Complement Activation Triggered by Biomaterial Surfaces

Mechanisms and Regulation

BY

JONAS ANDERSSON
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Abstract

Today there are a vast number of medical devices in temporary or permanent contact with human tissues. Blood-biomaterial contact is known to trigger the complement system and results in generation of fluid phase anaphylatoxins C3a and C5a, and surface-bound C3b and iC3b. All these products together are able to attract and activate leukocytes and trigger release of inflammatory mediators leading to a systemic inflammation indirectly causing hemostatic problems and even organ failure. The aim of this study was to identify how complement is triggered on a biomaterial surface and to find ways to regulate this activation.

The finding that complement activation on biomaterials can be divided into initiation and amplification will facilitate regulation of complement activation biomaterial surfaces. This concept is also compatible with the two techniques to regulate complement activation on a surface.

Keywords: Biomaterials, Biocompatibility, Blood, Complement

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Papers included in the thesis

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I  Andersson, Jonas ; Nilsson Ekdahl, Kristina ; Nilsson, Bo: Complement activation on a model biomaterial surface: Binding of C3b via the alternative pathway amplification loop to plasma proteins adsorbed to the surface. (manuscript)

II Andersson, Jonas ; Nilsson Ekdahl, Kristina ; Larsson, Rolf ; Nilsson, Ulf R. ; Nilsson, Bo: C3 Adsorbed to a Polymer Surface Can Form an Initiating Alternative Pathway Convertase. Journal of Immunology, 168(2002): no. 11, 5786-5791 (published)

III Andersson, Jonas ; Larsson, Rolf ; Richter, Ralf ; Nilsson Ekdahl, Kristina ; Nilsson, Bo: Binding of a model regulator of complement activation (RCA) to a biomaterial surface: surface-bound factor H inhibits complement activation. Biomaterials, 22(2001): no. 17, 2435-2443 (published)

IV Andersson, Jonas ; Sanchez, Javier ; Nilsson Ekdahl, Kristina ; Elgue, Graciela ; Nilsson, Bo ; Larsson, Rolf: Optimal heparin surface concentration and antithrombin binding capacity as evaluated with human non-anticoagulated blood in vitro. Journal of Biomedical Materials Research (accepted)
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## Abbreviations

<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AP</td>
<td>Alternative pathway of complement</td>
</tr>
<tr>
<td>AT</td>
<td>Antithrombin</td>
</tr>
<tr>
<td>β-TG</td>
<td>β-Thromboglobulin</td>
</tr>
<tr>
<td>C</td>
<td>Complement component</td>
</tr>
<tr>
<td>C1INH</td>
<td>C1 inhibitor</td>
</tr>
<tr>
<td>C3(H2O)</td>
<td>Inactivated C3</td>
</tr>
<tr>
<td>C3b,Bb</td>
<td>AP C3 convertase</td>
</tr>
<tr>
<td>C3b,Bb,C3b</td>
<td>AP C5 convertase</td>
</tr>
<tr>
<td>C4b,C2a</td>
<td>CP C3 convertase</td>
</tr>
<tr>
<td>C4b,C2a,C3b</td>
<td>CP C5 convertase</td>
</tr>
<tr>
<td>C5b-9</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>cC1q</td>
<td>Collagen domain of C1q</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CP</td>
<td>Classical pathway of complement</td>
</tr>
<tr>
<td>D</td>
<td>Dissipation</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>f</td>
<td>Frequency</td>
</tr>
<tr>
<td>FHL</td>
<td>Factor H-like protein</td>
</tr>
<tr>
<td>FHR</td>
<td>Factor H-related protein</td>
</tr>
<tr>
<td>gC1q</td>
<td>Globular domain of C1q</td>
</tr>
<tr>
<td>HAE</td>
<td>Hereditary angioedema</td>
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<tr>
<td>iC3</td>
<td>Inactivated C3</td>
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<tr>
<td>LP</td>
<td>Lectin pathway of complement</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
</tr>
<tr>
<td>MASP</td>
<td>MBL-associated serine protease</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannan-binding lectin</td>
</tr>
<tr>
<td>P</td>
<td>Properdin, factor P</td>
</tr>
<tr>
<td>PEO</td>
<td>Poly(ethylene oxide)</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear granulocyte</td>
</tr>
<tr>
<td>PNH</td>
<td>Paroxysmal nocturnal hemoglobinurea</td>
</tr>
<tr>
<td>PPO</td>
<td>Poly(propylene oxide)</td>
</tr>
<tr>
<td>PRP</td>
<td>Platelet-rich plasma</td>
</tr>
<tr>
<td>PVC</td>
<td>Poly(vinyl chloride)</td>
</tr>
<tr>
<td>R</td>
<td>Receptor</td>
</tr>
<tr>
<td>RCA</td>
<td>Regulator of complement activation</td>
</tr>
<tr>
<td>RHP</td>
<td>Rheumatoid arthritis protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SCR</td>
<td>Short consensus repeat</td>
</tr>
<tr>
<td>SPDP</td>
<td>N-succinimidyl 3-(2-pyridoldithio) propionate</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>sC5b-9</td>
<td>Soluble membrane attack complex</td>
</tr>
<tr>
<td>TAT</td>
<td>Thrombin-antithrombin complex</td>
</tr>
<tr>
<td>QCM-D</td>
<td>Quartz crystal microbalance with dissipation monitoring</td>
</tr>
</tbody>
</table>
Introduction

Today there are a vast number of medical devices that come in temporary or permanent contact with tissues of the human body. The purpose of these devices is to treat or overcome various disease states, but to be of benefit to the patient it is of major importance that the advantages of their use outweigh any adverse reaction produced by their contact with the patient’s tissue.

The majority of medical devices make contact with blood either during implantation or when functioning. Some examples of common blood-contact applications are blood pumps, oxygenators and hemodialyzers. Stents, vascular grafts, miniature pumps, sensors, pacemakers and heart valves are permanently in contact with blood. These devices are constructed from diverse types of materials that in many respects have little or nothing in common. All these materials intended to interact with biological systems are, however, often referred to as biomaterials (1). This term indicates only that the particular material is used in contact with the body and says nothing about whether that material is a good choice for the application.

The term biocompatibility is used to describe the ability of a material to perform with an appropriate host response in a specific application (1). This is not an absolute definition, and how biocompatibility should be assessed differs from one application to another. For example, what is a highly biocompatible material in terms of bone integration might be an unsuitable material for applications involving contact with blood.

Upon blood-biomaterial contact there is an instantaneous adsorption of proteins. The protein pattern of this adsorption depends on the particular material, and this event decides the outcome of the contact between blood and biomaterial. Since blood is important in our defense against foreign invaders, there is a great risk that a synthetic material will provoke an undesirable response. This response starts with activation of the cascade systems of blood, the most important ones being the coagulation, complement and contact systems. Activation of these systems will inevitably result in recruitment and activation of cells, which in turn are able to release inflammatory mediators (2).

The main focus of this work is on the initial blood-biomaterial contact and how the complement system is activated. Both the mechanisms that
control this initiation of complement activation and possible ways to regulate this activation have been studied.
Biomaterials

There are many different types of materials that are used as biomaterials. The most important are ceramics, composites, metals and polymers. In blood contact-related applications there is also a wide variety of materials in use, although polymers and metals are the most common. For example, polymers, and particularly poly(vinyl chloride), are used in tubing intended for extracorporeal circulation. Membranes in hemodialyzers are often made of regenerated cellulose, but chemically modified celluloses and completely synthetic membranes are also used (3-5). Oxygenator membranes are most often made from polypropylene (6, 7). Many types of implants are used in contact with blood. Artificial heart valves are typically made of pyrolytic carbon or titanium (8). Stents used to improve the result from angioplasty are mostly made of stainless steel, titanium and metal alloys (9).

Surface characteristics

The interaction between blood and biomaterials first takes place at the material surface. It is obvious that the properties of the material surface decide the outcome of this meeting. The surface can be considered from a physical, a chemical and a biological point of view.

The mechanical demands on a biomaterial vary widely among the various applications. Properties like hardness, tribology and roughness must always be taken into account when choosing the proper material. In terms of chemical properties, hydrophobicity is important, but the presence of specific chemical groups is also significant.

There are not many surfaces that are biologically active, but this is a growing field of great interest. By binding biologically active molecules to the surface it is possible to prevent unfavorable reactions from taking place. This strategy might also allow specific cell types, such as endothelial cells, to bind to ligands immobilized on the biomaterial surface.
Surface modifications

There are many established methods for optimizing the hardness and tribology of biomaterials. During the last decade there has been a focus on constructing materials with nanostructures, i.e., patterns on the nm scale. Patterns on the surface include pits, holes, stripes, pyramids and many others. There is also the possibility of combining this physical pattern with a chemical pattern (e.g., hydrophobic patches on a hydrophilic surface). The aim of this strategy is to achieve surfaces that will encourage proteins to adhere in a desirable conformation and cells to grow in a controlled manner.

One way to decrease protein binding is to make a hydrophobic surface more hydrophilic by binding poly(ethylene oxide) (PEO) to it (10). PEO is a flexible polymer, and when long PEO chains are effectively bound to a surface, they create a steric barrier that prevents proteins from reaching the surface. An additional advantage of this approach is that proteins on the surface tend to remain in the native state rather than becoming partly denatured. This protection from denaturation can be achieved by covalently binding PEO to the surface or by adsorption of block copolymers containing one or more PEO blocks and at least one poly(propylene oxide) (PPO) block (11). The use of block copolymers results in adsorption of the PPO block to the hydrophobic surface and leaves PEO blocks pointing into the aqueous solution. Copolymers of this type are called Pluronics, and previous studies have shown that Pluronic F108 is the most effective for reducing protein adsorption (12). The copolymer approach is advantageous because it can be applied to a number of different materials, and there is no need to create functional groups on the material to bind the PEO chains. Furthermore, it yields an even distribution of PEO chains. A limitation of this approach is the risk of desorption of the copolymer when the surface is exposed to certain solutions, such as blood, which contain lipids and lipoproteins (13).

Biologically active surface modifications

The most familiar biologically active surface modification is the binding of heparin to a surface. Heparin is present in the body as a proteoglycan where heparin is covalently attached to a protein core. Prepared in its soluble form, free from protein, this carbohydrate has been shown to be an efficient anticoagulant agent(14). Antithrombin (AT) is a known serine protease inhibitor and inactivates a broad range of coagulation factors. Binding of AT to a specific pentasaccharide motif in heparin induces a conformational change accelerating inactivation of coagulation enzymes(15). Immobilized heparin has been demonstrated to modulate activation of coagulation at the surface, but there is also an inhibitory effect on complement activation(16-
Possibly, this is due to the binding of factor H, a soluble regulator of complement activation, known to possess heparin-binding sites (15, 19, 20).

The aim of immobilized heparin is to create a surface that mimics that of endothelium in regard of blood compatibility. When considering heparin surfaces you must keep in mind that there are many different methods of immobilization! (21). It is of great importance that the AT-binding sequence is not compromised in the immobilization procedure. Every type of heparin surface must be tested for stability and biological activity.

Other types of bioactive surfaces are less commonly used. The end groups of Pluronic F108, for example, can be chemically activated to bind any protein (22), allowing one to bind an inhibitor of complement or coagulation to a surface while simultaneously preventing nonspecific protein adsorption.
Blood

Although it is a fluid, blood is considered to be a body tissue. About 45% of its volume is made up of cells, and the remainder is an aqueous solution, plasma, with a protein concentration of 80 mg/ml. The blood system fulfills many essential functions, such as distributing oxygen and nutrients. It also includes systems that provide defense against foreign invaders and maintain the integrity of the blood system (23, 24).

Cells of the blood

To be able to fulfill all the requirements of the blood system, many different cell types are required, all of which are specialized for different functions.

Erythrocytes

The main function of these cells, which lack a nucleus, is to transport oxygen to the tissues of the body and to remove harmful carbon dioxide (24). Although often considered to be an inert cell type in other respects, erythrocytes have been shown to be of importance in the amplification of coagulation. In platelet-rich plasma (PRP), which is basically blood lacking erythrocytes and leukocytes, activation of coagulation is much harder to trigger than in whole blood or in PRP supplemented with erythrocytes (25).

Platelets

Platelets also lack nuclei and are a crucial component in the formation of the clots induced by coagulation (23). Receptors for collagen and fibrin allow platelets to attach to wounded vessel walls and the fibrin fibers of a clot (26, 27). Degranulation of these bound platelets releases substances that are able to activate other platelets in the vicinity (28-30). In addition, phospholipids in the platelet cell membrane are able to act as cofactors for clotting factors (31).
Leukocytes

The main types of leukocytes are lymphocytes, monocytes and granulocytes.

Lymphocytes are involved in both innate and adaptive immunity: NK cells, involved in innate immunity, are able to fight tumors. T cells either kill cells that they recognize as foreign or act as helper cells to stimulate effector cells. B cells produce antibodies, the humoral arm of specific immunity.

Monocytes are large phagocytic cells that form part of the innate immune system. Upon migration from the blood into tissues they can be transformed into macrophages.

Granulocytes, also known as polymorphonuclear cells (PMN), can be divided into three different cell types: eosinophils, basophils and neutrophils. About 60% of the leukocytes and 95% of the granulocytes are neutrophils. This cell type is, together with monocytes, the most rapid responder upon complement activation and possesses a number of complement receptors. It acts both by phagocytosis and by release of inflammatory mediators (32).

Blood plasma and serum

Plasma is the fluid that remains when the cells are removed from blood. This fluid contains proteins and nutrients. Plasma has a protein concentration of about 80 mg/ml and is made up of thousands of different proteins, most of them present in trace amounts. These blood proteins can be divided into several functional groups:

The first group is the proteins that maintain the osmotic pressure of the blood, making certain that water does not leak out of the vessel. All the proteins present in blood contribute to this effect, but since albumin is the most common protein, it is considered to be of greatest significance. Present at 45 mg/ml in blood, albumin is also important as a transport protein.

Since transporting low-molecular weight compounds is a major responsibility of the blood system, it includes a number of transport proteins. Albumin is involved in the transportation of a wide variety of compounds, including fatty acids, bilirubin and calcium, copper and zinc ions. There are also numerous specific transport proteins with defined ligands.

The bulk of the plasma proteins participate in the defense systems of the blood. These defense proteins are involved in the coagulation, the fibrinolytic, the contact and/or the complement system. Also, the immunoglobulins are considered defense proteins. The defense proteins that are part of a specific system do not function on their own: They can also be arranged as part of cascade systems. In a cascade system, proteins are activated in a sequential manner to allow an initial activation to be amplified.
In many cases the term network system seems to be more appropriate, since there are more than sequential activations (32).

When working with complement it is most common to use serum instead of plasma (33, 34). Serum is a plasma-like fluid in which the components of the coagulation system have been allowed to clot and have been removed together with the cells. This treatment does not significantly activate components of the complement system.
The Complement System

The complement system is a part of the body’s innate immune system. Its aim is to eliminate microorganisms and other foreign substances that enter the body. About 30 soluble and membrane-bound proteins are part of the complement system; these include both effector and regulatory proteins (35-38). Because this part of our immune system is always active and does not need to be adapted before it is able to respond to an invader, it is able to provide a rapid response. The defensive functions of the complement system are achieved through several effector mechanisms:

- Labeling of foreign substances (=opsonization);
- Release of inflammatory mediators, anaphylatoxins;
- Mediating chemotaxis and release of anaphylatoxins;
- Lysis of cells through pore formation.

The central protein in the complement system is complement component 3 (C3). Activation of C3 can be achieved by any of three activation pathways: the classical pathway (CP), the alternative pathway (AP) and the mannan-binding lectin pathway (LP). At the point of C3 activation, these pathways converge and are able to trigger the terminal pathway, which may result in cell lysis.

It has been shown that the complement system is ancient in origin and by far precedes the adaptive immune system. The major components of adaptive immunity have been found only in the jawed vertebrates, such as sharks, and in higher vertebrates (39). Components of the complement system have been found as early as in jawless deuterostomes, such as sea urchins and ascidians (40, 41). No complement genes, however, have been found in the genomes of Drosophila or Caenorhabditis elegans (42, 43).
Complement Component 3

C3 is the central and most abundant protein of the complement system. This 185-kDa protein is present in plasma at a concentration of 1 mg/ml, but since it is an acute-phase protein, levels can be elevated during inflammation (44). C3 consists of two chains, an alpha chain (110 kDa) and a beta chain (75 kDa), that are connected by a disulfide bond (45). The main synthesis of C3 takes place in the liver, but multiple sources outside the liver are also known (46-48). This multifunctional protein or fragments thereof is known to interact with a number of different proteins (49). These proteins are complement receptors, complement components and regulators of complement activation (RCA).

C3 belongs to the alpha2-macroglobulin superfamily, which also includes alpha2-macroglobulin, C4 and C5. All of these proteins except C5 contain a unique concealed thioester bond between a cysteine and a glutamic acid residue (50). In native C3 the thioester bond is protected, but upon proteolytic activation by a C3 convertase the bond is exposed through a conformational change. The half-life of this metastable thioester bond, able to bind both hydroxyl and amino groups, is in the range of microseconds in the presence of water (51). The short half-life explains why binding only occurs close to the site of activation. In fact, only a small fraction of the activated C3b will bind before it is inactivated by water.

Upon activation of C3, a 9-kDa peptide fragment (C3a) is released from the alpha chain as a result of proteolysis by the C3 convertase (52). This convertase is made available by any of the activation pathways. The remaining part of C3, C3b, is conformationally changed and acquires new activities as it becomes bound to the surface (53). It is now able to interact with both components of the alternative pathway of complement and with RCAs (49). Interaction with factor I together with RCAs leads to cleavage, accompanied by additional conformational changes. The resulting iC3b is unable to participate in complement activation but is a known ligand for complement receptors (54-57). Further interaction with factor I and RCAs results in formation of two different fragments, soluble C3c and surface-bound C3dg. Finally, only C3d may remain bound if C3g is released by other serine proteases at the inflammatory site (58).

A surface opsonized with C3b and iC3b is made attractive for phagocytic leukocytes that have receptors for activated C3, mainly neutrophils (55). C3a remains soluble and is a potent anaphylatoxin that attracts leukocytes and induces release of inflammatory mediators (59).
Figure 1. Sequential cleavage of C3 by convertases and factor I.
The Classical Pathway

The name of the classical pathway refers to the fact that this was the first pathway to be discovered. The CP consists of three different complement components: the C1 complex (C1q, C1r, C1s), C4 and C2. Activation occurs upon binding of C1 to the Fc portions of IgM or IgG already bound to an antigen. Activated C1 is able to cleave C4 into C4a and C4b. C4a is a weak anaphylatoxin, and C4b is homologous to C3b and binds to the surface close to the site of activation. C2 binds to C4b and is then cleaved by C1 into C2a and C2b. C2a remains complexed with C4b, and C2b is released. The complex C4b,C2a forms the CP C3 convertase and promotes the proteolysis of C3. Some of the C3b will bind to C4b and form a new complex (C4b,C2a,C3b), which is called the classical C5 convertase (37, 60).

The Lectin Pathway

The LP is the most recently described pathway and is known to be activated by certain carbohydrates on microbial surfaces. Mannan-binding lectin (MBL) binds to these carbohydrate motifs. Together with MBL are MBL-associated serine proteases (MASP), which are bound in a complex, by analogy to the C1 complex. This complex is also able to form the CP convertase, C4b,C2a. There are also indications of direct cleavage of C3 (38).

The Alternative Pathway

The AP was discovered after the classical pathway and was first thought of as an activation pathway alternative to the classical pathway. This pathway includes four complement components: C3, factor B, factor D and properdin (factor P). The AP is triggered either by covalently bound C3b generated by the CP and LP or by soluble C3b-like C3, also called C3(H2O) or iC3, which is formed when C3 is spontaneously hydrolyzed in the fluid phase. This hydrolysis is a very slow process, and the half-life of the thiolester in native C3 is about 230 h. Factor B binds to C3b or C3(H2O) and is cleaved by factor D. Surface-bound C3b and activated B (Bb) form the alternative pathway C3 convertase, C3b,Bb. Properdin binds to the convertase and enhances its stability; it also binds to C3b and enhances its association with factor B (61). Since C3b participates in the C3 convertase, this association provides a powerful feedback loop. C3b that is generated through the AP can
bind to the C3b of the convertase and form the C3b,Bb,C3b complex, also called the alternative C5 convertase (36, 62, 63).

The Terminal Pathway

Complement can mediate lysis of foreign microorganisms through the formation of a membrane attack complex (MAC), also known as C5b-9. The classical or alternative C5 convertase cleaves C5 into C5a and C5b. C5b forms a complex with C6, and when C7 is bound to the complex, a metastable binding site is exposed, making membrane insertion possible. C8 and multiple copies of C9 are then sequentially associated with the complex to form a pore that is able to mediate lysis of the cell (64). Not all membrane attack complexes are inserted into the cell membrane: Sublytic concentrations of the MAC are known to stimulate various cells, including platelets (65).

Complement Regulation

Regulation of complement is needed to avoid uncontrolled activation of complement on host cells. This control is exerted at the levels of C1 activation, C3 convertase formation and, finally, MAC complex formation (35). A common motif in regulators of complement regulation (RCA) is the short consensus repeat (SCR). One such repeat contains about 60 amino acid residues, with the structure being stabilized by two disulfide bridges (66, 67).

C1 activation is controlled by C1 inhibitor, C1INH. It circulates in complex with C1 in the blood, and a deficiency of this regulator results in hereditary angioedema (HAE) (68).

Regulation of convertase formation is mediated by two different mechanisms: Surface-bound C3b and C4b can be inactivated by proteolytic cleavage, with their inactivated forms being unable to participate in the convertase complex. This cleavage is mediated by factor I and requires one of several cofactors (69). Some cofactors are able to participate in proteolytic inactivation of both C3b and C4b; others are able to act as a cofactor for only a specific substrate. In addition, proteins with decay accelerating activity dissociate the convertase or even prevent formation of the complex. This is achieved in a competitive manner, with the C3b or C4b being prevented from binding its ligand (35).

Finally, the control of MAC formation is mediated by the membrane-bound CD59. Through interference with the C5b-8 complex, C9 is prevented from binding in the polymeric fashion that is necessary to form the pore that
leads to cell lysis. CD59 is a GPI-anchored protein, and individuals unable to form GPI anchors have a high degree of hemolysis (35). This disorder is called paroxysmal nocturnal hemoglobinuria (PNH) and involves the lack of about 20 membrane-bound proteins, but the hemolysis seems to be caused by the lack of CD59 and DAF (70).

Membrane-bound regulators are, of course, present only on host cells, and soluble regulators have a much higher affinity for autologous cells than for microorganisms. This is one explanation for the ability of complement to distinguish between self and non-self. Soluble factor H, in particular, has been shown to be important for this distinction. There are, however, examples of microorganisms that have developed affinity for RCAs as a means of escaping complement (71).

<table>
<thead>
<tr>
<th>Regulator</th>
<th>Soluble or membrane bound</th>
<th>SCR</th>
<th>Decay activity</th>
<th>Cofactor activity</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td></td>
<td>AP</td>
<td>CP</td>
</tr>
<tr>
<td>C1INH</td>
<td>soluble</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAF</td>
<td>membrane</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MCP</td>
<td>membrane</td>
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<td>-</td>
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<tr>
<td>CR1</td>
<td>membrane</td>
<td>Yes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Factor H</td>
<td>soluble</td>
<td>Yes</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>C4bp</td>
<td>soluble</td>
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<td>-</td>
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</tr>
<tr>
<td>CD59</td>
<td>membrane</td>
<td>No</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Factor H

This RCA with a molecular weight of 155 kDa is present in plasma at 0.2-0.6 mg/ml. Its importance in the complement system may be indicated by the fact that it is the second most common complement protein. The protein consists of twenty SCR s linked together as a string of pearls with the dimensions 495x34Å (72). Factor H acts as a cofactor for C3b cleavage and also possesses decay accelerating activity. The N-terminal part of factor H is responsible for both of these activities (73).

The higher affinity of factor H for autologous cells is one of the most important factors responsible for complement discrimination between self and non-self (71). This ability has been assigned to regions of the protein that are distinct from the N-terminal portion, which is responsible for cofactor and decay accelerating activity. These regions contain sites with affinity for heparin and sialic acid, motifs that are found on endothelial cells.
Some pathogens are able to escape complement by expressing receptors for complement regulators on their surface, thereby mimicking an autologous surface (75).

Plasma contains several proteins that are related to factor H. Reconectin, or factor H-like protein 1 (FHL-1), contains the seven most N-terminal SCRs of factor H and is able to act as an RCA. There are also a group of proteins called factor H-related proteins (FHR) that are unable to act as RCAs and are expressed by individual genes, in contrast to FHL-1, which is an alternatively spliced product of the factor H gene (76).

Figure 3. Factor H with 20 SCR domains. The lines below indicate regions responsible for different activities of the protein.

Complement receptors

There are several different types of receptors for complement molecules. These are typically receptors for particular fragments of bound C3, anaphylatoxins or C1q.

The receptors for C3 fragments are named complement receptor (CR) 1-4. They have specificity for different fragments of C3 and are expressed on different cell types. These differences allow for the proper response to be mediated at a specific moment of complement activation.

Receptors for the anaphylatoxins C5a, C4a and C3a mediate both chemotaxis and activation of cells. The C5a receptor (C5aR) is the most thoroughly investigated, since C5a is the most potent complement anaphylatoxin (77).

There are also receptors for C1q. Two different types of C1q receptors are found in complex on the cell surface. They are directed against the globular and the collagen domains of C1q and are called globular C1q receptor (gC1qR) and collagen C1q receptor (cC1qR), respectively (78).
<table>
<thead>
<tr>
<th>Receptor</th>
<th>Receptor type</th>
<th>Ligand</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR1</td>
<td>CD35, RCA</td>
<td>C3b&gt;C4b&gt;iC3b</td>
<td>monocytes, neutrophils, B and T cells, erythrocytes, eosinophils and FDCs</td>
</tr>
<tr>
<td>CR2</td>
<td>CD21, RCA</td>
<td>C3d/C3dg&gt;iC3b</td>
<td>B-cells, FDCs</td>
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<tr>
<td>CR3</td>
<td>CD11b/CD18, Integrin</td>
<td>iC3b</td>
<td>monocytes, neutrophils, and NK cells</td>
</tr>
<tr>
<td>CR4</td>
<td>CD11c/CD18, Integrin</td>
<td>iC3b</td>
<td>monocytes, neutrophils, and NK cells</td>
</tr>
<tr>
<td>C3aR</td>
<td>G protein coupled receptor</td>
<td>C3a</td>
<td>Neutrophils, eosinophils, ECs</td>
</tr>
<tr>
<td>C5aR</td>
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<td>C5a</td>
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<tr>
<td>gC1qR</td>
<td>Collagen-like</td>
<td>gC1q, HMWK, fXII, fibrinogen</td>
<td>B and T cells, neutrophils, fibroblasts, platelets, ECs</td>
</tr>
<tr>
<td>eC1qR</td>
<td>-</td>
<td>eC1q</td>
<td>B and T cells, neutrophils, fibroblasts, platelets, ECs</td>
</tr>
</tbody>
</table>

**Monitoring Complement Activation**

Complement activity and activation can be measured in a number of ways (79). Activity refers to the presence of a functional complement system in the blood, and activation refers to the traces of a previous event of complement activation.

The activity of complement in blood is determined by using hemolytic assays specific for the alternative or the classical pathway. It is also possible to measure the levels of various complement components in blood.

As an indication of complement activation, the generation of activation products in the blood plasma is important. The anaphylatoxins C3a, C4a and C5a are such products and are therefore obvious candidates. C5a is the most potent anaphylatoxin, 20 times stronger than C3a and 2500 times stronger than C4a. Still, C5a is not a good indicator of activation, since it is rapidly bound to the C5aR. In contrast, the level of C3a is often assessed as an indicator of total complement activation using enzyme immunoassay (EIA) techniques (80). In addition, the formation of C4a can be used as an indicator of CP activation. To measure AP activation, the liberated Bα fragment of factor B is a common choice. An alternative approach to assessing total complement activation is to measure the level of the soluble MAC, sC5b-9 (81-83). This assay is also an indirect way of following the formation of C5a.
Therapeutic Complement Inhibitors

For many years there has been considerable interest in developing complement inhibitors for therapeutic treatment of complement activation. At this point, some complement inhibitors have been used clinically, but it has only been during the last decade that major efforts have been made. Strategies for inhibiting complement activation can be divided into several categories:

The most obvious strategy is to use physiological complement inhibitors already present in the blood. C1INH purified from plasma has been used for the treatment of HAE (68). There are also examples of other applications, such as myocardial infarctions and reperfusion injuries (84). C1INH is a serine protease inhibitor (serpin) that acts by covalently binding to the active site of the protease (85).

Other natural inhibitors of complement have been tested as recombinant proteins. The first to be produced was soluble CR1, sCR1, which is able to inhibit both the alternative and classical convertases (86). In several studies sCR1 has been shown to be effective in preventing injuries caused by reperfusion, asthma and biomaterial-induced injuries (55, 87, 88). Recombinant and soluble forms of DAF and MCP have also been used to inhibit complement-induced injuries (89, 90). Both proteins were shown to be active, but since they inhibit complement by different mechanisms, a MCP-DAF hybrid was shown to be more potent (91). Also, a soluble form of CD59 has been shown to work in vitro (92).

Another strategy for stopping complement activation is to simply block the proteins to be activated using antibodies(93). To be of clinical use these antibodies need to be humanized, and the Fc portion needs to be removed or shown not to activate complement (94). The most obvious targets to block are C3 or C5, and there are examples of antibodies against both of these proteins. Today a mAb against C5 developed by Alexion Pharmaceuticals Inc. (Cheshire, CT, USA) might be the most promising complement inhibitor in clinical trials (95). Many other complement proteins has been tested as targets, including factor D and properdin. An antibody against properdin has been shown to be efficient both in vivo and in vitro (96).

Production of therapeutics based on proteins is, however, both expensive and difficult. For this reason there is growing interest in developing small-molecule inhibitors of complement. This group can be further divided based on the mechanism of complement inhibition. There are anaphylatoxin receptor antagonists, serine protease inhibitors and blocking molecules (97). Today there are C5aR antagonists shown to be working in vivo, but there is no C3aR antagonist (98, 99). There are also some serine protease inhibitors, many directed against factor D. Factor D is a good target because of its low
concentration and the fact that it circulates in its active form in the blood. The problem with serine protease inhibitors, however, is that with these agents it is difficult to achieve the specificity that is needed (97). The small-molecule C3-blocking peptide compstatin has been shown to be efficient both in vivo and in vitro.

Compstatin is a 13-residue cyclic peptide that was originally selected using a phage-displayed random peptide library (100). Since originally described, this peptide has been modified to improve its stability and binding (101). Further studies have revealed that compstatin binds C3 and inhibits cleavage by the C3 convertase. Compstatin has been shown to be efficient in several studies. In an ex vivo model of xenograft rejection, survival of transplanted kidneys was significantly prolonged (102, 103). In a model of extracorporeal circulation, generation of C3a and sC5b-9 and binding of C3 fragments to the surface were inhibited (104). Also, complement activation caused by heparin-protamine complexes was blocked in baboons (105). It has also been shown that compstatin at low levels preferentially inhibits the alternative pathway of complement (104).
Blood-Biomaterial Interactions

Contact with blood is the initial event that occurs in extracorporeal circulation and when most implants are introduced into the body. Therefore, blood-biomaterial contact strongly influences the success of all types of biomaterials. This interaction is very complex, involving both proteins and cells.

Adsorption of plasma proteins

It must be mentioned that before protein adsorption there is a very short time period during which ions and water molecules of the plasma are becoming ordered at the surface. This event is, however, immediately followed by the adsorption of proteins.

Even adsorption of a single protein can be a complex situation involving initial interaction with the surface, binding and conformational change in the protein at the surface (106). When a mix of protein is adsorbed to the surface there is an additional level of complexity. The initial composition on the surface depends on the concentration and size of the proteins involved. Following this initial binding there is a period of time in which an exchange of proteins takes place to adapt the composition on the surface to the actual affinity of the proteins. Larger proteins most often have a higher affinity than do smaller proteins that bind more rapidly. This process has been termed the Vroman effect (107, 108). In plasma there are also other biomolecules such as lipids and carbohydrates to take into account.

Conformations of adsorbed C3

As mentioned previously, C3 changes its conformation when it is activated in sequential steps by proteolysis (53). These conformational changes are accompanied by changes in both the function of the protein and its recognition by receptors (49). A similar change in conformation can be achieved by denaturing soluble C3 in 3 mM SDS (109). Native C3 expresses both unique native (N) epitopes and stable (S) epitopes. Denatured C3
expresses both unique epitopes that are specific for denatured (D) C3 and also C3(S) epitopes. Upon denaturation the C3(N) epitopes are interchanged with C3(D) neoepitopes (110, 111).

There is a considerable epitope similarity between denatured soluble C3 and physiologically bound C3b. Bound C3b expresses C3(D), C3(N) and C3(S) epitopes, while SDS-denatured C3b expresses only C3(D) and C3(S) epitopes. Thus, C3b apparently undergoes a considerable conformational change upon activation and binding to a physiological surface (53, 112, 113).

What is more interesting from the perspective of biomaterials is the fact that C3(D) epitopes also become exposed upon adsorption of both C3b and C3 to the plastic surface of a microtiter plate (114). This fact has led to the suggestion that adsorbed C3 might be able to participate in an AP C3 convertase (114, 115).

Cascade Systems of Blood and Biomaterials

In addition to passive adsorption of proteins from plasma, there is also active binding of proteins to the surface by the cascade systems of blood. The coagulation and complement systems are both activated by surfaces (17, 55). The coagulation system is propagated in the fluid phase and on cell surfaces, both in blood and bound to the surface. Complement is both activated and propagated on the biomaterial surface (33).

Adsorption of whole blood

Blood cells express adhesion molecules and receptors on their cell surfaces. The repertoire of receptors differs among the various cell types and is also dependent on the state of activation of the particular cell. Both adsorbed proteins and proteins actively bound by the cascade systems are able to act as ligands for these receptors on the biomaterial surface (55, 116). Some proteins can act as ligands for these receptors in both in solution and when adsorbed, but there are also proteins that change their conformation upon binding and thereby increase their affinity for a particular receptor.

Platelets are activated by binding to a surface and will promote further activation of the coagulation cascade, both by the release of substances that promote platelet activation and by binding of further platelets (117, 118). Also, the activated platelet surface itself is pro-coagulatory. Platelets bind fibrinogen already bound to the surface, a reaction that is mainly mediated
by the GPIIb/IIIa receptor (CD41/61) (116). The presence of C1qR on platelets indicates the possibility of activation via C1q or fXII (119).

Polymorphonuclear leukocytes (PMNs) and monocytes bind via complement receptors and release inflammatory mediators induced by frustrated phagocytosis. The main receptor responsible for this binding is CR3, which binds iC3b as well as ICAM-1 and fibrinogen (30, 120). When complement is blocked by sCR1 in a model of cardiopulmonary bypass, binding of both PMNs and monocytes is inhibited. Platelet binding, however, remains unaffected (55).
Complement Activation on the Biomaterial Surface

Complement activation in vitro

At present, it is not understood how complement activation is triggered on the biomaterial surface. Some common explanations are adsorption of initiating proteins, a lack of complement regulators on the surface or the C3 tick-over process. Absence of complement regulators would explain the lack of control on the amplification loop rather than the actual initiation.

Activation of complement is initiated at certain sites at the surface. Deposition of C3 then spreads peripherally from this nucleus of activation (33). On surfaces pre-coated with IgG it has been shown that this C3 deposition covers the surface and eventually masks the expression of IgG and C1q (121, 122). Even if an IgG-coated surface is an extreme complement activator, this deposition is likely also to take place on a non-coated surface.

The general view of complement activation is that biomaterial surfaces activate the AP (33, 123). This is supposed to be initiated by the tick-over process in the fluid phase (124-126). There is, however, a strong report showing that the lack of C4 will delay the start of activation for 15-25 minutes upon contact with cuprophane membranes, suggesting the importance of CP in biomaterial-induced complement activation (4).

Different biomaterial surfaces possess different complement-activating properties. Physical properties such as hydrophobicity are important, and it has been shown that hydrophobic surfaces are more complement-activating (127), perhaps because of the conformation of adsorbed C3 on the surface (114). Also, the presence of different chemical groups on the surface is important (128). Hydroxyl and amino groups are known to bind activated C3. Recent studies, however, have shown that the surface is covered with a layer of protein and that C3 is actually bound to this layer (34). The chemical groups of the biomaterial surface are then covered and the importance of the
surface is rather to govern the composition and conformation of adsorbed proteins.

Complement activation upon blood-biomaterial contact in vivo

In the clinic, blood-biomaterial contact takes place in many different situations and involves many different types of materials (2). There is also a great diversity in the amount of surface area being exposed: areas from some square centimeters to areas up to several square meters. The smaller areas include stents and artificial heart valves (129-133). These devices are not known to cause any major complement activation. The risk is rather that these surfaces are able to act as initiation sites for thrombus formation (8). Local complement activation could, however, have a significant effect on the reactions of neighboring cells.

Extracorporeal circulation

The greatest challenge in the field of biomaterials is presented by extracorporeal circulation, when blood is circulated outside the body (134). Examples of extracorporeal circulation are cardiopulmonary bypass (CPB), membrane oxygenation and hemodialysis (134, 135). All of these applications involve the use of membranes to achieve gas exchange or removal of low molecular weight substances from the blood. To achieve optimal exchange, the area of these membranes can be at least two square meters. Although these procedures are usually accompanied by surgical trauma or a disease state, it has been shown that the complement-mediated tissue injury is to a great extent a consequence of bioincompatibility reactions toward the biomaterials used (136, 137).

Hemodialysis

Historically, hemodialysis has been associated with severe, even fatal, anaphylactic reactions (138). These events are less frequent today but can still take place. A concern in uremic patients undergoing maintained hemodialysis is accelerated (139, 140). The risk of myocardial infarction in these patients is 5-10 times higher than in healthy individuals (141). Many factors contribute to this increased risk: The uremic condition itself leads to disturbed lipoprotein levels, acidosis, toxins, free radicals and infections, all of which could contribute to accelerated arteriosclerosis (142, 143). Chronic inflammation, such as that found in patients with rheumatoid arthritis, systemic lupus erythematosus (SLE) and chronic rejections, has been shown
to be associated with accelerated arteriosclerosis and cardiovascular disease (144). Thus, it is likely that the chronic whole body inflammation triggered by hemodialysis is another factor contributing to accelerated arteriosclerosis in uremic patients.

Dialysis membranes activate complement to varying degrees (137). Cellulosic membranes are known to be strong activators, while polymeric membranes are considered to be less active in activating complement. This activation causes generation of membrane-bound C3 fragments (C3b, iC3b and C3dg) as well as soluble peptides (C3a and C5a) (145, 146). All of these fragments are ligands for leukocyte receptors and can trigger inflammation and release of cytokines. Complement activation also triggers up-regulation of cell-surface receptors on leukocytes (e.g., CD11b/CD18 and CD35), combined with down-regulation of L-selectin, making the cells more sticky and prone to interact with platelets and endothelial cells (104, 147). Also, this cellular activation leads to increased levels of acute-phase proteins such as CRP, allowing these molecules to bind endothelial cells and activate complement. CRP and complement components C3, C4 and C9 are present in atherosclerotic plaques, together with monocytes and macrophages (148, 149).

**Cardiopulmonary Bypass**

CPB is associated with side effects triggered by a number of events, including surgical trauma, blood-biomaterial contact, blood-gas contact, neutralization of heparin with protamine and also ischemia/reperfusion in the tissue disconnected during the procedure (heart and lungs) (150-152). The incidence of stroke during or after CPB is 1-4% (153). Another neurological problem often associated with CPB is prolonged cognitive dysfunction (154). Many of these problems are apparently caused by complement activation, a conclusion supported by in vivo studies using anti-C5 antibodies. Complement inhibition achieved by blocking C5 activation attenuates postoperative myocardial infarction, cognitive deficits and blood loss. There are several causes of complement activation during CPB. In particular, contact with both the biomaterial and the gas surface is known to activate complement. Complement is also activated by platelets in clotted blood, and a final activation occurs when soluble heparin is neutralized with protamine (155-157). This complement activation affects both leukocytes and platelets. In an in vitro study it has been shown that anti-C5 antibodies block both P-selectin expression by platelets and the formation of platelet/leukocyte complexes during extracorporeal circulation (158). Complement activation products, in particular C5a, are known to up-regulate receptor expression on both PMNs and monocytes (159). Other signs of an inflammatory response are the release of cytokines and chemokines (160).
Heparin surfaces in vivo

The most widely used surface modification of biomaterial surfaces in vivo is the heparin surface. In vitro this surface has been shown to be effective in reducing both complement and coagulation activation (16, 17, 161, 162). The most common application of heparin surfaces is extracorporeal circulation and in particular CPB (163). In many cases improved performance can be shown, but there are also reports from investigators failing to observe any benefit (164-167). This further underlines the need of using a well characterized heparin surface.

There are a number of reports showing reduced complement activation in CPB when using heparinized equipment. This is also accompanied by a decreased activation of granulocytes. When examining the mechanisms behind down-regulation of complement during CPB using immobilized heparin an increase in C1rs-C1INH complexes was observed. This increase presumably reflects the fact that heparin binds C1q. Formation of C3bc and sC5b-9 was, however, significantly lowered when a heparinized system was used (165).
Aims of the Investigation

This study was initiated to investigate the mechanisms of complement activation triggered by biomaterial surfaces and to develop techniques to specifically down-regulate complement activation at the surface. The AP is generally considered to be responsible for most of the complement activation that occurs at the biomaterial surface; in this project we wanted to investigate if this is indeed the case or whether the AP is a reflection of complement amplification.

The aim of paper I was to investigate whether complement activation occurs at the biomaterial surface or on top of the adsorbed protein layer. We also wanted to identify which mechanism initially triggers complement activation on a biomaterial surface.

Paper II was initiated to test whether adsorbed C3 was able to participate in an AP C3 convertase. This possibility had been suggested in previous reports, but no conclusive evidence had been presented.

The aim of paper III was to determine whether factor H can be immobilized to a biomaterial surface with retained activity.

The aim of the last paper was to test the influence of heparin surface concentration on both complement and coagulation parameters. It was also designed to test the influence of blood flow velocity.
Materials and Methods

The techniques in this work are mainly based on the use of enzyme immunoassays (EIAs) and quartz crystal microbalance with dissipation monitoring (QCM-D). In paper III a method to immobilize factor H to the surface was developed.

Enzyme Immunoassays

β-Thromboglobulin

β-Thromboglobulin (β-TG) was measured using the commercial Asserachrome™ β-TG EIA kit from Diagnostica Stago, Asnieres-sur-Seine, France. β-TG was captured in wells coated with specific rabbit anti-human β-TG F(ab’)2 fragments. Peroxidase-coupled rabbit anti-human β-TG antibody was used for detection. The values are given as IU/ml.

Thrombin-Antithrombin complexes

Thrombin-Antithrombin (TAT) complexes were measured using a commercial Enzygnost® TAT micro EIA kit from Behringwerke AG, Warburg, Germany. TAT was captured in wells coated with rabbit anti-human thrombin. Peroxidase coupled rabbit anti-human Antithrombin antibody was used for detection. The values are given as µg/ml.

Soluble C5b-9 complexes

Soluble C5b-9 (sC5b-9) complexes were measured using a modification of the previously described method by Mollnes et al (82). Plasma samples diluted 1/5 were added to wells coated with anti-neoC9 mAB McaE11 (81). Polyclonal anti-C5 antibodies (Dako, Glostrup, Denmark) diluted 1/500 were used for detection. Zymosan-activated serum, defined as containing 40,000 arbitrary units per ml (AU/ml), was used as a standard.
C3a

C3a was measured as follows: Plasma samples diluted 1/1000 were incubated in wells coated with mAb 4SD17.3, which served as capture antibody. C3a was detected using a biotinylated anti-C3a followed by horseradish peroxidase conjugated streptavidin. Zymosan-activated serum calibrated against a solution of purified C3a served as the standard, and the values are given in ng/ml (80).

Quartz Crystal Microbalance with Dissipation Monitoring

To measure the complement activation that takes place on a surface in real-time, a QCM-D instrument from Q-sense AB, Gothenburg, was used. Purified components of AP were used to assemble a C3 convertase. The ability of this convertase to perform proteolytic activation of C3 was monitored by the binding of C3b to the surface. 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 (ref), \( \Delta f = -n \Delta m C^{-1} \), where \( C \) (equivalent to 17.7 ng cm\(^{-2}\) Hz\(^{-1}\)) is the mass sensitivity constant and \( n (=1,3\ldots) \) is the overtone number (168). 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 (169). 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 (170-172).

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) instrument (Q-Sense AB, Gothenburg, Sweden), which is described in detail elsewhere (173). The volume of the chamber is 80 μl, and when the liquid in the chamber is exchanged, 0.5 ml is added from a temperature loop; the excess volume is allowed to overflow. Sensor crystals (5-MHz), spin-coated with hydrophobic polystyrene or sputtered with steel, were used. Changes in \( D \)
and \( f \) were measured on both the fundamental frequency (\( n=1 \), i.e. \( f \approx 5\text{MHz} \)) and the third (\( n=3 \), i.e. \( f \approx 15\text{MHz} \)) and fifth harmonic (\( n=5 \), i.e. \( f \approx 25\text{MHz} \)). Data from the measurements at the third harmonic are presented. All measurements were carried out at 25°C.

**Factor H Immobilization**

To immobilize factor H on a surface, the surface was first coated with a polymer supplying primary amino groups. A heterobifunctional crosslinker, N-succinimidyl 3-(2-pyridoldithio)propionate (SPDP), able to bind to the amino groups was then added (174). SPDP has a molecular size of 312 Da and is 6.8 Å long. In its unreduced state it can bind to thiol groups; if the bound SPDP is reduced, it provides thiol groups to the surface or the protein. Factor H was then able to bind to thiol groups on the surface supplied by SPDP.

**The Corline Heparin Surface**

In this study the heparin surface from Corline Systems AB was used. The Corline Heparin Surface was prepared by a conditioning layer of polymeric amine onto which a macromolecular conjugate of heparin was attached. The conjugate was prepared by covalent binding of approximately 70 moles heparin/mole carrier chain (21). This procedure results in a surface with a heparin surface concentration of 0.5 g/cm² and an AT binding capacity of 2-4 pmol AT/cm². Higher levels of heparin surface concentration and AT binding capacity were achieved by repeating the application of polymeric amine and heparin conjugate up to three times.

**The Chandler Loop Model**

In the Chandler loop model, extracorporeal circulation is mimicked using tubing loops. Fresh whole blood was drawn from healthy volunteers who had received no medication for 10 days. The blood was used with or without the addition of anticoagulant, depending on the material being tested. Pieces of PVC tubing furnished with immobilized heparin were used in the loops. Tubing with a total volume of 6.3 mL (inner diameter= 4 mm, length=500 mm) was filled with 4.5 mL of fresh whole blood, leaving a gas volume of 1.8 mL. The tubing was made into closed circuits using surface-heparinized connectors of thin-walled steel (length=20 mm). The ends of the connector
were tightly pushed into the lumen of the tubing and secured using copper seals. The tubing was then rotated vertically at desired speed, typically 33 rpm, in a 37°C water bath for the desired time. After incubation, the samples were collected in citrate or in EDTA, respectively, and centrifuged at 3,300xg for 25 min.
Experiments and Results

Four different studies have been carried out. In the first study, ways to identify the initiating pathway behind complement activation on biomaterial surfaces was explored. The second study investigated the mechanisms of alternative pathway activation on biomaterial surfaces. The two following studies examined the possibility of specifically down-regulating complement activation by conjugating two active biomolecules, factor H and heparin, to a biomaterial surface.

Paper I

In the first paper the question to be answered was whether complement activation takes place on the biomaterial surface or on the adsorbed protein layer. Also, the mechanism responsible for initiating complement activation triggered by the model biomaterial surface, polystyrene, was investigated. The initial protein layer on the polystyrene was estimated by QCM-D to measure approximately 8 nm. Complement activation following the adsorption of the protein film added 25% more mass to the surface than when complement activation was blocked.

AP activation was selectively blocked in serum with the complement inhibitor compstatin. In an EIA model it was shown that complement activation was immediately triggered by a self-limiting CP activation. This activation was immediately following the adsorption of the protein film.

Incubation with EGTA, which blocks the CP, indicated that AP activation began after a lag phase of approximately 5-10 min but subsequently provided the bulk of the C3b deposition onto the surface. In order to identify the types of protein film in whose presence AP amplification could take place, we assembled AP convertase complexes using purified C3 and factors B and D and monitored the assembly by QCM-D analysis. In addition to albumin, IgG, but not fibrinogen, allowed convertase assembly and amplification.

Western blotting of eluted proteins from the material surface demonstrated that the C3 fragments were bound to other proteins and that the most abundant fragment was iC3b. These results are consistent with a
model in which the main C3b binding is mediated by the AP on top of the initially adsorbed protein film. The activation is triggered by either CP- or AP-initiating convertases assembled from molecules found among the initially adsorbed proteins.

Paper II
In paper II the ability of adsorbed C3 to participate in an AP C3 convertase was investigated. The AP C3 convertase was assembled on a polystyrene surface and monitored using QCM-D. Adsorbed C3b was able to bind factor B, which in turn could be activated by factor D. This convertase cleaved C3 into C3b and C3a (as assessed by EIA). These results indicated that an active conventional C3 convertase, C3b,Bb, could be assembled on the surface.

When C3 itself was adsorbed to the polystyrene surface, the bound protein was recognized by monoclonal antibodies specific for bound C3b. Binding and activation of factor B by factor D also showed that the adsorbed C3 had taken on C3b-like properties, indicating the formation of a C3-containing AP C3 convertase, C3b,Bb.

In vivo the AP convertase is stabilized by the presence of properdin. When properdin was added together with factor B a C3,Bb,P convertase was formed, showing binding of C3b to the surface upon addition of C3.

Interestingly, adsorbed C3 was not down-regulated by factors I and H. Taken together, these results showed that adsorbed C3 can form a weak initiating AP convertase. The relatively weak proteolytic activity of the convertase is compensated by the fact that the it is not down-regulated by factors I and H.

Paper III
The third study examined the ability to immobilize factor H with retained activity on a model surface. Factor H was bound to the surface with both reduced and non-reduced SPDP.

By QCM-D it was shown that a higher amount of factor H bound to the unreduced surface, but the flexibility of factor H was higher on the reduced surface, indicating a more native state.

In both whole blood and plasma the surface with factor H generated less C3a and TCC in the fluid phase, and the binding of C3/C3 fragments was abrogated. When serum was incubated on the surface without immobilized factor H, C1s-C1INH complexes were generated, but no such generation of
C1s-C1INH complexes was observed when factor H was immobilized to the surface.

**Paper IV**

In paper IV the effect of the surface concentration of heparin on both coagulation and complement was studied. Tubing was coated with increasing concentrations of heparin, which produced antithrombin binding capacities of 6, 12 and 19 pmol/cm$^2$. The tubing was filled with blood, and the ends were connected to form a loop. The tubing loops were allowed to rotate for 1h at three different velocities (13, 28 and 43 cm/s).

When the binding capacity for antithrombin was raised from 6 pmol/cm$^2$ to 12 pmol/cm$^2$ at 28 cm/s, the generation of TAT and the release of ß-TG decreased, reflecting the fact that both coagulation and platelet activation were inhibited. The complement parameters C3a and TCC decreased when the antithrombin binding capacity was raised from 6 pmol/cm$^2$ to 12 pmol/cm$^2$. A further increase to 19 pmol/cm$^2$ did not result in any further improvement regarding either coagulation or complement. The decreased activation of coagulation activation could be explained by an increased antithrombin binding capacity. The factor H binding capacity did not increase in parallel with decreased complement activation, and the increased complement compatibility remains to be explained.

Interestingly, there was an increased generation of C3a when the flow rate was raised. TCC showed no significant increase, and TAT was lower at 13 cm/s than at the two higher velocities.

In summary, by increasing the surface concentration of heparin, blood compatibility can be improved. An increased concentration might also be necessary when the flow rate is increased, since the complement activation seems to be dependent on the flow rate of the blood.
Discussion

Contact between blood and biomaterial is known to trigger activation of the complement system (175). There are many possible explanations for this activation, but there is at present no extensive theory on the matter. One explanation for this phenomenon is that complement activation triggered by biomaterials has been considered to be of AP nature only (33, 123). This could lead to the conclusion that the “tick-over” process in the fluid phase is responsible for the initiation of complement activation and that the degree of activation depends only upon the amount of binding sites on the surface. There are also numerous publications suggesting that the complement-activating properties are dependent upon the quantities of hydroxyl and amino groups, which are known to be acceptors for covalent binding of C3b, on the surface (128).

The fact is, however, that upon contact with blood, the biomaterial surface is rapidly coated with adsorbed plasma proteins. Most of the chemically reactive groups on the surface will be hidden beneath this layer of proteins. The biomaterial surface determines the composition of the protein layer and also the conformation of the adsorbed proteins. This situation indicates a more indirect role of the biomaterial surface in complement activation.

In paper I, complement activation on the polystyrene surface was shown to be initiated by the CP. This finding was also confirmed in paper III, in which a generation of C1s-C1INH complexes was shown in serum incubated on polystyrene. This complex is formed when there is activation of the CP, suggesting that the polystyrene surface possess CP-activating properties. Further support for an important role for the CP has come from a report showing a 15-min delay in the initiation of complement activation when serum lacking C4 is incubated on cuprohane (4).

If the AP is blocked, there is a rapid activation that is, however, unable to be amplified. On adsorbed albumin there is no such rapid activation. Instead there is a slow complement activation that does not take place until about 10 min after serum exposure. Blocking the alternative pathway totally inhibits complement activation, indicating that the initiation of complement activation is triggered by either the CP or the AP, depending on the surface in question. The following amplification of complement initiation is,
however, driven by the AP. This also explains why complement activation triggered by biomaterials has been considered to be of AP origin. When complement is measured in the fluid phase, AP markers predominate.

The triggering mechanism behind the initiation of complement activation is probably related to conformational changes occurring in adsorbed proteins that produce a more activating state. CP activation could be triggered by adsorbed IgG, IgM, C1 or even C4. When considering AP-induced initiation from the generally accepted point of view, the most likely explanation would be the tick-over process. This view implies that the initiation takes place in the fluid phase through a slow conversion of C3 into iC3, a more C3b-like state that is able to participate in a fluid-phase AP C3 convertase(124-126). The activation would then be amplified on the biomaterial surface. Paper II presents evidence that an alternative explanation is possible. These experiments have indicated that adsorbed C3, as previously suggested, is able to initiate AP activation (114, 115).

We have shown that adsorption of C3 also converts it into a C3b-like molecule that is able to participate in a C3,Bb,P convertase. The possibility that adsorption is the actual tick-over mechanism cannot be ruled out, since there is always a surface present. The most important feature of this convertase is that it does not seem to be regulated by factors I and H. This is a strong argument for the importance of this convertase, even though it has a much lower activity than does the C3b,Bb,P convertase. If there is a lack of regulation, even a low activity might be able to initiate complement activation.

The QCM-D technique has been shown to be suitable for monitoring the assembly of AP convertases of purified complement components in real time. Although its sensitivity was lower than that of surface plasmon resonance (SPR), it was still high enough to allow us to measure the weak interactions of the C3,Bb,P complex. In the case of QCM-D, it is also easy to modify the surfaces to be tested. Several studies of complement activation have previously been carried out using SPR!(104, 176). The design of these experiments, however, did not allow a direct comparison to make it possible to determine the best technology to test the in vitro assembly of AP components.

Factor H is a soluble inhibitor of the AP. Immobilization of functional factor H on a surface was postulated to specifically inhibit AP. The factor H surface did indeed inhibit complement activation, as indicated by lower levels of binding of C3 fragments to the surface. There was also a reduced generation of C3a and sC5b-9, and no C1s-C1INH could be detected in the fluid phase. This latter finding suggests that factor H is able to inhibit CP, a feature that had not previously been assigned to factor H. There are, however, reports that rheumatoid arthritis protein (RHP), later identified as
factor H, interacts with the globular head of C1q, a finding which supports the possibility that factor H really interferes with CP activation. Since complement activation was shown to be initiated by CP on polystyrene, this ability could be most important. Factor H could then interfere already at the level of the initiation of complement activation instead of regulating only the AP-mediated amplification.

An already available technique to inhibit both coagulation and complement activation is covalent immobilization of heparin onto a surface intended for contact with blood. This type of surface works by binding AT and accelerating its inhibitory effect on coagulation factors. Complement is inhibited by factor H, which has a high affinity for heparin. An increased surface concentration of heparin further inhibits coagulation and complement activation. The activation of complement is increased with increased flow rates, and increased heparin surface concentrations might be a way to meet these more demanding conditions. The finding that increased complement inhibition is not associated with increased binding of factor H does not mean that factor H is not of importance at heparin surfaces. Instead, only the additional compatibility might be mediated by another factor. The Corline heparin surface used in this study is known to consist of unfractionated heparin. This unmodified form still possesses all the physiological properties of heparin, which are far from completely understood.

The results of this study indicate that there are some key events taking place during complement activation at a biomaterial surface. First, plasma proteins are adsorbed to the surface, with a composition and conformation that are dependent upon the biomaterial involved. Some of the proteins are adsorbed in a conformation that triggers initiation of complement activation: IgG, IgM, C1 or C4 could trigger the CP, or on surfaces that do not bind any of these proteins in a proper conformation, C3 could activate the AP. After the initiation step, complement activation is amplified by an AP feedback loop that generates the bulk of the C3b on the surface and may also trigger the terminal pathway of complement. The result is the generation of fluid phase anaphylatoxins C3a and C5a, soluble C5b-9 complexes and surface-bound C3b and iC3b. All these products together are able to attract and activate cells and also trigger further release of inflammatory mediators from them (55, 104). This response is a good thing if it takes place at the proper site, directed against a foreign invader of the body. If instead we are dealing with a large area of a biomaterial surface, this reaction could lead to a systemic release of inflammatory mediators. If not treated properly, it could lead to systemic inflammation, indirectly causing hemostatic problems or even organ failure (177, 178).
Figure 4. Summary of the complement activation on a biomaterial surface. Exposure of blood to the surface results in an immediate adsorption of proteins that play an important role in initiation of complement activation. This results in binding of C3b to the surface and is amplified by the AP amplification loop. Formation of anaphylatoxins results in activation of leukocytes and C3 fragments on the surface acts as ligands for leukocyte binding.
Conclusions

Initiation of complement activation on a biomaterial surface can be triggered by either the CP or the AP.

When complement activation is triggered, amplification is mediated by the AP feedback loop.

Adsorbed non-activated C3 can form an initiating convertase that escapes factor H and factor I regulation. This is achieved by a conformational change in C3 upon adsorption that generates a C3b-like form.

It is possible to immobilize factor H on a polymer surface with retained biological activity.

The initial trigger of complement activation can be the CP, LP or AP, even if the major activation of complement on a biomaterial surface is mediated by the AP.

Increased surface concentrations of heparin further increase the inhibition of complement activity and coagulation. Complement activation is increased by increasing blood flow rates.

Quartz crystal microbalance with dissipation monitoring (QCM-D) is an excellent technique for monitoring complement activation on a surface in real time.
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