

# Binding of C3 fragments on top of adsorbed plasma proteins during complement activation on a model biomaterial surface

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## Abstract

In the present study we investigate whether complement activation in blood in contact with a model biomaterial surface (polystyrene) occurs directly on the material surface or on top of an adsorbed plasma protein layer. Quartz crystal microbalance-dissipation analysis (QCM-D) complemented with enzyme immunoassays and Western blotting were used. QCM-D showed that the surface was immediately covered with a plasma protein film of approximately 8 nm. Complement activation that started concomitantly with the adsorption of the protein film was triggered by a self-limiting classical pathway activation. After adsorption of the protein film, alternative pathway activation provided the bulk of the C3b deposition that added 25% more mass to the surface. The build up of alternative pathway convertase complexes using purified C3 and factors B and D on different protein films as monitored by QCM-D showed that only adsorbed albumin, IgG, but not fibrinogen, allowed C3b binding, convertase assembly and amplification. Western blotting of eluted proteins from the material surface demonstrated that the C3 fragments were covalently bound to other proteins. This is consistent with a model in which the activation is triggered by initiating convertases formed by means of the initially adsorbed proteins and the main C3b binding is mediated by the alternative pathway on top of the adsorbed protein film.

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## 1. Introduction

The complement system consists of approximately 30 plasma and membrane-bound proteins (receptors and regulators) that play a primary role in host defense. These proteins destroy invading foreign cells and substances, either through direct lysis or by mediating leukocyte function. The main event in the activation of complement is the proteolytic cleavage of C3 into C3b and C3a. Activation is achieved by two multi-subunit enzyme complexes, the convertases, that are assembled by three different activation pathways: the classical

pathway (CP) is triggered by the formation of antigen–antibody complexes; the mannan-binding lectin pathway (LP) is activated independently of antibodies and is triggered by the binding of MBL to specific carbohydrates; and the alternative pathway (AP) is triggered by foreign surfaces that do not provide adequate down-regulation of the convertase [1]. The nascent C3b molecule is able to bind specifically to proteins and carbohydrates via free hydroxyl or amino groups, forming covalent ester or amide bonds, respectively. The amide bond is stable, but the ester bond is more labile because of the esterolytic activity of C3b [2]. The covalent bond is essential for the biological activity of C3b [3]. C3b is cleaved at two sites in the  $\alpha'$ -chain by factor I to iC3b, and additional cleavages by factor I in the former  $\alpha'$ -chain separate the C3c fragment from the covalently bound C3d,g fragment [4].

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In most instances, biomaterials in extracorporeal devices come in contact with undiluted blood plasma. Also, biomaterials implanted into soft or hard tissue encounter extracellular fluids that contain plasma proteins at similar concentrations. Several reports have indicated that biomaterial surfaces, both *in vitro* and *in vivo*, trigger the AP upon exposure to plasma or blood [5–7]. Some reports have also demonstrated CP activation [7]. However, the mechanisms by which complement activation is triggered and amplified on biomaterial surfaces are not yet clear.

It is well established that different biomaterial surfaces have different complement activating properties, depending upon their degree of hydrophobicity or hydrophilicity [8,9] and the presence or absence of amino, hydroxyl [10], or other chemical groups [11]. It has been assumed that the presence of particular substituents, especially amino or hydroxyl groups on the biomaterial surface, influences the activation of complement, since these groups are essential for the covalent binding of C3b. However, these studies have not addressed the fact that when a biomaterial comes in contact with blood plasma or other body fluids, the plasma proteins immediately bind and cover the surface of the biomaterial. The occurrence of this binding process suggests that the biomaterial surface affects the composition of the adsorbed proteins, rather than being directly involved in binding of the complement activation products [12].

## 2. Materials and methods

### 2.1. Serum and serum preparations

Blood drawn from 10 healthy donors and 2 individuals with MBL deficiency was allowed to clot for 1 h at room temperature. After centrifugation at  $3300 \times g$  for 25 min at 4°C, the serum was collected and pooled. The final pool was stored at –70°C. In order to specifically block the CP, EGTA and  $Mg^{2+}$  were added to bring the final concentration of the serum to 10 mM in EGTA and 2.5 mM in  $Mg^{2+}$  (Mg-EGTA); to specifically block the AP, the serum was made 7  $\mu$ M in Compstatin analog peptide ICWQDWGAHRCT, a cyclic peptide that inhibits complement activation by binding to C3 and blocking its interaction with the convertases [13,14]. It is 40 times more active than its parent molecule (a detailed description will be published elsewhere) [15]. Compstatin inhibits the AP at a lower concentration than that required to inhibit the CP. Seven  $\mu$ M Compstatin was the lowest concentration at which Mg-EGTA gave a total inhibition of complement activation. Total inhibition of complement activation was achieved by bringing the serum to a final concentration of 40  $\mu$ M Compstatin or 10 mM EDTA.

Serum was depleted of immunoglobulins by passing 25 ml of serum containing 10 mM EDTA over a column containing 20 ml of equal amounts of Protein A and Protein G Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). The serum was thereafter dialyzed against veronal-buffered saline (VBS) containing 0.15 mM  $Ca^{2+}$  and 0.5 mM  $Mg^{2+}$  (VBS $^{2+}$ ). The content of IgG, IgM and IgA was reduced by 97%, 70% and 30%, respectively, as determined by nephelometry. The serum was diluted to 70% of its original protein concentration.

### 2.2. Purified proteins and antibodies

Fibrinogen was purchased from Chromogenix (M-öndal, Sweden), IgG from Centeon Pharma (Marburg, Germany), human serum albumin (HSA) from Aventis Behring (Marburg, Germany), and bovine serum albumin (BSA) from Intergen (Oxford, UK). Factor D was purified from peritoneal dialysis fluid of patients with renal failure, as described by Catana and Schifferli [16]. All other proteins were purified from human plasma. C3 and factor H were purified according to Hammer et al. [17], except that the factor H purification was preceded by an euglobulin precipitation [18]. Factor B was purified according to Lambris et al. [19]. The proteins were frozen and stored at –70°C and were thawed only once. C3 was digested with 1% (w/w) trypsin (Sigma Chemical Co, St. Louis, MO, USA) for 5 min at room temperature to give C3a and C3b. The fragments were separated by gel filtration on a Sephadex G-100 column (Amersham Biosciences, Uppsala, Sweden) equilibrated in PBS.

### 2.3. Quartz crystal microbalance-dissipation (QCM-D) analysis

The QCM-D technique relies on the fact that a mass adsorbed onto the sensor surface of a shear-mode oscillating quartz crystal causes a proportional change in its resonance frequency,  $f$ . Changes in  $f$  reflect the amount of mass deposited onto the surface of the crystal. For thin, evenly distributed and rigid films, an adsorption-induced frequency shift ( $\Delta f$ ) is related to mass uptake ( $\Delta m$ ) via the Sauerbrey relation,  $\Delta f = -n\Delta m C^{-1}$ , where  $C$  (equivalent to  $17.7 \text{ ng cm}^{-2} \text{ Hz}^{-1}$ ) is the mass sensitivity constant and  $n$  ( $=1,3,\dots$ ) is the overtone number [20]. However, for proteins adsorbed from the aqueous phase, one must also be aware that water hydrodynamically coupled to the adlayer is included in the measured mass uptake. In addition, when the adsorbed material is non-rigid, additional energy dissipation (viscous loss) is also induced. The dissipation factor ( $D$ ) reflects frictional (viscous) losses induced by deposited materials, such as proteins adsorbed on the surface of the crystal. Hence, changes

in the viscoelastic properties of adlayers (e.g., those induced by conformational changes) as well as differences between various protein-surface interactions can be monitored [21].

Analysis of adsorption kinetics by simultaneous measurement of both the frequency,  $f$ , and the energy dissipation,  $D$ , was performed using a quartz crystal microbalance with dissipation monitoring (QCM-D) (Q-Sense AB, Gothenburg, Sweden), which is described in detail elsewhere [22]. The volume of the chamber was 80  $\mu$ l, and when the liquid in the chamber was exchanged, 0.5 ml was added from a temperature loop; the excess volume was allowed to overflow. Sensor crystals (5 MHz) spin-coated with hydrophobic polystyrene (Q-Sense) were used. Changes in  $D$  and  $f$  were measured at both the fundamental frequency ( $n=1$ , i.e.  $f \approx 5$  MHz) and the third ( $n=3$ , i.e.  $f \approx 15$  MHz) and fifth harmonics ( $n=5$ , i.e.  $f \approx 25$  MHz). Data from the measurements at the third harmonic are presented. All measurements were carried out at 25°C.

#### 2.4. Adsorption of plasma proteins from serum to polystyrene, monitored by QCM-D

Adsorption of plasma proteins from serum to polystyrene was monitored by QCM-D. Before the addition of serum, the surface was incubated with VBS<sup>2+</sup> for 10 min. Serum was incubated for 1 h, and the chamber was then incubated with VBS<sup>2+</sup> for 10 min.

#### 2.5. Monitoring of the assembly of alternative pathway convertases using QCM-D

Protein dilutions and QCM-D assessments were performed in PBS containing 1 mM Ni<sup>2+</sup> (PBS-Ni). Ni<sup>2+</sup> was used to stabilize the AP convertase [23]. The sensor crystal was precoated with 200  $\mu$ g/ml of fibrinogen, IgG, or HSA for 50 min, followed by a 10-min incubation with PBS-Ni. C3b (200  $\mu$ g/ml) was then added and incubated with the crystal for 50 min. Finally, each surface was washed with PBS-Ni for 10 min. After the coating procedure, the surface was incubated with factor B (38  $\mu$ g/ml) for 10 min, followed by a 10-min incubation with factor D (10  $\mu$ g/ml) before C3 (133  $\mu$ g/ml) was added for 50 min as previously described [24]. All proteins were diluted in PBS-Ni, and each cycle ended with a 10-min PBS-Ni rinse. Each of the described cycles of incubation with purified complement components was performed three times on each surface coat.

#### 2.6. Serum incubation

Serum (undiluted or diluted in VBS<sup>2+</sup>) was incubated for up to 60 min in wells of polystyrene microtiter plates [25]. Complement activation was stopped by transferring the serum to wells of new plates containing EDTA

to give a final concentration of 10 mM. The original plate was immediately washed three times with washing buffer (PBS containing 0.05% Tween 20 and 0.02% Antifoam [Pharmacia Diagnostics, Uppsala, Sweden]). In order to investigate the binding of C3 fragments to polystyrene-adsorbed plasma proteins, the wells of microtiter plates were precoated with HSA (40 mg/ml), IgG (55 mg/ml), or mixtures thereof, as indicated in the legend of Fig. 6 for 5 min at 37°C before serum incubation.

#### 2.7. Enzyme immunoassays (EIA) for the detection of surface-bound C3 and C4 fragments

Washing buffer and working buffer (washing buffer containing 1% [w/v] BSA) were used. After the serum incubation, the wells were saturated with 300  $\mu$ l working buffer for 15 min at 37°C. C3 and C4 fragments were detected with 100  $\mu$ l horseradish peroxidase (HRP)-conjugated anti-C3c diluted 1/400 (Dako A/S, Glostrup, Denmark) and anti-C4 diluted 1/200 (The Binding Site, Birmingham, UK), respectively, in working buffer for 30 min at 37°C. The plates were stained with a color solution consisting of 10 mg phenyldiamine dihydrochloride (Sigma) dissolved in 40 ml 0.1 M citrate-phosphate buffer (pH 5.0) and containing 10  $\mu$ l 30% H<sub>2</sub>O<sub>2</sub>. Staining was carried out for 5 min. The absorbance was measured at 492 nm.

#### 2.8. Western blot analysis of plasma proteins adsorbed to a polystyrene surface exposed to serum

Serum samples (1 ml each) were incubated in polystyrene tubes for 1 h at 37°C. The tubes were washed five times with PBS and then incubated for 1 h at room temperature on a rocker with 0.5 ml PBS containing 2% SDS [26]. The eluted proteins were analyzed under non-reducing and reducing conditions by SDS-PAGE using 7.5% gels, followed by Western blot analysis using HRP-conjugated rabbit antibodies against human C3c. The anti-C3c antibody used detects the  $\beta$ -chain and the 40 kDa polypeptide chain of C3c, including the corresponding epitopes in the  $\alpha$ -chain of C3 [27].

### 3. Results

#### 3.1. Adsorption of serum proteins to a polystyrene surface as detected by QCM-D

Human serum containing 10 mM EDTA or 40  $\mu$ M Compstatin was exposed to the polystyrene surface in a QCM-D chamber. There was an instantaneous binding of proteins to the surface, which leveled off after less than 1 min (Fig. 1). The initial rapid binding from serum resulted in a normalized frequency shift of  $-100$  Hz at F3 (15 MHz)/3. Since 100% serum was

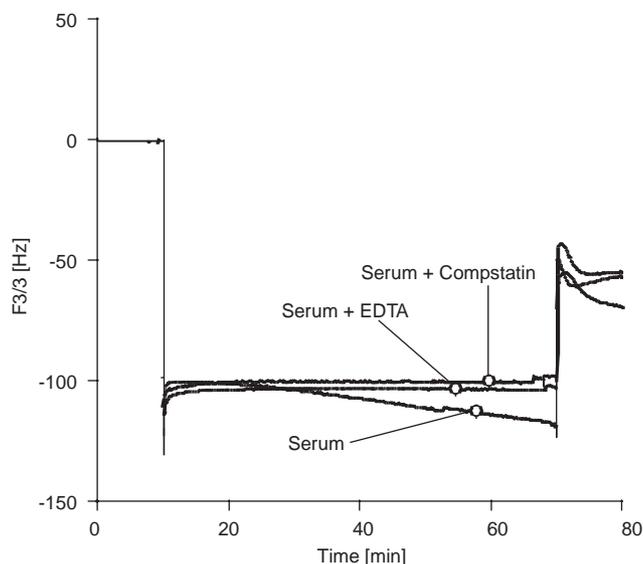


Fig. 1. Adsorption of serum proteins to polystyrene. Adsorption of plasma proteins from undiluted human serum to polystyrene at 25°C was monitored by QCM-D. The surface was initially exposed to VBS containing  $Mg^{2+}$  and  $Ca^{2+}$ . After 10 min, serum, serum containing 10 mM EDTA or serum containing 40  $\mu$ M Compstatin was added. The incubation was stopped after 70 min by exchanging serum for buffer in the chamber.

applied and a significant buffer effect was expected, we have used the value after rinsing (–55 Hz). Using the Sauerbrey equation, this corresponds to a surface concentration of 970 ng/cm<sup>2</sup>. Assuming a density of 1200 kg/m<sup>3</sup>, which is between that of water (1000 kg/m<sup>3</sup>) and protein (1400 kg/m<sup>3</sup>), an average thickness of about 8 nm was calculated. Normalization of F3, F5, and F7 revealed that F3/3, F5/5, and F7/7 differed, indicating that the actual amount of bound proteins was underestimated by the Sauerbrey equation (not shown). In the absence of EDTA and 40  $\mu$ M Compstatin, which totally inhibit complement activation, there was continuous binding for at least 60 min (Fig. 1). The binding level after the rinse was about 25% (–70 Hz) higher than for the complement-inhibited controls.

### 3.2. Assembly of AP convertases on polystyrene surfaces coated with HSA, IgG or fibrinogen

The ability of a specific plasma protein to act as an acceptor for binding of C3b was assessed by assembling AP convertases on plasma protein-coated polystyrene surfaces in a QCM-D chamber. AP convertases were assembled by the sequential addition of the AP components B, D, and C3 after an initial coating with C3b. As shown in Fig. 2, functional convertase complexes were formed on IgG and HSA but not on fibrinogen, indicating that fibrinogen is a poor acceptor of C3b, while both albumin and IgG can bind C3b.

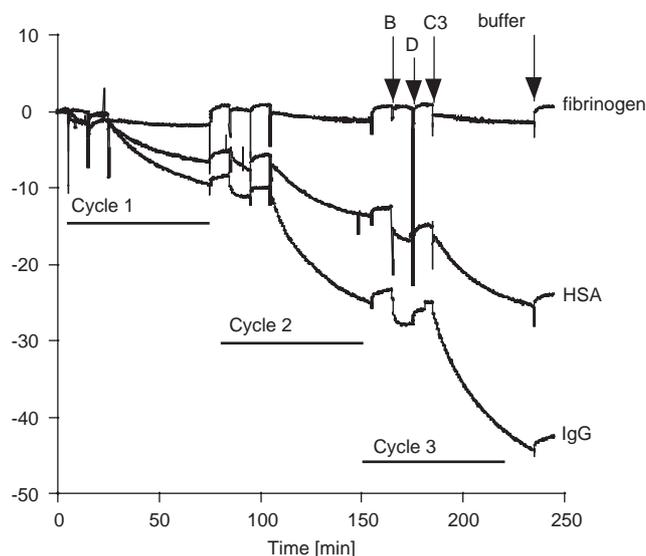


Fig. 2. Generation of AP convertases on polystyrene coated with HSA, IgG, or fibrinogen, as monitored by QCM-D. Polystyrene surfaces were precoated with 200  $\mu$ g/ml of HSA, IgG, or fibrinogen, followed by 200  $\mu$ g/ml of C3b. For each experiment the AP convertase was assembled by sequentially adding factor B (10 min), factor D (10 min), C3 (50 min), and buffer (10 min) in three identical cycles. Each bar in the figure represents one cycle. All steps were carried out in PBS containing 1 mM  $Ni^{2+}$ .

### 3.3. Western blot analysis of eluted plasma proteins from polystyrene treated with normal serum

We treated polystyrene tubes with normal serum and then eluted the bound plasma proteins with 2% SDS. When the samples were subjected to SDS-PAGE under non-reducing conditions, followed by Western blotting with anti-C3c antibodies, four distinct C3 bands were observed (Fig. 3). One band corresponded to monomeric C3, while the other three had higher molecular sizes, indicating that C3 was bound to other molecules. Since the electrophoresis was performed under non-denaturing conditions, this binding was most likely covalent. After reduction with mercaptoethanol, only the  $\beta$ -chain and (faintly) the 40-kDa polypeptide chain of C3c were seen. This finding suggested that the surface-bound C3 fragments were iC3b.

### 3.4. Binding of C3 and C4 fragments to a polystyrene surface

When undiluted serum was exposed to the polystyrene surface of wells of microtiter plates for 60 min, a continuous binding of C3 fragments to the surface was observed (Fig. 4A). In serum containing Mg-EGTA, in which activation of C4 had been blocked, this rapid binding disappeared and was delayed by approximately 15 min, but the quantity of C3 fragments deposited was

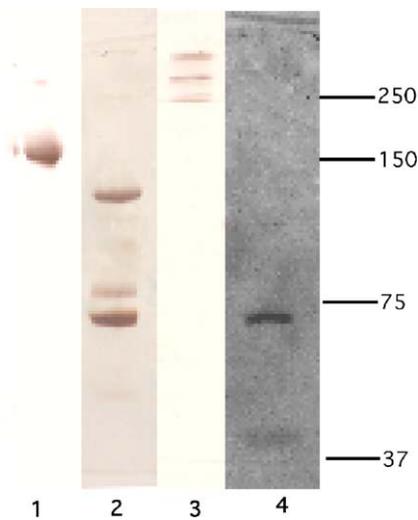


Fig. 3. *Adsorbed serum proteins eluted from polystyrene.* Eluted proteins were detected by SDS-PAGE under non-reducing (N) and reducing (R) conditions, followed by Western blotting using anti-C3c. Lane 1 show purified C3 (N); lane 2, purified C3 (R); lane 3, eluted proteins (N); lane 4, eluted proteins (R).

the same as in normal serum after 45 min. In contrast, in serum containing  $7\ \mu\text{M}$  Compstatin in which the AP was inhibited, the binding of C3 fragments began as rapidly as in normal serum, but the extent of the binding was substantially lower. Serum that contained both Mg-EGTA and Compstatin resembled serum containing 10 mM EDTA. In both these sera, no noteworthy complement activation was observed (Fig. 4A).

The binding of C4 fragments tested under the same conditions showed that the binding in undiluted serum in the presence or absence of  $7\ \mu\text{M}$  Compstatin had peaked already after 2 min and then tapered off. No binding occurred in serum containing Mg-EGTA or EDTA (Fig. 4B).

Kinetic studies of the binding of C3 fragments in normal undiluted and diluted serum in the presence of  $7\ \mu\text{M}$  Compstatin to block AP activation showed that the binding was immediately triggered and that maximal binding occurred in 25% serum dilution (Fig. 4C). In undiluted serum, low-level binding of C3 fragments still occurred, and the trigger rate was not different from those of the dilutions.

### 3.5. *Binding of C3 fragments to a polystyrene surface in contact with different dilutions of serum*

Binding of C3 fragments to the polystyrene surface was further investigated using serially diluted serum incubated for 30 min (Fig. 5). The binding of C3 fragments was considerably increased when the serum concentration was lowered from 100% to 50%. Further dilutions resulted in lower binding. The binding of

undiluted Mg-EGTA serum was higher than that of undiluted serum without additives, but it rapidly decreased to the background level of EDTA-serum at concentrations under 10%. Over a concentration range of 100% to 0.1%, passive binding from the EDTA-serum continuously increased.

### 3.6. *Identifying the trigger of complement activation on a polystyrene surface*

Sera from individuals with MBL deficiency did not behave differently from those from normal individuals, an observation which ruled out the possibility that the LP was the trigger of complement activation on polystyrene (not shown). In order to establish that the trigger was instead the CP, reconstitution of an IgG-deficient serum would be ideal. When we depleted sera of IgG by using Protein A and Protein G Sepharose, we obtained sera that only activated the AP. However, reconstitution of these sera with physiological doses of IgG did not produce sera that were comparable to unmanipulated sera, despite the addition of C1q (not shown). Therefore, we took an alternative approach and assessed binding of C3 fragments from serum to wells precoated with HSA, IgG, or mixtures thereof (Fig. 6). Unlike the results obtained with uncoated surfaces, the binding to HSA was slow and disappeared in the presence of  $7\ \mu\text{M}$  Compstatin, indicating that only the AP was operative. In contrast, different concentrations of precoated IgG alone or combined with 40 mg/ml of HSA triggered a very rapid and strong binding indicative of an initial CP activation.

## 4. Discussion

Our QCM-D studies showed that when plasma proteins were deposited onto a polystyrene surface in contact with undiluted human serum under conditions in which complement activation was totally blocked, the passive adsorption of proteins occurred much more rapidly than the active binding of proteins that was mediated by complement activation in serum without additives (a relatively slow and continuous process). We calculated that the average mass deposited by the initial adsorption was  $970\ \text{ng}/\text{cm}^2$ , corresponding to a protein layer of approximately 8-nm thickness. These values obtained for the rate of binding and the thickness of the adsorbed protein layer are in good agreement with previously published data obtained by ellipsometry for undiluted serum [12]. Provided that the proteins are not deposited in clusters, this thickness corresponds to more than a monolayer of proteins of the size of the most common proteins in serum (i.e., HSA [4 nm] and IgG [8 nm]). Complement activation added approximately 25% to the initially adsorbed protein mass after 60 min

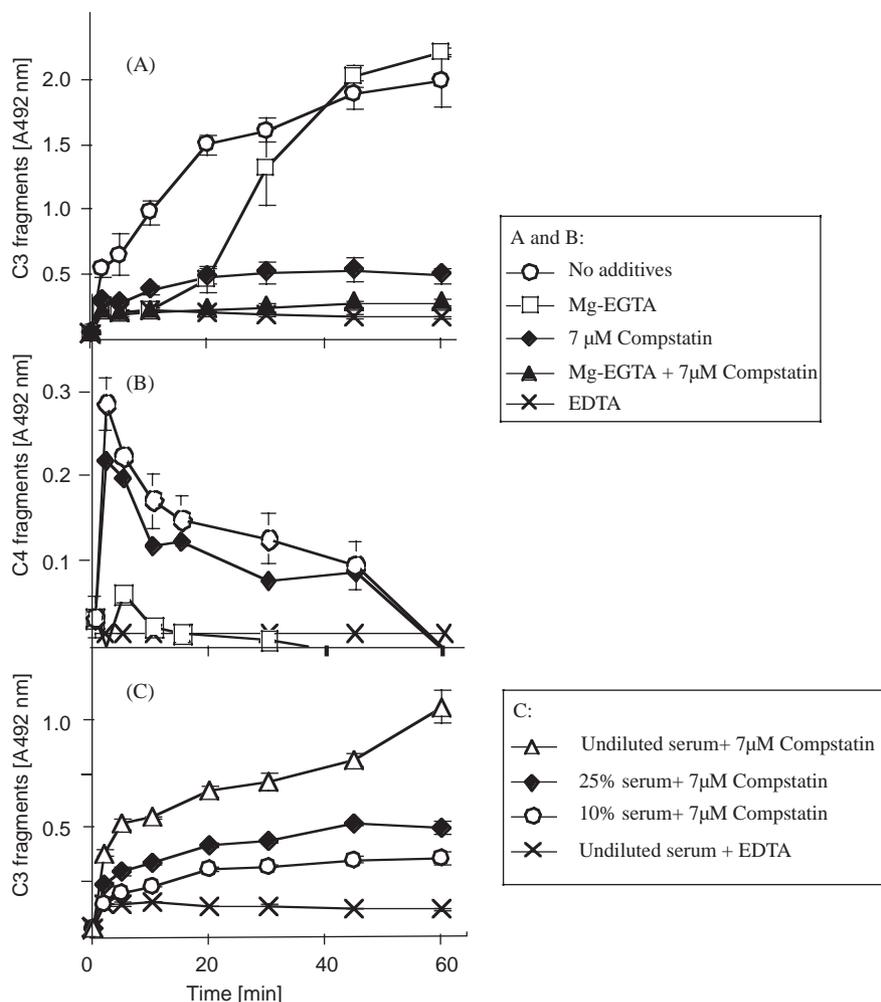


Fig. 4. Complement activation on polystyrene. Binding of C3 and C4 fragments to polystyrene in contact with serum at 37°C was monitored by EIA using anti-C3c and anti-C4. Dilutions were performed in VBS<sup>2+</sup>. Panel A, kinetics of the binding of C3 fragments in undiluted serum; panel B, kinetics of the binding of C4 fragments in undiluted serum. Serum without additives (open circles) or serum containing 10 mM EDTA (crosses), Mg-EGTA (open squares), or 7 μM Compstatin (closed diamonds) to block either total complement activation or only CP or AP activation, respectively, was used. Panel C, kinetics of the binding of C3 fragments in undiluted serum (open circles), 25% serum (filled diamonds) and 10% (open triangles) containing 7 μM Compstatin. Serum containing 10 mM EDTA (crosses) was used as control.

of incubation; this layer of complement fragments probably consists mainly of C3 fragments, since its deposition was inhibited by Compstatin. Taken together, these findings strongly suggest that complement activation occurs on top of a layer of plasma proteins. The most abundant of these proteins, HSA (42 mg/ml), IgG (11 mg/ml), and fibrinogen (3 mg/ml), have all been reported to bind to biomaterial surfaces [28].

The concept that complement activation may occur on top of adsorbed plasma proteins has led us to hypothesize that C3b, a prerequisite for AP activation, binds covalently to plasma proteins such as HSA, IgG, and fibrinogen. We therefore investigated whether the AP convertase could be assembled on a layer of adsorbed HSA, IgG, or fibrinogen, with purified AP proteins C3, B, and D being added in sequence to the QCM chamber. Both IgG and HSA functioned as

acceptor molecules for the binding of C3b, allowing amplification of C3b binding to the surface. As we have previously reported, fibrinogen did not have this property [24]. The same study also showed that the less abundant C3 and C3b molecules can act as acceptors for covalent C3b binding.

In order to confirm that C3b covalently binds to an adsorbed layer of plasma proteins, undiluted serum was incubated in polystyrene tubes and, after washing, bound plasma proteins were eluted with 2% SDS. The samples were subjected to SDS-PAGE, followed by Western blotting using anti-C3c. By using non-reducing conditions and room temperature, covalent ester bonds were maintained. This analysis demonstrated that a significant portion of the C3 fragments had a molecular size greater than that of monomeric C3. Thus, it appears that the C3 fragments were covalently bound to other

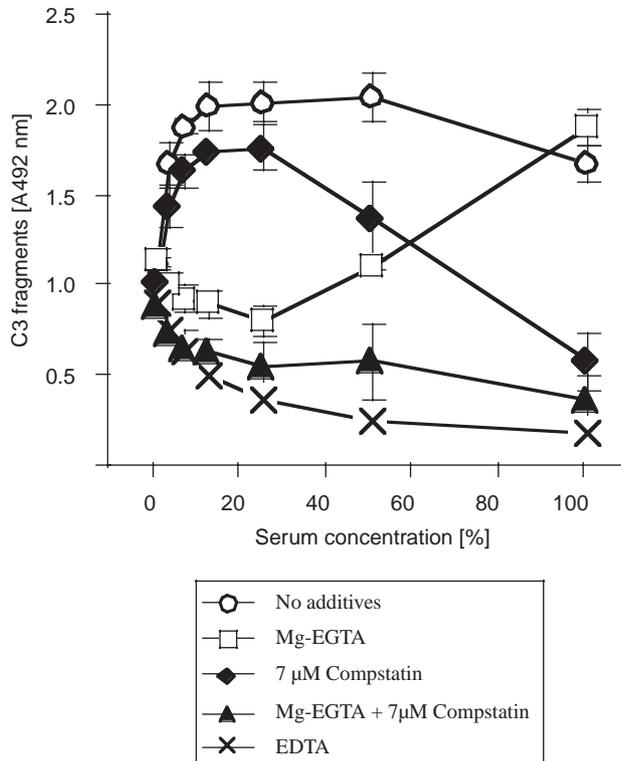


Fig. 5. Complement activation on polystyrene in dilutions of serum. Binding of C3 fragments to polystyrene in contact with dilutions of serum was monitored by EIA using anti-C3c. Serum without additives (open circles), or serum containing Mg-EGTA, 7 μM Compstatin or 10 mM EDTA to block the CP, AP or total complement activation, respectively, was used.

molecules, i.e., plasma proteins adsorbed to the polystyrene surface. The bands similar in size to monomeric C3 seen on the Western blots performed using non-reducing conditions may have been monomeric C3, C3b, or iC3b. Under reducing conditions the high molecular size bands disappeared, suggesting that the bonds were of ester origin and that the polypeptide pattern was compatible with iC3b. The instability of the ester bond does not rule out the possibility that a larger number of molecules were originally ester-bonded to plasma proteins [2].

In order to define which complement pathway is involved in C3b deposition onto a polystyrene surface, we have exposed the surface to various concentrations of human serum or to serum containing EGTA or 7 μM Compstatin to block CP or AP activation, respectively. The activation of complement in undiluted serum was mainly driven by the AP. The contribution made by CP activation was found to increase continuously as the concentrations of serum decreased, while the AP activation reciprocally tapered off and disappeared at serum concentrations under 10%.

In our kinetic studies of uncoated polystyrene and undiluted serum to which we added Compstatin to

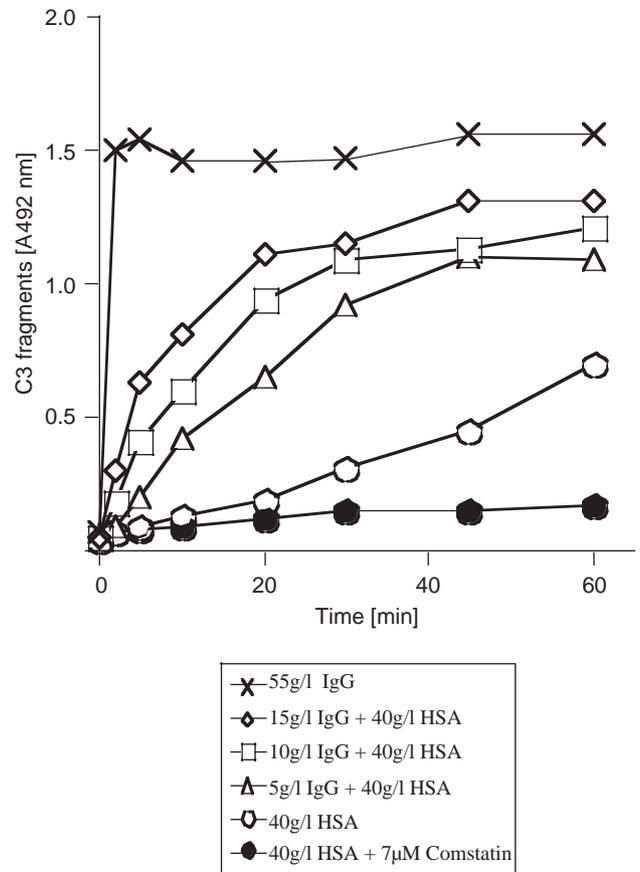


Fig. 6. Complement activation on preadsorbed proteins. Kinetic binding of C3 fragments in undiluted serum on polystyrene precoated with HSA, IgG, or mixtures thereof as detected by EIA using anti-C3c. The polystyrene was precoated with IgG (55 mg/ml, crosses), IgG (15 mg/ml, open diamonds; 10 mg/ml, open triangles; 5 mg/ml, open squares) and HSA (40 mg/ml), or HSA (40 mg/ml, open circles) alone for 5 min at 37°C. Serum was also incubated on HSA (40 mg/ml; closed circles) in the presence of 7 μM Compstatin.

block the AP, the CP leveled off after 10–20 min, after which no further C3b deposition took place. In unmanipulated serum, binding of C4 fragments was immediately triggered but tapered off within 10 min. One explanation for these observations comes from earlier studies demonstrating that during initial complement activation, C3b binding seems to physically shield the underlying proteins, including C1q and IgG, and may also block available acceptor sites for the binding of C4b [25,29], thereby halting further activation via the CP. However, this limited CP activation might also reflect transient conformational changes in IgG caused by the adsorption to and denaturation of the molecules at the surface. The IgG molecules would thus be biologically available for only a limited time. An additional third explanation could be the Vroman effect [30] according to which adsorbed plasma proteins are exchanged for larger proteins. However, this latter explanation is contradicted by other studies showing

that IgG preadsorbed to polystyrene is not released by incubation in serum [25]. Even though the CP deposits only minute amounts of C3b on the surface, the deposition is rapid and sufficient to instantaneously trigger the AP amplification loop. The combined sequential activation of the two pathways results in the total deposition of C3b molecules on the surface.

The fact that serum depleted of IgG only promoted AP is consistent with a limited activation of the CP. In vivo studies have also shown that no CP activation occurs when patients with congenital C4 deficiency or acquired IgG deficiency undergo hemodialysis [7]. In such patients the initiation of complement activation was delayed, but the activation finally reached the same amplitude as in patients with normal levels of C4. These findings suggest that the C1 complex or, more likely, IgG adsorbed to the biomaterial surface is able to trigger CP activation, a conclusion that is supported by the present study and by previous in vitro studies indicating that IgG adsorbed to surfaces mediates a vigorous activation and binding of C3b to the surface [25,31].

In order to determine whether IgG in a plasma protein film precoated on a polystyrene surface could trigger the CP, we precoated polystyrene with IgG, HSA, or mixtures thereof, and reacted these surfaces with undiluted serum; our results confirmed that complement activation occurred on the adsorbed protein layers. In the case of HSA, initiation was very slow, with a prolonged lag phase typical of AP activation. Blockade with 7  $\mu$ M Compstatin confirmed that the activation was almost completely mediated by the AP. When IgG was tested, a pronounced complement activation was immediately triggered by the CP. Mixtures of IgG and albumin produced an intermediate result, and a mixture with only trace amounts of IgG in albumin activated the serum in a manner similar to serum alone, thereby mimicking the initial layer of plasma proteins that is bound when serum comes in contact with a polystyrene surface.

These findings have allowed us to construct a model to explain how complement activation and C3b binding take place on a biomaterial surface: the initiation of complement activation, which provides the initial C3b molecules that bind to the plasma protein coat on the material surface, is probably triggered by molecules of the initially bound protein film. Adsorbed IgG has been shown in the present study and others [25,31] to initiate the CP, and we have previously demonstrated that adsorbed and conformationally changed C3 is able to serve as a nucleus for initiating the AP convertase [24]. Once C3b is generated and covalently bound to the protein coat, the AP amplification loop can be triggered. This amplification loop then generates the majority of the C3b molecules that bind to the material surface. Important consequences of this model, therefore, are

that initiation of the AP amplification loop can be triggered by either CP or AP convertases and that a biomaterial surface which binds low levels of plasma proteins, will also be a poor activator of complement; these predictions have, indeed, been verified empirically in a previous study [8].

## 5. Conclusions

In the present study, using undiluted blood serum in contact with polystyrene, we have demonstrated that: the AP amplification loop is instantaneously triggered by plasma proteins in the initially adsorbed plasma protein layer. This initiation is mainly mediated by the CP, but AP activation may also be operative. The main bulk of C3b is deposited by the AP amplification loop and covalently bound to the initially adsorbed protein layer. We believe that the hypothesis derived from these findings will be of importance in understanding complement activation on other biomaterial surfaces.

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