Quantitative Measurement of C3 Activation at Polymer Surfaces

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Activation of C3 by nylon 6,6 and polymethyl methacrylate (PMMA) polymers in whole canine serum was investigated using Laurell electroimmunoassay and antigen–antibody crossed immunoelectrophoresis (XAA). Canine sera were utilized because most artificial blood contact materials used in prosthetic devices are tested in dogs. The measurements were made possible by converting the polymers to microparticles, increasing the available surface area to approximately 200,000 sq cm/g. Both polymers activated C3 within a few minutes of serum contact at 39°C, the canine biologic temperature, after which no further development was seen. Nylon activation, studied in detail, increased with increasing surface area up to a saturation level of about 20% conversion reached at approximately 350 sq cm/ml polymer surface/serum volume. At saturation, XAA plates from nylon and control zymosan particles appeared identical. The nylon-induced activation was highly temperature dependent and was inhibited by at least a factor of 5 at 30°C, relative to that at 39°C. Both nylon and zymosan particles aggregated canine platelets in citrated plasma in vitro. Addition of Mg-EGTA inhibited nylon-induced C3 activation and platelet aggregation, but did not affect zymosan-induced C3 activation or platelet aggregation. Rapid activation of C3 by nylon 6,6 and PMMA suggest a role for complement activation at polymer surfaces in the early phase of the blood/surface interaction in the canine model. Results of ex vivo blood flow experiments using these same polymer surfaces are consistent with this hypothesis.

Despite the widespread use of artificial blood contact surfaces in prosthetic devices, no such surface is completely inert. Increased platelet turnover has been documented both for left heart assist pumps and prosthetic heart valves, and a common finding in intraaortic balloon pumped patients is sharply reduced platelet count beginning just after balloon insertion. Granulocyte adhesion to surfaces of explanted devices has also been documented, and a consistent finding of a canine ex vivo blood flow experiment at Avco Everett Research Laboratory is platelet and polymorphonuclear leukocyte (PMN) adhesion to all surfaces tested, encompassing more than 30 different types of surface composition. The consistent finding of PMNs at polymer surfaces suggests the involvement of complement components in the cell adhesion, and complement activation at nylon leukapheresis filters and dialysis membrane blood contact surfaces has already been demonstrated.

In this study we investigated the dose–response characteristics, the time dependence, and some aspects of the mechanism of complement activation at polymer surfaces in contact with whole serum in vitro. Canine sera were utilized because most short-term evaluations of biomaterials and devices are carried out in dogs, and because the blood surface interaction has been extensively studied with the canine model.

Polymers of interest were first converted to microparticulate form so as to vastly increase the available surface area to greater than 100,000 sq cm/g of material and to thus potentially involve a measurable fraction of available complement proteins in serum-polymer interaction. In an experiment, serum was exposed to an amount of polymer suspension at the canine biologic temperature (39°C) for a fixed period of time. The reaction was quenched by cooling to 0°C, the polymer particles removed by centrifugation, and the serum assayed for C3 level and C3 conversion using Laurell electroimmunoassay and antigen–antibody crossed immunoelectrophoresis respectively.

Nylon 6,6, and polymethyl methacrylate (PMMA) surfaces were studied. Nylon activation was studied in detail, including variation of the amount of converted and bound C3 with polymer surface area, the time evolution of the conversion response, and the temperature dependence of the process. Protein diffusion times to the dispersed microparticulate polymer surface were negligible (small fraction of a second) compared to times measurable in the system (minutes) and any measured time dependence of conversion or binding is attributable to the surface reaction itself rather than to diffusion. Dependence of complement activation on Ca++, and Mg++ was also studied using ethylene glycol tetracetic acid (EGTA) and Mg-EGTA chelating agents.

In all experiments zymosan particles were used as a control (alternative pathway) complement activator. C3 activation by polymers and zymosan was also compared with that due to a stable C3 convertase prepared by addition of cobra venom factor (CVF).

In the vitro response of canine platelets to zymosan and nylon particles was also investigated. Nonprimate platelets exhibit immune adherence, and a response
to complement-activating particles is plausible. This was investigated using standard nephelometric aggre-
gomentry, with a small amount of particle sample added to citrated platelet-rich plasma. The effects of
EGTA and Mg-EGTA on aggregation were also inves-
tigated as in the C3 activation studies.

MATERIALS AND METHODS

Polymer Particles

Nylon 6,6 pellets (Dupont Zytel 101) were obtained without lubricant or other additive. The pellets were extracted with 100% ethanol to remove any trace additive, dried, and then dissolved in 90% formic acid to produce a 1.5% w/v solution.

Nylon microspheres were produced by a method developed by Herzlinger. The process involves infusion of a polymer solution into a nonsolvent bath via a submerged turbulent jet. Particles rapidly precipitate from the polymer solution as it mixes with water in the receiving vessel, forming a milky colloidal suspension. A fresh 1.5% (w/v) nylon 6,6/formic acid solution infused at 24.9 cc/min from a 30-cc glass syringe connected via silastic tubing to a submerged no. 22 standard needle filed to 1 cm length, (50 cc into 500 cc water) typically yields a polydisperse suspension of submicron size spheres (see Fig. 1). Most of the surface area is contributed by spheres in the range 0.15 - 0.4 microns.

Turbulence is not necessary for microparticle formation. Slow laminar infusion of the polymer solution through an identical orifice produces a stream of droplets that settle in the water receiving bath due to the higher density of the formic-acid-nylon solution. The droplets, which are of macroscopic size, break up during settling to form a fine colloidal suspension. The resulting polymer particles are in the micron and submicron size range, but are larger than those obtained under high flow conditions.

Upon formation, the nylon suspension was immediately washed by repeated centrifugation at 25,000 g and resuspended with fresh distilled water. The suspension was then concentrated to 10 mg/ml and stored frozen. Some of the suspension was vacuum dried and resuspended in distilled water with sonication. No difference in C3 conversion or binding was observed for the vacuum dried particles.

The particle formation process uses no surfactant or other surface active substance, in contrast to other polymer particle methods. The only impurity to be removed is the original solvent, which is

NYLON 6, 6 PARTICLES

Fig. 1. Scanning electron micrograph of nylon 6,6 microspheres at 20,000 x.
accomplished by exhaustive washing for the nylon/formic acid system.

Particles can be formed from any polymer for which a suitable solvent can be found, which is completely miscible with a nonsolvent receiving bath. Nylon particles were also prepared using hexafluoroisopropanol (HFIP) as a solvent, under conditions similar to those for formic acid. Excess HFIP was readily removed from the washed suspensions by vacuum drying. Polymer suspensions prepared from both types of solvent were evaluated in preliminary C3 activation studies. Results from each were substantially the same, indicating little possibility of an effect due to residual solvent. The nylon particles prepared from HFIP solutions had a greater tendency to form aggregates during particle purification, and particles prepared from formic acid solution were utilized for the bulk of the study.

Poly(methyl methacrylate) (PMMA) particles were prepared in a similar manner to the nylon particles. PMMA (Eastman, Rochester, N.Y.) was dissolved in redistilled tetrahydrofuran (THF) and precipitated twice in methanol. The purified polymer was redissolved in redistilled THF to make a 0.4% solution (w/v). Twenty-five milliliters of polymer solution was then infused into 500 ml water under flow conditions similar to those used with nylon 6.6. The resulting microsphere suspension tended to form aggregates upon standing that could be broken up by agitation. The microspheres were washed exhaustively, as for nylon, and vacuum dried to ensure complete removal of THF. The dried particles were suspended in distilled water with sonication and stored frozen. The nylon particles prepared from HFIP solutions had a greater tendency to form aggregates during particle purification, and particles prepared from formic acid solution were utilized for the bulk of the study.

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**Specific Surface Area of Polymer Particles**

The specific surface area of both polymer particle samples was determined from analysis of scanning electron micrographs (weighted area to volume measurement). For the nylon microspheres, Brunauer-Emmett-Teller analysis of argon gas adsorption at liquid argon temperature was also used. The gas adsorption measurements were carried out using a gravimetric method by Professor F. J. Micale of Lehigh University.

**Zymosan Particles, Cobra Venom Factor**

Zymosan was obtained from Sigma Chemical Co., St. Louis, Mo. and prepared according to the protocol of Mayer.14 Dark field reflected light microscopy of a wet zymosan particle suspension indicated hollow cell wall structures approximately 5–6 μm in diameter. However, it was not possible to quantitatively determine the specific surface area of the zymosan particles due to uncertainty in the wall thickness of the zymosan particles.

Cobra venom factor (Naja haje) was obtained from Cordis Laboratories, Miami, Fla. and reconstituted according to directions to yield 100 U/ml. It was incubated with serum in vitro at approximately 2 U/ml, near that recommended by the manufacturer for in vivo deactivation. In previous work, we used somewhat lower CVF concentrations to achieve complete deactivation in vivo (98% removal of canine antigenic C3) over a 24-hr period.

**Chelating Agent**

The effect of Ca++ and Mg++ was investigated using ethyleneglycol bis(aminooxyether) tetracetic acid (EGTA) (Pfaltz and Bauer, Stamford, Conn.). The free acid was first neutralized to pH 7.4 with sodium hydroxide and utilized at a final serum concentration of 8 mM. At this concentration, nearly all of the Ca++ and much of the Mg++ is chelated. EGTA with Mg++, added to produce a final serum concentration of 8 mM, was also utilized.

**Blood Handling**

For serum samples, blood from conditioned dogs was withdrawn via venapuncture into plastic syringes, transferred to siliconized glass tubes, and allowed to clot at room temperature. Blood for platelet-rich plasma (PRP) was withdrawn from the jugular vein into plastic syringes containing sodium citrate (final concentration 0.38%). The blood was transferred to capped polystyrene tubes, centrifuged for 5 min at 60 g, and the PRP removed with a plastic tuberculin syringe.

**Polymer/Serum Exposure**

Serum was dispensed in 1-ml aliquots into capped sterile polystyrene tubes. The aliquots were preheated to the incubation temperature for 3–5 min, after which an amount of particle suspension was added, the mixture vortexed gently, and held at the preset (± 0.2°C) temperature for a fixed period of time. Most of the runs took place at canine biologic temperature. After completion of the polymer/serum incubation, the tubes were immediately cooled to 0°C in an ice bath and the particles removed by centrifugation in ice-packed bacteriological tubes. Control samples containing no added polymer but proceeding through all other mixing, temperature cycling, and centrifugation steps were run with each experiment. After centrifugation, all serum samples were transferred to screw-top polypropylene vials, frozen by immersion into a dry-ice-acetone bath, and stored at –70°C prior to assay.

Most of the measurements with nylon 6.6 were carried out using a particle sample having a specific surface area of 1.91 × 10^5 sq cm/g as determined by SEM analysis, assuming a nylon density of 1.14. Argon gas adsorption analysis yielded 1.74 × 10^5 sq cm/g for the same sample; the two types of measurement thus agree to within 10%. The PMMA particles had a specific surface area of approximately 3.5 × 10^5 sq cm/g as determined by SEM analysis. There was a greater tendency of the PMMA particles to clump, and there is some uncertainty in surface area due to this factor.

**C3 Assays**

Anti-dog C3 serum obtained from Miles Laboratories, Elkhart, Ind., and from Cappell Laboratories, Cochranville, Pa. was utilized in the assays. The monospecificity of the antisera was first analyzed by conventional immunoelectrophoresis against whole canine serum. The Laurell electroimmunoassay (EIA)15 was used to determine the C3 level remaining in the sera after exposure to polymer surfaces. Crossed antigen–antibody electrophoresis (XAA),16 in which proteins are separated in one dimension and quantitated in the orthogonal direction using EIA, was utilized to determine the relative amount of antigenic C3 split products remaining in solution after polymer exposure. For each XAA curve, C3 split product levels were quantitated as a fraction of total available C3 by the ratio of the area under the conversion peaks relative to the total area under the curve. Areas were measured by xerographic projection of the XAA plates onto a millimeter grid. In order to compare C3 conversion in serum samples from different dogs, with different levels of total C3, conversion was normalized to a reference C3 level, that of a pooled serum sample, as follows:

\[
\text{Normalized sample conversion} = \left( \frac{\text{EIA Sample C3}}{\text{EIA Std. C3}} \right) \times \left( \frac{\text{C3 Assay C3}}{\text{C3 Assay Std. C3}} \right)
\]

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Correction factors\(^7\) for the effect of molecular weight and mobility on relative precipitate area of conversion products are not known for the canine system and were not applied.

**Cellulose Acetate Protein Electrophoresis**

A rapid protein electrophoresis assay was used with some of the serum polymer experiments to determine that particle exposure did not significantly affect the main serum protein fractions. Cellulose acetate membrane electrophoresis using Ponceau S general purpose protein stain was carried out to analyze control and particle exposed sera and the results compared via densitometry. No effects due to particle exposure could be seen.

**RESULTS**

**Nylon-6,6-Induced C3 Conversion**

*Dose–Response Characteristics, Time Evolution, and Comparison With Zymosan and Cobra Venom Factor Induced Conversion*

The main observable effect was the formation of C3 split products, which remained in solution after polymer/serum contact. C3 remaining bound to the particle surface, as measured by depletion using EIA, was found to be a negligible fraction of total available C3. This result does not rule out binding of a low molecular weight C3 fragment or a non-antigen-bearing fragment to the nylon surface. However, the main observable effect was C3 conversion (activation).

Both nylon and zymosan particles induced C3 conversion within a few minutes of serum contact at 39\(^\circ\)C. Two conversion peaks were generally observed that were slightly more anodic than the main C3 peak. The mobilities correspond roughly to those of human C3b and C3c and suggest these conversion products. For each type of activator the amount of conversion increased with increasing particle surface up to a maximum saturation level. The saturation conversion level was the same for both nylon and zymosan, and at saturation, the XAA patterns for zymosan and nylon appeared to be identical. No evidence of conversion was ever seen for the control serum samples (see Fig. 2).

Nylon-induced conversion saturated at approximately 2 mg/ml or 350 sq cm/ml nylon surface/serum volume. The mean normalized saturated conversion level was 18% of the total C3 present, with a standard deviation \(\sigma(n-1) = 3.5\%\), for \(n = 7\) experiments. The dose–response curve for nylon-induced (conversion response to increasing surface area) is summarized in Fig. 3. Brackets, where present, indicate measured standard deviations from data representing serum samples from several dogs. The data clearly show a saturation phenomenon but are not sufficiently precise to indicate the exact form of the curve at low particle surface concentration. The data also can be expressed on a unit area basis as conversion of 0.05% of the C3 in 1 ml of serum for each square centimeter of nylon surface in serum contact.

The entire conversion process occurred during the first few minutes of polymer/serum contact at 39\(^\circ\)C. Data showing the time evolution of the conversion response at saturation concentration are summarized in Table 1. Very little conversion is seen during the first minute of polymer/serum contact, while the bulk of the reaction is complete at 5 min. The initial delay is not due to
temperature; all serum samples were brought to 39°C prior to the addition of the nylon polymer.

Both nylon and zymosan induced rapid C3 conversion, which for all surface concentrations and exposure times was limited to less than about 20% of total available C3. To ascertain whether this limitation is due to control proteins or other serum factors and not some artifact of the system, C3 conversion induced by CVF, which forms a stable C3 convertase in serum, was also measured. CVF (Naja haje) at 2 U/ml, incubated with whole canine serum at 39°C for 10 min, produced conversion levels exceeding 40% of total available C3, more than double that seen for any nylon or zymosan sample. The converted C3 had peaks at the same mobilities as were seen for nylon and zymosan, as shown in Fig. 2. Whatever control mechanism is present in both nylon- and zymosan-activated conversion can be bypassed by CVF.

**Dependence on Ca²⁺ and Temperature**

Nylon-6,6-induced conversion was completely inhibited by 8 mM EGTA and EGTA-Mg²⁺, while zymosan, when acting on the same serum sample and analyzed on the same XAA plate, induced conversion in both the EGTA and EGTA-Mg²⁺ samples, as shown in Table 2. The data clearly indicate Ca²⁺ dependence for nylon-6,6-induced process, which suggests activation that originates with C1.

Despite the apparent difference in origin of nylon and zymosan conversion, alternative pathway versus probable classical pathway, the conversion pattern at saturated amount of activating agent appear identical, and both reactions apparently cease at about 20% conversion of the total C3.

A relevant concern with artificial surface induced blood reactions is temperature dependence, since blood temperature is often altered during extracorporeal circulation. The nylon-induced reaction was found to be highly temperature dependent. Incubation of serum with 2 mg/ml nylon for 10 min at 30°C resulted in at least a fivefold reduction in C3 activation compared to that observed under identical conditions at 39°C. More precise quantitation at this low level of conversion was not possible.

**PMMA-Induced Conversion**

PMMA particles also produced measurable C3 conversion in short-term polymer/serum exposure at 39°C. Saturated PMMA-induced conversion averaged about 6%-7% of total available C3 (n = 4), much less than for nylon. The PMMA activation process was not investigated in detail.

**Platelet Aggregation**

Both nylon 6,6 and zymosan particles aggregated canine platelets in citrated plasma, as shown in Fig. 4. Upon adding suspension of either particulate, optical transmission abruptly decreases due to the additional light scattering by the particles. After a delay of about 30–45 sec, an aggregation response is detected that proceeds for about 7–10 min until an endpoint is reached. Because of the higher optical density of the particle-platelet aggregates, the final optical transmission level is well below that of platelet poor plasma, which is not shown in Fig. 4.
Nylon-induced aggregation was completely inhibited by 8 mM Mg-EGTA, while zymosan-induced aggregation proceeded with an additional delay, as shown in Fig. 4. Ionized calcium in 0.38% citrated plasma is estimated at 0.1 mM.18 This level is apparently sufficient for nylon-particle-induced aggregation; its removal with Mg-EGTA inhibits aggregation due to nylon but not zymosan. This is consistent with a complement-dependent process with a similar dependence on Ca\(^{2+}\) and Mg\(^{2+}\) demonstrated for C3 activation.

No platelet response to soluble complement activator (CVF), at 2 U/ml, was seen. The slow aggregation response to nylon and zymosan and lack of response to soluble activator suggest direct particle-platelet attachment rather than a release reaction.

**DISCUSSION**

Rapid C3 activation induced by polymer surfaces in whole sera, and aggregation of platelets by polymer particles in platelet-rich plasma, suggests a role for complement activation in mediating the initial cellular reactions at artificial blood contact surfaces in the canine model in vivo. Cell adhesion and thrombus formation at the same polymer surfaces as were studied here have been investigated using an ex vivo blood flow experiment.11 In the experiment, native canine blood impinges upon a test surface in a flow configuration that avoids reactivation and permits direct microscopic monitoring of cell adhesion under dynamic conditions. The blood flow experiment has been extensively used at moderate flows (surface shear rates less than 50 sec\(^{-1}\)). The phenomena observed are consistent with the present results in terms of:

(A) Time dependence.11,19 After an initial delay of about 30–40 sec, platelets and leukocytes (primarily PMNs) rapidly adhere to polymer surfaces. When test surfaces are maintained at canine biologic temperature, they are covered with a dense layer of aggregated platelets and adherent leukocytes after 10 min exposure to blood flow.

(B) Temperature dependence.19 Reduction of surface temperature to 30°C inhibits leukocyte adhesion and substantially reduces the rate of platelet deposition.

(C) Inhibition by CVF.11 Administration of cobra venom factor (Naja haje) a day prior to the experiment results in approximately 98% reduction in C3, as measured by EIA, and 90% reduction in CH50, while not affecting leukocyte or platelet counts, coagulation times, or ADP levels required to aggregate platelets in vitro. Substantial reductions in both platelet and leukocyte adhesion to nylon and PMMA surfaces have been observed for the CVF-treated animals; in some experiments involving nylon 6,6 surfaces, platelet and leukocyte adhesion were completely inhibited.

Thus, the immunochemical and platelet aggregation data presented here are consistent with cell adhesion phenomena observed in ex vivo blood flow experiments, and the combined results suggest a role for complement activation in mediating cell adhesion to polymer blood contact surfaces in the context of the canine model.

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