Assessment of complement activation in human disease
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Chapter 1

General Introduction
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The complement system
As a major effector mechanism, the complement system plays a central role in innate immunity. It consists of more than 30 plasma- and membrane bound proteins constituting a first line of defence against many pathogens (1, 2). The importance of complement is illustrated by the fact that patients with complement deficiencies show an increased predisposition for infections and auto-immune disease.

Activation of the complement system can occur via three pathways, which are initiated via separate mechanisms and eventually converge in a common terminal pathway (Fig. 1). The classical pathway of complement is activated by binding of the recognition molecule C1q to IgG- or IgM-containing immune complexes or to other structures, such as dsDNA (3, 4), amyloid beta (5) or pentraxins like SAP and CRP (8). Furthermore, it has been shown that C1q can directly bind to apoptotic cells and thereby mediate complement activation (9-11). This implicates that C1q may be involved in the clearance of apoptotic cells. The lectin pathway is initiated by binding of mannan binding lectin (MBL) or ficolins to a wide array of carbohydrate structures on bacterial or viral surfaces (12). Finally, activating structures of the alternative pathway are present on the cell walls of bacteria, viruses and yeasts, whereas IgA-containing immune complexes can also activate the alternative pathway (13, 14).

Recently, in mouse models for rheumatoid arthritis (collagen induced arthritis (CIA) model and anti-GPI serum transfer model), evidence was found for IgG mediated complement activation, that was completely dependent on the alternative pathway while bypassing the requirements for the classical pathway (15, 16).

The classical pathway
The first component of the classical pathway of complement is C1, which is a complex of three proteins, C1q, C1r and C1s. In vivo, two molecules of each of the pro-enzymes C1r and C1s, forming a C1r2C1s2 tetramer, are associated with one C1q molecule, forming the macromolecular C1 complex (17, 18). C1q is a cationic plasma glycoprotein with a molecular weight of 460 kDa. The molecule consists of six subunits, resembling a bunch of tulips (Fig. 2). The subunits are composed of an N-terminal globular head region and a C-terminal collagenous tail. Each subunit consists of 3 polypeptide chains, designated A, B and C. Thus, the complete molecule comprises 6A, 6B and 6C chains. A and B chains of each subunit are connected to each other by disulfide bonds, whereas C chains are non-covalently linked to an A-B heterodimer and disulfide bonded to a C chain of an adjacent subunit (19-21).
**General Introduction**

**Classical**
- Immune complexes (IgG, IgM)
  - CRP
  - SAP
  - DNA

**Lectin**
- Microbial polysaccharides
  - MBL/Ficolins
  - MASP1 MASP2

**Alternative**
- Microbial surfaces
  - C3(H2O)
  - C3(H2O)Bb
  - Properdin (P)

**Terminal pathway**
- C5b, C6, C7, C8, C9 (C5b-9)
  - Membrane Attack Complex (MAC)

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**Figure 1.** The complement system can be activated via three different pathways; the classical, lectin and alternative pathway that eventually converge into the terminal pathway. Activation of the terminal pathway results in formation of the lytic membrane attack complex (MAC). The complement system is tightly regulated by fluid phase and membrane bound complement regulators which are indicated in grey boxes.
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C1q links the humoral immune response to the complement system by binding to the Fc-regions of IgG- or IgM-antibodies within immune complexes, thereby activating the classical pathway of complement. Multivalent binding of the C1q globular heads to the target surface fixes the “arms” of C1q in such a way that intramolecular changes in the pro-enzyme C1r are induced, resulting in functional protease activity. Next, C1r cleaves C1s, thereby inducing its proteolytic activity (22). The natural substrates for the C1s protease are C4 and C2. Upon binding of C1s, C4 is cleaved leading to the release of the C4a anaphylatoxin and deposition of the larger C4b fragment near C1q on the pathogenic surface. Subsequently, C2 binds to C4b on the surface. When C2, attached to C4b, is cleaved by C1s, the smaller fragment C2b will be released while C2a forms a complex with C4b (C4b2a) attached to the pathogenic surface. The C4b2a complex is the classic C3 convertase on the surface of the pathogen. This C3 convertase cleaves C3, which results in release of the anaphylatoxin C3a and binding of C3b, either directly to the pathogenic surface or to the C4bC2a complex (24).

A key step in the propagation of complement activation and elimination of pathogens from the body is the covalent binding of C3 and C4 to target surfaces. C4 and C3 both contain a highly reactive thio-ester which is easily hydrolyzed by water molecules and is protected within the native molecule. Therefore these proteins circulate as inert proteins until they are proteolytically activated. Activation of C3 and C4 results in a conformational change and exposure of the internal thio-ester which may react with amino- or hydroxyl-groups to form amide or ester bonds. Only a small proportion of activated C3b and C4b will bind to the target surface that initiated complement activation. The majority of the molecules will be hydrolyzed by surrounding water molecules and remain in the fluid phase. This rapid inactivation limits the efficiency of the reactive proteins to the site of activation (23-26).

Figure 2. Schematic representation of the C1q molecule.
The lectin pathway

The lectin pathway is an antibody-independent route of complement activation on bacteria and other micro-organisms. The lectin pathway essentially uses the same molecules as the classical pathway; e.g. C4, C2 and C3, except that its recognition molecules are MBL or ficolins rather than C1q. MBL is structurally related to C1q, being a high molecular weight multimeric molecule with globular binding regions and a collagenous stalk (27-29). Both MBL and ficolins are members of the collectin family of proteins, which are characterized by the presence of both a collagen-like region and a sugar-binding C-type lectin domain. MBL can be associated with the MBL-associated serine proteases (MASPs), MASP-1 (30), MASP-2 (31), MASP-3 (32) and a truncated form of MASP-2, called Map-19 (33). MBL binds to a wide variety of carbohydrate structures such as mannose and fucose on bacterial and viral surfaces, thereby activating the complement system (12). MASP-2 appears to be the major complement-activating enzyme of this pathway. It resembles the classical pathway protease C1s in cleaving both C4 and C2, eventually leading to the formation of the C3 convertase C4b2a (34, 35).

Next to MBL, two types of human serum ficolins, L-ficolin and H-ficolin, are associated with MASPs and activate the lectin pathway in a similar manner to MBL (36, 37). A third type of human ficolin, M-ficolin, is a non-serum ficolin which is present in lungs and leukocytes (38). Ficolins may bind to patterns of acetyl-groups, either presented by carbohydrates such as GlcNAc or GalNAc, or non-carbohydrate acetylated compounds. Binding has also been observed towards other bacterial components such as lipoteichoic acid, which is a cell wall component of Gram-positive bacteria (39). Ficolins are, just as MBL and C1q, capable of binding to apoptotic cells and thereby activating complement. This has led to the idea that these recognition molecules are directly involved in the disposal of dying cells (40, 41).

The alternative pathway

The alternative pathway does not require the action of antibodies to initiate the activation cascade; instead, it can be initiated by spontaneous cleavage of complement component C3. Key to the activation of the alternative pathway is that the internal thio-ester in C3 not only serves to link activated C3b to an activator, but also to stabilize the conformation of native C3. Upon disruption of the thio-ester the conformation of C3b changes to become able to interact with factor B (fB). As indicated in a previous section, the internal thio-ester is sensitive to water molecules and is therefore “hidden” in a hydrophobic region of C3 (42-44). Yet, in time water molecules are able to penetrate some C3 molecules and to hydrolyze the thio-ester. The conformation of C3(H2O) changes to become similar to that of C3b. Hence, intact C3 with a
disrupted thio-ester has also been called “C3b-like C3” (45). Via this mechanism of spontaneous hydrolysis of native C3 small amounts of C3b-like C3 are continuously generated in the fluid phase (“tick over” of C3). This C3(H2O) binds to fB, which subsequently can be cleaved by factor D (fD). Cleavage of fB results in formation of the subunits Bb and Ba. The Ba subunit diffuses away and this results in an active alternative pathway C3 convertase (C3(H2O)Bb). C3(H2O)Bb is stabilized by binding to properdin (P), and in its turn can activate native C3 into C3b (Fig. 1). In a similar way as described above for C3(H2O), the C3bBb(P) complex is formed, which also is a C3 convertase that can cleave C3, thus forming an amplification loop resulting in more C3b deposition. Amplification of the alternative pathway is thought to take place exclusively on activating surfaces, on which bound C3b is not inactivated by complement inhibitors. In case of fixation of C3b at sites where the breakdown of C3b into C3c and C3d,g is prevented (for example on microbial surfaces), this amplification may result in extensive activation of C3. Target specificity of the alternative pathway is largely determined by the carbohydrate environment of bound C3b, which influences the outcome of the competition between fB and the major alternative pathway inhibitor factor H (fH) for binding to C3b on membranes. On activating surfaces, fB will bind to C3b, but on non-activating surfaces fH will bind which abrogates further activation (46).

C3b has affinity for IgG molecules. Two C3b molecules can be sequentially bonded to each other and to one IgG heavy chain, forming C3b2-IgG complexes. In covalent complex with IgG, C3b is partially protected from enzymatic inactivation by the fluid phase inhibitor factor I because of impaired factor H binding. Apparently, IgG provides a protected surface, which enables alternative pathway activation (47). Therefore, C3b2-IgG complexes are very efficient activators of the alternative complement pathway (48, 49).

Recently, properdin has been reported to act as recognition molecule within the alternative pathway of complement. By direct binding to microbial targets, properdin provides a platform for the formation of C3 convertases (50). Properdin is a multivalent protein composed of identical subunits (51) which may enable binding to polyvalent ligands clustered on microbial surfaces. This is similar to C1q and MBL, the recognition molecules of the classical and lectin pathway, which are also multivalent proteins that bind polyvalent ligands.

The alternative pathway is not only an activation pathway by itself, but also provides an important amplification loop for the classical and lectin pathway of complement. C3 which is activated via these pathways may initiate the alternative pathway. Amplification via this route accounts for more than 80% of initial classical or lectin pathway induced terminal complement activation (52).
The terminal pathway

The three complement activation pathways merge at the level of C3, finally resulting in the formation of the membrane attack complex (MAC) through a common terminal pathway. The terminal pathway is entered when C5 binds non-covalently to a site on C3b in the C4b2a3b complex (C5 convertase). Cleavage of C5 occurs by the C2a fragment and results in C5a and C5b. C5a is the most powerful anaphylatoxin of the complement system, attracting leukocytes expressing a C5a-receptor (e.g. neutrophils and mast cells). C5b contains a labile hydrophobic surface binding site and a binding site for C6. In contrast to the initiation pathways, in the terminal pathway none of the components is enzymatically cleaved. Binding of each following component in this route is achieved by conformational changes in the acceptor molecule. A complex of components C5b, C6, C7, and C8 mediates the polymerization of up to eighteen C9 molecules into a tube-like pore that is inserted into the plasma membrane of unwanted organisms, such as gram-negative bacteria and virally infected cells. This channel through the lipid bilayer is called membrane attack complex (MAC) and finally results in osmotic lysis of the target cell.

Effector mechanisms of complement

Biological activity of the complement system is not restricted to MAC formation and subsequent cell-lysis. An important action of complement is to facilitate phagocytosis and destruction of pathogens by phagocytes. Pathogens may be opsonized with complement components (C4b, C3b) that are recognized by so called complement receptors on phagocytic cells (CR1, CR2, CR3). Complement receptors are also important for the clearance of immune complexes from the circulation. For example, CR1 present on erythrocytes binds to immune complexes opsonized with the activated complement components C3b and C4b and transports them to the liver and spleen where they are removed by macrophages (53).

Activation of complement also contributes to inflammation by the release of anaphylatoxins. C5a and, to a lesser extent, C3a trigger release of histamine and other mediators upon binding to specific receptors on mast cells or basophils, resulting in vasodilatation. C5a, a potent chemoattractant, recruits neutrophils, monocytes, eosinophils and mast cells to the site of inflammation (54).

More recently, it has been established that complement collaborates with the adaptive immune system by augmenting the humoral response to antigens and thereby lowering the threshold for B cell activation. In mice, CR2 forms a co-receptor complex with CD19 and CD81. Co-ligation of this complex with the B-cell receptor (BCR) occurs when a C3d-opsonized antigen binds via the
antigenic part to the specific BCR and via C3d to CR2 in the co-receptor complex. This results in enhanced B cell responses where lower amounts of antigen are required for proper B cell activation (55, 56).

**Complement regulation**

To control the process of complement activation, complement regulatory proteins are present in plasma and on host cell membranes. Membrane-bound complement regulators are expressed on most host cells to prevent innocent bystander killing by complement activation in the neighbourhood of these cells. The complement system is tightly regulated to prevent excessive activation on a single target, fluid phase activation and activation on self-molecules and cells. Foreign surfaces lacking control proteins are attacked by complement, while host cells are protected. Deficiencies of control proteins may lead to excessive complement activation and disease.

The initiation step of the classical pathway is inhibited by the soluble regulator C1-inhibitor (C1-inh). C1-inh blocks the active site of C1r and C1s and dissociates them from C1q. Hereby, fluid phase C1 activation and excessive activation on a target are prevented (57). C1-inh is also able to inhibit the action of MASP-2, the complement activating protease of the lectin pathway (58).

At the level of C3 convertases, several inhibitors (both fluid phase and membrane bound) are known. The fluid phase serine protease factor I (fI) requires a cofactor for proteolytic degradation of C3b and C4b (59). Soluble cofactors for fI are the regulators factor H (fH) and C4 binding protein (C4bp). Membrane bound fI cofactors are the regulators CR1 (CD35) (60) and membrane cofactor protein (MCP, CD46) (61). Proteolytic cleavage of C3b into C3c and C3d,g by factor I prevents C3 convertase formation and decreases its affinity for CR1. C4 is degraded by fI in a similar manner as C3. Factor H is the most important inhibitor of the alternative pathway. Next to cofactor activity for fI in the fluid phase, it also acts on C3b on membranes. It inhibits the formation and accelerates decay of C3 convertases by competing with fB for binding to C3b. A key to the regulation of C3 activation is whether fH binds to C3b deposits on membranes. This is influenced by the carbohydrate environment of deposited C3b; fH has affinity for negatively charged molecules on host cells, such as sialic acid (62).

The membrane bound regulator decay accelerating factor (DAF (CD55)) accelerates the decay of C3 and C5 convertases (63, 64). MCP (CD46), which is another membrane bound complement regulator, acts as cofactor for fI in the cleavage of C3b and C4b. The activity of the anaphylatoxins C3a, C4a and C5a is regulated by plasma carboxypeptidase N, which degrades these molecules into less potent variants.
The terminal pathway of complement is regulated by the fluid phase regulators S-protein (vitronectin) and clusterin. Both S-protein and clusterin bind to the C5b-9 complex, thereby preventing insertion of this complex into the cell membrane (65). CD59 is a membrane bound inhibitor of MAC formation. It inhibits the formation of the MAC by preventing the binding of C9 to the C5b-C8 complex. (66).

**C4 polymorphism**
Complement component C4 is the most polymorphic protein of the complement system. It is a large plasma protein (200 kDa) consisting of three chains (α, β and γ) that are connected via disulphide bonds (Fig. 3). C4 is encoded by two closely linked genes that are located within the MHC class III region on chromosome 6 and which give rise to two isotypic variants, C4A and C4B. Although these isotypes only differ in four amino acids, C4A and C4B are functionally different. After proteolytic cleavage by C1s or MASP-2, C4A preferentially binds via its exposed thio-ester to amino groups, whereas C4B reacts preferentially with hydroxyl groups (23, 67).

**Figure 3.** (A) Intact C4 is a 200 kDa protein that consists of three disulphide-bridged polypeptide chains. (B) Proteolytic cleavage results in release of C4a and exposure of the reactive thio-ester in C4b. (C) The thio-ester of C4b may react with surrounding water molecules or with free amino- or hydroxyl groups.
This implicates that C4A may be functionally advantageous to ensure the solubilization of immune complexes, and clearance of immune complexes through binding to CR1 on erythrocytes. C4B however, would be mainly important for the removal of bacteria by propagating the classical and lectin complement activation pathways that eventually lead to the formation of MAC.

Most individuals have both C4 isotypes, but partial C4 deficiency is quite common in humans. Heterozygous or homozygous C4A or C4B deficiencies are reported to be associated with a great variety of autoimmune or infectious diseases. An increased prevalence of C4A deficiencies has been found in several populations of patients with systemic lupus erythematosus (SLE), which is a typical immune complex disease (68-70). It has been hypothesized that because C4A is more relevant in clearance of immune complexes, deficiency of C4A results in impaired processing of immune complexes which, as discussed below, may be important in the pathogenesis of SLE. Furthermore, it has been suggested that C4B deficiency predisposes for bacterial infections (71, 72).

**Complement and disease**

The complement system plays a pivotal role in human disease. On the one hand, complement activation has many protective functions in immunity and deficiencies within the complement system may lead to increased susceptibility to invasive bacterial infections or development of autoimmune diseases. On the other hand, undesired or excessive complement activation is a major cause of tissue injury in many pathological conditions.

Deficiencies in the early components of the classical pathway (C1q, C4, and C2) are strongly associated with development of SLE (73, 74). MBL deficiency is associated with increased susceptibility to infections, particularly when immunity is already compromised: for example, in infants and young children, patients with cystic fibrosis and after chemotherapy and transplantation (75). C3 deficiency increases the risk for recurrent pyogenic infections, because of lack of opsonization and inability to use the membrane attack pathway (76). Moreover, C3 deficiency predisposes for membranoproliferative glomerulonephritis (77). Deficiencies in constituents of the alternative pathway (factor B, factor D and properdin) as well as deficiencies in terminal pathway components (C5, C6, C7, C8 and C9) lead to increased susceptibility to Gram-negative bacteria such as Neisseria, as a result of the inability to attack the outer membrane of these organisms (78-81).

Deficiency in C1-inh leads to recurrent angioedema attacks. C1-inh deficiency may result from a genetic defect (hereditary angioedema, HAE) or may be caused by an acquired condition
(acquired angioedema, AAE), such as the formation of auto-antibodies towards the reactive site of C1-inh. In type I HAE C1-inh protein synthesis is defective, which leads to low serum levels. In type II HAE normal C1-inh quantities are produced, but with functional impairment of the protein. Both HAE and AAE are associated with decreased C4 levels, due to uncontrolled activity of C1s (82).

Deficiencies in the fluid phase complement regulators factors H and I may result in a state of acquired, severe C3 deficiency (76). Absence of either of these control proteins leads to uncontrolled cleavage of C3. Therefore, deficiency of either of these regulatory proteins gives rise to similar problems as inherited C3 deficiency, such as increased susceptibility to bacterial infections (83). Moreover, mutations in these complement regulators are associated with atypical haemolytic uremic syndrome (aHUS) (84, 85). More recently, variants of fH have been identified as major risk factor for age-related macular degeneration (AMD) (86-88). These findings have been supported by observations that mutations in fB also predispose to AMD (89).

The complement regulators DAF and CD59 are linked to the membrane via GPI-anchors. A mutation in the PIG-A gene leads to incomplete synthesis of GPI-anchors and absent or reduced surface expression of GPI-linked proteins, such as DAF and CD59 (90). This genetic disorder leads to paroxysmal nocturnal hemaglobulinuria (PNH), a disease in which affected erythrocytes and platelets are more vulnerable to complement mediated lysis (91, 92). The critical role of C5 in this disease is illustrated by observations that upon administration of C5 blocking antibody, disease symptoms of PNH can be largely reduced (93). Erythrocytes of individuals who are genetically deficient in DAF (Inab phenotype) but express CD59, are not more susceptible to complement mediated lysis as erythrocytes from healthy controls (94). This demonstrates that susceptibility to lysis is controlled primarily by CD59 on the erythrocytes.

Inappropriate complement activation may cause tissue injury. Excessive activation of complement has been implicated in the pathogenesis of a large number of diseases, including cardiovascular, neurological and autoimmune disease. Most often complement activation is not the cause of disease, but it can exacerbate clinical symptoms and sustain the inflammation. The pro-inflammatory effects of complement activation products contribute to host tissue injury. Complement activation leads to the release of anaphylatoxins (C3a and C5a), which have many pro-inflammatory effects such as attraction, activation and degranulation of neutrophils. Furthermore, the production of sublytic amounts of MAC may lead to upregulation of adhesion molecules on endothelial cells and synthesis of pro-inflammatory cytokines (95). Activation of complement plays an important role in development of ischaemia/reperfusion injury, such as in acute myocardial infarction (96, 97). Moreover, it has been shown that complement activation is
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associated with severity of disease in autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis (98-101) and it plays a role in the onset of Alzheimer’s disease (102).

Complement and autoimmunity
Complement deficiency is associated with autoimmune disease. Mainly the lack of early classical pathway components (C1q, C4, and C2) strongly predisposes for the development of autoimmune disease, such as SLE. Healthy individuals have natural IgM antibodies directed against neo-epitopes on late apoptotic and necrotic cells (103). Furthermore, the acute phase proteins CRP and SAP are also capable of binding to apoptotic cells (104, 105). Upon binding of these adaptor molecules to damaged cells, the classical pathway of complement is activated which contributes to removal of immune complexes and cellular debris derived from apoptotic cells (9, 106-108). In addition, the recognition molecule of the classical pathway, C1q, has been demonstrated to bind directly to apoptotic cells and activate complement as well (9, 11). Deficiencies in the classical pathway may therefore lead to impaired clearance of apoptotic material and release of intracellular auto-antigens such as nuclear components. Prolonged exposure of these auto-antigens to the immune system increases the risk for auto-antibody formation. This is called the “waste disposal hypothesis” (2). The presence of auto-antibodies against nuclear constituents (anti-nuclear antibodies, ANAs) is a common feature in SLE. Immune complexes consisting of auto-antibodies and auto-antigens may accumulate in highly vascularized tissues such as skin, kidneys and lungs, where they become pathogenic. Deposited immune complexes induce inflammation through local activation of the complement system, which leads to infiltration and activation of neutrophils (56, 74).

Next to disposal of apoptotic debris, complement plays a role in the regulation of self-reactive B cells. “The tolerance hypothesis” is an alternative hypothesis that might explain how defects in complement result in development of SLE (109). This hypothesis states that complement-coated self-antigens are delivered to developing self-reactive B cells to enhance their negative selection. Defects in complement might therefore result in failure of negative B cell selection, allowing auto-reactive B cells to survive and propagate when they would normally undergo apoptosis or anergy (109, 110). In conclusion, the combination of increased presence of auto-antigens because of impaired clearance of apoptotic cells and escape of B cell tolerance might explain the relationship between classical pathway deficiencies and development of SLE.
Scope of this thesis
The classical pathway of complement activation plays an important role in autoimmune disease. To evaluate the involvement of complement in disease it is important to be able to measure complement activation in patients. Chapter 2 describes a novel assay to measure classical pathway mediated complement activation in plasma (C1q-C4 ELISA). During classical pathway activation, covalent complexes are formed between activated C4 and the recognition molecule C1q. These complexes appear to be highly specific for the classical pathway and are very stable. Chapter 3 reviews the assays that have been described to measure complement activation in bodily fluids. The most important characteristics of the various available assays are discussed here. The newly developed C1q-C4 ELISA is used in Chapter 4, in which classical complement pathway activation is evaluated in patients with rheumatoid arthritis (RA). C1q-C4 complexes are analyzed in patients with either active or inactive disease. C1q-C4 levels are significantly higher in patients with active RA compared to patients with inactive disease which indicates that C1q-C4 complexes may be useful as novel diagnostic marker for RA disease activity. Chapter 5 demonstrates the characteristics of circulating C1q-complement complexes in more detail. It appears that in healthy individuals C1q-complement complexes circulate as part of the intact C1 complex. Moreover, deposition of complement activation fragments on C1q seems to have a regulatory effect, since it lowers haemolytic activity of the C1q molecule. In chapter 6 the C4 polymorphism is studied on both genetic and protein level in a healthy study population using different methods to discriminate between C4A and C4B, the two major C4 isotypes. Since C4 protein levels correlate very well with the C4 genetic profile, we conclude that the combination of MLPA and isotype-specific ELISAs is a good approach to study the C4 polymorphism. Finally, Chapter 7 contains a general discussion and a summary of the results.
Chapter 1

References


