsorption of fibrinogen might be an important element in the attraction of inflammatory cells to implant surfaces. Indeed, PET disks coated with human fibrinogen accumulate numerous phagocytes as do disks incubated with fibrinogen-repleted serum, supporting a central role for fibrinogen in this response. We recognize that the "purified" fibrinogen employed (human fibrinogen, Type I, from Sigma Chemical Co.) may contain contaminants (such as plasmin[ogen]) that might influence responses to coated materials. However, the results of a number of additional experiments discussed below all support a central role of surface-adsorbed fibrinogen in acute inflammatory responses to PET implants.

Although the major difference between plasma and serum is the presence or absence of fibrinogen, there are several other components which, although they only comprise a small fraction of total plasma protein, might be affected by the processes of clotting. To control for any possible effects of these minor constituents (which include coagulation factors and fibronectin), we employed plasma from an afibrinogenemic patient. Material coated with afibrinogenemic plasma failed to attract phagocytes. Furthermore, when physiologic levels of purified fibrinogen were added to afibrinogenemic plasma, disks incubated in this mixture prompted normal recruitment of phagocytes. The results indicate that fibrinogen is of predominant importance in mediating inflammatory responses to implanted PET.

The proinflammatory effect of adsorbed fibrinogen would appear to hold in vivo as well. Mice having almost undetectable amounts of plasma fibrinogen were produced by repetitive injections of ancrod, a thrombinlike protease derived from the venom of the Malayan pit viper (*Agkistrodon rhodostoma*) (43, 44, 49, 65–67). These hypofibrinogenemic mice showed almost no phagocyte accumulation on untreated PET disks but did mount a normal phagocyte response to fibrinogen-coated disks. Inflammatory cell recruitment was also normalized in hypofibrinogenemic mice given injections of purified fibrinogen. It should be noted that plasma fibrinogen levels in these animals rebounded from undetectable at the time of implantation to near normal 16 h after implantation. However, it is likely that the composition of the adsorbed protein layer is determined shortly after implantation and that later adsorption of fibrinogen was minimal (as it evidently is on material precoated with albumin). Thus, the results of these experiments fully support the proposition that fibrinogen, which spontaneously adsorbs to plain Dacron® surfaces after implantation, is a critical determinant of subsequent inflammatory responses.

This is, to the best of our knowledge, the first solid evidence for a critical role for fibrinogen in attracting inflammatory cells to implanted biomaterials. In other circumstances, the physical presence of fibrin(ogen) has long been recognized as coeval of inflammatory responses, with fibrin(ogen) deposition typical of both acute and chronic inflammatory processes (68–71). Similarly, large amounts of fibrin(ogen) accumulate within or around biomaterial implants (11, 16–21, 27, 72). This surface-adsorbed fibrinogen may be a most important element in the thrombogenic and embolic events triggered by material surfaces in vivo (73, 74).

Perhaps of direct pertinence to the present findings, Shinoda and Mason (63) found that the deposition of phagocytes and platelets on hemodialysis membrane was far less than normal in an "afibrinogenemic" patient being hemodialyzed. Interactions between phagocytes and fibrin(ogen)-coated surfaces may well involve specific binding sites for fibrin on macrophages (75–77) and for fibrinogen on PMN (68, 78–80). However, the apparent proinflammatory effects of fibrin(ogen) may involve indirect interactions. For example, some fibrin degradation products (81–85) and fibrinopeptide B (86) are strongly chemotactic for PMN, monocytes and fibroblasts. In addition, lower molecular weight degradation products of fibrin(ogen) are known to promote granulocyte infiltration and vascular permeability (82, 87).

Unfortunately, the present work provides no indication of the state of the adsorbed fibrinogen associated with proinflammatory activities. Thus, adsorbed fibrinogen may directly trigger inflammatory responses or do so indirectly (e.g., through conversion to fibrin or formation of degradation products). Nonetheless, the present work does support the importance of spontaneous fibrinogen adsorption as a critical precedent to subsequent inflammatory cell responses to the surfaces of implanted biomaterials. A more comprehensive understanding of the ensuing sequence of events may lead to the rational design of more compatible implantable and blood-contact biomaterials.

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